Nutritional Enhancement of Cereal Milling Wastes using Enzymes.

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Abstract

Wheatfeed is a low value by-product of the cereal milling industry and has potential as an ingredient of pet foods. However, it has a high fibre content and this renders it unsuitable for non-ruminants unless some means can be found to convert the fibre to more digestible substances. This work considers enzyme hydrolysis as a means for improving the nutritional value of wheatfeed.

Preliminary investigations focused on evaluating mixtures of enzymes in various combinations. The extent of hydrolysis was routinely assayed by measuring the release of sugars and proteins. Further experiments were performed to establish the optimal conditions under which a mixture of enzymes, comprising cellulase, hemicellulase and pectinase, hydrolysed wheatfeed. Studies were also conducted where these enzymes were added sequentially to wheatfeed and useful information was gained on the composition of the susceptible components. Steam explosion was investigated as a pretreatment of wheatfeed to make subsequent enzyme treatment more effective. However, the results were inconclusive. Trials were carried out using commercially available enzymes to compare their effectiveness on the wheatfeed. A cellulase, was selected for further investigation into the effects of particle size, extent of agitation, and enzyme concentration on sugar release. An empirical mathematical model describing the action of this enzyme was developed. Enzyme treatment of wheatfeed was also performed under conditions of reduced water content, or 'solid state'. However, enzyme action was limited, yielding lower quantities of sugars and protein.

The treatment of wheatfeed with enzymes was shown to increase digestibility of the substrate. However, the high costs of enzymes would effectively rule this out as a commercial option and alternative methods such as for example a form of composting using cellulolytic fungi might prove more economic.

Keywords: Enzymes, animal feeds, milling by-products, wheatfeed, steam explosion, digestibility.

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Nomenclature

A	Molarity of acid (M)
a,b,c,	Constant
B_t	Amount of blank titrant (ml)
C	Cellulase
C_{A0}	Total sugar available (mg/ml)
C_{E0}	Enzyme loading (ml/ml)
C_{P0}	Initial concentration of sugar (mg/ml)
C_P	Sugar concentration (mg/ml)
C_{pmax}	Maximum concentration of sugar which can be obtained at a given enzyme loading (mg/ml)
E	Enzyme/enzyme treated
E_0	Initial concentration of enzyme
ES	Enzyme-substrate complex
G	β-glucanase
H	Hemicellulase
I	Inhibitor
K	Constant $(ml/mg)^n$
K_m	Michaelis constant (mg/ml)
M	Mass sample (g)
$\%N$	Percent nitrogen
n	Number of moles of product
n	Constant (dimensionless)
P	Product
P	Pectinase
R_0	Severity
R_P	Rate of Production of sugar (mg/ml)/hour
S	Substrate
S	Steam exploded wheatfeed

S_t	titrant of sample (ml)
T	Temperature (°C)
t	Time
T_b	Base temperature (°C)
T_r	Temperature of reaction (°C)
V	Velocity
V_m	Half of maximum velocity
v	Steady state velocity of reaction
X	Xylanase

1 Introduction

Feed processors are continuously seeking to reduce costs of production in order to maintain profitability. One way of achieving this is to substitute cheaper sources of nutrients for the more costly ones. By-products of the food industry, particularly those considered as wastes, may have potential as low cost feed additives. If the nutritional properties of such materials can be improved through appropriate treatment, they could constitute an important source of ingredients for the feed industry. One such material is 'wheatfeed' a by-product of flour milling. Wheatfeed consists mainly of wheat bran. The cell wall material of wheat bran comprises about half of the dry weight of the bran, with arabinoxylans being the major components (Voragen et al., 1994). Wheatfeed in its natural state is indigestible by dogs owing to its high fibre content.

The research described here has, as its objective, the task of enhancing the nutritional value of wheatfeed. The approach taken is to make use of enzymes to digest the fibres contained within the wheatfeed thereby releasing metabolisable sugars and proteins. This type of processing is widely accepted for the treatment of wood, and materials derived from wood, primarily for the production of renewable energy, but its use for nutritional enhancement has been less well studied.

The structure of wheatfeed is complex and it was accepted from the outset that no single enzyme was likely to result in the maximum release of sugars and proteins from the indigestible components. Initial studies were conducted to evaluate the action of a number of different enzymes (in purified form) in various combinations with a view to identifying combinations that may lead to significant fibre digestion. Further studies were undertaken to identify operating conditions that may lead to the optimal release of sugars and proteins. The order in which enzymes were added would result in differential patterns of sugar release, and

experiments in which various enzymes were added in different orders were undertaken so that information on the action of individual enzymes as well as the composition of the susceptible wheatfeed components could be obtained. Included alongside these experiments were those in which the wheatfeed was subject to a form of pretreatment by steam explosion. The latter process was chosen because it was essentially a thermal treatment and did not involve the addition of substances that may have proved injurious to the health of the animals consuming the treated feed.

Commercialization of an enzyme-based treatment process would ultimately be reliant on the types of enzymes available in the market place and comparative experiments were conducted with a number of commercial enzyme preparations. Allied to these experiments were more detailed investigations into the mode of action of a selected commercial cellulase. Also reported here are attempts to model the behaviour of this enzyme.

Conventional modes of treating a solid substrate in dilute suspension in water with enzymes, would incur high costs in reducing the water content of the finished product. Consequently, enzyme digestion experiments were conducted using wheatfeed under reduced water conditions similar to the 'Koji' fermentations of the Far East.

The ultimate test of the acceptability of the enzyme-treated wheatfeed would be to feed it to animals under laboratory conditions. However, evaluations of this sort were beyond the scope of the present work and as an alternative, a series of digestibility tests were conducted as a measure of the nutritional valorization of the final product.

2 Literature Review

2.1 Wheat

Wheat is the most widely cultivated (Leonard and Martin, 1963) and most important cereal crop (Kent-Jones and Amos, 1967). It has been used as a food source since prehistoric times (Kent-Jones and Amos, 1967). The exact dates as to when it was first grown are unknown (Faridi and Finley, 1989), but it has been grown for at least the last 12,000 years and there is even some archaeological evidence that it was used around 15,000 BC (Gooding and Davis, 1997).

It is believed that wheat first evolved from wild grasses (Mattern, 1991) and was first grown in the Middle East, but the cultivation has now spread (Scade, 1975) and it is grown in most parts of the world (Leonard and Martin, 1963).

The best conditions for growing wheat are a cool and moderately moist early growing season. This is followed by a warm dry sunny period during which the plant can mature (Scade, 1975). Probably for this reason wheat is grown in all temperate countries (Leonard and Martin, 1963). With special breeding and selection some varieties have been grown from near tropical areas to those bordering the Arctic circle (Scade, 1975). Wheat can also be grown in almost any kind of soil (Kent-Jones and Amos, 1967). It is therefore perhaps no surprise that wheat is an important foodstuff, as it is available to many people.

Commercially three different types of wheat are grown; *Triticum aestivum*, *Triticum compactum* and *Triticum durum*. *Triticum aestivum* dominates world production and can be used for making bread flour, pastries and biscuits (Mattern, 1991). *Triticum compactum* is used to produce flours for cakes and pastries and *Triticum durum* is used for macaroni and pastas (Mattern, 1991).

Wheat can also be classified by grain hardness, grain colour and the time of year it is planted. Winter wheat is planted late in the summer or autumn and matures in early spring, whereas spring wheat is planted in spring and matures in late summer (Faridi and Finley, 1989). In the United States, *Triticum aestivum* is divided into four classes for marketing purposes; hard red winter wheat, hard red spring wheat, soft red winter wheat and common white. Hard wheat is mainly used for bread whilst the soft variety is used for cakes and pastries (Mattern, 1991). Nearly all English wheat is soft wheat and is used to produce flour for biscuits and cakes rather than bread. The wheat has a high moisture content and needs drying before storage or milling (Scade, 1975).

The grains of common wheats range from lengths of 5 to 8 mm and widths of 2.5 to 4.5 mm. The weights range from 20 to 60 mg, with an average of weight 37 mg (Mattern, 1991). A grain of wheat consists of a pericarp and seed. The seed can be further split up into three parts; the seedcoat, the endosperm and the germ (Kent, 1966). These are further subdivided as shown in Figure 2.1. It should be made clear at this point that different texts refer to the same layers of the grain using different nomenclature.

2.1.1 Anatomy of The Grain

2.1.1.1 Pericarp

The tissues of the pericarp form a protective layer over the entire wheat grain (Leonard and Martin, 1963). It is made up of several layers; the epidermis, the epicarp and the endocarp (Scade, 1975). The pericarp represents about 5% by weight of the grain, the constitution of which can be seen in Table 2.1.

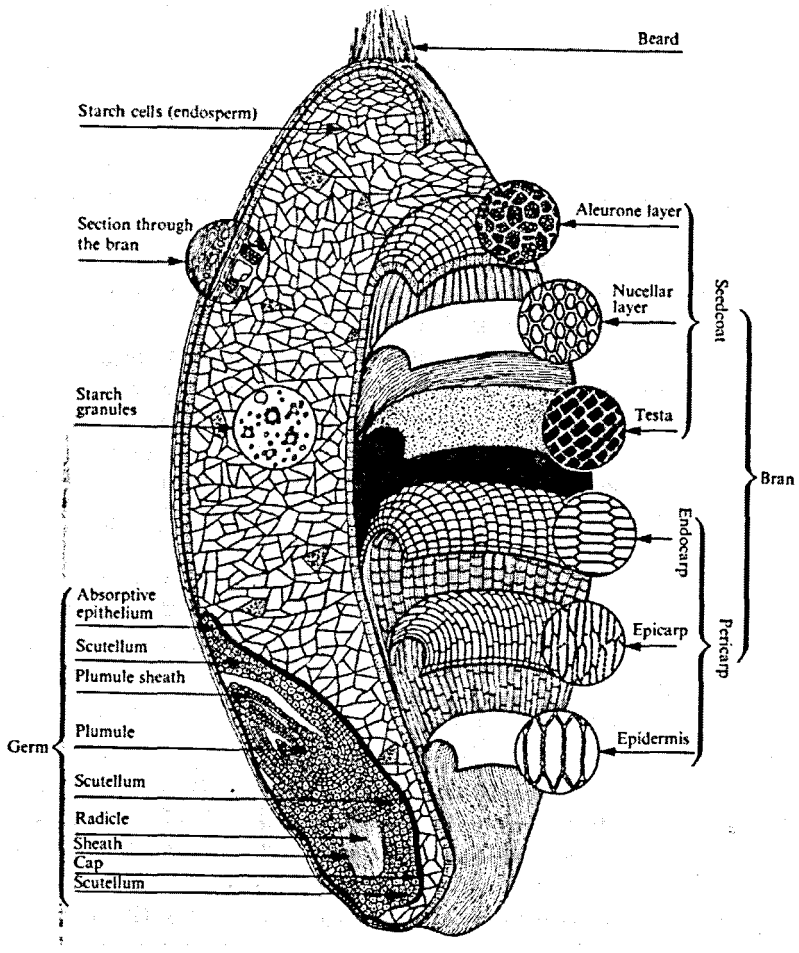


Figure 2.1 A Longitudinal Section of a Wheat Grain (Scade, 1975).

Table 2.1 The Constitution of the Pericarp (Laszity, 1999).

Component	**Percentage (by weight)**
Cellulose	20
Protein	6
Ash	2
Fat	0.5
Other Polysaccharides	70

2.1.1.2 Seedcoat

The seedcoat forms a cover over the embryo and endosperm. It is firmly joined to the innermost layer of the pericarp (Leonard and Martin, 1963). The nucellar layer is a single row of compressed cells (Leonard and Martin, 1963) which are moisture proof and protect the endosperm from moisture effects. Together the nucellar layer and testa represent 2 – 3% of the grain (Kent-Jones and Amos, 1967).

The aleurone layer consists of thick walled cubical cells (Kent, 1966). It is relatively high in protein, ash, phosphorous, vitamins and enzymes compared to the rest of the grain (Laszity, 1999). The aleurone layer is the innermost layer of bran, that is all the layers above it (i.e. those previously described) are all known as bran (Kent, 1966). This is shown in Figure 2.1. The bran is about 13-17% by weight of the wheat grain (Laszity, 1999).

2.1.1.3 Germ

The germ is partly embedded in the endosperm at the base of the grain (Leonard and Martin, 1963). It accounts for 2-3% of the grain (Laszity, 1999) and is made of the embryo (plumule), primary root (radicle) and the scutellum (Leonard and Martin, 1963).The latter is used for food storage (Laszity, 1999).

2.1.1.4 Endosperm

The endosperm is the major part of the grain (Leonard and Martin, 1963). It has thin walled cells that vary in size, shape and composition depending on the position of the cell within the grain (Kent, 1966). The starch granules within the endosperm are embedded in a matrix of proteinaceous material (Leonard and Martin, 1963). There is more protein in the outer layers of the endosperm than the inner layers. The protein content per unit volume of the endosperm is lower than that of the germ and the aleurone layer, but because it is such a large part of the grain it contains approximately 73% of the grain protein content (Pace, 1959).

The protein content of wheat usually exceeds 12% by weight of the grain (Johnson *et al.*, 1972). The wheat grain has four types of protein; albumin, globulin, gliadin and glutenin. Combined gliadin and glutenin are referred to as gluten. It is gluten and its water absorbing properties which give many baked products their characteristics. The main proteins in the wheat grain are the storage proteins, gliadins and glutenins in the endosperm (Kent, 1966).

As it is the starchy endosperm which is used for flour, the bran is removed from the grain by milling and is a waste product.

2.1.2 Use of Wheat as a Feedstuff

A significant amount of wheat is used for animal feed. The actual amount varies from year to year depending on the price of the wheat in relation to other grains (Faridi and Finley, 1989). Wheat bran is composed of the pericarp, seedcoats and aleurone layer and remnants of endosperm (Antoine et al., 2003). The milling by-product, bran and intermediates between bran and flour are often called 'wheatfeed'. They are often used as ingredients of swine feed (Huang et al., 2001). If wheatfeed is to be used in animal feed the amount of fibre in the diet must be controlled, as excessive fibre may render a food injurious to some animals (Kent-Jones and Amos, 1967). As the milling by-products also include the aleurone layer, wheat bran is also a potential source of nutrients (Antoine et al., 2003). The bran waste consists of 12% protein; if this could be utilized, then it could become a cheap protein source. Unfortunately, little is known about the proteins in the aleurone layer. It is known however, that a high proportion of the proteins are soluble in water or dilute neutral salt solutions. More protein could be released and used if the links between the fibre and protein could be broken.

Cereal brans are rich in cell wall polysaccharides (Voragen et al., 1994), which are mainly insoluble celluloses and hemicelluloses (Klopfenstein, 1990). These cell wall polysaccharides have a heterogeneous nature and are poorly

characterized (Wen et al., 1988). The composition of wheat bran is shown in Table 2.2.

Table 2.2 Composition of Wheat Bran (Fisher, 1985).

Component	**Percentage (By weight)**
Starch	22.0
Hemicelluloses	24.5
Cellulose	10.2
Lignin	4.3
Free sugars	7.6
Protein	12.4
Non-Protein, non-nucleic nitrogen containing compounds	3.6
Nucleotides	0.2
Lipids	4.4
Ash	6.1
Phytate	4.0
Total recovered	**99.3**

2.1.3 Cellulose

Cellulose is one of the main components of the plant cell wall (Walker, 1993). It is a linear structural polysaccharide, made of D-glucose units which are β (1 → 4) linked (Garrett and Grisham, 1995) by glucosidic linkages (C-O-C) (Walker, 1993). Its structure is shown in Figure 2.2. The disaccharide cellobiose is the repeating unit as each glucose unit is rotated by 180° relative to its neighbour. Therefore cellulose may also be thought of as a polymer of cellobiose (Walker, 1993). The size of cellulose molecules can vary from 7,000 to 14,000 glucose units in secondary plant walls, but can be as low as 500 units in primary walls (Leschine, 1995). The most soluble celluloses have the smallest molecular weights and conversely the least soluble the highest (Immergut, 1963).

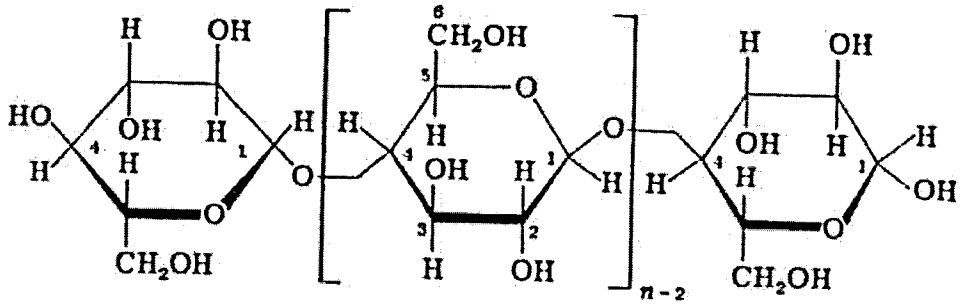


Figure 2.2: Cellulose (Wenzl, 1970).

The two glucose units at either end of the chain differ to the other units, one has a reducing hemiacetyl, the other has a non-reducing alcoholic hydroxyl (Walker, 1993).

Cellulose has both amorphous and crystalline structures. In the former the molecules are arranged randomly and in the latter they are arranged in a lattice-like form (Wenzl, 1970). Crystalline cellulose occurs in long filaments, called microfibrils. These are separated from one another by amorphous cellulose (Walker, 1993). Microfibrils consist of bundles of cellulose molecules which are in a regular alignment, these make up larger cellulose fibres. The microfibrils lie in almost the same plane as the larger fibre but at a slight angle to it (Farmer, 1967). Figure 2.3 shows how the microfibrils build up on the cellulose fibre.

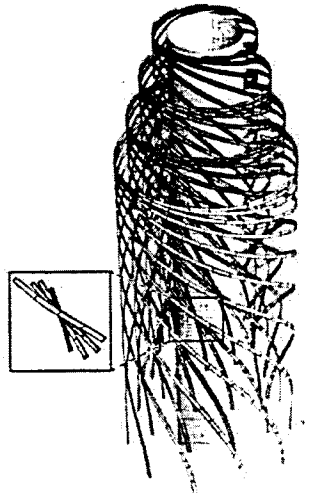


Figure 2.3 The Build-up of Microfibrils of Cellulose (Emons and Mulder, 2000).

Microfibrils have a diameter of about 100Å. The average diameter depends on the origin of the cellulose, with the smallest being wood cellulose and then cotton, bacterial cellulose and finally, ramie (a plant with a flax-like stem). This order is reflected in the resistance of hydrolytic degradation of the cellulose, although this also depends on crystallinity (Immergut, 1963).

All cellulose fibres have a similar structure consisting of a primary wall, a secondary wall and a lumen, or central canal. The primary wall is quite thin and is mainly made up of noncellulosic material like waxes and pectin. The thickness of the secondary cell wall may vary depending on the maturity of the fibre. This is where most of the cellulose material is contained (i.e. the microfibrils). The central canal is mainly proteinaceous material (Immergut, 1963). Figure 2.4 shows this arrangement for a cotton fibre.

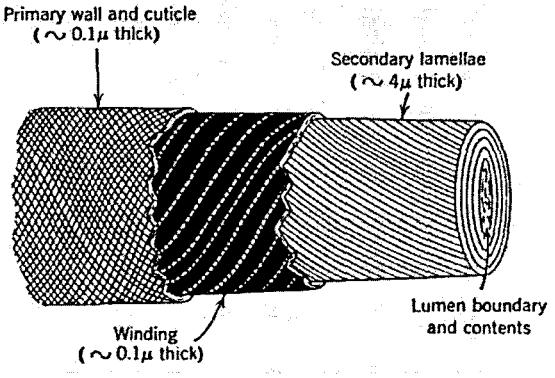


Figure 2.4 The Structure of Cotton Fibre (Immergut, 1963).

The configuration of the cellulose chains allow hydrogen bonding to take place between residues of the same chain. The chains run parallel to one another and therefore hydrogen bonding can occur between cellulose chains. Intramolcular and intermolecular bonds allow all the available hydroxyl groups to exhibit hydrogen bonding. A covalent glucosidic bond is stronger than a hydrogen bond, but there are a large number of hydrogen bonds along the chain. The hydrogen bonded cellulose chains form sheets which pack on top of each other to form the

three-dimensional crystal structure. These sheets are held together by weak Van der Waals forces (Walker, 1993).

Cellulose is very resistant to hydrolysis by acids, or enzymes of the digestive tract and consequently most animals can not digest it. Cattle, deer and other ruminants are able to digest cellulose as they have bacteria in the rumen which secrete cellulases (Garrett and Grisham, 1995).

2.1.4 Hemicellulose

Many different terms have been suggested to describe this fraction. For example, hemicellulosic polysaccharides are sometimes characterized according to their sugar components and referred to as pentosans or hexosans (Wenzl, 1970). The term hemicellulose is unsatisfactory, but it has generally been and can be defined as, the non-cellulosic cell wall polysaccharides (Farmer, 1967). Unfortunately, some quite different terminologies are in use and this can make the study of this subject confusing.

Hemicelluloses are the major constituents of plant cell walls, filling the voids between the cells in the middle lamella (Wilkie, 1979), this is shown in Figure 2.5. They consist of long chains of sugar residues, but unlike cellulose they contain several different sugar residues including both hexoses and pentoses. More than one sugar can also be found in the main chain. Hemicelluloses have much shorter chains than those of cellulose, they are often branched and may have uronic acid and acetyl groups attached. The main chain is not always (1→4) linked. Because the molecules are less regular and branched, they will not pack into bundles easily and therefore do not have marked fibre-forming properties, but they are more gelatinous. They are fairly readily hydrolysed by acid and a large amount of those in wood can be extracted by alkali (Farmer, 1967).

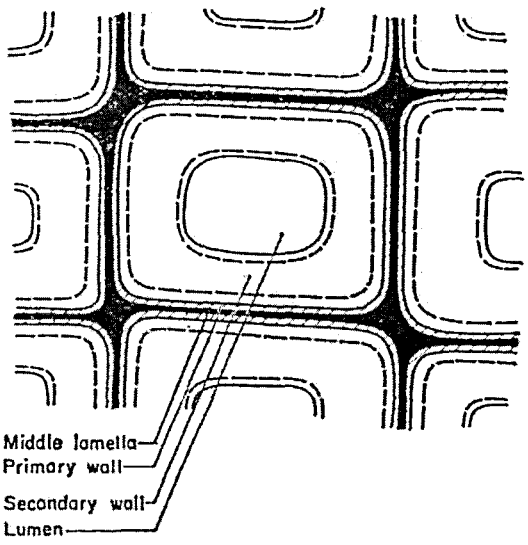


Figure 2.5 Cross Section of a Group of Wood Cells (Farmer, 1967).

Hemicelluloses typically have molecular weights from 60,000 to 600,000 Daltons (Low, 1994). Differences in solubility occur due to differences in the average molecular weight, the distribution of the molecular weight, molecule structure, the functional groups, the homogeneity of the composition and the relationship with the cell walls (Wenzl, 1970).

Doree (1950) reported that after fractionating the hemicellulose in wheat bran it was found that the chief products obtained on hydrolysis were xylose, arabinose, uronic acid and a small amount of glucose. Fincher and Stone (1986) reported that wheat bran was composed of 39% xylose, 34% glucose, 26% arabinose, 0.1% mannose and 1.1% galactose by weight. With the major polysaccharides present being arabinoxylan (64%), cellulose (29%) and non-cellulosic glucan (6%).

The function of hemicellulose is uncertain, but it is possible it could form the link between cellulose and lignin (Walker, 1993). Some workers have suggested that arabinoxylans may aid the movement of nutrients through the porous gel, or even that they may inhibit intercellular ice formation (Izydorczyk and Biliaderis, 1995).

2.1.4.1 The Xylans

Although arabinoxylans are a minor part of the entire cereal grain, they make up an important part of the cell walls. The thin walls that surround the endosperm cells and the aleurone layer walls are mainly arabinoxylan (60-70%) (Izydorczyk and Biliaderis, 1995).

Arabinoxylans have highly branched structures (Edwards et al., 2003) and are made up from a linear β-D-(1→4) linked xylopyranose backbone with α-L-arabinofuranose substitutions attached by α-(1→3) or α-(1→2) linkages (Edwards et al., 2003). The ratio of arabinose to xylose varies (Schuerch, 1963) as do the sequences of the linkages of these sugars and the presence of other substituents (Edwards et al., 2003). A fairly high ratio of arabinose to xylose gives the molecule a rigid rod-like conformation (Izydorczyk and Biliaderis, 1992). The hemicelluloses from the pericarp of wheat bran contain both arabinose and xylose singly and doubly branched (Schuerch, 1963).

It has been suggested that wheat arabinoxylans are heterogeneous and that it is difficult to assign them a single structure (Izydorczyk and Biliaderis, 1995). Edwards *et al.* (2003) suggest however, that the distribution of the arabinose substituents along the backbone is probably as important as the extent of the substitution, as it will affect the conformation of the chain and therefore the ability of the arabinoxylans to interact. They also found that although there is a large amount of structural heterogeneity among cereal arabinoxylans, recent studies would suggest that the distribution of the arabinosyl residues along the xylan backbone is non-random. Edwards *et al.* (2003) went on to propose a structure for arabinoxylan in wheat bran this is shown in Figure 2.6.

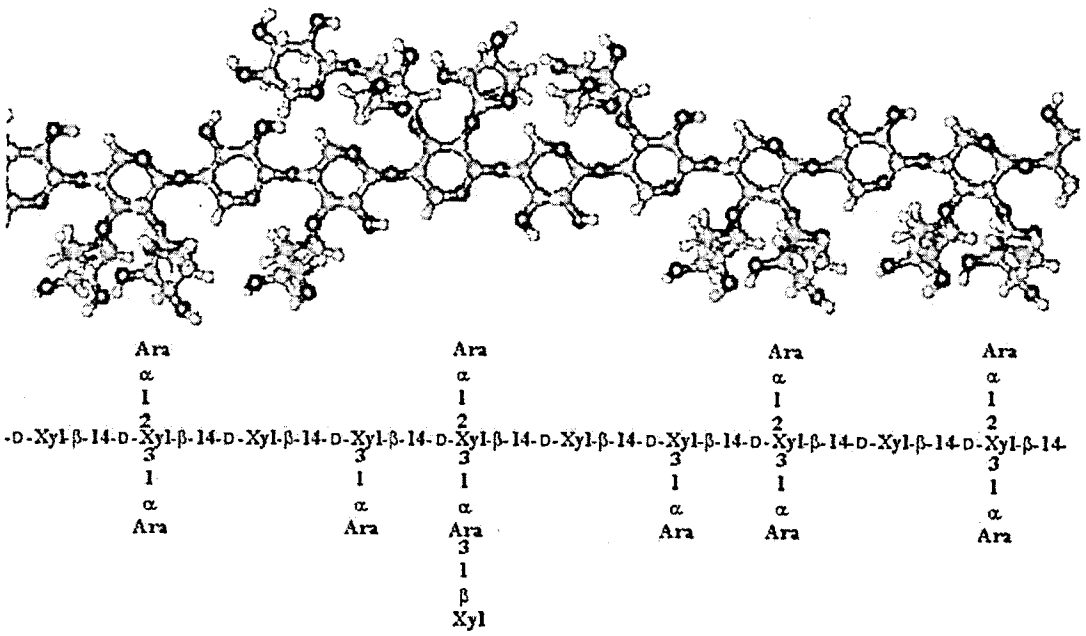


Figure 2.6 The Proposed Structure for Arabinoxylans of Wheat Bran (Edwards *et al.*, 2003).

Arabinoglucuronoxylans contain both arabinose and uronic acids. The hemicelluloses from the pericarp of wheat bran contain both arabinose and xylose, in addition to D-Glucuronic acid and its 4-O-methyl derivative (Schuerch, 1963).

2.1.4.2 Mannans

Glucomannans may be present in wheat cell walls, as some residues of glucose and mannose have been isolated after extraction with concentrated alkali, However, if present at all, they will account for only a small percentage of wheat endosperm and aleurone cell walls.

2.1.4.3 β-Glucans

β-Glucans are found in the endosperm and aleurone cell walls of wheat. They consist of linear chains of β-glucosyl residues joined by (1→3) and (1→4) glycosidic linkages (Fincher and Stone, 1986).

2.1.5 Proteins

Protein is known to be present in the aleurone layer (Amrein et al., 2003). The aleurone cells have thick walls inside which protein bodies are densely packed (Autio, 1996), the cell wall matrix may account for the low digestibility of protein in wheat bran (Saunders et al., 1972). It has been shown that protein digestibility of the aleurone cells increases once the walls are physically broken (Saunders et al., 1972). The proteins in the aleurone layer are albumins and globulins, hence they are water or salt soluble. The aleurone layer is the main storage location for amylases, proteases (for storage proteins) and hemicelluloses (Laszity, 1999).

2.1.6 Pectin

Wheat bran also contains pectic substances (Hwang et al., 1993). These are thought to consist of linked units of D-galacturonic acid, D-galactose and L-arabinoses (Stephen, 1983).

2.1.6.1 Arabinogalactans

These polymers have a galactose backbone with arabinosyl branches. They are also water soluble and form part of the pectic substances (Southgate, 1995).

2.1.6.2 Galacturonans

Galacturonans have linear backbones formed by galacturonic acid residues, they are α 1$\rightarrow$ 4 linked and are soluble in water. These polymers are the major part of pectic substances and are found in the middle lamellae (Southgate, 1995). The pure polymer is rare in nature, most have rhamnose residues at intervals along the chain and are known as "Rhamnogalacturonans".

2.1.7 Lignin

Lignin is the second most abundant renewable material on Earth, second only to cellulose and is the most abundant renewable aromatic substance (Kirk and Farrell, 1987). It is present in most plant tissues (Sarkanen, 1963) and is the third major component in the wood cell wall (Farmer, 1967) where it is interspersed

with hemicelluloses to form a matrix which surrounds the cellulose microfibrils (Kirk and Farrell, 1987). Lignin is present in wheat bran and as Table 2.2 shows, it accounts for 4.3% by weight of the bran. In wood it is the major constituent of the middle lamella (Farmer, 1967; Kirk and Farrell, 1987) which forms the boundary of adjacent cells (Farmer, 1967). It protects the cellulose and hemicelluloses in the plant cell wall from enzyme attack (Kirk and Farrell, 1987).

Lignification is a process where hydrophobic lignin replaces water in the cell wall, encrusting cellulose, other polysaccharides and protein and primary cell wall. This wood-like substance results in cells with extra strength and makes them impenetrable to water and enzymes (Iiyama et al., 1994). The deposition of lignin will over fill the spaces between the cell material and therefore causes the cell wall to thicken and swell (Walker, 1993). Lignification is an irreversible process which will stop the living functions of the cell by the time the process is completed (Sarkanen, 1963). Plant lignins can be separated into three groups, softwood (gymnosperm), hardwood (dicotyledonous angiosperm) and grass and annual plant (monocotyledonous angiosperm) (Pearl, 1967). They contain different base units but the term "lignin" is used for the family of related polymers (see Figure 2.7), (Kirk and Farrell, 1987).

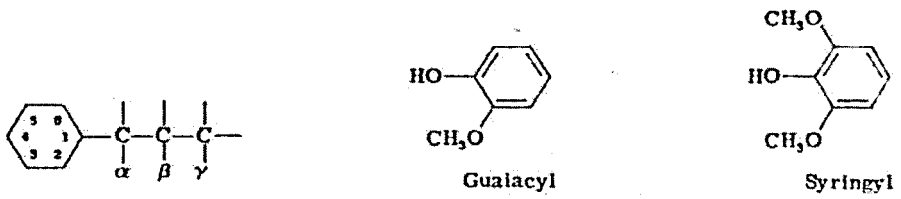


Figure 2.7 The Structure of Some Moieties Isolated from Lignin (Wenzl, 1970).

Studying the chemical structure of lignin is difficult as it is insoluble (Farmer, 1967) and can not be easily hydrolysed into smaller units. Studies on the structure of lignin are often based on the modified fragments which have been extracted from finely ground wood (Walker, 1993).

No regular structure has been established for lignin (Walker, 1993). It is a polymeric material with a basic structural unit built up from the phenolpropane nucleus i.e. an aromatic ring with a three carbon side chain (Farmer, 1967). Lignin is amorphous which means that the structure can only be described generally and depends on the way the basic monomer units are linked together.

These linkages can be (see Figure 2.8);

Head to tail. For example linkages between C4 and a side chain carbon of another unit, or a carbon to carbon C5 to Cβ linkage.

Head to head. For example linkages between the α and α' β', or γ' positions and α–α and β–β carbon linkages.

Tail to tail. For example C5-C5 linkages (Walker, 1993).

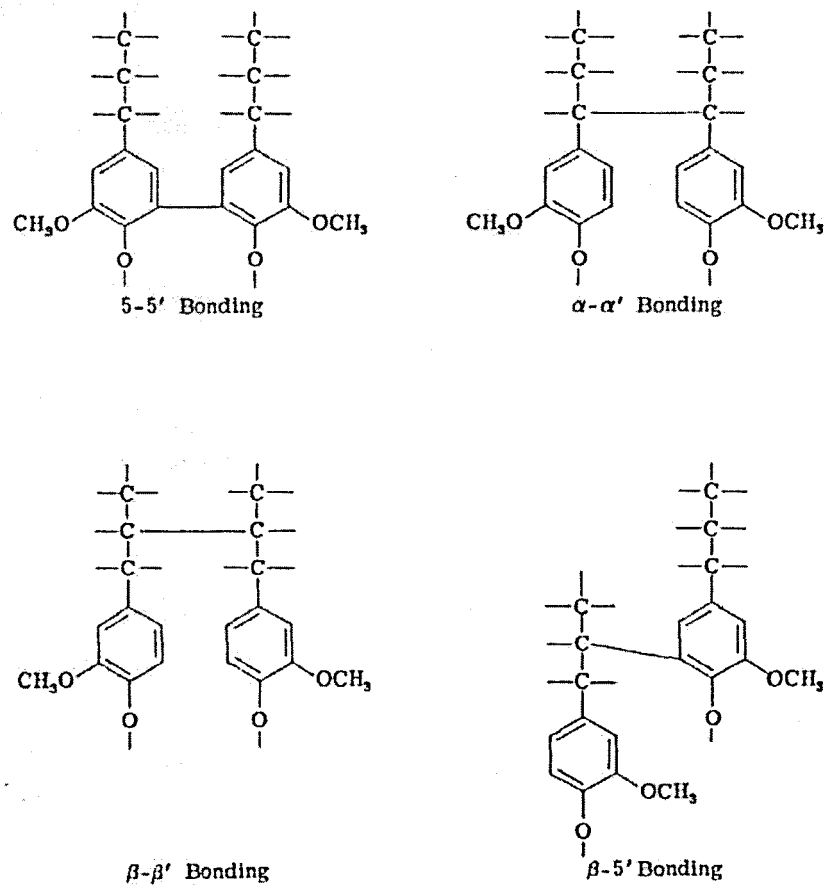


Figure 2.8 Some linkages in Lignin (Wenzl, 1970).

The question as to whether lignin-carbohydrate bonds occur puzzled investigators for some time. It was known that there was lignin in the cell and also carbohydrate, but it is impossible to extract carbohydrate from unhydrolysed wood by using carbohydrate solvent cuprammonium hydroxide and also impossible to extract more than a trace of lignin using well known lignin solvents. Other methods isolated compounds that contained no free sugars, then liberated both lignin and sugars after further hydrolysis.

More recent postulations are that the surfaces of the lignin are associated with and cover the wall polysaccharides and proteins giving the chance for covalent cross-linking to occur. Three types of cross-links are shown in Figure 2.9 (Iiyama et al., 1994).

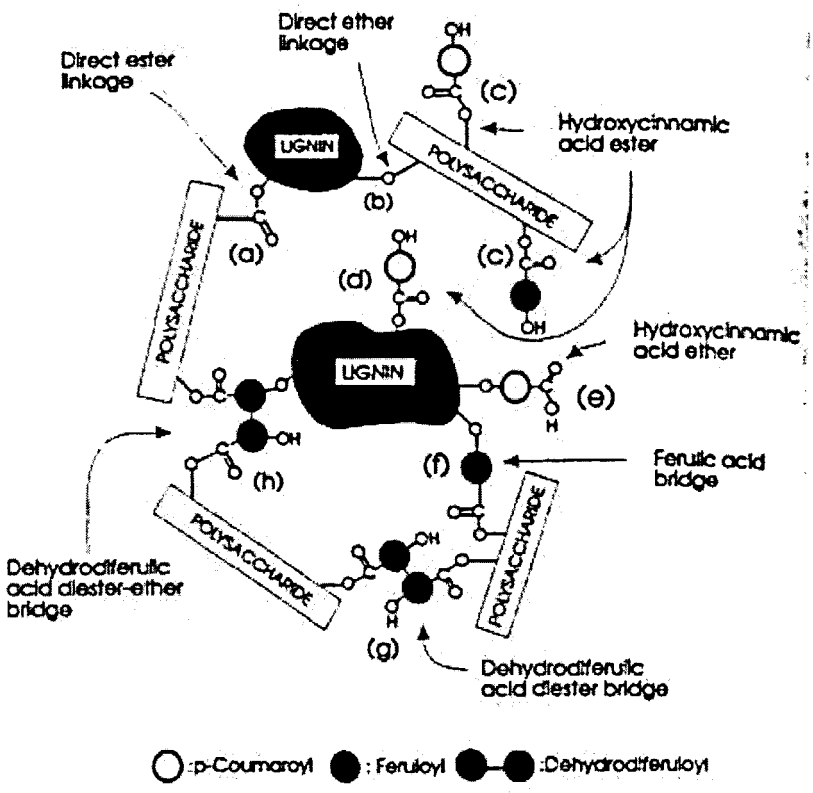


Figure 2.9 Possible Covalent Cross-Links Between Polysaccharides and Lignin (Iiyama *et al.*, 1994).

2.1.8 Inorganic Materials

Plant cell walls usually contain some inorganic materials, calcium, potassium and magnesium for example (Southgate, 1995).

2.1.9 Fibre

2.1.9.1 Introduction

The definition of the term fibre has changed over the years. For many years the fibre content was expressed as "crude fibre" which was measured by a test referred to as the Weende method. This method was used to analyse fibre in animal forages in the United States for many years (Trowell, 1985). Unfortunately, this test results in the loss of about fifty percent of the cellulose

and eighty five percent of the hemicellulose during the extraction process and therefore the measurement is widely considered to be inaccurate (Trowell, 1985).

Some workers have preferred to split plant sources into two groups, the digestible group is referred to as 'available carbohydrates' and the indigestible groups are 'unavailable carbohydrates'. The former are susceptible to the endogenous enzymes of the upper digestive systems of humans to produce energy within the body. Glucose, fructose, sucrose, lactose, maltose, raffinose and stachyose are available carbohydrates, as are starch and dextrins (partly hydrolysed starch molecules) (Trowell et al., 1985).

Some starch, referred to as 'resistant starch,' is not susceptible to enzymic attack and is therefore not part of the available carbohydrates as defined above (James, 1995). As discussed later, resistant starch has to be taken into account in methods for analysing dietary fibre.

Unavailable carbohydrates are not susceptible to the actions of endogenous enzymes (James, 1995). It is now known that these polysaccharides can be fermented by bacteria in the large bowel and will contribute a small amount of absorbed energy (Trowell et al., 1985) so the term 'unavailable carbohydrates' is essentially obsolete. The term mainly used today is 'dietary fibre' which is defined as the sum of the polysaccharides and lignin which are not digested by the endogenous secretions of the human gastrointestinal tract, this fraction has a variable composition and is made up of different types of polysaccharides (cellulose, hemicelluloses and pectic substances) and the non carbohydrate lignin (Trowell, 1985).

This definition has however not been universally accepted and variations of it exist in the literature. More importantly, without unanimous agreement as to the definition of what fibre is, it is difficult to make sense of some experimental measurement, particularly where there are large discrepancies in the protocols.

2.2 Enzymes

Enzymes are very effective and highly specific biocatalysts. Enzymes are proteins, or to be more precise, their main components comprise proteins. Generally, one enzyme will catalyse the reaction of one, or a pair of substrates (Simon, 1996). The enzyme will react with the substrate at specific areas referred to as active sites see Figure 2.10.

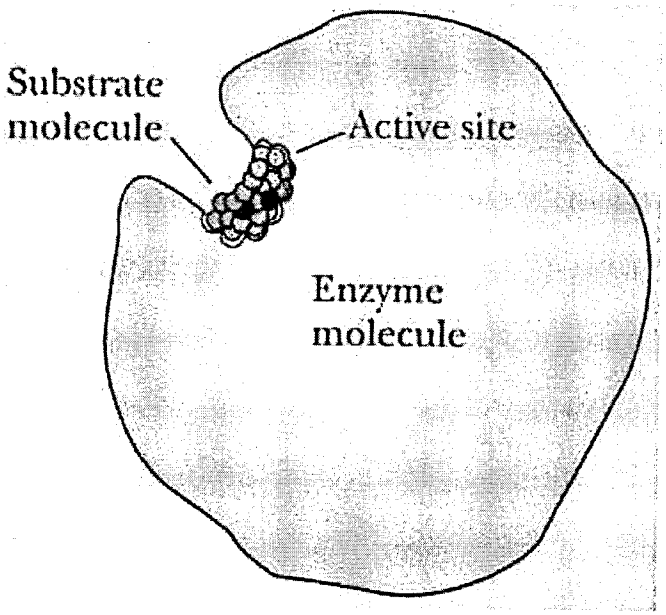


Figure 2.10 A Simplified Diagram of an Active Site (Garrett and Grisham, 1995).

The enzyme (E) will bind with the substrate (S) to give an enzyme substrate complex (ES), this complex is usually unstable and decomposes to give a product whilst regenerating the free enzyme.

$$E + S \leftrightarrow ES \rightarrow E + P \tag{2.1}$$

The enzyme-substrate interaction has been likened to the operation of a key in a lock to explain the high levels of specificity which some enzymes display.

2.2.1 Enzyme Inhibition

Some compounds are able to inhibit the activity of enzymes. This manifests itself by a decrease in the rate of the reaction (Garrett and Grisham, 1995). There are two different kinds of inhibition, reversible and irreversible.

2.2.1.1 Reversible Inhibition

Reversible inhibition can be sub-divided further into competitive, non-competitive and uncompetitive inhibition.

Competitive Inhibition

Where competitive inhibition takes place an inhibitor (*I*) can bind to the enzyme at the active site and thus competes with the substrate for this site. The substrate and the inhibitor are likely to have a similar structure allowing them both to bind (Garrett and Grisham, 1995). In the lock and key analogy described earlier, for a different key to fit the lock, it must be similar in shape to the key made for the lock (Figure 2.11). The reactions that occur when competitive inhibition takes place comprise those described above (Equation 2.1). In addition a reaction where the inhibitor binds to the enzyme must also be considered (Equation 2.2).

$$E + I \leftrightarrow EI \tag{2.2}$$

Increasing the substrate concentration decreases the effects of inhibition, as the increased substrate means it is more likely to be a substrate molecule rather than an inhibitor which binds with any given enzyme (Garrett and Grisham, 1995).

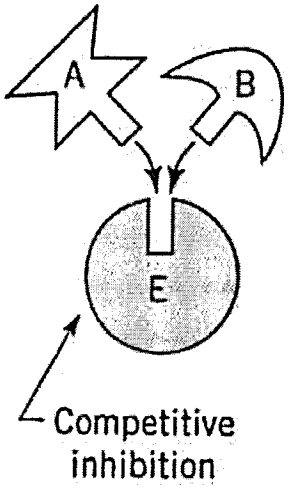


Figure 2.11 A Simple Representation of Competitive Inhibition (Levenspiel, 1999).

Non Competitive Inhibition

In non-competitive inhibition the inhibitor can react with both the enzyme and the enzyme-substrate complex. As it can react with both, it can be concluded that the inhibitor is not binding to the same site on the enzyme as the substrate. However, in binding to the enzyme the inhibitor reduces the catalytic power and the ES substrate cannot form, or decompose to give the products at the normal rate (Garrett and Grisham, 1995). The reactions that take place include those previously described (Equations 2.1 and 2.2) but also the following ones:

$$EI + S \leftrightarrow ESI \tag{2.3}$$

$$ES + I \leftrightarrow ESI \tag{2.4}$$

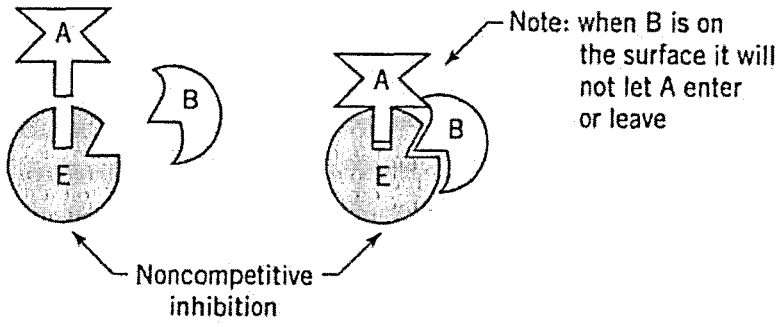


Figure 2.12 A Representation of Non–competitive Inhibition (Levenspiel, 1999).

Uncompetitive Inhibition

In uncompetitive inhibition, the inhibitor binds to the enzyme–substrate complex which gives a complex which can not undergo a reaction to produce the product. In this case inhibition may increase when the substrate concentration is increased (Lehninger, 1975). The reactions concerned are the same as those shown by Equations 2.1 and 2.4.

2.2.1.2 Irreversible Inhibition

In irreversible inhibition the inhibitor comes into contact with the enzyme and attaches covalently, modifying the enzyme so that it can no longer bind with the substrate in the manner for which it was to be used. Enzyme activity will decrease over time. In this case the inhibition reactions are the same as those described by Equations 2.2 and 2.4 but the reaction is irreversible (Garrett and Grisham, 1995).

2.2.1.3 Activation energy

Any reaction can be described by a reaction coordinate diagram which is a picture of the free energy changes. The free energy of a system is plotted against the progress of the reaction. Each reactant has an energy level called the ground state, to get from one ground state to another via a reaction may require an increase in the free energy of the system even if the ground state of the product is less than

the ground state of the reactant. This energy is required for alignment of reacting groups, bond rearrangements and other transformations required for the reaction to proceed in either direction. Figure 2.13 shows the ground states for the reaction

$$S \leftrightarrow P \tag{2.5}$$

the increase in energy required can be seen. For the reaction to go ahead the molecules must have an energy level raised to the top of the free energy peak. At this point both the forward and the backward reactions can and are equally likely to take place. This point is the transition state. The difference between the ground state and the transition state is called the activation energy. The higher the activation energy the slower the reaction will be (Lehninger, 1975).

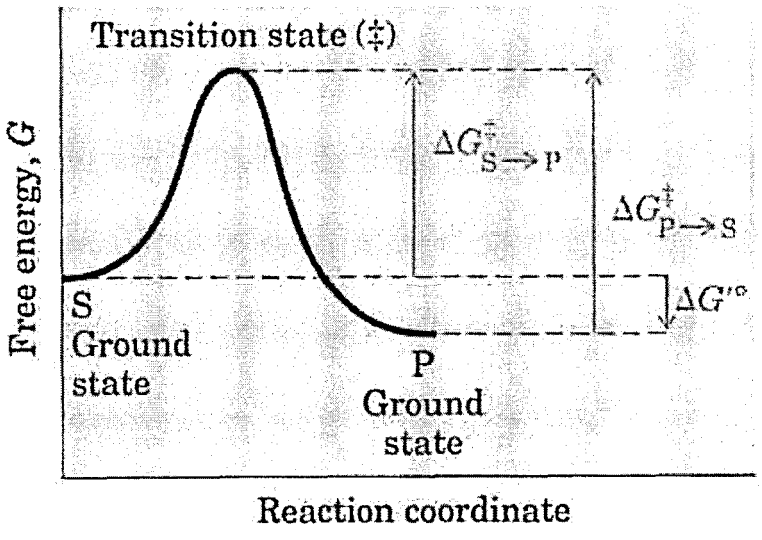


Figure 2.13 Reaction Coordinate Diagram (Lehninger, 1975).

Using a catalylst, such as an enzyme is a way of altering the reaction pathway. If an enzyme is used to catalyse the reaction described above the following equation describes the reaction.

$$E + S \leftrightarrow ES \leftrightarrow EP \leftrightarrow E + P \tag{2.6}$$

In this case the reaction pathway is altered. The activation energies required to form *ES* then *EP* and finally *E+S*, is low compared to the uncatalysed activation energy and therefore the reaction will happen faster. This is shown in Figure 2.14 (Lehninger, 1975).

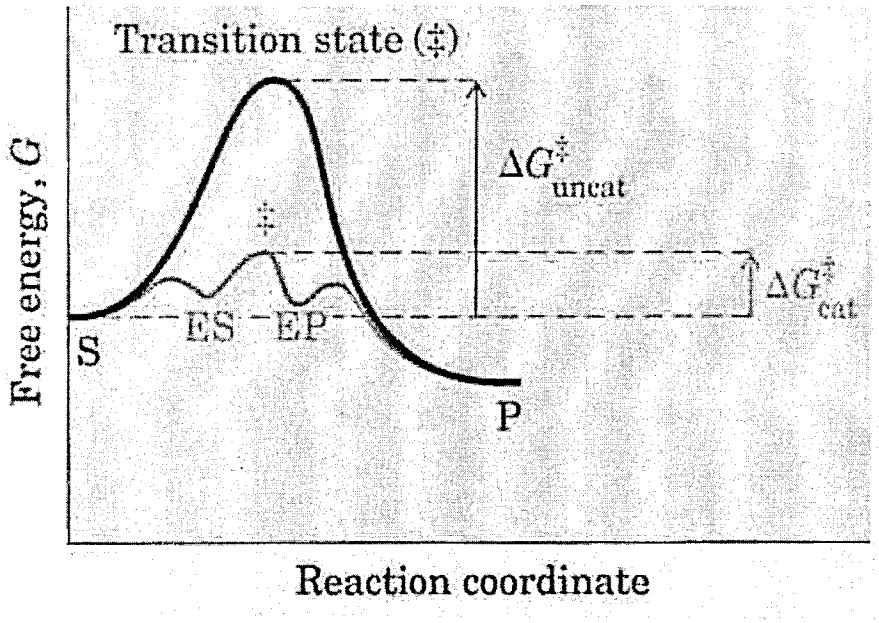


Figure 2.14 Reaction Coordinate Diagram Comparing Enzyme Catalysed and Uncatalysed Reactions (Lehninger, 1975).

2.2.1.4 Michaelis-Menten Kinetics

For most enzyme systems the variation in the initial reaction rate can be described by Michaelis-Menten kinetics. If it is assumed that the pH, non-substrate components and buffer have no effect on the reaction, then the following equation describes the system

$$(E)+(S)\leftrightarrow(ES)\rightarrow(E)+n(P) \tag{2.7}$$

where: (E) = enzyme concentration

(S) = initial substrate concentration

(ES) = cellulose-cellulase complex concentration

n = number of moles of product

(P) = product concentration

The steady state rate of the reaction v is given by:

$$v=\frac{V(S)}{(S)+K_m} \tag{2.8}$$

where: V $= K_3\,(E_0)$

(E_0) = initial concentration of enzyme

(S) = initial concentration of substrate

K_m = Michaelis constant = $\frac{K_2+K_3}{K_1}$

K_m represents the substrate concentration at which half of the maximum rate. (V_m) is reached under the experimental conditions (Ghose and Das, 1971).

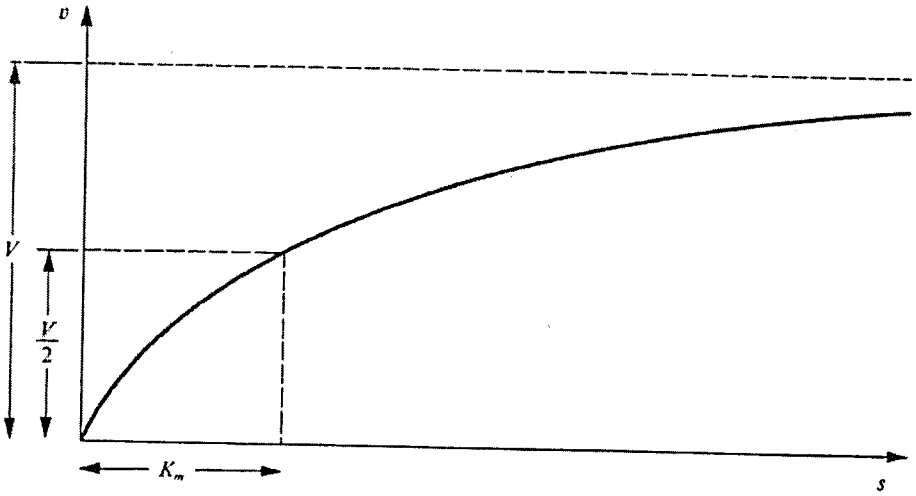


Figure 2.15 The Hyperbolic Relationship Between Initial Reaction Rate and Substrate Concentration of a Simple Catalysed Reaction (Yudkin and Offord, 1973).

If the Equation 2.6 is linearised

$$\frac{1}{v} = \frac{K_m}{V} \cdot \frac{1}{(S)} + \frac{1}{V} \qquad (2.9)$$

A plot of $1/v$ against $1/(S)$ (a Lineweaver-Burk plot) is a straight line with a gradient of K_m/V and an intercept of $1/V$. The gradient is dependent on the reaction mechanism and any inhibition (Ghose and Das, 1971; Yudkin and Offord, 1973) and is steeper for inhibited reactions. The value where the line crosses the x axis = $(-1/K_m)$.

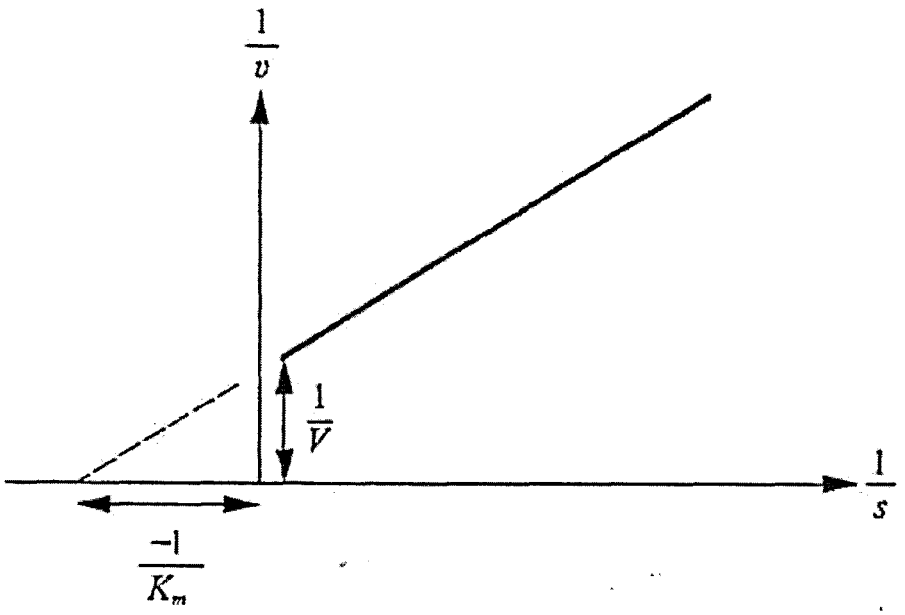


Figure 2.16 Lineweaver-Burk Plot (Yudkin and Offord, 1973).

2.2.2 Enzymes in the Feed Industry

In the 1880s Takamine developed the idea of ingesting an enzyme to help aid digestion. His enzyme Takadiesterase was the first relatively sophisticated commercial enzyme to be produced (Dunne, 1991; Gharpuray *et al.*, 1983). The enzyme industry is now highly sophisticated and enzymes are used throughout the food and beverage industry, although Dunne claimed in 1991 that the overall successful usage of enzymes in the food, beverage, and pharmaceutical industries has yet to be realized in the feed industry.

Between the late 1980s and mid 1990s, feed enzyme supplements specifically for animal diets became more readily available. In this time feed enzyme supplementation increased, but mainly in pig and poultry diets (Officer, 2000). Certainly the literature on the subject seems biased towards these areas of study and still reflect Takamine's original idea of adding enzymes directly to feed as an aid to digestion.

The fibre content of a feedstuff will ultimately limit its productive value (Hutcheson, 2002). The carbohydrases produced in the digestive tracts of animals will not be able to completely degrade the complex mixtures of carbohydrates typically found in commercial feed (Simon, 1996). Ruminant animals (i.e. those which have more than one stomach) consume diets which are high in fibre and which consist mainly of cellulose, hemicellulose and lignin. Enzymes produced by several predominantly ruminal bacteria can attack the xylan molecules in the fibre and release sugars which are then fermented by other bacteria (Martin et al., 1998). Monogastric animals can not utilize fibre. Enzymes can be used to degrade specific bonds in the feedstuff that would not be broken down by endogenous enzymes in the animal (Hutcheson, 2002). They act at a molecular level but they can also generate changes in the microstructure and the functional properties of cereal foods (Poutanen, 1997). In this way, the enzymes will release more nutrients which the animal can then use (Hutcheson, 2002). This would obviate the need to add such nutrients exogenously.

It is also believed that the water holding capacity of soluble and insoluble fibre fractions in grain and grain products, can result in water-soluble nutrients being trapped in the fibre matrix and therefore not being available for digestion. If the fibre matrix is disrupted by enzymes, these nutrients may be released (Partridge and Hruby, 2002).

When choosing the enzyme for a particular application, it is important to match the enzyme activity and the feed composition and target the parts of the food which will be harmful, or of no nutritional value to the animal without enzyme treatment. If a foodstuff can be treated in such a way, then there is the possibility of using cheaper ingredients which were previously unacceptable (Officer, 2000).

As previously described a large amount of wheat bran is cellulose. *Trichoderma reesei* (formerly *Trichoderma viride*) has been found to be amongst the most highly effective fungi for hydrolysing cellulosic materials, producing at least five

different cellulases, two cellobiohydrolases and three endoglucanases (Medve et al., 1994). These components work to provide a synergistic effect. Converse and Optekar (1993) measured the degree of synergy which is defined as the ratio of glucose produced when both cellobiohydrolase and endoglucanase enzyme components are present to the sum of the glucose produced when they are present individually. It is widely accepted that the endoglucanase breaks internal glucosidic bonds in the chain, after which the cellobiohydrolase cleaves cellobiose units from the nonreducing ends of the chain (Medve et al., 1994). The degree of synergy is less at lower enzyme concentrations as the number of chain ends available for the cellobiohydrolase is sufficient for the reaction to continue. As the enzyme concentration increases, the cellobiohydrolase is more dependant on the endoglucanase to produce more chain ends for the reaction, therefore the synergy of the system increases (Converse and Optekar, 1993). At very high enzyme concentrations there is a decrease in the degree of synergy as the enzymes are acting competitively against one another (Converse and Optekar, 1993).

Results showing the benefit of adding enzyme preparations to ruminant feed are inconsistent (Bhat, 2000). Some researchers report improvements in feed digestibility, yet others report no difference. Variable responses have been found when feed products have been given to animals of the same age in a similar diet. This is either due to variability in the enzyme formulation or in the feedstuff itself (Officer, 2000). In regard to plant feedstuffs, Dunne (1991) reported that the complex structure of plant cell walls and the variability in the composition from plant to plant makes the job of enzymic hydrolysis very difficult, this may account for the inconsistency of research results.

By-product feeds have been used extensively in dairy cattle rations in many parts of the world as economical substitutes for corn and soybean meal. There is increasing interest in the nutritive value of by-product feeds as nutritionists seek to manipulate undegraded intake protein and non-fibre carbohydrate concentrations of dairy cattle rations (Batajoo and Shaver, 1998).

Work has previously been carried out by Waszczynskyj et al. (1981) using cellulase, pectinase and hemicellulase to break down wheat bran in a pre-treatment step for a process to extract protein using alkaline conditions. Their report confirmed that there was potential for carbohydrases to increase the yield of intracellular constituents from a solid substrate such as wheat bran. Weinburg *et al.* (1995) showed that cellulase alone was not effective at hydrolysing alfalfa cell walls and a mixture containing hemicellulase and pectinase was necessary. Bedford (2000) in his work concluded that the enzymes used must have broad hydrolytic activity, as the carbohydrates comprising the cell wall structures and the indigestible starch/protein complexes are extremely heterogeneous.

Enzyme inhibitors have been reported in several cereal grains, for example protein inhibitors of amylases are naturally present in the endosperm. Rouau and Surget (1998) showed that there is a water extractable and thermo-labile compound which inhibits extrogenous hemicellulases present in wheat grains, especially in the endosperm.

Enzymes used in processing feed must undergo toxicity testing as specified by EU regulations, Council Directive 70/524/EEC (The Commission 2002). Issues concerned are the evaluation of toxicity to the animal and the consumer via residues in human food (Simon, 1996).

One disadvantage of using enzymes in feed processing is their high cost. According to (Ishihara et al., 1991) enzymes make up 60 to 80 % of the total process cost. Process costs need to be tightly controlled in instances where it is proposed to treat low-value by-products with enzymes. This is because high processing costs will eliminate the advantages of using low value by-products. Slow reaction kinetics and the heterogeneous nature of reactions in solid-liquid systems renders the process an unlikely candidate for continuous processing. In addition, the mechanisms of hydrolytic reactions in heterogeneous systems are difficult to model.

2.2.3 Enzyme Kinetics.

The hydrolysis of cellulose has been studied by many workers and a number of models for the kinetics of the reaction have been proposed.

Huang (1975) claimed that the initial absorption of the cellulase from *Tricoderma reesei* onto the cellulose is fast, but that the subsequent reaction is slow, after which the enzyme will gradually be released back into the liquid. The slow kinetics lead to high processing costs and this makes enzymic treatment uneconomic (Gan et al., 2002b; Gharpuray et al., 1983). The slow reaction rates also render the process unsuitable for continuous reactor systems, also high enzyme concentrations are needed to achieve high cellulose conversions (Eriksson et al., 2002).

In the hydrolysis of cellulose by cellulase, it has been observed that the reaction rate decreases rapidly over time. Initially there is a rapid release of sugars, this is then followed by a second phase in which the sugar production follows a declining rate (Ortega et al., 2001). This decrease in reaction rate has been the subject of much discussion and a number of different theories have been proposed to explain it.

Some explanations focus on the structure of the substrate. As previously discussed in section 2.1.3, cellulose in plant materials exists in two forms, amorphous and crystalline. Katz and Reese (1968) suggested that in the initial stages of the reaction the more susceptible amorphous cellulose is hydrolysed first to produce reducing sugars, leaving the more resistant crystalline cellulose intact. The more resistant crystalline material will then be hydrolysed at a reduced rate. This phenomenon was demonstrated by Desai and Converse (1997) who hydrolysed cellulose for a given period of time, then contacted the partly hydrolysed cellulose with fresh enzyme (non-deactivated). A reduced initial rate of hydrolysis was observed supporting the view that the remaining cellulose was more resistant to enzymic attack. However, the rate of reaction in this experiment

was higher than that of an experiment allowed to continue with the original enzyme solution, this lead them to conclude that the loss of substrate reactivity was not the principle cause for long residence times required for complete conversion.

Caufield and Moore (1974) indicated that milling increased the susceptibility of crystalline and amorphous cellulose to enzymic attack, but that the digestibility of the crystalline fraction was improved more than that of the amorphous fraction, suggesting that the increase in the digestibility was probably due to a decrease in particle size. The structural features of the cellulose are probably interlinked, thus physical pre-treatments to reduce the crystallinity will inevitably also increase the surface area so it is difficult to find a meaningful relationship between an individual structural feature and the hydrolysis rate without taking all the physical characteristics into account (Gharpuray et al., 1983).

Product inhibition of cellulases has been studied by a number of workers (Gan *et al.*, 2002b; Ghose and Das, 1971; Howell and Mangat, 1978; Huang, 1975; Ortega *et al.*, 2001). Cellobiose, an intermediate product in the production of glucose from cellulose, inhibits the cellulase as does glucose. Medve *et al.* (1998) proposed that the enzyme binds to the surface of the cellulose and successively cleaves off cellobiose units from a single cellulose chain without being released from the enzyme substrate binding site. Gan *et al.* (2002b) suggest that the enzyme adsorbs onto the surface of the cellulose substrate and then desorbs again after completing it's catalytic action

Movagharnejad et al. (2000) modelled enzyme action by using a shrinking particle model, this decreases the amount of binding sites available on the surface of the cellulose. This shrinkage will continue until there are no available binding sites on the surface at which point the reaction ceases.

The time dependent change in the molecular weight of the substrate coupled with the observed loss of enzyme activity, render it difficult to model cellulase action mathematically (Gan et al., 2002a). Many workers have assumed Michaelis-Menten kinetics (Gan et al., 2002b). The models of cellulose degradation proposed differ in the assumptions made relative to the structure of the substrate, product inhibition and enzyme stability, however Zhang et al. (1999) suggest that this approach is inadequate at describing how cellulase digests insoluble cellulose. Another factor adding to complexity of the models is the synergism of the components in the cellulase. The synergistic effect of the cellulases varies depending on the cellulase mixture and the substrate composition (Ortega et al., 2001). Despite the level of interest in enzymic cellulase hydrolyses the mechanism of cellulase action still remains unknown (Ortega et al., 2001).

2.2.3.1 Substrate Inhibition

It is generally observed that for relatively low substrate concentrations the initial rate of an enzyme reaction will increase until a maximum is reached. At high substrate concentrations a decrease in rate is often observed. This phenomenon is known as substrate inhibition (Palmer, 1993). This could occur for several reasons, firstly there could be two types of substrate binding sites on the enzyme, the first substrate molecule binds to the enzyme, then a second also binds, this forms a dead end complex (Palmer, 1993). Secondly, high levels of substrate could generate an altered reaction pathway, causing partial (or hyperbolic) substrate inhibition. In this case, an infinite amount of substrate will give a reduced, but finite rate of reaction. Thirdly, the substrate could act as an allosteric inhibitor (Cleland, 1979), binding at a different site to usual changing the reaction characteristics. The plot of the initial rate of the reaction against the initial substrate concentration gives a sigmoidal plot (Palmer, 1993), with either partial or total inhibition (Cleland, 1979). Fourthly, a high level of substrate could cause inhibition by an increase in ionic strength or a higher concentration of toxic counterions (Cleland, 1979).

2.2.3.2 Product Inhibition.

Mandels and Reese (1963) found that of 36 cellulases they tested, most were competitively inhibited by cellobiose. Glucose was also shown to inhibit cellulase. Susceptibility to inhibition by the end products depends on the source of the cellulase (Ghose and Das, 1971). For finely milled and heat-treated cellulose the efficiency of *Trichoderma reesei* is reduced by 40% in the presence of 30% glucose (Ghose, 1969).

2.3 Pretreatment of Lignocellulosic Material

One of the main problems in using lignocellulosic materials as a fermentation substrate is their resistance to hydrolysis. Over the last twenty years, many different methods of pretreating lignocelluloses have been developed and evaluated to assess whether they can enhance the enzymic hydrolysis of carbohydrates in lignocelluosic feeds (Belkacemi et al., 2002). As previously discussed in section 2.1, lignocelluloses comprise a matrix of crystalline and amorphous cellulose and hemicellulose which is closely associated with lignin. This configuration and composition of these constituents confer protection from enzymic attack. Pretreatment methods which disrupt the highly ordered cellulose structure, reduce the lignin content and increase the surface area available for hydrolysis, will increase the rate and degree of hydrolysis (Fan et al., 1982). There are various forms of pretreatment and these are classified as physical, chemical and biological pretreatment (Fan et al., 1982).

2.3.1 Physical Pretreatment

2.3.1.1 Steam Explosion

The first steam explosion experiments were carried out by (Mason, 1928). It is now classed as one of the major processes for pretreating cellulose (Wood and Saddler, 1988). Steam explosion is most often used in the wood industry, wood chips, or shavings are treated with pressurized steam. This is then quickly

released through a valve (Josefsson et al., 2002). During the steam explosion process the structure of the lignocellulosic material disintegrates, the hemicellulose is partially hydrolysed to sugars and the lignin melts, increasing its susceptibility to enzyme hydrolysis (Vlasenko et al., 1997). Unfortunately, side reactions during lignin degradation may form toxic compounds (Belkacemi et al., 2002). During steam pretreatment the pentoses and hexoses formed from hydrolysed hemicellulose and cellulose may be degraded to furfural and 5-hydroxymethylfurfural, levullinic acid and formic acid together with other substances such as aliphatic acids, furan derivatives and phenolic compounds. The latter three could inhibit subsequent fermentation (Soderstrom et al., 2003). Under very severe conditions sugar degradation during pretreatment causes a loss of substrate, as well as undesirable by-products (Soderstrom et al., 2003).

The most important operational conditions in steam explosion pretreatment are time, temperature and chip size (Negro et al., 2003). The severity of the steam pretreatment *(R_0)* allows a comparison of steam pretreatment compared to a standard rate. It is given by the following equation of Overend and Chornet (1987).

$$R_0 = \exp\left[\frac{(T_r - T_b)}{14.75}\right] \times t \qquad (2.10)$$

Where:

R_0 = severity

t = time (mins)

T_r = temperature of reaction (°C)

T_b = base temperature (i.e. 100°C)

Equation 2.8 was modified by Heitz et al. (1987) to take into account slow indirect heating whilst the steam explosion chamber reaches temperature. It was predicted that the long time taken to reach the stable temperature (30-60 minutes)

would have a significant effect compared to the short time at which the material is held at a constant temperature (0-3 minutes).
The modified equation is given as:

$$R_0 = \int_0^t \exp\left(\frac{T-100}{14.75}\right)dt \qquad (2.11)$$

Where

R_0 = modified severity
T = temperature of the reaction (°C)
t = time of the reaction (s)

An increase in crystallinity has been observed in steam exploded samples and was attributed to the degradation and dissolution of easily accessible components of lignocellulose i.e. hemicellulose and lignin thus resulting in a product with increased concentration of crystallites (Puri, 1984).

Fernandez-Bolanos et al. (2001) reported that pretreating olive stones by steam explosion converted hemicelluloses into soluble carbohydrates and that the susceptibility to hydrolysis with cellulase improved. Dekker and Wallis (1983) found steam exploded bagasse produced a material highly susceptible to enzymic hydrolysis by *Trichoderma reesei.*

Josefsson et al. (2002) found that steam pretreating aspen wood gave a resulting material with lower molecular weight cellulose with increased time and temperature of the pretreatment. The cellulose content increased with increased temperature and explosion time which was interpreted as a consequence of the removal of lignin and hemicellulose (Josefsson et al., 2002).

Some workers have carried out acid-catalysed steam explosion and ammonia fibre explosion of wheat straw. They found that both methods of pretreatment

increased the enzymic digestibility of the straw (Vlasenko et al., 1997). It was reported that the acid-catalysed steam explosion reduced the amount of hemicellulose by 2.1 times, but the ammonia fibre explosion treatment only reduced the hemicellulose content slightly (Vlasenko et al., 1997).

Saddler et al. (1982) reported that air drying reduced the enzymic hydrolysis of all steam exploded wood samples as the structure of the material shrank and collapsed and thus became less accessible to the enzyme. The contraction reduces the internal surface area of the substrate (Fernandez-Bolanos et al., 2001). They also determined that grinding samples with a pestle and mortar increased the accessibility of the samples (Saddler et al., 1982).

2.3.1.2 Milling and Grinding

Lignocelluloses can be ball milled to reduce both the particle size, the degree of crystallinity and the amount of polymerisation (Fan et al., 1982).

2.3.2 Chemical Pretreatment

2.3.2.1 Hydrogen Peroxide

Hydrogen peroxide is an oxidizing agent which can be use to pretreat lignocelluloses. Oxidizing agents penetrate into the cellulose and then oxidize causing structural modification (Fan et al., 1982). With most oxidants the reaction is confined to the amorphous regions of the cellulose (Han and Callihan, 1974).

Hydrogen peroxide releases lignin from the lignocellulose matrix (Gould, 1985b), the hemicelluloses are also soluble in H_2O_2 if the pH is above 11.5 (Curreli et al., 1997). Gould (1984) reported that after hydrogen peroxide treatment of wheat straw, enzymic hydrolysis with *Trichoderma reesei* yielded glucose with an efficiency of almost 100%, based on the cellulose content of the insoluble residue. He also reported that the straw loses some of its integrity during the pretreatment and that the changes observed in the physical and morphological properties of the

cellulose fibres, suggest that some of the glucose units may also be modified during the treatment. The percentage of glucose units affected must be small due to the large glucose yield. He went on to predict that a change in a small amount of the glucose units would be enough to disrupt the hydrogen bonding pattern between the chain resulting in an open structure which could not reform a crystalline structure. This loss in integrity is accompanied by a large increase in the water absorbency of the residue (Gould, 1985b).

Alkaline peroxide pretreatment are most effective with monocotyledonous plants, especially members of the family Graminae (cereal grasses, eg. wheat, rye, oats). One difficulty with this pretreatment is that the solubilised hemicellulose will be lost in the liquor and will therefore be more difficult to recover. For feed applications it may be beneficial to keep both the hemicellulose and the cellulose (Gould, 1985a).

Hydrogen peroxide pretreatment occurs at a fairly rapid rate at room temperature and so is an effective pretreatment method which does not require a costly energy input. Furthermore, the lignin degradation products released are not toxic in the subsequent enzyme saccharification (Gould, 1984).

2.3.2.2 Sodium Hydroxide Treatment

Treating lignocellulosic materials with dilute sodium hydroxide will cause the material to swell, thus increases the internal surface area, decreases the degree of polymerisation, decreases crystallinity, disrupts the lignin structure and separates the linkages between the lignin and carbohydrates (Fan et al., 1982). All of these effects will enhance the enzymic hydrolysis.

2.3.2.3 Acid Treatment

Dilute acid can be used to pretreat lignocellulosic materials. There are two types of dilute acid pretreatment; high temperature (greater than 160°C) continuous

flow for low solids (5-10%) loadings, or low temperature (less than 160°C) batch process for high solid loadings (10-40%) (Sun and Cheng, 2002).

Choteborska et al. (2004) found that first treating wheat bran to eliminate residual starch, then pretreating using dilute sulphuric acid increased the amount of sugars released during enzymic hydrolysis. They reported that the best results were achieved using 1% sulphuric acid at 130°C for 40 minutes giving a sugar yield of 52.1g/100g of substrate. Moreover, under these conditions there was only a low level of furfural and 5-hydroxy-methyl-2-furaldehyde produced, these compounds are toxic to the enzymic hydrolysis as previously discussed in section 2.3.1.1. Other acids also used for dilute acid pretreatment of lignocelluloses include hydrochloric acid and phosphoric acid (Fan et al., 1982).

Although dilute acid pretreatment increases the cellulose hydrolysis it is usually more expensive than steam explosion and the pH has to be neutralized downstream prior to enzymic hydrolysis (Sun and Cheng, 2002).

2.3.2.4 The Organosolv Process

This uses organic or aqueous organic solvent mixtures sometimes with an acid or an alkali catalyst to disrupt the internal lignin and hemicellulose bonds (Sun and Cheng, 2002). The solvents mostly used are acetone, methanol, ethanol, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Duff and Murray, 1996). The solvents need to be removed from the process as they may be inhibitory to the enzymes in the enzymic hydrolysis step (Sun and Cheng, 2002).

The organsolv process gives three fractions; dry lignin, aqueous hemicellulose and cellulose. The cellulose fraction is fairly susceptible to enzymic hydrolysis, which increases with the removal of hemicellulose as this opens the pores in the cellulose structure (Duff and Murray, 1996).

2.3.3 Biological Pretreatment

Biological pretreatment uses micro-organisms to degrade the lignin and hemicellulose. White, brown and soft rot fungi are used. Brown rot fungi mainly attacks cellulose whilst white rot and soft rot attack lignin and cellulose. A disadvantage with this type of pretreatment is the slow rate of hydrolysis (Sun and Cheng, 2002).

2.4 Extrusion

Extrusion is used to either cook or form foods into products having particular characteristics such as novelty shapes. This process generally occurs at high temperatures for a short period of time, and is capable of generating temperatures of up to 180°C, pressures up to 2000 psi and relatively high shear rates. Residence times are typically between three and five minutes (Sharma et al., 2000).

On entering the extruder, the feed gradually loses granular identity under the influence of shear. The temperature and pressure increase rapidly, then the product is forced through a die which shapes the final product (Figures 2.15 and 2.16) (Sharma et al., 2000). After the material is fed through the die, it expands rapidly and loses some moisture due to the release in pressure (Harper, 1981). The process will denature proteins including many natural enzymes and gelatinise starch (Wang et al., 1993). Proteins denature, or change their structure at around 60 to 70°C and starch gelatinises at around 51 to 78°C (Kearns, 1999). Gelatinised starch forms an elastic, inflatable mass and the whole formulation will puff when it is discharged through the die (Williams, 1999). Extrusion will inactivate anti-nutritional factors and helps stop bitter flavours and this renders the food more palatable and digestible (Williams, 1999).

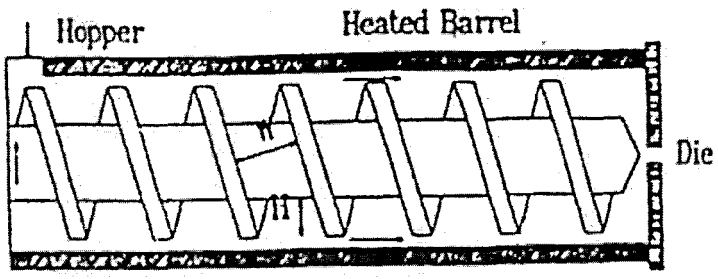


Figure 2.17 Diagram of a Single Screw Extruder (Jaluria and Wang, 1999).

Extruding a product will add texture and shape as desired (Sharma et al., 2000). Dog food is sometimes extruded through novelty shaped dies to produce a shaped final biscuit, formed into a bone for example. After extrusion, the product is dried and it may also be coated with fat, or a gravy powder to add to the palatability of the final product (Williams, 1999). Extrusion will allow the size of the product to be varied (Stratford, 1996), this allows the producer to cater for animals of all sizes.

Extrusion may be conducted either with single or twin-screw extruders. The former are mainly used by pet food manufacturers, as they are simpler mechanically and cheaper than twin-screw extruders. However, some difficulty with mixing raw ingredients may be encountered in single screw services (Starer, 1999). For foods with high fat levels (above 17%) a twin-screw extruder should be used (Kearns, 1999). Single screw extruders can not process low viscosity material (such as that with a high oil content) because the material must stick to the screw to be pumped forward (Starer, 1999).

The goal of the extrusion process is to make a product which has a maximum of 12% moisture, this will help to avoid spoilage during storage (Kiang, 1999). Many extruded dog foods have a shelf life of 12 –18 months which exceeds that of some other forms of dog foods (Stratford, 1996).

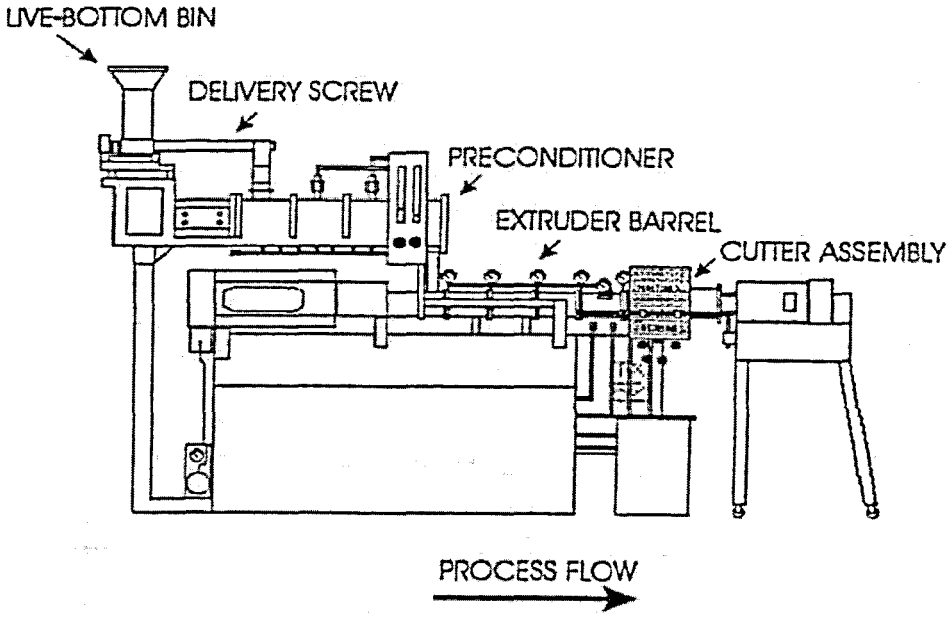


Figure 2.18 Flow Diagram of an Extruder (Brent, 1999).

2.5 Dog Food and Nutrition

2.5.1 Food Requirements of the dog

The diet required by an animal will vary depending on its lifestyle and life stage. For example, a working animal, will need a different diet from one who is kept as a companion. A pregnant animal will have different requirements to a puppy, or young male adult (Burrows, 1988). In all cases a balanced diet is important.

The food which is consumed by the animal will provide the nutrients for energy and materials which can be used for growth or repair of the body (Simpson et al., 1993). The nutrients can be split into groups of proteins, fats, carbohydrates vitamins and minerals (Simpson et al., 1993).

2.5.1.1 Carbohydrates

There is no known minimum dietary carbohydrate for the dog (Burger, 1988), the carbohydrates are mainly used as an energy source, but may also be converted into body fat and stored, or used as a material from which the metabolism of other

compounds can be started (Simpson et al., 1993). If a dog is suddenly given a large amount of a disaccharide such as lactose or sucrose it may become ill with diarrhoea, however low levels of carbohydrates are well tolerated (Burger, 1988).

2.5.1.2 Proteins

Protein in the diet is broken into the amino acids and reformed into new proteins which regulate the metabolic processes (as enzymes), or as structural proteins required for the growth and repair of the tissues. Amino acids can be classed as essential or non-essential. Essential amino acids can not be made by the body of the animal and must therefore be present in the food. The essential amino acids for the dog are shown in Table 2.3.

Table 2.3 The Essential Amino Acid Requirements of the Dog (Simpson *et al.*, 1993).

Amino Acid	Amount Required (g/1000Kcal of metabolisable energy)
Arginine	1.37
Histidine	0.49
Isoleucine	0.98
Leucine	1.59
Lysine	1.40
Methionine + cystine	1.06
Phenylalanine + tyrosine	1.95
Threonine	1.27
Tryptophan	0.41
Valine	1.05
Total	11.57

2.5.1.3 Fat

Fat in the food is a concentrated energy source, which makes the food more palatable, and provides an acceptable texture (Burger, 1988).

2.5.2 Dog food

Dog food is available in several types. Canned dog food is a wet food and has a long shelf life, it is very digestible and the nutrients are highly available. Most canned dog foods are formulated to provide a balanced diet with sufficient amounts of nutrients in relation to energy. In order to derive the recommended amount of energy large amounts of this type of food will need to be consumed. Therefore, although it is possible to feed a dog solely on canned food they are mainly intended to be used in conjunction with another cheaper source of energy such as biscuits (Rainbird, 1988).

Completely dry dog foods are usually made from cereals, or cereal by-products, protein concentrates and fat, mineral and vitamin supplements. These will then provide a balanced diet when mixed with canned food. Dry dog foods are less palatable than moister foods (Rainbird, 1988).

Dry dog foods are available as biscuits, or mixtures of meals and flakes. They can be formulated to be complete foods or intended to be used with fresh meat or canned foods. Dry products look similar, but contain different ingredients depending on the life stage of the target animal. They will have a long shelf life (several months) as there is insufficient moisture for the growth of micro-organisms (Rainbird, 1988).

2.5.3 The Digestive System of the Dog

The dog is a monogastric animal. Food is received into the stomach where it is stored and digested. Different glands in the stomach secrete mucous, hydrochloric acid, pepsinogen and the hormone gastrin (Reece, 1997). Pepsinogen is converted to pepsin by the presence of hydrochloric acid. As the stomach empties into the

small intestine a release of the hormone entergastrone causes the stomach to stop producing acid. Prior to entering the small intestine the antral mucosa produces an alkaline solution which is low in enzymes, this is mixed with the food.

In the small intestine more enzymes are added, some from the dudodenal mucosa and some from the pancreas. The pancreatic enzymes are proteases, lipases and amylase. The digestion is completed in the small intestine i.e. the proteins, fats, and carbohydrates are broken down into amino acids, dipeptides, glycerol, fatty acids and monosaccharides. These components are absorbed as they are released (Burger and Blaza, 1988).

The stomach of ruminant animals have four compartments, this enables them to digest both cellulose and hemicelluloses. The rumen of these animals is host to a number of micro-organisms inside that can utilize cellulose and hemicellulose to produce acetic acid, lactic acid, butyric acid and propionic acid. These acids can then be absorbed by the animal and used as an energy source (Svendsen and Carter, 1984).

2.6 Solid State Bioreactors

There are various different types of enzyme bioreactors which can be used for process scale up. In industry it would be beneficial to use a solid state reaction, a reaction carried out on solids in the absence (or near absence) of free water (Pandey, 2003) for wheatfeed digestion as this saves on costs of drying the final product.

2.6.1 Batch Bioreactors

2.6.1.1 Tray Bioreactor

Tray bioreactors are the simplest bioreactors (Pandey, 1991). Traditional tray Koji bioreactors were made from bamboo baskets, although now larger trays with

a wire mesh base (Cannel and Moo-Young, 1980) or perforated bottoms are used (Pandey, 1991). The trays have a thin layer of substrate (about 9 cm thick) spread over them and they are placed in a stack with a small gap between them to allow air to pass through. The stack is held in a chamber under controlled conditions (see Figure 2.17) (Cannel and Moo-Young, 1980). Mixing may be done occasionally - this is usually manual (Chisti, 1999). Tray bioreactors are labour intensive and take up a large amount of space (Pandey, 1991).

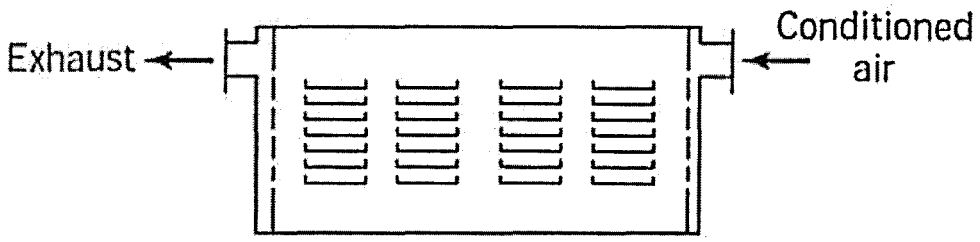


Figure 2.19 A Tray Stack (Chisti, 1999)

2.6.1.2 Rotary Drum Bioreactors

A rotary drum bioreactor consists of a horizontal cylindrical vessel rotated around the long axis (Chisti, 1999), causing the substrate to mix (Figure 2.18). Various workers have used drum bioreactors with modifications such as more than one chamber and internal baffles. A difficulty encountered with some substrates however, is that they tend to agglomerate into balls (Pandey, 1991).

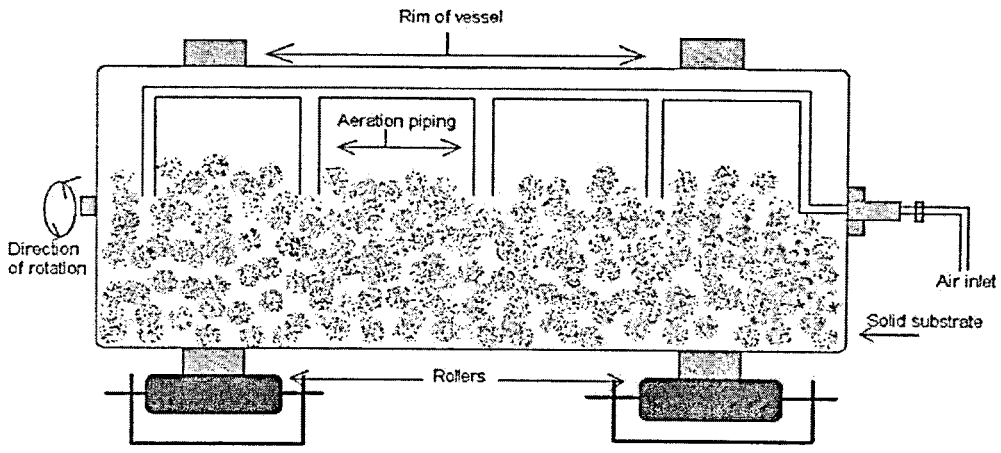


Figure 2.20 A Rotating Drum (Robinson and Nigam, 2003).

2.6.1.3 Fluidized Bed.

A fluidized bed has a relatively shallow bed of substrate through which air is passed causing fluidization (Chisti, 1999). The fluidization causes the substrate to tumble and mix (Figure 2.19).

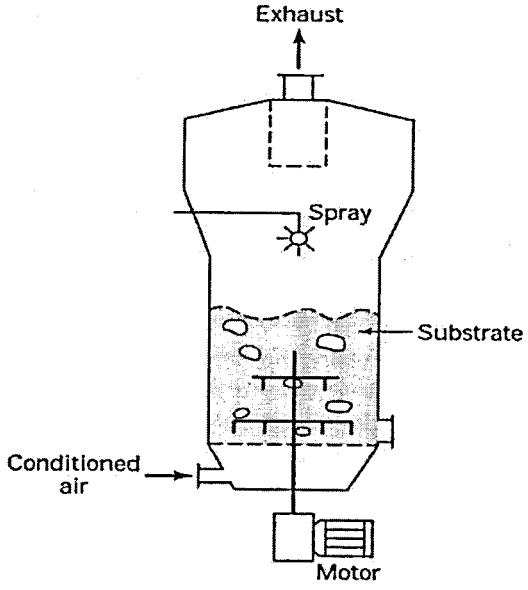


Figure 2.21 A Fluidized Bed Bioreactor (Chisti, 1999).

2.6.1.4 Packed Bed

Packed bed reactors usually comprise columns made of either glass or plastic. The solid substrate sits on a perforated base through which a flowing stream of air is introduced (Robinson and Nigam, 2003) (Figure 2.20).

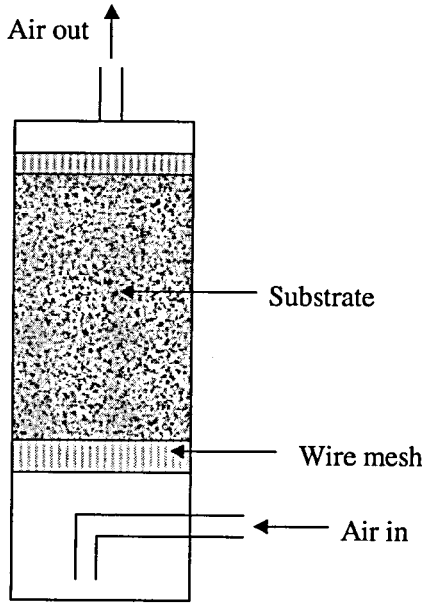


Figure 2.22 A Packed Bed Bioreactor (adapted from Robinson and Nigam, 2003).

2.6.1.5 Agitated Tank Bioreactors.

These are stationary batch bioreactors which have paddles to stir and mix the substrate shown in Figure 2.21.

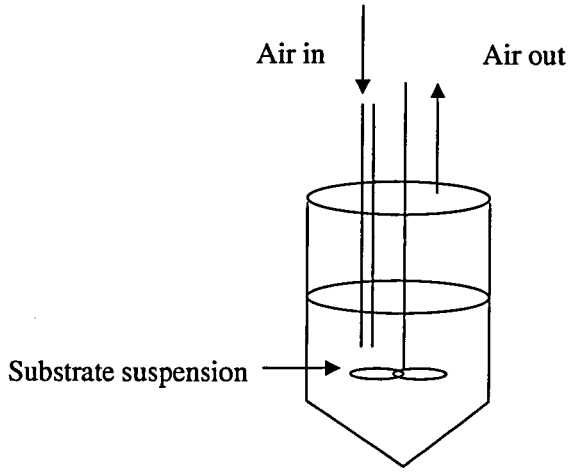


Figure 2.23 Agitated Batch Reactor.

2.6.2 Continuous Bioreactors

2.6.2.1 Continuous Screw Bioreactors

In the continuous screw bioreactor the substrate is fed in at one end of the screw and undergoes a reaction along the length of the screw as it moves along until it reaches the outlet (Chisti, 1999) (Figure 2.22). The screw bioreactor does not compact the substrate and gives good mixing (Tengerdy, 1985).

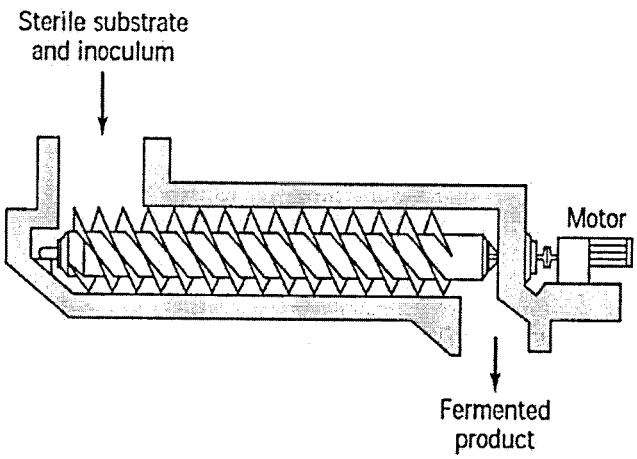


Figure 2.24 Continuous Screw Bioreactor (Chisti, 1999)

2.6.2.2 Tower Bioreactor

Tower bioreactors have stacks of trays which rotate. The substrate enters at the top of the bioreactors and slowly falls down to lower and lower trays until it exits at the bottom as reacted product (Tengerdy, 1985).

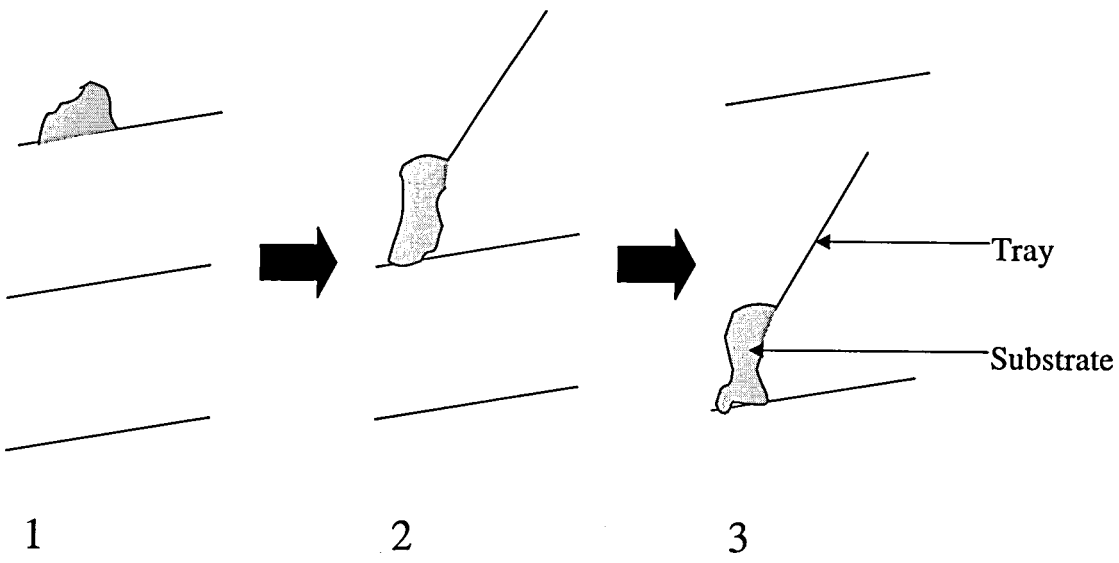


Figure 2.25 A Diagram of Three Trays Within a Tower Bioreactor.

3 Materials and Methods

This study aims to build on the knowledge obtained from the literature to characterize a low value by-product of the cereal milling industry (wheatfeed) and reduce its fibre content using enzymes to digest it into sugars. The reduction in fibre would allow wheatfeed to be used in petfood in the place of more expensive components.

Work was carried out using enzymes in both purified forms and commercially available enzyme mixtures. There follows a description of these enzymes and the optimum conditions for their usage. Protocols used for the preparation of wheatfeed prior to enzymic digestion are described. The experimental work was carried out in both free liquid and solid state digestion environments. The basic protocols for these experimental methods are given here. A method of steam pretreating the wheatfeed prior to enzymic digestion was also investigated to evaluate if this would give increased digestion during the enzyme treatment. The protocols for this treatment are described here.

The amount of sugars and proteins released from the wheatfeed during enzymic hydrolysis was measured to compare the effectiveness of different treatments. The sugars were measured using several different analysis methods employing a glucose test kit, a glucose meter and high performance liquid chromatography (HPLC). Total protein content was measured by both the Lowry-Peterson and the Kjeldahl methods. The methods used in this PhD work are described in this chapter.

3.1 Wheatfeed

Wheatfeed is a by-product of the milling process and was provided by Witworths (Wellingborough, Northamptonshire, UK). Wheatfeed is a wheat bran and published compositions of wheat bran were used throughout this work.

3.2 Enzymes

Cellulase (C-9422), hemicellulase (H-2125), pectinase (P-2401) and xylanase (X-3876) were obtained from Sigma (Poole, Dorset, UK), and B-glucanase (49101) was obtained from Flukka (Gillingham, Dorset, UK). These enzymes were supplied in solid form and are referred to here as 'single enzymes'. Celluclast, viscozyme, and ultraflow were obtained from Novozymes (Bagsvaerd, Denmark) and ronozyme and roxozyme from Roche (Basel, Switzerland). These enzymes were supplied as aqueous solutions and are referred to here as 'commercial enzymes'.

3.2.1 Cellulase (C-9422)

This cellulase is a crude powder from *Tricoderma viride*. It has an activity of 8.4 units/mg solid and 1 unit will liberate 1.0 μmole of glucose from cellulose in one hour at pH 5.0 and 37°C.

3.2.2 Hemicellulase (H-2125)

This hemicellulase is a crude powder from *Aspergillus niger*. It has an activity of 1.5 units/mg and 1 unit will liberate 1.0 μmole of product from hemicellulose in one hour at pH 5.5 and 37°C.

3.2.3 Pectinase (P-2401)

This is a crude powder from the *Rhizopus* species. It has an activity of 711 units/g and 1 unit will liberate 1.0 μmole of galacturonic acid from polygalacturonic acid per minute at pH 4.0 and 25°C.

3.2.4 β-glucanase (49101)

This enzyme is cultivated using *Aspergillus niger* it contains 1.1 U/mg, where the international unit (U) is 'the amount of enzyme needed to catalyse a rate of 1μmol of substrate per minute (Garrett and Grisham, 1995).

3.2.5 Xylanase (X-3876)

This powder will liberate 1.0 μmole of xylose per minute at pH 4.5 and 30°C.

3.2.6 Celluclast 1.5L

This is a liquid cellulase preparation produced by Novozymes using a selected strain of *Trichoderma reesei.* It catalyses the breakdown of cellulose into glucose, cellobiose and higher glucose polymers, and is supplied with an activity of 700 endoglucanase units (EGU)/gram. Celluclast is designed to be used for the breakdown of cellulosic material into fermentable sugars, or to increase the extraction yield of products from plants. The optimum pH is 5.0 and temperature is 65°C, after which the activity decreases rapidly. The influence of pH and temperature on the activity of celluclast are shown in Figures 3.1 and 3.2.

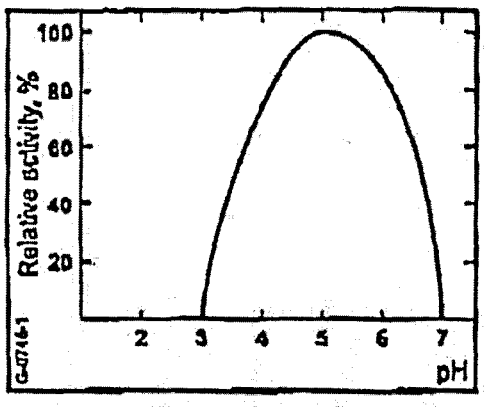


Figure 3.1. The Effect of pH on the Activity of Celluclast (Novozymes).

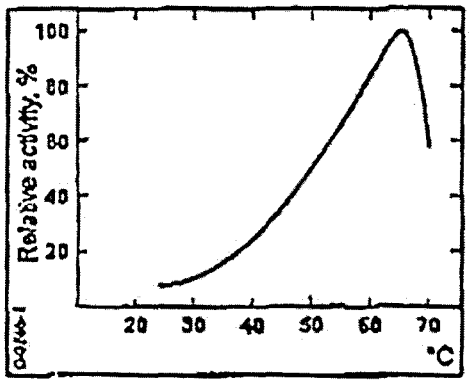


Figure 3.2. The Effect of Temperature on the Activity of Celluclast (Novozymes).

3.2.7 Viscozyme L

Viscozyme L is a liquid preparation produced by Novozymes from a selected strain of *Aspergillus aculeatus*. It contains a mixture of carbohydrases; including arabinase, cellulase, β-glucanase, hemicellulase and xylanase. It also has some activity on branched pectin substances. Viscozyme is designed to break down plant cell walls to allow extraction of useful plant material and also for use in cereal and vegetable processing. The activity is 100 fungal β-glucanase units (FBG)/gram. The optimum conditions are quoted as pH in the range of 3.3-5.5 and temperatures between 25-55°C.

3.2.8 Ultraflow

This is a liquid carbohydrase preparation produced by Novozymes from fermentation of a selected strain of *Humicola insolens*. Ultraflow is a heat stable multi-active β-glucanase. The most important activities are cellulase, xylanase, pentosanase and arabinase. It is designed for use in the brewing industry to break down pentosans with a xylan backbone and single arabinose side chains attached. Ultraflow contains 45 FBG/gram along with some side activities. The enzymes in ultraflow have optimal conditions at the typical temperatures and pH for the mashing part of the brewing process, i.e. a pH in the range of 5.2-5.6 and a temperature in the range of 45-65°C (Bamforth, 2003)

3.2.9 Ronozyme

This is a liquid carbohydrase preparation produced by Roche from the fermentation of *Humicola insolens*. The main activities are endo-1,4-β-xylanase and endo-1,4-β-glucanase activity, in addition to cellobiase, hemicellulase and cellulase. It is designed as a feed additive to be sprayed onto feed pellets.

3.2.10 Roxozyme

The main activities in this enzyme mixture produced by Roche, are endo-1,4-β-glucanase (minimum of 8,000 units/ml), endo-1,3-β-glucanase (minimum of 18,000 units/ml), and endo-1,4-β-xylanase activity (minimum of 26,000 units/ml). It is designed to be used as a feed additive.

3.3 Substrate Preparation Methods

3.3.1 Riffling

Prior to all experiments the wheatfeed was riffled. The riffler conveys wheatfeed from a hopper to rotating containers. The wheatfeed is distributed evenly into the containers, helping to ensure that it is well mixed, eliminating settling effects and ensuring that the experiments were conducted with a consistent product. The wheatfeed was processed using two rifflers. The first one, manufactured by The Pascall Engineering Company Limited (Crawley, Sussex UK), separated it into larger fractions (approximately 200 g) which were then further processed through a second riffler, manufactured by The Triton Engineering Company Limited (Ashford, Kent, UK) into smaller fractions (approximately 50 g). The Triton riffler had a speed control, this was set at 80. The rifflers used are shown in Figures 3.3 and 3.4.

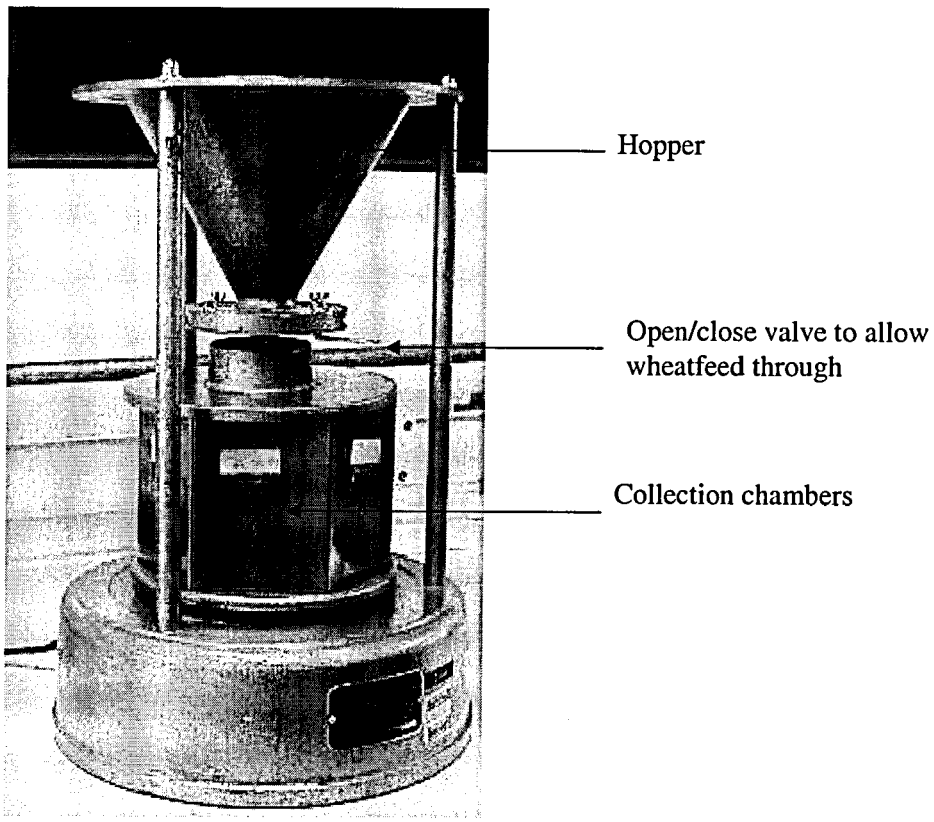


Figure 3.3 Pascall Riffler.

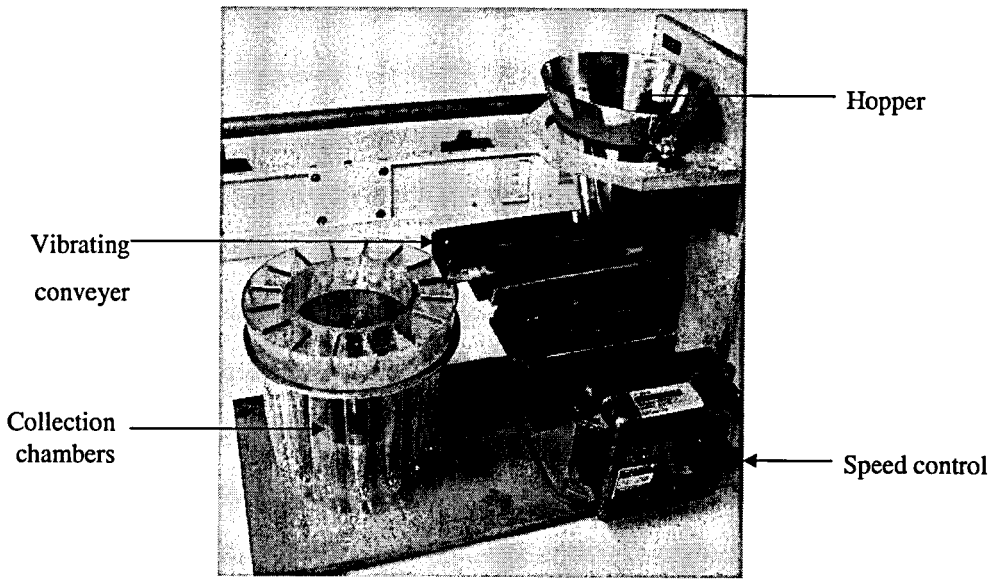


Figure 3.4. Triton Riffler

3.3.2 Sieving

For particle sizing experiments the substrate was sieved into fractions using an automatic sieve shaker (Model 03502, Fritsch Analysette, Idar-Oberstein, Germany) at an amplitude setting of '9.8' with permanent shaking for 10 minutes and sieves (Endecotts Limited, London) of sizes 839, 553, 348 and 197 μm were used in the sieve shaker.

3.4 Substrate Processing

3.4.1 Milling

For experiments where the particle size of the substrate had to be reduced a rotating knife mill was used (ABM GmbH., Marktredwiz, Germany). The substrate was passed through the mill until the particles would pass through a 197 μm sieve.

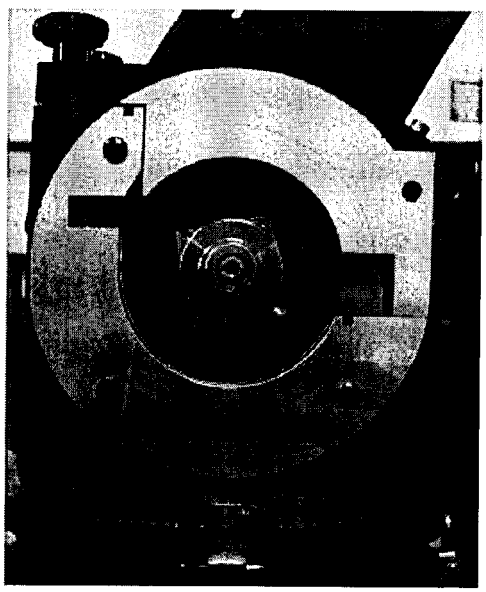


Figure 3.5 View of the Interior of the Mill.

3.4.2 Steam Explosion

Steam explosion was carried out in a stainless steel pressure vessel of capacity 270 ml which was placed in a chromatography oven, (Model PU 4500, Philips, Blackburn, Lancashire, UK). A schematic diagram of the experimental set-up is shown in Figure 3.6.

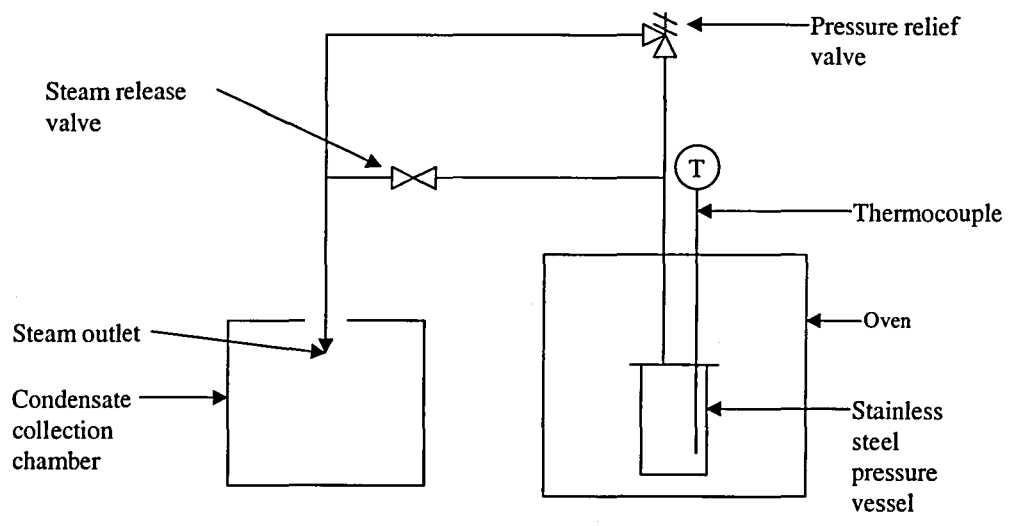


Figure 3.6. Steam Explosion Experimental Equipment.

The pressure release valve was set at 12 BarG and was tested using nitrogen.

Wheatfeed and distilled water were mixed then loaded into the pressure vessel, the lid sealed and the steam release valve closed. The temperature of the oven was set and quickly reached. The temperature in the pressure vessel slowly increased, as it did so, the pressure inside rose as the water evaporated to form steam.

After the required holding time had elapsed, the steam release valve was opened releasing the pressure. The steam released was caught in the condensate collection chamber. The set point temperature was reduced to 0°C and the oven

door opened allowing the whole system to cool for twenty minutes before the pressure vessel was removed.

Unless otherwise stated, 10 g of riffled wheatfeed was steam exploded using 10 ml of distilled water, an oven temperature of 130°C and a holding time of 45 minutes.

The steam exploded wheatfeed was either used as generated, or after drying in a vacuum oven by Townsend and Mercer, Limited (Croydon, UK) overnight (at a vacuum of 600 mm Hg and a temperature of 55°C). It was then ground with a pestle and mortar to produce a substrate with a particle size of between 35 μm and 1000 μm using sieves manufactured by Endecotts (test sieves) Limited (London, UK). This material was frozen until required.

3.5 Citrate Buffer

Citrate buffer was used for all of the experiments. It was produced using solutions of 0.1 M citric acid and 0.1 M sodium citrate. To make one litre of citrate buffer at pH 5.0, 205 ml citric acid and 295 ml sodium citrate were added to 500 ml distilled water and mixed.

3.6 Analytical Methods

A comparison of some of the analysis techniques used for this work, with other well know techniques can be found in Appendix 1.

3.6.1 Moisture Analysis

The moisture of samples was found by measuring the weight of the samples before and after drying overnight at 105°C in an oven (Gallenkamp, Loughborough, Leicestershire).

3.6.2 High Performance Liquid Chromatography (HPLC).

HPLC was used to analyse the post treatment liquor for xylose, arabinose, fructose and glucose.

A column 25 cm long by 4.6 mm (inside diameter) containing a reverse phase silica material was used at 37°C. The column was manufactured by Arganaut Technologies Limited (Hengoed, Mid Glamorgan UK). The mobile phase through the column was a mixture of 80% acetonitrile and 20% distilled water. A Waters 410 differential refractometer manufactured by Millipore (Stonehouse, Glouscestershire) was used to detect and quantify the sugars. Sample (0.35 ml) was injected into the system to ensure the 0.02 ml sample loop was sufficiently filled. Between injections the needle was cleaned using fresh eluent. The flowrate of the mobile phase was 2 ml/minute and each run took 25 minutes, this allowed higher molecular weight components to leave the column.

The sugars used as standards comprised D-glucose, Fisher scientific (Loughborough, Leicestershire, UK), D-xylose, Fisher Scientific (Loughborough, Leicestershire, UK), D-fructose, BDH Chemicals (Lutterworth, Leicestershire, UK) and D-arabinose, Aldrich, (Gillingham, Dorset, UK). The acetonitrile eluent was obtained from Fisher Scientific (Loughborough, Leicestershire, UK).

Calibrations were carried out for all the sugars by first preparing stock solutions by dissolving 4 g of the individual sugar in 100 ml of distilled water. Dilutions of the stock solution were prepared as required and injected into the HPLC. Peak areas were plotted against concentration to verify that the relationship was linear (Figures 3.7 to 3.10).

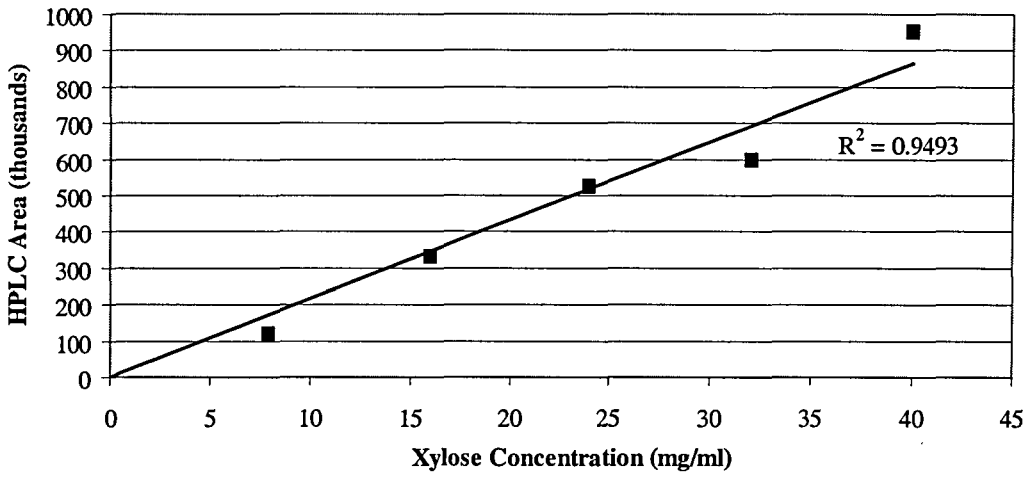


Figure 3.7 HPLC Calibration Results for Xylose.

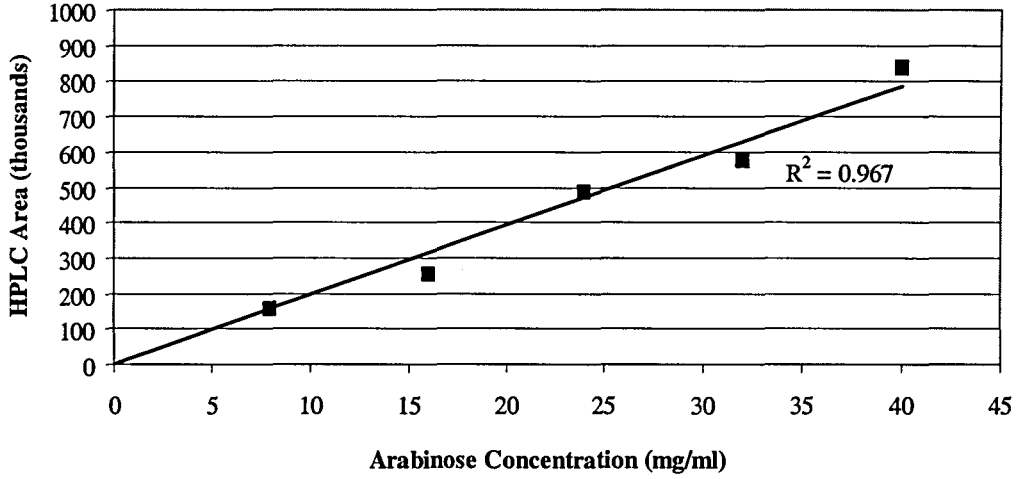


Figure 3.8 HPLC Calibration Results for Arabinose.

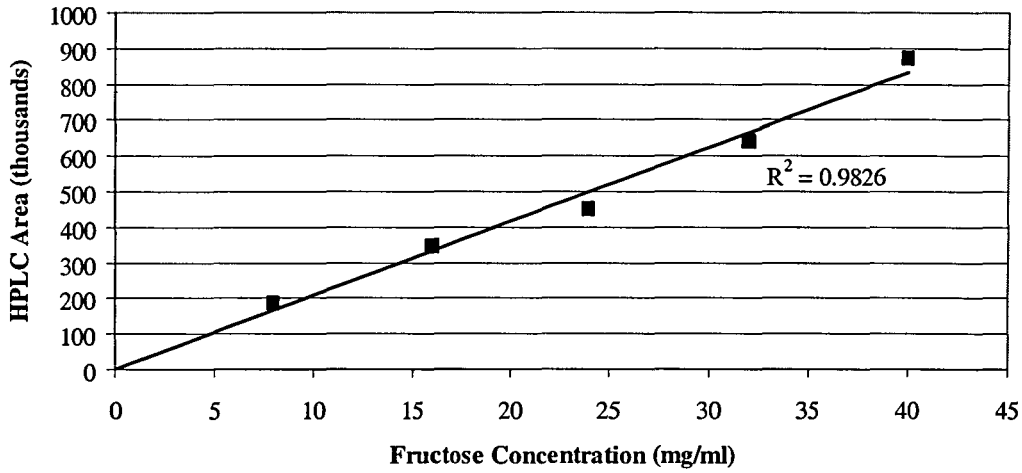


Figure 3.9 HPLC Calibration Results for Fructose.

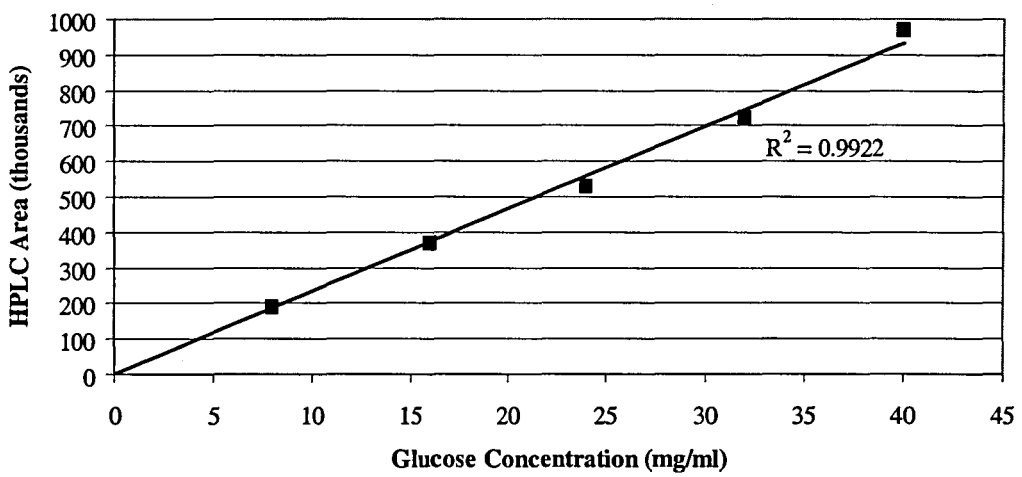


Figure 3.10 HPLC Calibration Results for Glucose.

3.6.3 Glucose Determination Using Glucose Oxidase.

A Sigma Glucose kit (catalogue number 510-DA) was used, this comprised an enzyme preparation, barium hydroxide and zinc sulphate solutions (0.1M), o-Dianisidine Dihydrochloride and a glucose standard. The kit was obtained from Sigma (Poole, Dorset, UK).

The test measures glucose colourmetically. The principle is based on two enzymic reactions which are coupled (Sigma, 1990).

$$Glucose + 2H_2O + O_2 \xrightarrow{Glucose\ \ Oxidase} Gluconic\ \ acid + 2H_2O_2 \qquad (3.1)$$

$$H_2O_2 + o-Dianisidine \xrightarrow{Peroxidase} Oxidised\ \ o-Dianisidine \qquad (3.2)$$

(colourless) (brown)

The intensity of the brown colour was measured on a spectrophotometer, Model number 510269, by Shimadzu Europa (Milton Keynes UK), at a wavelength of 450 nm and compared to a blank with no glucose present. This should be prepared at the same time as the samples. A calibration chart (Figure 3.11) was prepared using samples with known glucose concentrations between 100 µg/ml and 3000 µg/ml. A new calibration graph was produced each time new kit reagents were used.

The glucose kit was used to analyse the amount of glucose present for the experiments to determine which mixture of single enzymes gave the best digestion.

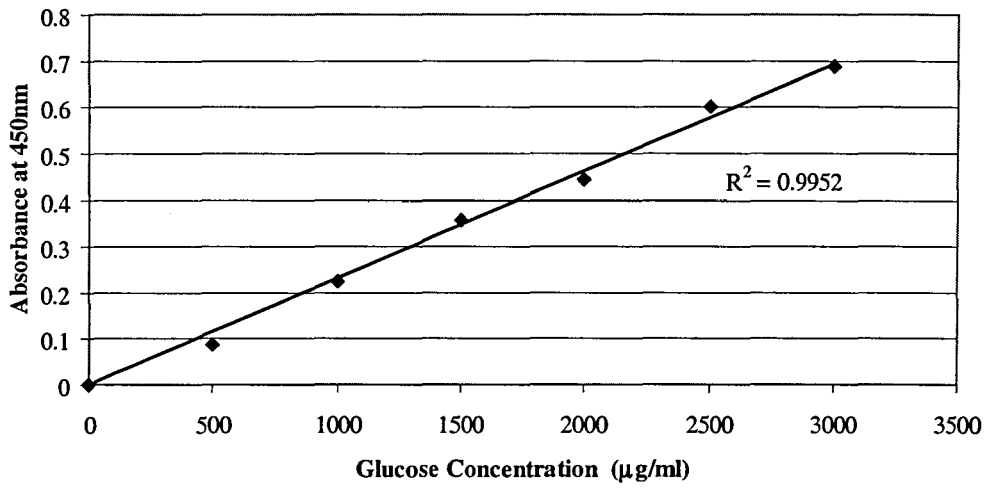


Figure 3.11 A Typical Sigma Glucose Kit Calibration Graph.

3.6.4 Measurement of Glucose using the Accu-chek Advantage II Meter

The Accu-chek Advantage II meter (Roche Diagnostics, Lewes, East Sussex, UK) is designed as a quick home test for diabetics to use and can be obtained over the counter at a chemist shop. The test measures the level of glucose in a liquid and gives an estimate within forty seconds. A test strip is inserted into the machine and then a spot of sample squeezed into a small slit in the side of the strip (Figure 3.12). The glucose is oxidized in reactive glucose dehydrogenase during which hexacyanoferrate (III) is reduced to hexocyanoferrate (II). The hexocyanoferrate (II) produced is reoxidized by a palladium containing electrode and the electron flow released is proportional to the glucose concentration in the sample.

The Accu-chek meter was used as a rapid means to determine approximate glucose levels released into solution. The range of the meter is from 0.6 mmole/l to 33 mmole/l glucose.

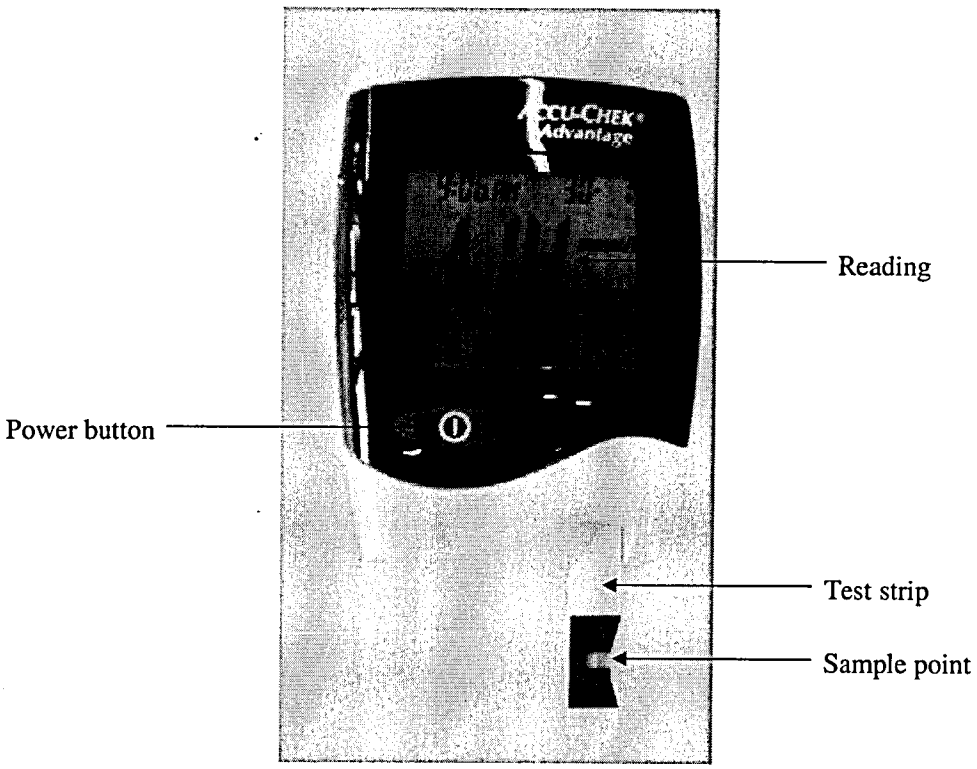


Figure 3.12. Accu-chek Advantage II Glucose Detector.

3.6.5 Lowry-Peterson Method for Protein Determination.

Protein analysis was carried out on the sample supernatants according to the method of Lowry *et al.* (1951) incorporating the changes proposed by Peterson (1977). Bovine serum albumin (BSA) was obtained from Fisher Scientific (Loughborough, Leicestershire, UK). Sodium carbonate, copper sulphate, potassium sodium tartrate, sodium hydroxide and Folin-Ciocalteu Reagent used for the Lowry-Peterson reagents were also from Fisher Scientific.

In this assay 1 ml of reagent (A), containing 0.25 mg/ml copper sulphate, 0.5 mg/ml potassium tartrate carbonate, 25 mg/ml sodium carbonate and 0.25 ml/ml 0.8 M sodium hydroxide (see Appendix 2 for composition of reagents) were added to the 1ml of the protein sample, mixed and allowed to react for 10 minutes at 25°C. Reagent B (0.5 ml), which is Folin-Ciocalteu Phenol reagent diluted

with distilled water in the ratio 1:5, was then added and the samples immediately vortexed. The samples were left for another 30 minutes at 25°C, before the solutions are transferred to micro-cuvettes. The absorbance at 750 nm was measured in relation to a blank sample containing 1 ml citrate buffer processed at the same time as the samples.

Protein in the sample reacts with copper to produce a protein copper complex, this then reduces the Folin-Ciocalteu Phenol reagent (phosphomolybdate-phosphotungstate reagent) to give a blue colour (Lowry *et al.*, 1951).

The calibration for the Lowry-Peterson method was carried out using bovine serum albumin (BSA). BSA (0.05 g) was dissolved in 100 ml of citrate buffer solution at the same pH as the samples to be analysed. A 2 ml aliquot of this solution was then taken and made up to 10 ml with citrate buffer solution and mixed.

The Lowry-Peterson analysis was executed in the same way as previously discussed for samples and a graph plotted of log absorbance at 750 nm versus log concentration of protein.

Reagent A is only stable for three weeks. A calibration was performed whenever new reagents were prepared and also for all the pH values used in experiments to be analysed. A typical Lowry-Peterson calibration graph can be seen in Figure 3.13.

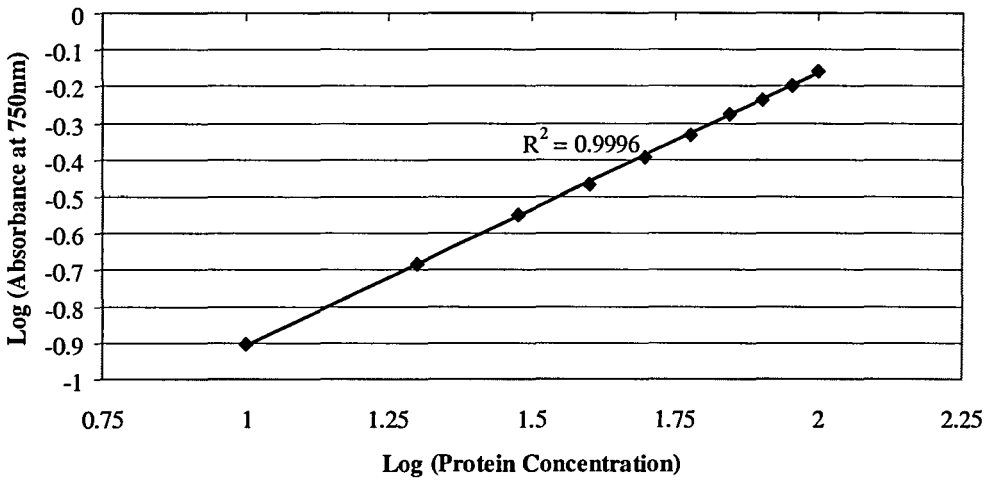


Figure 3.13 A Typical Lowry-Peterson Calibration Graph at pH 5.0.

3.6.6 Kjeldahl Method for Nitrogen Determination.

In the Kjeldahl method, the nitrogen from the protein in the sample tested, was converted into ammonium sulphate by sulphuric acid. Sodium hydroxide is added to make the solution alkaline, after which the ammonia was distilled into an excess of boric acid. The sample was then titrated to estimate the level of total nitrogen in the initial sample. Total nitrogen is multiplied by a factor dependent on the sample material to find the protein content (Pearson, 1962). In the case of wheat bran, this factor is 6.25 (James, 1995).

Ammonium sulphate was obtained from Hopkins and Williams (Cheadwell Heath, Essex, UK), and the catalyst tablets, hydrochloric acid, boric acid and sodium hydroxide were obtained from Fisher scientific (Loughborough, Leicestershire, UK).

Sample (0.5 g) was placed into a glass digestion tube on ashless filter paper, a catalyst tablet was added to each tube together with 15 ml concentrated sulphuric acid. The tubes were placed into a Buchi (Manchester UK) digestion chamber unit and the digestion was allowed to take place for approximately 40 minutes at heat setting 7 on the unit. The tubes were allowed to cool. Each tube was placed

into a distillation unit, (Buchi, Manchester UK) where water and sodium hydroxide was added and the sampled distilled over into a conical flask containing 50 ml 0.2 M boric acid and methyl red indicator. This mixture changes colour from grey to light green. The mixture was titrated with 0.1 M hydrochloric acid, the end point was when the when the mixture changed back to the original grey colour.

An ammonium sulphate control (using 0.0.5 g ammonium sulphate) and a blank (just filter paper) were processed along with the samples.

The percentage nitrogen of the sample can be calculated using the following equation.

$$\%N = \frac{14.01 \times \{S_t - B_t\} \times A}{M} \qquad (3.3)$$

Where

$\%N$ = percentage nitrogen
S_t = ml of titrant of sample
B_t = ml of titrant of blank
A = molarity of acid
M = mass of sample (g)

The ammonium sulphate control has a known nitrogen content, the percent recovery of nitrogen can be calculated and the figures adjusted to allow for this.

3.6.7 Englyst Fibre Method

This is a method which will report the total, soluble and insoluble non-starch polysaccharides in a sample.

For total fibre, the sample (0.25 g) was de-starched by adding 2 ml dimethyl sulphoxide (DMSO) which disperses the starch. This was then gelatinised by

boiling in water for 65 minutes after which 8 ml acetate buffer at pH 5.2 and 50°C was added. Following this, starch was digested to glucose using 0.5 ml amylase solution and 0.1 ml pullulanase solution. The samples were incubated at 42°C for 16-18 hours before the tubes were cooled in cold water for 10 minutes. Approximately 40 ml industrial methylated spirit (IMS) was added and the mixture allowed to stand for 60 minutes to precipitate out soluble fibre. The sample was then centrifuged at 1500 rev/minutes for 10 minutes and the supernatant (IMS/aqueous layer) removed. The solids were washed with 85% IMS and 15 % water, then 100% IMS. Acetone (20 ml) was added, the mixture vortexed, and the supernatant taken and dried at 80°C. Sulphuric acid (5 ml of 12 M) was added vortexed and then left at 35°C at for 60 minutes to digest the non-starch polysaccharides into sugars. A 3 ml aliquot of this solution was added to a test tube containing 1 mg/ml allose as an internal standard. The solution was adjusted to an alkaline pH using 12.5 M ammonium hydroxide. Sodium borohydride (0.2 ml) was added to convert the sugars to their corresponding alditols. These were then changed to alditol acetates using 5 ml acetic anhydride in the presence of 0.5 ml methylimidazole, extracted into 0.9 ml IMS for 5 minutes and analysed by gas chromatography. The results for the sugars could then be related to fibre.

Uronic acids may also be present and are also non-starch polysaccharides. These cannot be measured by gas chromatography so after the extraction into IMS, some of the sample was assayed for uronic acids using a colourmetric method. Sulphuric acid (0.3 ml at 2 M) was added to 0.3 ml sample, 0.3 ml sodium chloride and boric acid mixture was added and vortexed. The tubes were left for 40 minutes at 70°C and cooled in water for up to 1 hour. Dimethylphenol (0.2 ml) was added, then the absorbance measurement could be made using a spectrophotometer at a wavelengths of both 400 and 450 nm. The reading at 400 nm is subtracted from that at 450 nm to correct for hexose interference.

The results for both tests were included for the total fibre results.

The insoluble fibre was analysed in the same way as the total fibre, with the modification that instead of adding IMS to precipitate out the soluble fibre, 40 ml phosphate buffer (pH 7.0) was used to wash this fibre off. The resulting fibre was therefore insoluble. The soluble fibre is found by calculating the difference between the total and insoluble fibre results (Hebbs, 2004).

3.6.8 Total Starch Method

The sample (2.5 g) was filtered and extracted into 80 ml 40% IMS for 1-1.5 hours, which helps prevent shorter chain polysaccharides from dissolving into the solution. This allowed for sugars present in the sample initially. The sample was filtered and 2.1 ml of 27% hydrochloric acid was added to 50 ml filtrate. This was boiled for approximately 15 minutes to break the starch down into glucose. Next, clarification was carried out using 10 ml carrez solution, to precipitate out protein and the sample filtered. The optical rotation was measured using a polarimeter which calculates the glucose concentration (Hebbs, 2004).

3.6.9 Gel Starch Method

Gel starch is described as 'digestible starch,' in pet food it is important that the gel starch to total starch ratio is high.

For gel starch analysis, the sample (approximately 0.5 g) was digested with 10 ml enzyme amyloglucosidase at 37 °C for 3 hours to convert the gel starch to free glucose. The protein was precipitated using carrez solution and the samples filtered. Glucosidase (4 ml) was added to 1000 µl sample to oxidize the glucose which produces hydrogen peroxidase. A 4-amino phenazone and phenol indicator turns pink in the presence of peroxidase, this can then be measured using a ultra violet spectrophotometer to indicate the level of glucose present, which in turn indicates the initial level of starch present (Hebbs, 2004).

For samples where sugar is present prior to starch digestion, a sugar correction must be carried out by running the gel starch method without adding amyloglucosidase. This gives the background glucose level. A sugar correction was performed for all the samples in this study (Hebbs, 2004).

3.7 Experimental Methods

The experiments are split up into two types, firstly using single enzymes (and mixtures of single enzymes) and secondly using industrial enzymes. The single enzyme experiments are all small scale as the cost of the enzymes limited scale up.

3.7.1 Single Enzyme Treatment

The basic method for the single enzymic treatment of wheatfeed is described. Various modifications of the basic method were employed and these are described in the chapters relating to experiments.

Experiments were carried out in a heated water bath, (Grant Instruments Cambridge Limited, Cambridge, UK) at 50°C with a shaker at 150 rpm, using 2 g of wheatfeed and 10 ml citrate buffer at pH 5.0 in 50 ml conical flasks. The enzymes were mixed into the buffer and placed in the water bath to heat for 30 minutes, before being added to the wheatfeed in the conical flask. This was then covered with parafilm (Fisher Scientific, Loughborough Leicestershire, UK) to avoid evaporation and allowed to digest for 4 hours. After digestion the sample was centrifuged at 15,000 rpm for 10 minutes at 10°C, (Model 2383K, Hermle, Huddersfield UK) and the supernatant collected and frozen for HPLC and Lowry-Peterson protein analysis.

3.7.2 Commercial Enzyme Treatment

The work with the commercial enzymes was carried out in 50 ml conical flasks, using a stirred bioreactor, or in petri dishes, depending on the nature of the experiment. Some work was also carried out on the mode of the enzyme action, this was performed in a 500 ml conical flask.

For work carried out using 50 ml conical flasks the basic procedure is the same as that used for work with the single enzymes.

3.7.2.1 Stirred Bioreactor Experimental Method

For larger scale work, a 2 litre glass bioreactor manufactured by Quickfit, (Fisher Scientific, Loughborough, Leicestershire UK), with a metal stirrer was used. The stirrer consisted of a U-shaped paddle and a small Rushton impeller, (Figure 3.14). The entire bioreactor was placed into a water bath, screwed onto a stand and connected to a drive motor (Model 502D, LH Fermentation, Emeryville, USA). The water bath was plastic and jacketed with insulating wrap to reduce heat loss. A water heater, (Gallenkamp, Loughborough, Leicestershire, UK) was clamped to one side of the bath. The bath had plastic balls floating on the water to reduce water evaporation.

A sampling point was fitted through the lid of the bioreactor which enabled samples to be withdrawn into the sample collector. This can be seen in Figure 3.14.

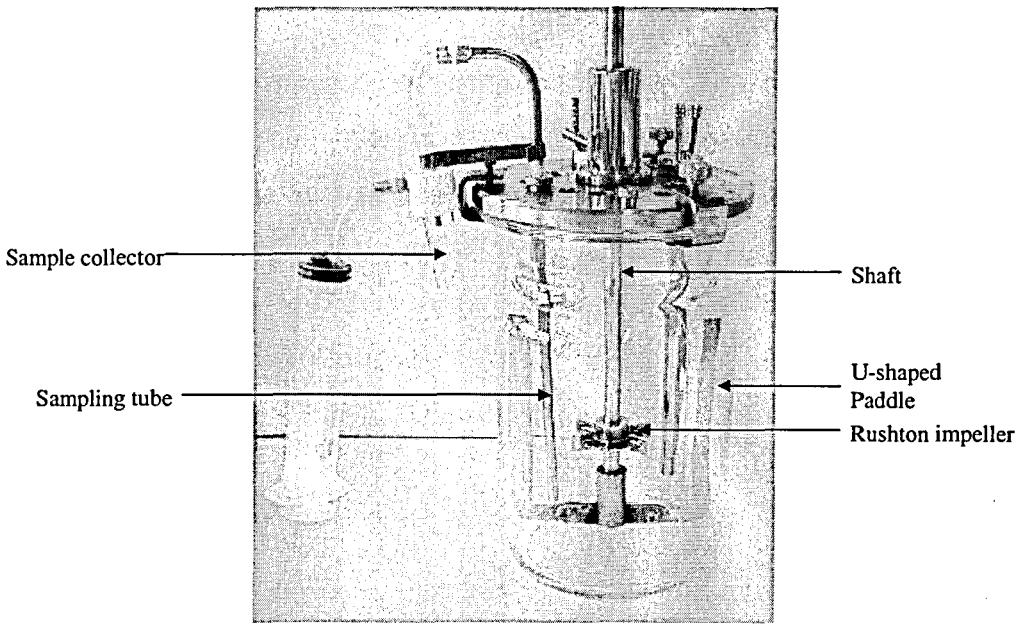


Figure 3.14. Glass Bioreactor.

For all the experiments carried out in this equipment, the water bath was set at 50°C. Citrate buffer (1500 ml) was preheated in a microwave to approximately 50°C, after which it was poured into the bioreactor through an opening in the lid. Stirring was commenced at this stage and 25 ml enzyme was added and mixed at approximately 200 rpm to distribute the enzyme, before the stirrer was slowed to 50 rpm. The system temperature was allowed to equilibrate for 30 minutes, after which 300 g wheatfeed was added through an addition port in the lid using a funnel. Addition of the wheatfeed represented 'zero time'. Variations in the method for individual experiments are discussed in the relevant chapters.

3.7.2.2 Solid State Digestion Experimental Method

Small scale experiments were carried out using 5 g wheatfeed and 5 ml buffer-enzyme solution in petri dishes which were sealed and placed in an incubator. The enzyme used was celluclast.

There were two sets of solid state experiments, but both follow the same basic method. Firstly the enzyme-buffer solutions were mixed then heated in an

incubator at 50°C for 30 minutes, to allow the solution to reach thermal equilibrium prior to being mixed with 5 g riffled wheatfeed. After the addition, the mixture was placed into a petri dish which was then sealed with parafilm. The dishes were then placed in an incubator for 24 hours. After 24 hours, the petri dishes were removed from the incubator and placed in ice. Samples were put into a stomacher bag with 40 ml citrate buffer at pH 5.0 and treated for 1 minute in a laboratory stomacher Model 400, Steward (Norfolk, UK). When all the samples had been processed in this way, they were centrifuged at 15,000 rpm for 10 minutes at 10°C. Supernatant (1 ml) was placed into a test tube for Accu-chek analysis; the remainder was frozen and later analysed by HPLC for sugars and Lowry-Peterson for protein.

On defrosting for analysis, the samples were centrifuged and filtered before analysis was performed.

3.7.2.3 Enzyme Mode of Action Experiments

Citrate buffer (300 ml) at pH 5.0 was dispersed into a 500 ml conical flask with 0.3 ml celluclast. This was covered with parafilm and placed into a bioreactor with a shaker at 15°C for 1 hour to allow thermal equilibrium to be reached. A 5 ml sample was taken filtered and subjected to immediate Lowry-Peterson protein analysis. Fibrous cellulose powder (20 g) (from Whatman) was added to a second 500 ml conical flask, the buffer enzyme solution was added to this and the flask placed in the bioreactor with shaking. Samples (5 ml) were taken filtered and subjected to immediate Lowry protein analysis at regular intervals of time.

3.7.2.4 Digestibility Tests

In standard digestibility tests it is the solid remaining after digestion which is of primary interest. However, for experiments using a variation of the basic 50 ml conical flask digestion method described previously (section 3.7.1), the liquid fraction could not be disregarded after digestion as it contained released sugars

and protein. If the solution were removed the fibre result would show an artificially high level. To resolve this problem all the contents of the conical flask were mixed with a readily digestible component of pet food (i.e. poultry meal) and dried in a vacuum oven (Townsend and Mercer, Limited, Croydon, UK) overnight (at a vacuum of 600 mm Hg and temperature of 55°C) prior to analysis. It was then ground with a pestle and mortar to produce a sample with a particle size of between 355μm and 1000 μm using sieves manufactured by Endecotts (test sieves) Limited (London, UK).

3.7.3 Basic Liquid Sample Preparation for Analysis.

Most of the experiments analysed the liquor after treatment. This was often frozen for storage, then defrosted and filtered before analysis. The filters used were 0.45μm polypropylene Whatman filters (Fisher Scientific, Loughborough, Leicestershire, UK).

3.7.4 Extrusion

The ultimate objective of this work was to establish whether enzyme treated wheatfeed could be incorporated into dog food. Following standard practice with formulation of this type of food, the digested wheatfeed would be blended with other ingredients and then extruded through a twin-screw Clextral Extruder, (Model 21, Firminy, France). Maximum temperatures in the range of 130°C–140°C were used, along with a feed flowrate of approximately 5 kg/hr and a water feed flowrate in the range of 30-50 l/hour.

4 Digestion of Wheatfeed Using Enzyme Mixtures

4.1 Introduction

The composition of wheatfeed was discussed in Chapter 2. As there is no single 'target' substrate against which an individual enzyme could be directed at, it was decided to pursue a strategy of using multi-enzyme mixtures to break down the indigestible elements of wheatfeed. For ease of analysis the latter are defined as breaking down into proteins and simple sugars. However, it is likely that short chain oligomers of both carbohydrates (arising from the incomplete digestion of complex polymers such as cellulose and hemicellulose) and the amino acids (produced from the breakdown of proteins) would also be digestible but very much harder to quantify.

The choice of enzymes used was largely determined by the composition of the wheatfeed; it was therefore decided to investigate the action of a cellulase, a hemicellulase, a pectinase, a xylanase and a β-glucanase.

Initially these enzymes were used in various combinations to obtain a rapid comparison of the yields of glucose using a rapid method of analysis. In subsequent experiments the spectrum of sugars obtained was compared using HPLC. In the final series of experiments, the yields of protein resulting from the treatment of wheatfeed with various enzyme mixtures were compared.

4.2 Method

All the experiments were carried out in 50 ml conical flasks using 2 g of wheatfeed and 10 ml of citrate buffer (pH 5.0) following the basic method detailed in section 3.7.1.

In the initial experiments the following quantities of enzymes were used:
Cellulase 40 mg, hemicellulase 50 mg, pectinase 1.2 mg, xylanase 32 μg

In subsequent experiments the xylanase was replaced by β-glucanase. The quantity added to the reaction mixture was 2 mg.

The solutions were analysed for glucose using the Sigma glucose kit (glucose oxidase) method outlined in section 3.6.3. Table 4.1 shows the enzyme combinations used for the first set of experiments.

Table 4.1. Enzyme Mixtures Investigated.

Experiment number	**Enzymes**			
	Cellulase	**Hemicellulase**	**Xylanase**	**Pectinase**
C	+	-	-	-
H	-	+	-	-
X	-	-	+	-
P	-	-	-	+
HC	+	+	-	-
CX	+	-	+	-
CP	+	-	-	+
HX	-	+	+	-
HP	-	+	-	+
PX	-	-	+	+
HCX	+	+	+	-
HCP	+	+	-	+
CPX	+	-	+	+
HPX	-	+	+	+
HCPX	+	+	+	+
Control	-	-	-	-

Once the preliminary experiment with combinations of both single and enzyme mixtures had been performed, four enzyme treatments were selected for more intensive investigation. This involved comparing the spectrum of individual sugars produced using HPLC.

For the third set of experiments another enzyme was introduced into the study, this enzyme was β-glucanase. Another matrix was drawn up so that mixtures of β-glucanase could be tested. The resulting solutions for analysis for these experiments were analysed by HPLC. The matrix of experiments are shown in Table 4.2.

Table 4.2. Combinations Including β-glucanase.

Experiment number	**Enzymes**			
	β-Glucanase	**Hemicellulase**	**Cellulase**	**Pectinase**
G	+	-	-	-
GH	+	+	-	-
GC	+	-	+	-
GP	+	-	-	+
GCH	+	+	+	-
GCP	+	-	+	+
GHP	+	+	-	+
GHCP	+	+	+	+

From all the results gained so far, four enzyme mixtures were chosen and the solutions tested for protein using the Lowry-Peterson assay.

There was some concern as to the reproducibility of the experiments and the performance of the HPLC. To evaluate possible problems a more thorough investigation was carried out using HCP and GC mixtures along with a xylanase treatment as a comparison and a control. Duplicates of these treatments were

compared using the Students two tailed t-test. The comparison was carried out at the 5% level. This work is shown in Appendix 3.

4.3 Results

The results obtained for the first matrix of experiments using the Sigma glucose kit analysis are shown in Table 4.3, ranked in order of highest glucose concentration.

Table 4.3 Glucose Release from Wheatfeed using Different Enzyme Mixtures.

Enzyme Mixture	Glucose mg/ml
HP	24.8
HCX	21.7
CP	21.5
HCP	17.5
HCPX	16.7
HPX	16.5
HX	15.9
PX	15.3
HC	15.1
H	15.0
CPX	13.9
C	11.9
P	11.4
CX	11.0
X	10.4
Control	7.3

Where

X = Xylanase, C = Cellulase, H = Hemicellulase, P = Pectinase.

Table 4.4 shows the HPLC results for the four enzyme treatments selected for further investigation. It can be seen from the results that xylose was mainly released from samples contacted with cellulase. Some xylose was released from the HP and X and control treatments, however, this was less than 1 mg/ml, whereas both the HCP and HCPX treatments where cellulose was present, released xylose in the region of 6 mg/ml. The results obtained for fructose show that the liberation of this sugar was similar for all treatments giving results in the range of 3.8 to 5.8 mg/ml. Glucose was the sugar which showed the greatest release for all of the enzyme treatments. The control released the lowest amount of glucose (4.77 mg/ml). The two highest glucose liberations were obtained from the treatments with cellulase in the enzyme mixture.

Table 4.4 HPLC Sugar Release from Wheatfeed using Different Enzyme Mixtures

Enzyme mixture	**Reducing sugars mg/ml**			
	Xylose	Fructose	Glucose	**Total**
HP	0.25	3.88	8.24	12.37
HCP	6.08	4.83	15.25	26.16
HCPX	5.72	4.58	14.67	24.97
X	0.49	5.78	7.04	13.31
Control	0.20	4.13	4.77	9.10

Comparing the results it was decided that xylanase did not give high sugar yields and therefore was unlikely to be successfully digesting the wheatfeed. At this stage it was eliminated from the experiments.

The enzyme mixtures were supplemented with another enzyme, β-glucanase, this was tested alone and in mixtures with the other enzymes already used (excluding xylanase). Table 4.5 shows the results gained.

Table 4.5 HPLC Sugar Release from Wheatfeed using Different Enzyme Mixtures Including β-Glucanase.

Enzyme mixture	**Reducing sugars mg/ml**			
	Xylose	Fructose	Glucose	**Total**
G	0.44	5.30	9.39	15.13
GC	6.29	5.95	12.75	24.99
GH	0.81	5.84	20.40	27.05
GP	0.42	5.57	10.49	16.48
GHC	7.58	4.73	20.53	32.84
GHP	0.72	4.47	22.14	27.33
GCP	7.33	6.20	12.81	26.34
GHCP	6.81	4.78	18.95	30.54

Where G = β-glucanase

It can be seen from the results (Table 4.5) that xylose was generally released most after it had been contacted with cellulase. The fructose liberation results were similar for all the treatments. Glucose was the sugar released most for all of the treatments tested, the highest results were generally obtained from samples with hemicellulase, cellulase, or both in mixture with β-glucanase.

From all the results gained so far, four enzymes mixtures were chosen, they were HCP, HCPX GC and GHC. These four enzyme mixtures were used for further experiments using the Lowry-Peterson assay, to evaluate the protein content of the buffer solutions after the digestion. The results gained are shown in Table 4.6. The highest result was obtained by the GHC mixture with a protein yield of 34.6 mg/ml, this was followed by HCP (30.7 mg/ml). The lowest two results were only just over 1 mg/ml apart in the protein yield and still gave fairly high results.

Table 4.6 Results from the Lowry-Peterson Assay.

Enzyme mixture	Protein Released (mg/ml)
HPCX	27.9
HCP	30.7
GHC	34.6
GC	26.8
Control	6.5

4.4 Discussion

From the results obtained from the preliminary experiments (see Table 4.1) (not including β-glucanase), four enzyme mixtures were chosen for further work. The mixtures chosen were HP, HCP, HCPX and X (where H denoted hemicellulase, C, cellulase and X, xylanase). These were chosen for different reasons. HP was chosen as it was the mixture that gave the highest amount of glucose in the solution. Xylanase was chosen, as although it gave the lowest glucose concentration its products of digestion would not have been detected by the assay method used here. Analysing the solution by high performance liquid chromatography (HPLC) would enable the spectrum of sugars released by this enzyme to be revealed.

The HCPX and HCP mixtures were chosen, partly because they gave relatively high glucose yield, but also to establish whether the addition of the xylanase could improve the overall sugar yield. The inclusion of hemicellulose seemed to be beneficial and to result in high glucose yield (Table 4.4).

For the second series of experiments using these four enzyme mixtures and analysing by HPLC, it can be seen by comparing the results of the control with the xylanase experiments, that the xylanase was not effective at breaking down

carbohydrates. It released a little glucose though, which concurred with the glucose kit data. These results also showed that the HCPX and HCP mixtures yield similar amounts of reducing sugars including xylose. The results show that xylanase was less effective at releasing xylose than some of the other enzymes. There was however a noticeable difference between the HP results and the HCPX and HCP results showing that the latter two enzyme mixtures were more successful than the HP mixture. This was in contrast to the findings obtained using the Sigma glucose kit. However, it could be argued that the HP mixture gave unusually low results as it can be seen that the fructose released was less than that released by the control.

The β-glucanase was introduced into the study and another matrix of experiments, was drawn up so that mixtures of β-glucanase could be tested. In this matrix (shown in Table 4.2) xylanase was omitted for the reasons stated above.

The final series of experiments incorporated β-glucanase (G). The first mixture chosen was GC as this had yielded a high fructose concentration in combination with a relatively high xylose release. The second mixture chosen was GCH as this gave the highest glucose result coupled with the highest xylose result, but a low fructose result. It also had a high total reducing sugar result. The work continued using the new four chosen enzyme mixtures, these were HCPX, HCP, GC, GCH for protein analysis after which the best enzyme combination was determined.

It was concluded that the most promising two enzyme mixtures were β-glucanase, cellulase (GC) and hemicellulase, pectinase, cellulase (HPC) mixtures. This is in the light of both sugar and protein experimental results and economic considerations, that is they may not give the best results but the difference in the results is small compared to the much higher cost of more expensive enzymes (cellulase and β-glucanase are the most expensive). From these two enzyme

mixtures the best mixture to use is HCP as the hemicellulose and pectinase are fairly cheap compared to β-glucanase.

The final chosen enzyme combination, of hemicellulose, cellulose and pectinase was a combination used as a pretreatment step in work by Waszczynskyj *et al.* (1981), who found that using this combination of enzymes had potential to increase the yield of protein from wheat bran when followed by an alkaline extraction process.

4.5 Conclusion

It is difficult to choose a 'best' enzyme mixture for the further work as HPLC analysis gives results for more than one reducing sugar. The aim of the project is to use an enzymic mixture to digest the fibre of wheatfeed to produce reducing sugars and render the wheatfeed more digestible. The breakdown of the fibre will release protein bound up in the fibre matrix and therefore it will be free for use by the animal when it is incorporated into animal food. It is not known which sugars released correspond to a more digestible product, or to a high protein release. The hemicellulose, cellulose and pectinase combination of enzymes was effective at breaking down fibre to release sugars and protein, this combination was the most economically viable from those which gave good results.

The t-test results (Appendix 3) show that the experiments are repeatable. Generally there was no significant difference between duplicates of the same treatment, the main exception to this rule was the HPLC xylose results. The HPLC fructose results showed that this is not a good sugar to use for different treatment comparisons, therefore the best sugar to compare is glucose. The protein results showed that experiments were repeatable and reliable using the Lowry–Peterson analysis.

5 Digestion of Wheatfeed by Sequential Addition of Enzymes.

5.1 Introduction

The series of experiments described in this chapter were conducted in order to determine whether the sequence in which individual enzymes were added to wheatfeed was significant and whether by comparing patterns of sugar and protein release, clues could be gained about the susceptibility to enzyme attack of the various components of wheatfeed. In the experiments described below, digestion of the wheatfeed was achieved by adding single enzymes in sequence. Three enzyme types were incorporated- cellulase, pectinase, and hemicellulase. In each case digestion was allowed to proceed for 2 hours before adding the second enzyme; after a further 2 hours the third enzyme was added. The experiments were designed so that every permutation of enzyme addition was examined. In all, two series of experiments were carried out. In the first, untreated wheatfeed was used as the substrate whilst in the second series, both un-pretreated wheatfeed and steam exploded wheatfeed were employed.

5.2 Method

The experiments were carried out in 50 ml conical flasks and followed the basic method detailed in section 3.7.1. There were a few minor variations to the basic method however, the most significant being that 20 ml citrate buffer (at 50°C and pH 5.0) was used. All the experiments were performed in duplicate.

The citrate buffer contained the first enzyme to be added. Once added, the flask was covered with parafilm and allowed to digest for 2 hours. After this time, the conical flasks were taken from the water bath and placed in ice to allow the wheatfeed to settle so that a sample (2 ml) could be taken. The second enzyme was added in 2 ml buffer (at room temperature) and allowed to digest at 50°C for

2 hours before the sampling process and enzyme addition was repeated. Finally, after a further 2 hours the third sample was taken. The samples obtained were filtered and frozen for high performance liquid chromatography (HPLC). It was decided not to analyse for protein for the first series of experiments.

The combinations of enzymes used can be seen in Table 5.1, where 'H' denotes hemicellulase, 'C' denotes cellulase and 'P' denotes pectinase.

Table 5.1.Notation for Sequential Digestion Experiments.

Experimental Label	First Enzyme	Second Enzyme	Third Enzyme
HCP	Hemicellulase	Cellulase	Pectinase
HPC	Hemicellulase	Pectinase	Cellulase
CPH	Cellulase	Pectinase	Hemicellulase
CHP	Cellulase	Hemicellulase	Pectinase
PCH	Pectinase	Cellulase	Hemicellulase
PHC	Pectinase	Hemicellulase	Cellulase
Control	None	None	None

A second sequential addition experiment was carried out using a selection of the enzyme combinations (HCP, CPH, PCH and Control experiments). This set of experiments were performed as a repeat of the first set for un-pretreated wheatfeed, but the same treatments were also applied at the same time to steam exploded wheatfeed, which had previously been dried and frozen for storage. The experimental method was the same as that previously described, using 2 g steam exploded wheatfeed in the place of wheatfeed for the steam exploded experiments. The resulting solutions from these experiments underwent both HPLC and Lowry-Peterson protein analysis. The steam explosion was carried out as detailed in section 3.4.2.

The experiments are described in Table 5.2. In this table the labels are the same as previously used, (Table 5.1). 'S' is used to denote steam exploded wheatfeed.

Table 5.2. Selected Sequential Treatment Experimental Design.

Experiment Label	**Steam Exploded**	**First Enzyme**	**Second Enzyme**	**Third Enzyme**
HCP	NO	Hemicellulase	Cellulase	Pectinase
CPH	NO	Cellulase	Pectinase	Hemicellulase
PCH	NO	Pectinase	Cellulase	Hemicellulase
Control	NO	None	None	None
HCP (S)	YES	Hemicellulase	Cellulase	Pectinase
CPH (S)	YES	Cellulase	Pectinase	Hemicellulase
PCH (S)	YES	Pectinase	Cellulase	Hemicellulase
Control (S)	YES	None	None	None

5.3 Results

The results for the sugars released for the un-pretreated wheatfeed experiments are shown in Figures 5.1-5.3.

Figure 5.1 shows the results for xylose liberation. By comparing the two treatments in which cellulase was added first (CPH, CHP) to all the other treatments it is evident that xylose is only liberated once the cellulose has been added. This is confirmed by examining the other results, for example PCH, where there is no sugar release after the first addition, but where xylose is only detected after the second addition. Further confirmation comes from the results for HPC where xylose is only detected after the final enzyme addition. No xylose was detected in the control. The maximum xylose detected after enzymic treatment lay within the range of 8.0 to 10 mg/ml.

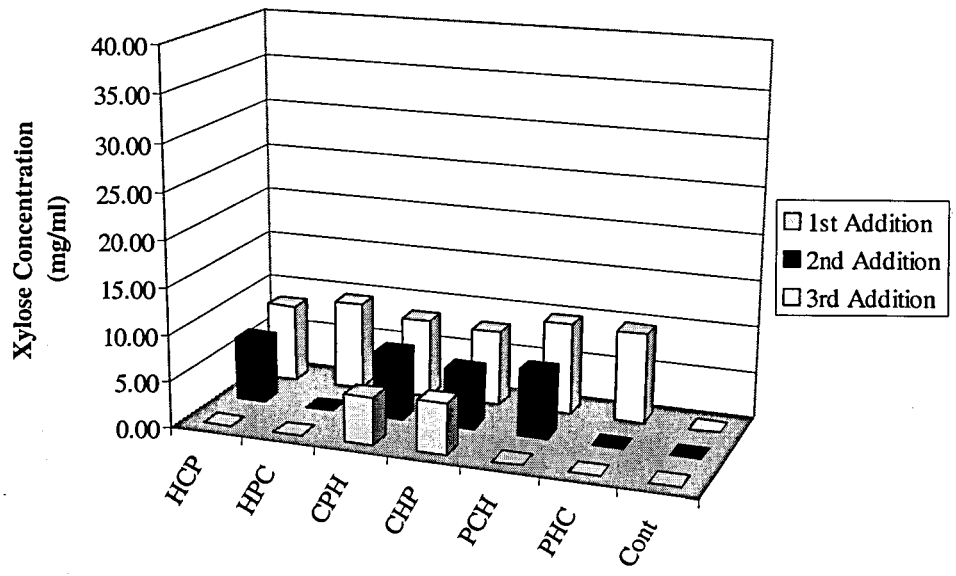


Figure 5.1 Xylose Released By Sequential Addition of Enzymes.

The arabinose results are shown in Figure 5.2. Similar results were gained for both the control and the enzymic treatments of wheatfeed regardless of the order of enzyme addition. The results after the final stage of enzyme digestion were in the range of 4.0 to 4.5 mg/ml.

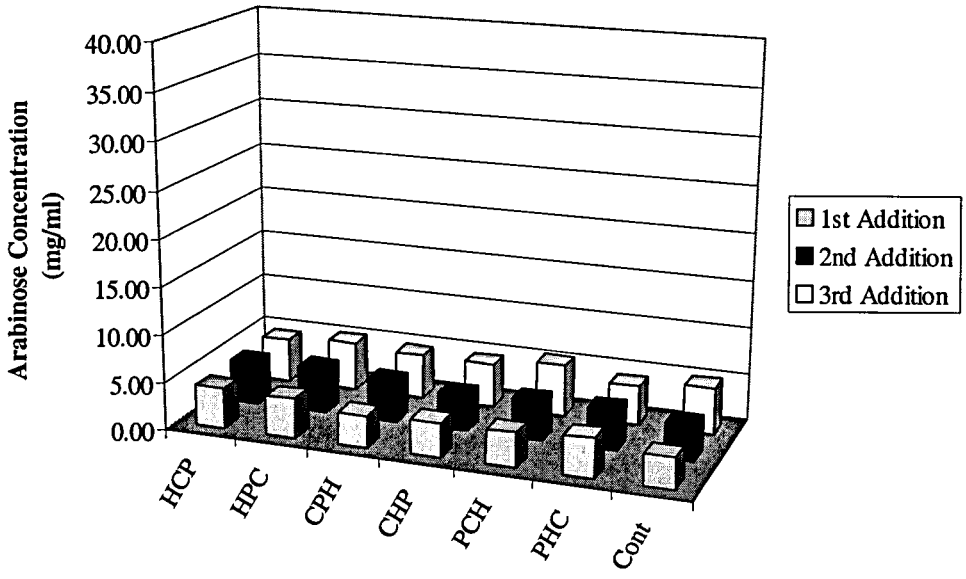


Figure 5.2 Arabinose Released By Sequential Addition of Enzymes.

The results for glucose are shown in Figure 5.3. Glucose is liberated for all of the treatments and also for the control, with the final values ranging between 17.5 and 31.0 mg/ml. The glucose results for the control seem high, with more glucose released than for any of the treatments after the first addition. The variation between the duplicates was also high for these experiments with 4 out of the 21 duplicate results having an error of over 50%. Only 5 out of the 21 duplicate results have an error of less than 10 %.

The enzyme treatment results for the first addition stage show that most glucose was released from the wheatfeed treated with cellulase (CPH and CHP). If further comparisons are made between the second addition results for the HCP and HPC samples, it can be seen that the result for the HCP shows more glucose has been released than for the HPC result, however, once the cellulase is added in the third stage for HPC the glucose liberation greatly increases. This suggests that cellulose addition has a pronounced effect on glucose release. Further confirmation can be gained from the PCH and PHC results where it is clearly seen that after the second addition the glucose liberated is greater for the PCH treatment than for the PHC treatment. However, it is also evident that the glucose released during the PHC treatment increases after the third treatment where cellulase is added. Furthermore, if the final results for the HCP and HPC are compared it can be seen that the greatest final glucose liberation occurs when cellulase is added last, with a glucose increase of 20.9 mg/ml between the second and third addition for the latter case. This is confirmed by the results from the PCH and PHC treatments where the final result is again greatest for the PHC treatment where cellulase is added last, in this case the glucose increase is 12.2 mg/ml.

A comparison of the CPH and CHP reveals that the results are similar however, the greatest final sugar liberation is achieved from the CHP results suggesting that more glucose is liberated from the wheatfeed if hemicellulose is added before pectinase. This is confirmed by comparing the results from the PHC and HPC

treatments. Taken together these findings would suggest therefore that greatest sugar release would occur for the HPC treatment and this is confirmed by the results, as 6.1 mg/ml more glucose was obtained than for any other treatment.

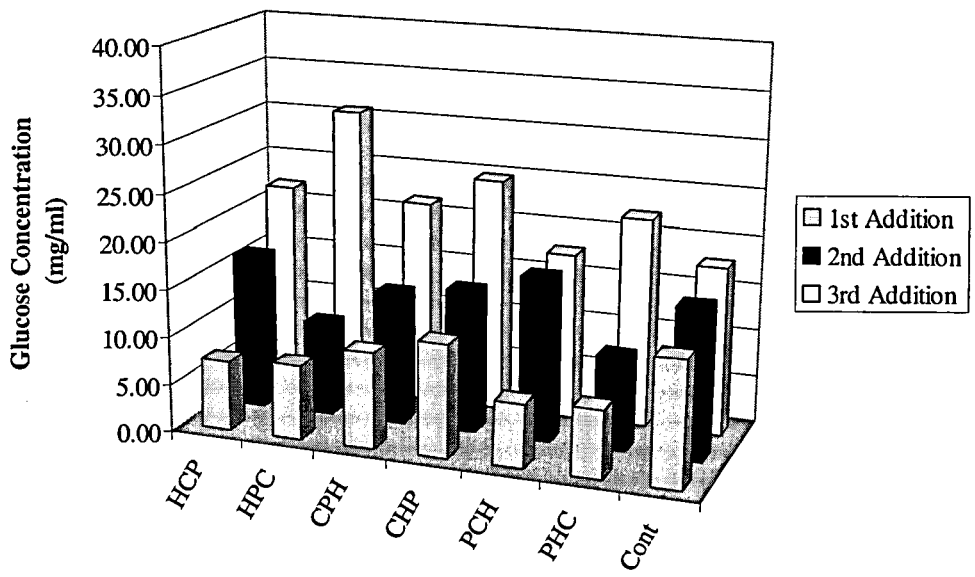


Figure 5.3 Glucose Released By Sequential Addition of Enzymes.

For the second experiment only a selection of the treatments were used. These were HCP, CPH, PCH and control. The repetitions were carried out for two reasons, firstly, as a contrast to the steam exploded wheatfeed, ensuring that the conditions were the same for both the un-pretreated wheatfeed samples and steam exploded samples. Secondly there was a problem with the column used to analyse the samples from the first experiment and a new column was fitted. Repeating the experiment allows a comparison between the results gained with the old analysis equipment and the new. The treatments repeated were chosen in such a way that each enzyme was the first added once.

For the second experiment where both un-pretreated wheatfeed and steam exploded wheatfeed were used, no xylose was liberated from either of the controls

(Figure 5.4). Xylose was liberated during all the other treatments in the range between 8.5 to 12 mg/ml. The results for the treatment of un-pretreated wheatfeed shows the pattern of xylose liberation is broadly similar to the first series of experiments. The results show that no xylose was detected for the HCP and PCH treatments until after the second enzyme cellulase was added, whereas for the CPH treatment, xylose was detected after the first enzyme addition. This again confirms findings from the first set of experiments (Figure 5.1). Xylose is also not detected after the first stage for both the HCP and PCH treatments of steam exploded wheatfeed, but is detected after the second enzyme addition. In both cases the second enzyme to be added is cellulase. The steam exploded CPH sample liberates xylose after the first addition of cellulase. These results suggest, that xylose is not released until cellulase has been added.

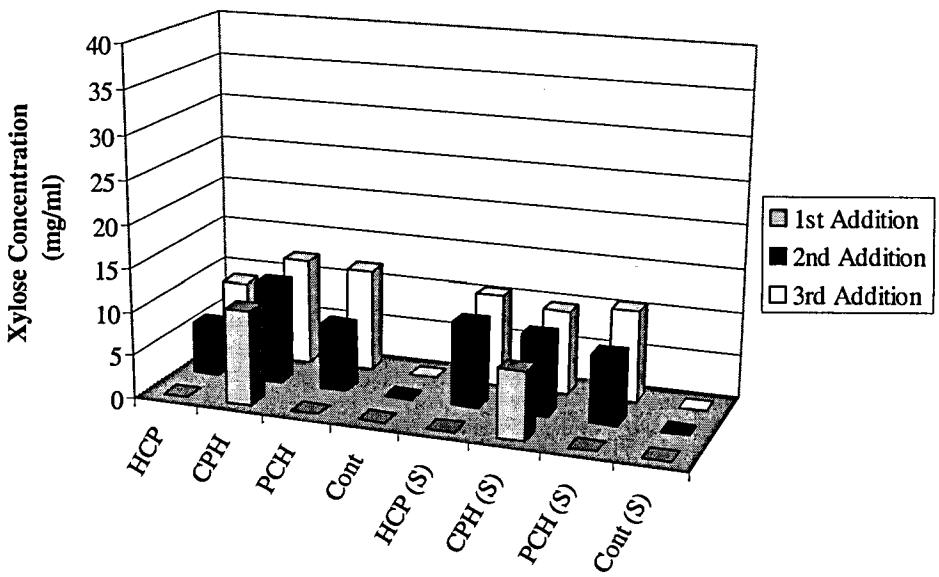


Figure 5.4 Xylose Released By Sequential Addition of Selected Enzyme Mixtures for Steam Exploded and Un-pretreated Samples.

(s) indicates steam exploded wheatfeed.

Figure 5.5 shows the results for arabinose release. Arabinose is released from the un-pretreated wheatfeed samples and the control in the range of 4.5 to 9.5 mg/ml. This range is higher than was previously observed (Figure 5.2). The first addition for the CPH experiment gives an unusually high result for arabinose compared to the earlier experiment (Figure 5.2).

For the steam exploded wheatfeed samples no arabinose is detected for any of the treatments, or the control at any stage of the experiment.

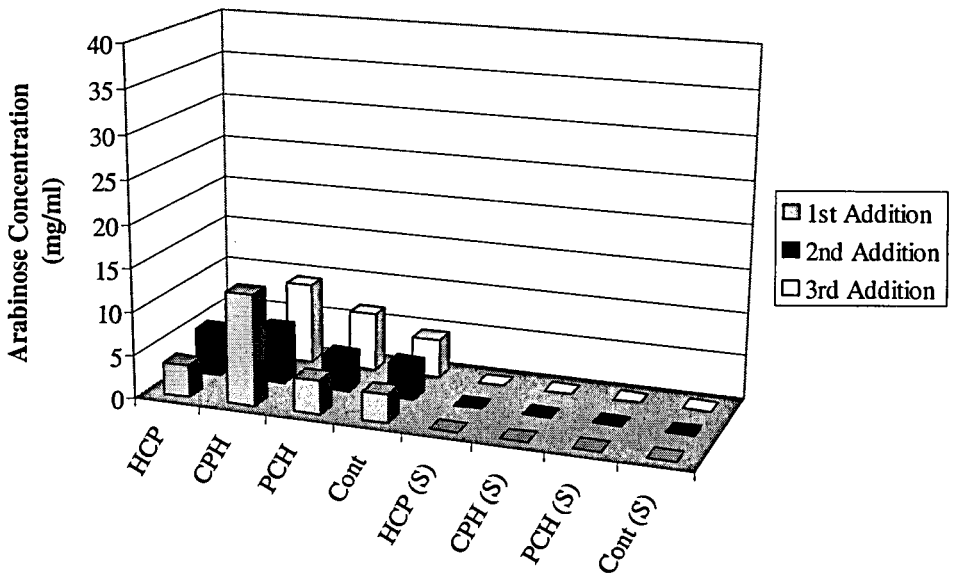


Figure 5.5 Arabinose Released By Sequential Addition of Selected Enzyme Mixtures for Steam Exploded and Un-pretreated Samples.

(s) indicates steam exploded wheatfeed.

Both sets of results give a similar trend for glucose (Figure 5.6). The graph gives the impression that there is not much difference between the results for the un-pretreated wheatfeed. If however, the results from the first experiment are revisited (Figure 5.3) it can be seen that for the treatments repeated here, the difference in the final glucose released is slight. Some glucose is released with all the enzyme treatments and also in the controls, but the greatest increase is after

cellulase has been added to the mixture, this is shown very clearly by the steam exploded results with glucose release increasing by over 21 mg/ml for all the treatments after cellulase addition. Samples which have been steam exploded release much more glucose than the un-pretreated samples, except in the case of the control where less glucose is released.

If the un-pretreated wheatfeed control results are compared to those gained in the first experiment (Figure 5.3) it can be seen that the results here are much lower than those gained in the first experiment. Because the HPLC column was changed after the first series of experiments were conducted, greater reliance is placed on the second set of results.

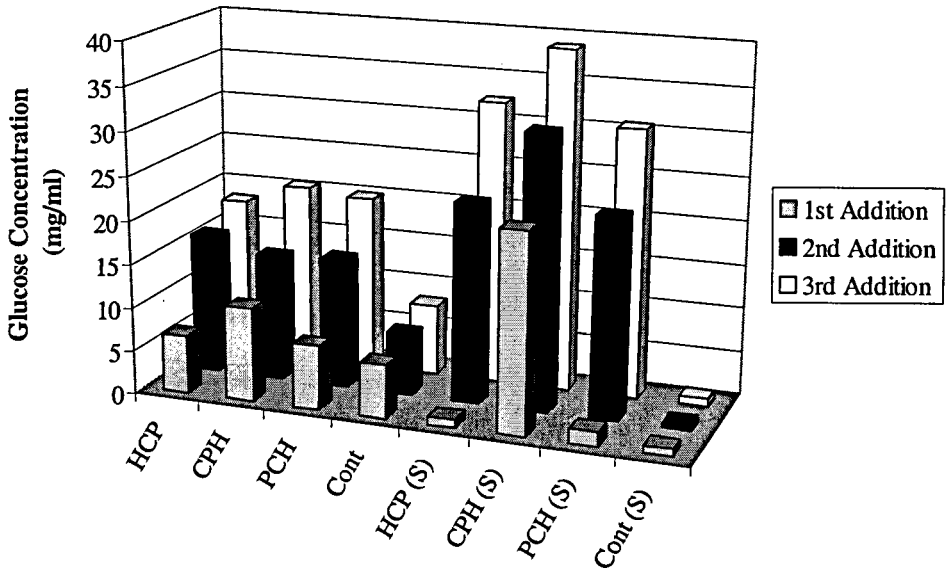


Figure 5.6 Glucose Released By Sequential Addition of Selected Enzyme Mixtures for Steam Exploded and Un-pretreated Samples.

(s) indicates steam exploded wheatfeed.

The protein results, Figure 5.7, show that some protein is released for all enzyme combinations and also by the controls. However, it is apparent from comparing

the HCP and PCH results with the CPH results for first addition, that most protein is released after cellulose has been added to the mixture for both steam pretreated and un-pretreated samples. This is further confirmed by comparison of the first addition results for the HCP and PCH treatments with their respective second addition results after cellulase has been added, where the protein detected is greatly increased. The steam exploded treatments release less protein than the un-pretreated wheatfeed treatments. Both the wheatfeed control and the steam exploded control release less protein than the treated samples.

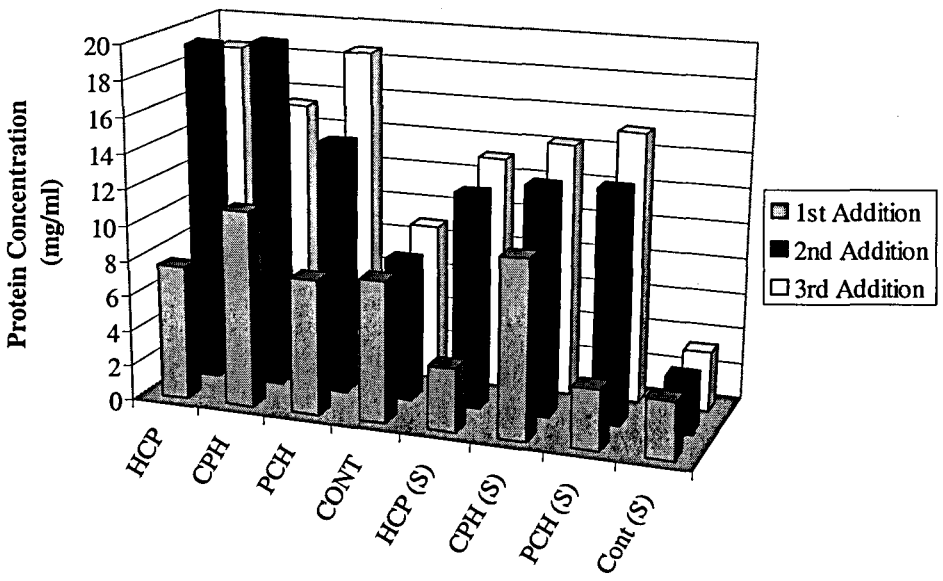


Figure 5.7 Protein Released By Sequential Addition of Selected Enzyme Mixtures for Steam Exploded and Un-pretreated Samples.

(s) indicates steam exploded wheatfeed.

5.4 Discussion

Reference has already been made to the discrepancies in the sugar levels between the first and second experiments in which un-pretreated wheatfeed was used. This was almost certainly due to problems occurring with the HPLC analysis and resulted ultimately in the fitting of a new column. The new column gave better

separation of the sugars and this allowed better detection. The results for the first experiment controls were high and the duplicates gave values which for the final sample had a difference of over 17 mg/ml for the glucose release. These were the last samples to be analysed on the old column. These results must therefore be viewed as suspect. The errors calculated using the two duplicate results for glucose for the first experiment, were over 50% for 4 out of 21 duplicate pairs (19% of results) using the old column, only 5 samples gave results with errors of less than 10% (23.8% of results). The new column used for the second experiment analysis, for both steam exploded and un-pretreated wheatfeed, gave a difference of over 50% mg/ml for 3 out of 24 results (12.5% of results) and errors of less than 10 % for 14 out of 24 duplicates (58.3% of results). Notwithstanding, some useful results were obtained from the first experiment, in particular more enzyme combinations were tested in the first set of experiments which allowed an insight into the best order of enzyme addition. The results gave an indication for patterns of the release of sugars and the repeats show the same trends in the results.

A particularly interesting result was that there was no xylose release until cellulase had been added. Xylose is present in the hemicellulose of wheat bran which is mainly made up from arabinoxylans (Fincher and Stone, 1986). The arabinoxylans are made up from a xylopyranase β-D-(1→4) linked linear backbone with α-L-arabinofurnanase units attached (Edwards *et al.*, 2003). Wilkie (1979) suggested that hemicellulose filled the void between the cellulose fibres, and Walker (1993) predicted that although the function of hemicellulose was unknown it could form a link between cellulose and lignin. The results obtained here suggest that the xylose is in fact entrapped in the bran within cellulose fibres or closely linked to the cellulose and that until the cellulose fibres are digested by cellulase the xylose is not released. The results obtained would therefore support the view that the hemicellulose is linked to the cellulose and the linkage is through the xylose chain. The xylose results could also be explained if the cellulase enzyme demonstrated xylanase activity. Both Kim, (1995) and Kim

et al. (1997) report that on producing a cellulase from *Bacillus stearothermophilus* and *Bacillus circulans* respectively, they found that it also acted on xylans. Notenboom *et al.* (1998) reported that a cellulase (β-1,4-gycanase) hydrolysed xylan 40-fold more efficiently than cellulose.

In the first set of experiments (un-pretreated wheatfeed only) similar amounts of arabinose was released regardless of which enzymes were added, or with no enzymes present. This indicates that arabinose is not trapped within a matrix and is either much nearer the surface of the wheatfeed, or less closely related to other constituents in the fibre and is released into solution under the reaction conditions selected. If the arabinose is present in the arabinoxylans as side chains then it may be easier for this to break free into solution. If arabinose was widely distributed within the fibre matrix, then one might expect that some additional arabinose would be released into the solution after digestion with enzymes, but this was not the case.

The addition of cellulase unambiguously stimulated glucose release. It was noted from the results for the first experiment (Figure 5.3) that the best order of addition of enzymes was hemicellulase, pectinase then cellulase for the un-pretreated wheatfeed glucose release. As previously mentioned, the void between cellulose fibre is filled with hemicellulase, therefore adding the hemicellulase first would start to degrade some of this material allowing better access to the cellulosic fibres. The cellulose fibres are composed of a primary wall a secondary wall and a lumen. The primary wall is the outer layer and is mainly made up of non cellulosic material such as waxes and pectin. The secondary wall is where most of the cellulosic material is contained and the lumen comprises mainly proteinaceous material (Immergut, 1963). This explains why more glucose is liberated if pectinase is added after the hemicellulose, but before cellulase. Once the hemicellulase has allowed better access to the fibres then the pectinase is able to digest the pectin in the primary cell wall, allowing the cellulase better access to the cellulosic material in the secondary cell wall. It could also be possible that

better results are gained if hemicellulose is added before pectinase if the hemicellulase needs a longer reaction time, if it is added last it only has 2 hours to react. As only a selection of the enzyme treatments were performed on the steam exploded wheatfeed then this 'best' order of addition can not be concluded for the steam pretreated wheatfeed, however from the treatments carried out the best results were obtained from the CPH treatment.

Glucose was released in the control experiments for both steam exploded and un-pretreated wheatfeed. This glucose may have come from starch present in the bran fraction possibly from endosperm still attached to the bran. The digestibility tests (section 10.3) show that 22% of the wheatfeed is composed of starch.

The results from the steam explosion experiments show the same trend as was observed for the untreated wheatfeed with respect to xylose release, with no xylose released into solution until cellulase has been added. There is a difference however with regard to the arabinose. Steam exploded wheatfeed showed no arabinose release regardless of the enzymes added. This suggests that the arabinose is either more labile than the other sugar constituents or that it is more accessible during the pretreatment step and therefore degraded during steam explosion. It is possible that if the xylose part of the arabinoxylans are more closely linked with the cellulose fibres they may be protected from the steam explosion by the cellulose.

The release of glucose from steam exploded samples clearly shows the same trend as the un-pretreated samples, with more glucose released after the addition of cellulase. In the case of steam exploded wheatfeed this trend is shown more clearly than for the un-pretreated wheatfeed. The release of glucose is greater for the steam exploded samples than from the un-pretreated wheatfeed. This agrees with findings from the initial steam explosion experiments, shown in Appendix 4 and may be due to other components of the fibre being degraded allowing the cellulase better access. In both cases glucose is the predominant sugar released.

Most of the protein in the wheatfeed is released after cellulase has been added to the mixture for both un-pretreated and steam exploded wheatfeed. Protein is present in the aleurone cells in the inner layer of the bran and also inside the cellulose fibrils (Immergut, 1963). In the former case the aleurone cell wall must be degraded before the protein is released, in the latter case the cellulose fibrils must be degraded before the protein is free. This would explain why cellulose must be added before protein is released. Figure 5.7 shows that overall, less protein is released from the steam exploded wheatfeed than from the un-pretreated wheatfeed. This is probably due to protein degradation during steam explosion.

5.5 Conclusion

The experiments described here revealed important information about the action of the three enzymes hemicellulase, pectinase and cellulase, on wheatfeed. Xylose is either entrapped in the fibre in such way that cellulose must be digested before xylose is released, or the cellulase used also has some xylan activity. Arabinose is present in an easily soluble form and is released into solution without the use of enzymes. Some glucose is released into solution without the use of enzymes this is possibly due to the presence of starch. Most is released however, after cellulase is added. The order of enzymes which release the most glucose from un-pretreated wheatfeed is hemicellulase, pectinase and then cellulase. Steam exploding the wheatfeed prior to performing the experiment degrades arabinose and none is present in solution after digestion. More glucose was released when the wheatfeed was steam exploded. Although some protein was solubilised in the absence of enzymes, peak protein release occurred after the addition of cellulase. Steam exploding the wheatfeed led to reducing levels of protein release presumably because protein degraded during steam explosion. However, the breakdown products of proteins, oligopeptides and amino acids, although not detectable by the Lowry-Peterson assay would nonetheless have nutritional value. For the second experiment the highest total reducing sugars

released by the un-pretreated wheatfeed was obtained from the CPH treatment which released 42.0 mg/ml. The highest total reducing sugars from the steam exploded wheatfeed was obtained by the CPH treatment which liberated 48.5 mg/ml. Therefore steam explosion pretreatment will allow liberation of 6.5 mg/ml more sugar.

A limitation of the experiments described is that the enzymes all had differing optimum conditions. Experiments employed one temperature and one pH and therefore not all the enzymes were at their optimum conditions.

6 Optimising the Digestion of Wheatfeed Using Enzyme Mixtures.

6.1 Introduction

The performance of all enzymes is strongly dependent on the environmental conditions in which they function. Outside of living cells the two parameters most easily varied are temperature and pH.

Each of the enzymes used here was derived from a different source and had optimal conditions (as defined by pH and temperature) that differed from one another. The cellulase was from *Trichoderma reesei* and optimal performance was achieved at pH 5.0 and temperature 37°C. The hemicellulase was derived from *Aspergillus niger* and functioned optimally at pH 4.5 and 40°C. Finally, the pectinase used here was that produced by a species of *Rhizopus* and displayed optimum conditions of pH 4.0 and 25°C. It was proposed to use these enzymes in combination, in order to achieve high yields of sugars and proteins from the digestion of wheatfeed and therefore it was necessary to determine the conditions under which this occurred optimally. There was also a possibility that synergistic effects may be revealed by investigating the combined performance of the three enzymes mentioned above over a range of temperatures and pH. For example the action of one of the enzymes might result in increased availability of substrate for the next.

In this Chapter, the effects of changing temperature in the range 30 to 55°C and pHs in the range 3.0 to 5.8 were investigated. The initial conditions investigated were temperatures of 50°C and 37°C and pHs of 3.4 and 5.0. Most of the previous experiments were carried out at pH 5.0 and 50°C. The intervals between the parameters investigated were chosen to be sufficiently distant from one another so as to reveal differences above the level of experimental errors in

determining the concentration of sugars and protein, but not so great as to permit significant peaks or troughs to be overlooked. Another important parameter in industrial enzymic processes is the time of digestion and in the experiments described two digestion times were investigated, 8 and 24 hours.

6.2 Method

Experiments were carried out using a cellulase, hemicellulase and pectinase mixture of single enzymes to evaluate the optimum conditions of the combination. All experiments were carried out in 50 ml conical flasks following the basic procedure outlined in section 3.7.1. Initial experiments were carried out using citrate buffer at pH 5.0 and 3.4, temperatures of 50°C and 37°C and times of 8 and 24 hours. These experiments were carried out in duplicate and included controls (to which no enzymes were added) for each set of parameters. All samples were centrifuged and the supernatants taken and frozen for high performance liquid chromatography (HPLC) and Lowry-Peterson protein analysis.

After these initial experiments had been conducted, surface graphs of total reducing sugar and protein released were plotted, this gave an indication as to whether to increase, or decrease the values of the parameters under investigation in order to move towards an optimum. Further experiments were therefore designed to more closely approach conditions at which the highest yields of sugars and protein release could be achieved. These later experiments were carried out at a wider range of conditions using pH 3.0 and 5.8 and temperatures of 30°C and 55°C as well as the original parameters. A final experiment was carried out at the discovered optimum conditions.

Table 6.1 shows all of the experiments performed to produce the surface graph. Table 6.2 shows the amounts of citric acid and sodium citrate necessary to produce buffers of the desired pH.

Table 6.1. Experimental Conditions.

Time (hours)	Temperature (°C)	pH
8	30	3
8	30	3.4
8	**37**	**3.4**
8	**37**	**5**
8	**50**	**3.4**
8	**50**	**5**
8	50	5.8
8	55	3.4
8	55	5
8	55	5.8
24	30	3
24	30	3.4
24	**37**	**3.4**
24	**37**	**5**
24	**50**	**3.4**
24	**50**	**5**
24	50	5.8
24	55	3.4
24	55	5
24	55	5.8

The initial experiments carried out are shown in bold type.

Table 6.2. Formulation of Citrate Buffers from Cruickshank, (1960).

pH	0.1 M Citric acid (ml)*	0.1 M Sodium citrate (ml)*
3.0	465	35
3.4	400	100
4.8	230	270
5.0	205	295
5.8	118	382

*Quantities added to 500 ml of distilled water.

6.3 Results

Figure 6.1 shows the surface plot for total reducing sugars released after 8 hours digestion. It is immediately apparent that there are no prominent peaks or troughs. There appears to be one more or less well defined peak at intermediate pH (between 4.0 and 5.0) and at high temperature 55°C and a ridge giving high sugar concentration at lower temperatures (between 35 and 40°C) over the entire pH range examined, with the possible exception of the lowest pH (3.5) which reveals a small minimum.

The corresponding control experiment (Figure 6.2) shows that the highest concentrations of sugars were released at the lowest temperatures investigated, with a possible maximum at 35°C, or below and at pHs above 5.0.

In the corrected plot (i.e. the values from Figure 6.2 subtracted from those of Figure 6.1) shown in Figure 6.3, the maximum at high temperatures (55°C) and intermediate pH persists, but the ridge which was observed in Figure 6.1 is now a more definite peak and occurs in a region of low pHs (approximately 3.5) and at low temperatures (35 - 40°C).

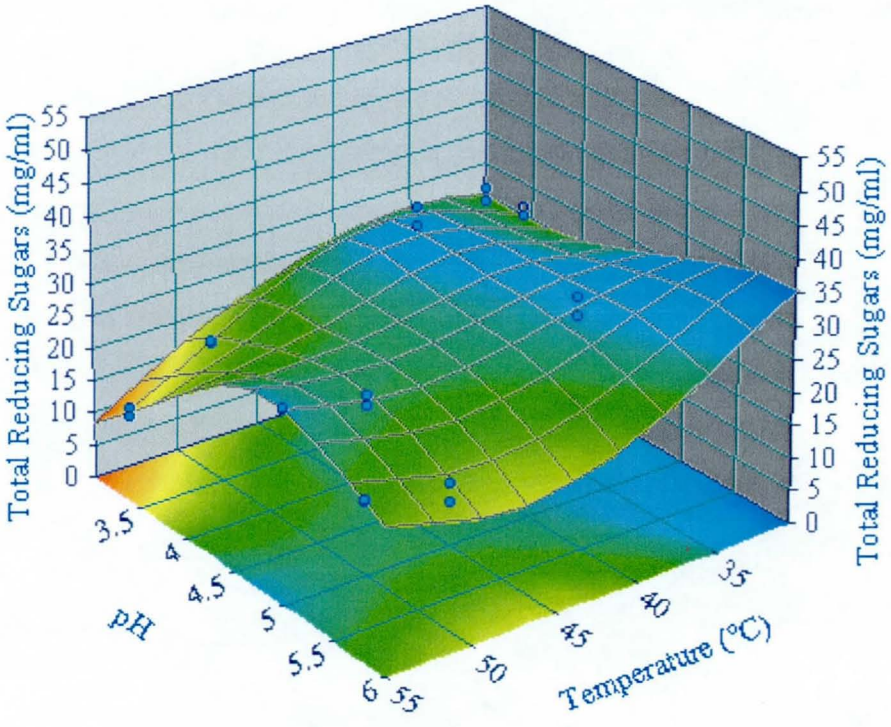


Figure 6.1 Surface Plot of Total Reducing Sugars Released from Wheatfeed after Enzymic Digestion for 8 Hours.

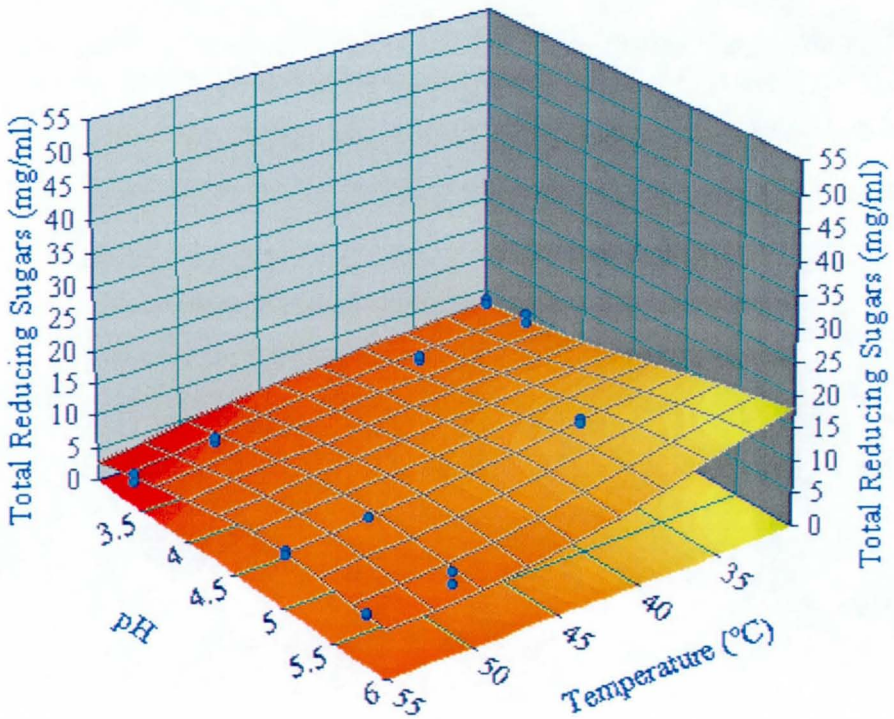


Figure 6.2 Surface Plot of Total Reducing Sugars Released from Wheatfeed in the absence of Enzymes for 8 Hours.

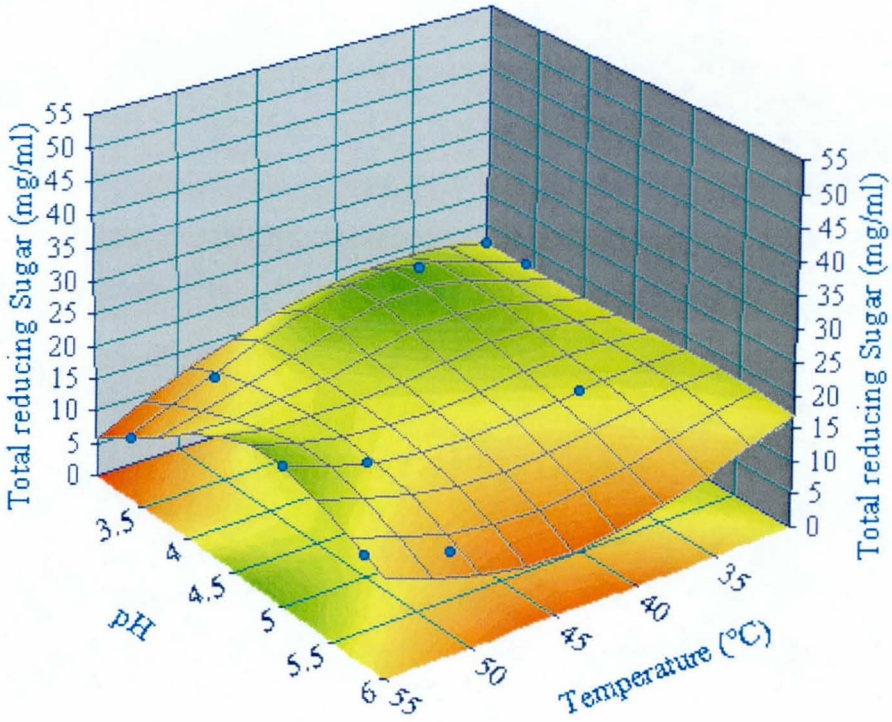


Figure 6.3 Surface Plot of the Corrected* Total Reducing Sugars Released from Wheatfeed after Enzymic Digestion for 8 Hours.

* These were obtained by subtracting the concentrations detected in the control experiments conducted at identical conditions.

The surface plot for 24 hour digestion is shown in Figure 6.4. Two maxima are revealed; one at low pH (3.0 to 4.0) and low temperature (between 35 and 40°C) and a lesser one at the higher temperature of 55°C and pHs in the range of 4.0 to 5.0. A pronounced minimum is revealed at pHs above 5.5 and temperatures in the region of 42°C and below.

The controls (Figure 6.5) reveals a 'diffuse' maximum, at intermediate pHs and intermediate to higher temperatures (45 to 50°C) and a somewhat more distinct maximum at very low pHs and temperatures.

The corrected plot (Figure 6.6) reveals a very similar profile to that of the uncorrected plot (Figure 6.4) albeit with reduced sugar yield. These results suggest that optimal sugar release occurs at pH 3.7 and at a temperature of 37°C. Moreover it is also clear that the longer digestion time of 24 hours is beneficial in resulting in increased yields.

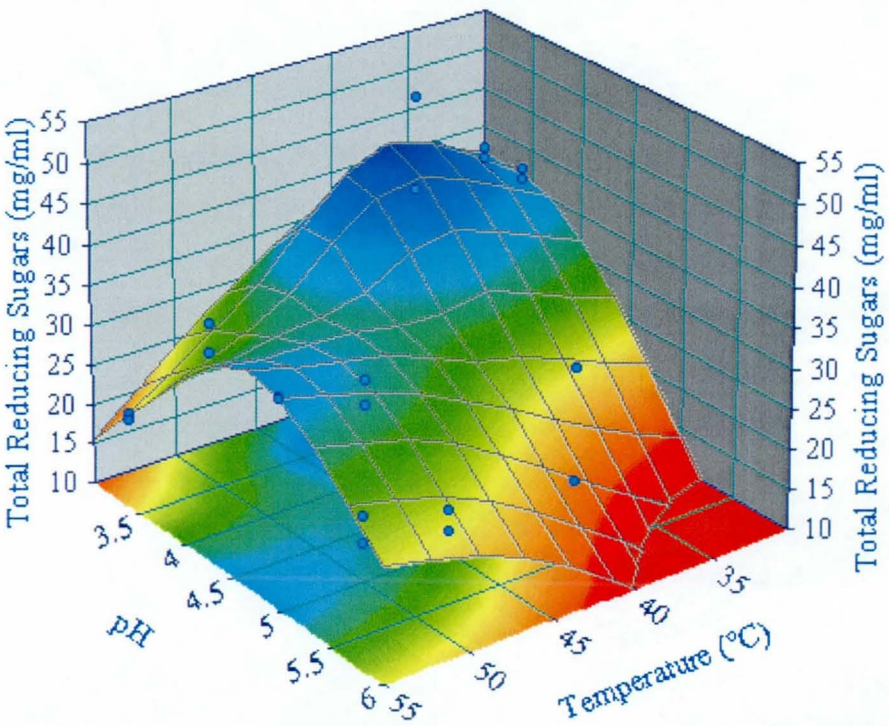


Figure 6.4 Surface Plot of Total Reducing Sugars Released from Wheatfeed after Enzymic Digestion for 24 Hours.

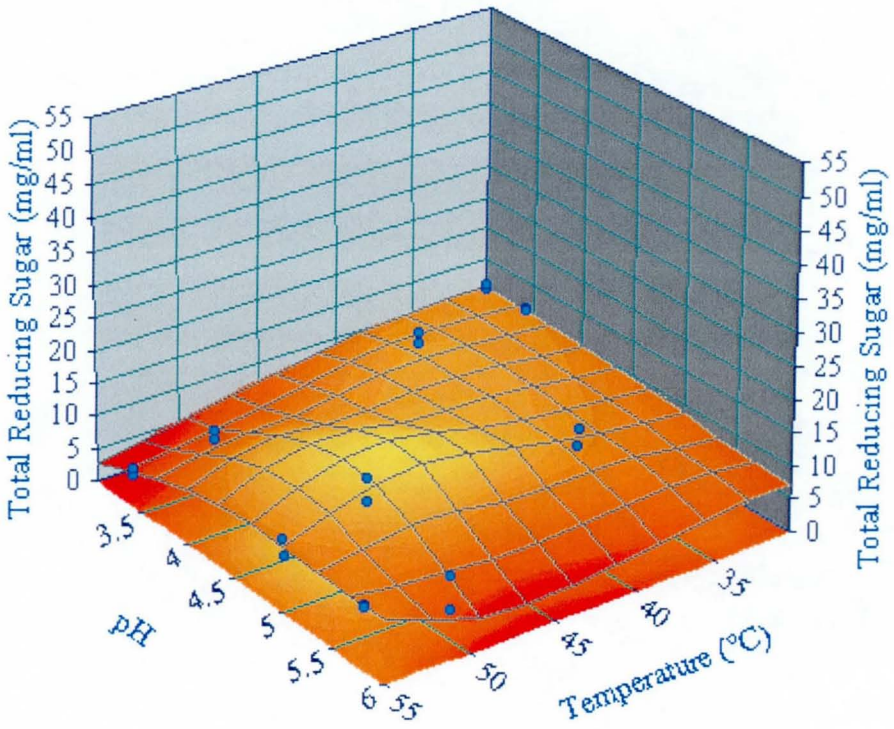


Figure 6.5 Surface Plot of Total Reducing Sugars Released from Wheatfeed in the absence of Enzymes for 24 Hours.

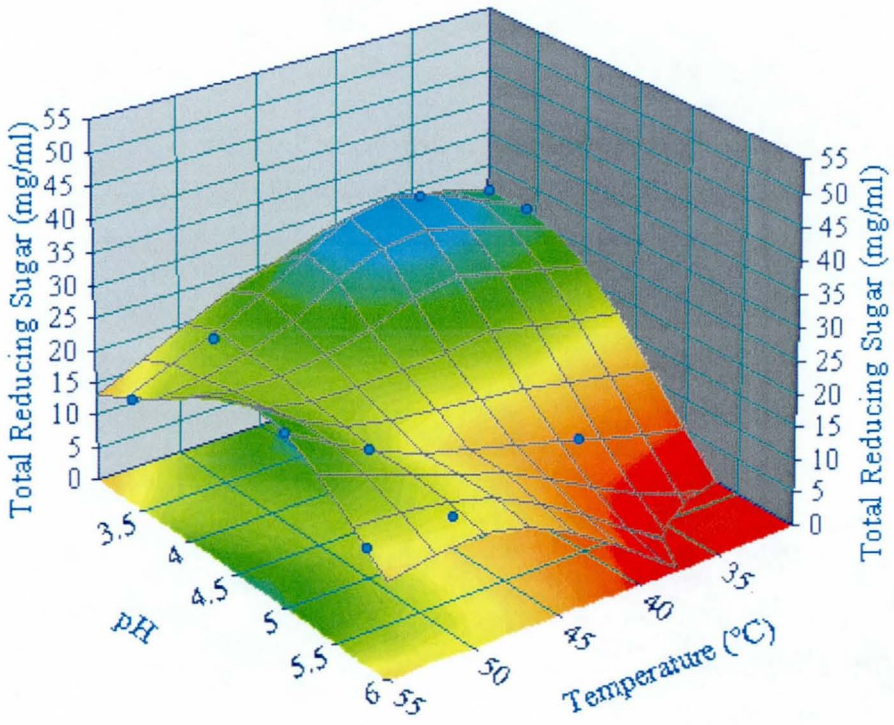


Figure 6.6 Surface Plot of the Corrected* Total Reducing Sugars Released from Wheatfeed after Enzymic Digestion for 24 Hours.

* These were obtained by subtracting the concentrations detected in the control experiments conducted at identical conditions.

The results for individual sugars are contained in Appendix 5. Arabinose and xylose were consistently released in small amounts during both enzymic digestion and control experiments and there is no obvious difference between the 8 and 24 hours experiments. Glucose was released in the greatest amount and followed the pattern of the total reducing sugars shown here.

Figure 6.7 shows the surface plot for protein release after 8 hours digestion. The most obvious feature of this figure is the pronounced minimum at pHs in the region of 4.5 and temperatures below 40°C. High concentrations of proteins were released at low temperatures and pHs. A less pronounced region of high yield is that at low pH and high temperature. The controls (Figure 6.8) reveal an essentially featureless plane with protein yields of 10 mg/ml more or less uniformly spread over the entire region of interest. Figure 6.9 shows the corrected plot, the surface is very similar in appearance to the surface for the uncorrected data.

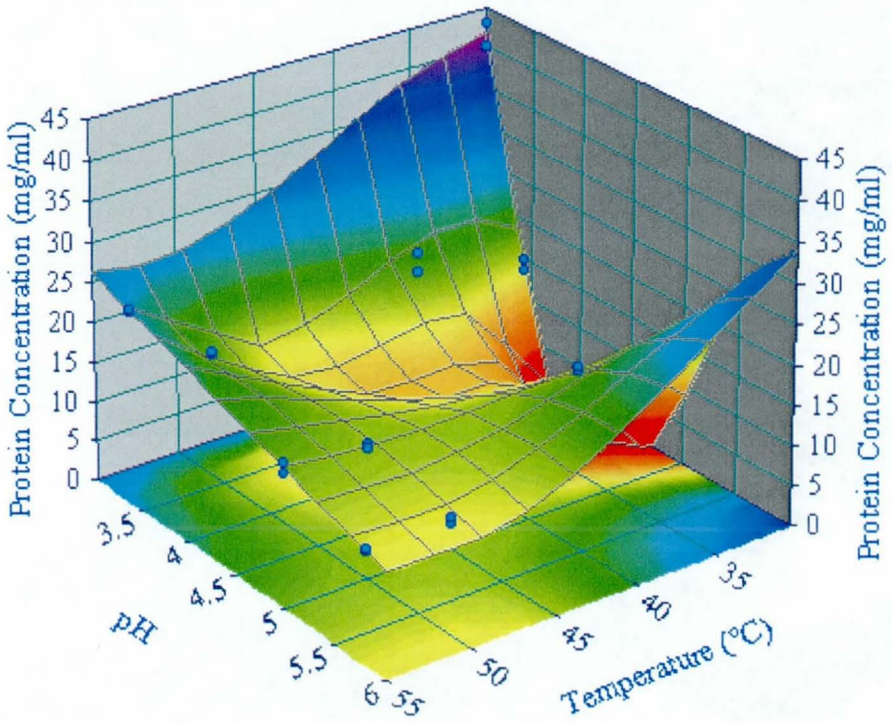


Figure 6.7 Surface Plot of Protein Released from Wheatfeed after Enzymic Digestion for 8 Hours.

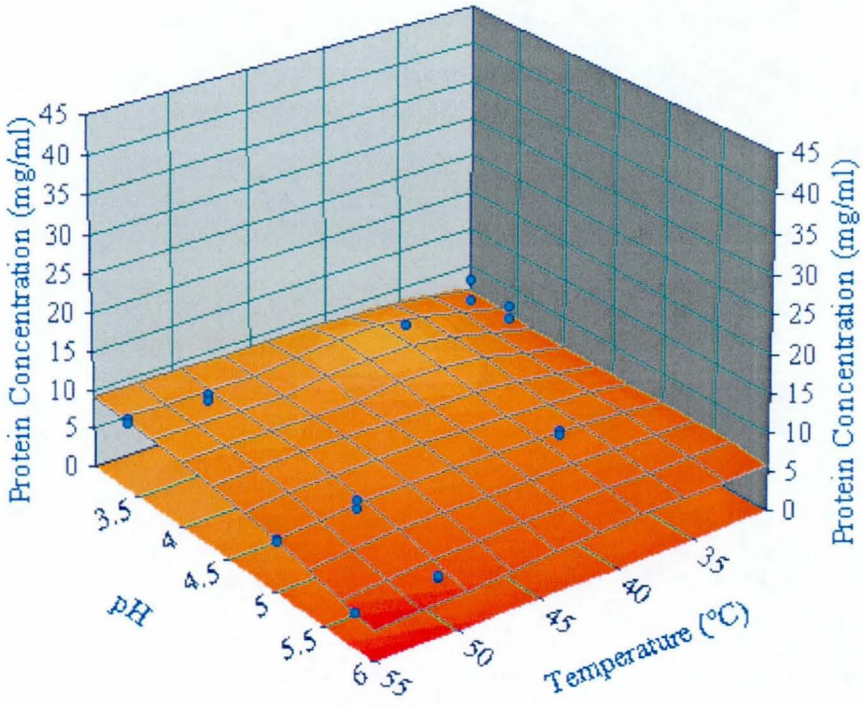


Figure 6.8 Surface Plot of Protein Released from Wheatfeed in the absence of Enzymes for 8 Hours.

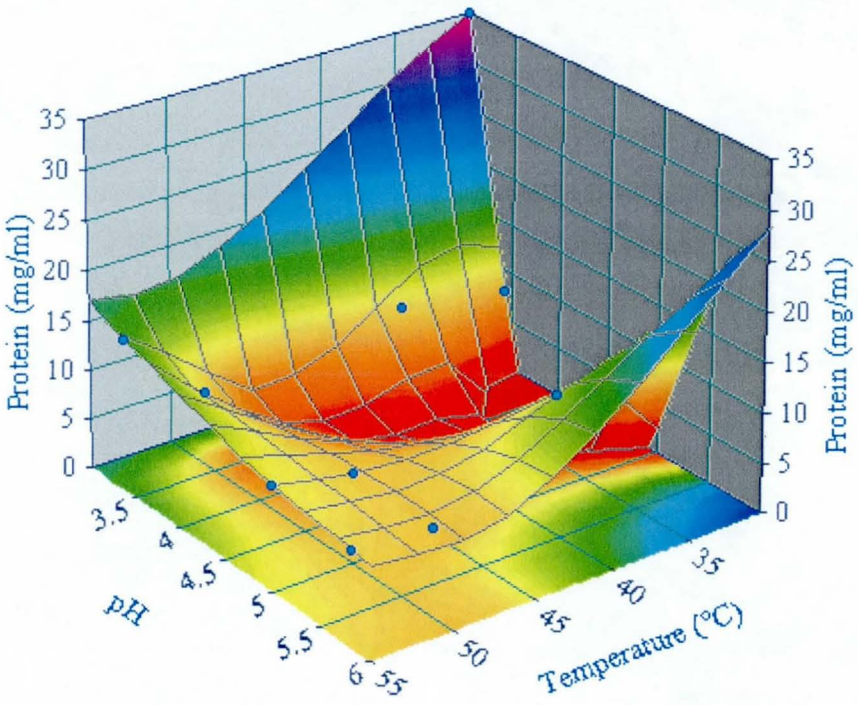


Figure 6.9 Surface Plot of the Corrected* Protein Released from Wheatfeed after Enzymic Digestion for 8 Hours.

* These were obtained by subtracting the concentrations detected in the control experiments conducted at identical conditions.

Figure 6.10 shows the surface plot for protein release after 24 hours digestion. The shape of the surface is similar to that obtained from the 8 hour data, however, the peak at low temperature and high pH gives a result about 10 mg/ml lower. Figure 6.11 shows the corresponding control surface, the data shows a slight maxima at low pH (3.0) and intermediate temperatures (35 to 45°C). As

previously, once the data has been corrected by subtraction of the control data, the overall shape of the surface plot (Figure 6.12) remains essentially unchanged although the drop in protein release as pH increases from low to intermediate pH is steeper.

The suggested optimum conditions for protein release is therefore at pH 3.0 and 30°C, with a sharp decrease in the amount of protein released as the pH increases.

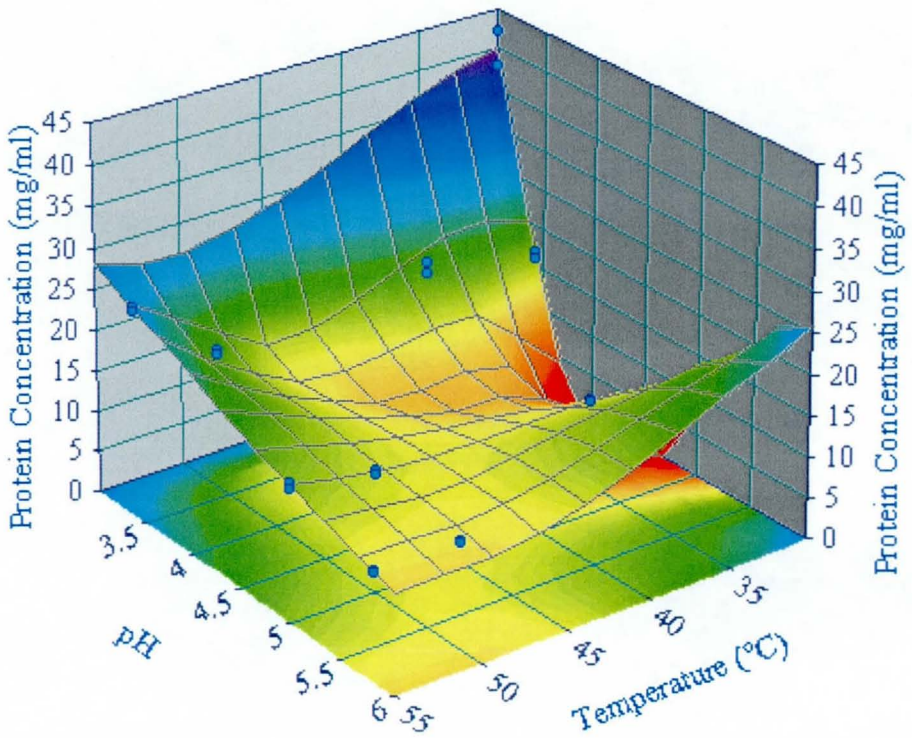


Figure 6.10 Surface Plot of Protein Released from Wheatfeed after Enzymic Digestion for 24 Hours.

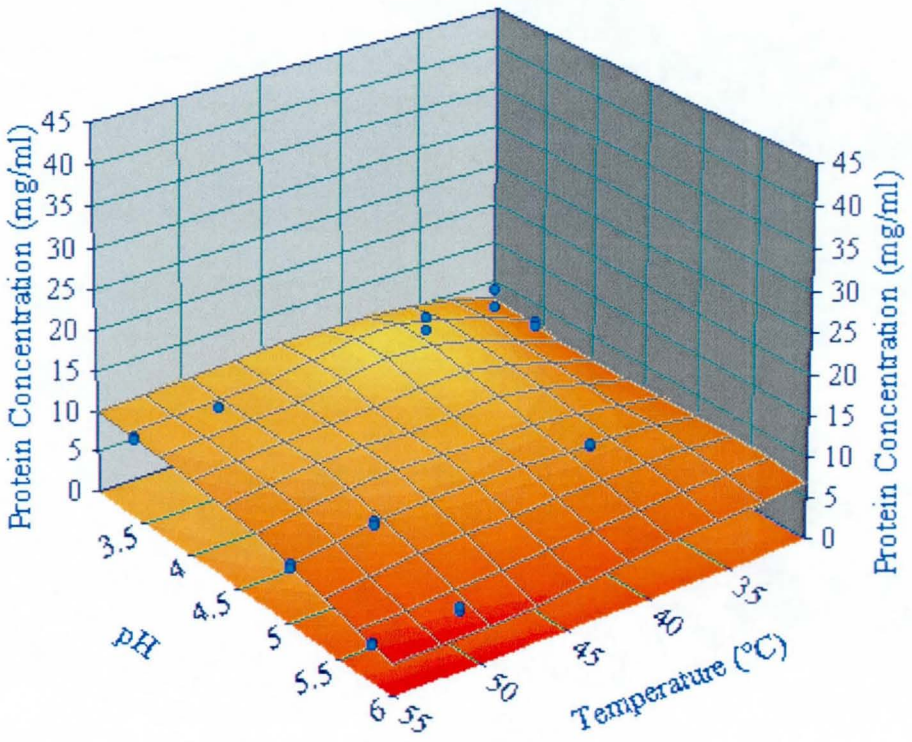


Figure 6.11 Surface Plot of Protein Released from Wheatfeed in the absence of Enzymes for 24 Hours.

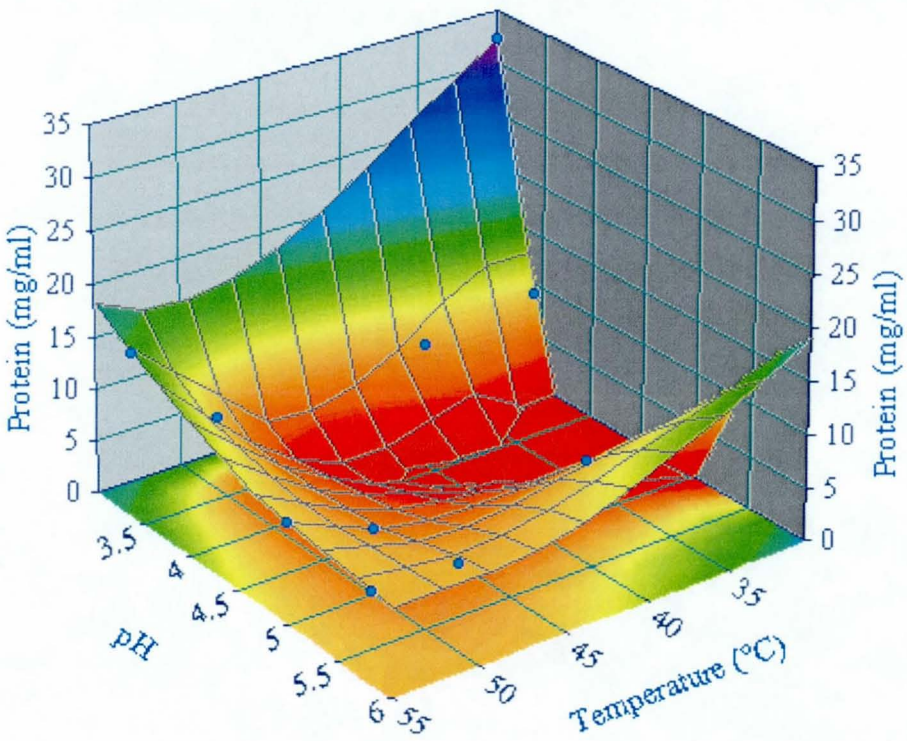


Figure 6.12 Surface Plot of the Corrected* Protein Released from Wheatfeed after Enzymic Digestion for 24 Hours.

* These were obtained by subtracting the concentrations detected in the control experiments conducted at identical conditions.

Using the two optimum conditions for total reducing sugar and protein release a combined optimum was estimated. This was a pH of 3.2 and a temperature of 35°C. The results for this experiment gave a total sugar release of 25.6 mg/ml for the 8 hour experiment and 34.2 mg/ml for the 24 hour experiment and protein concentrations of 21.3 and 22.0 mg/ml respectively.

6.4 Discussion

The experiments described here successfully revealed the presence of optimal conditions for the release of sugars. Both the 8 hour and 24 hour results show a peak at low pH (about 3.5) and low temperatures (about 35 to 40°C) with a somewhat less prominent maximum at conditions described by a temperature of 55° and a pH of 4.5 to 5.0 however, the latter conditions may represent those of a 'descending slope' from a maximum that lies at temperatures above 55°C and possibly at intermediate pHs.

Whereas sugars were released from wheatfeed under the conditions investigated here in the absence of enzymes (Figures 6.2 and 6.5), significantly greater amounts of sugar were solubilised when enzymes were added (corrected Figures 6.3 and 6.6.). There was moreover a distinct advantage in prolonging the digestion time above 8 hours. Figure 6.3 shows that at 8 hours the maximum concentration of sugar solubilised was 18.0 mg/ml but after 24 hours (Figure 6.6) it was 30.0 mg/ml.

Similarly, some protein was released in the absence of enzymes (Figures 6.8 and 6.11) however, substantially greater amounts were solubilised in the presence of enzymes. In contrast to the findings for sugars a higher maximum was obtained after 8 hours digestion rather than after 24 hour digestion (by approximately 10 mg/ml). This could be explained by the released protein denaturing over time in harsher conditions. Proteases are known to be stored in the aleurone layer of the bran (Laszity, 1999) and these might have degraded some of the protein released. The resulting peptides and amino acids would still be nutritionally available but would not be detectable by the Lowry-Peterson assay.

The protein results (Figures 6.9 and 6.12) reveal a region at intermediate pH (4.5) and low temperature (35°C) where the conditions are apparently totally unsuitable for protein solubilisation. Although the apparent descent to zero is clearly an

artefact of the software used to obtain the surface plots, as even in the absence of enzyme (Figures 6.8 and 6.11) at least 5 mg/ml of protein was released at the most unfavourable combination of pH and temperature - and not uncommonly values in the region of 10 mg/ml were achieved. Protein is apparently released at all the extremities of the region investigated, however, the highest yield was obtained after 8 hours at low temperatures and low pH (28 mg/ml).

The combined optimum conditions for sugar and protein release were discovered to lie at pH 3.2 and temperature 35°C. An experiment carried out at these conditions gave results similar to those suggested by the surface graphs with 25.6 mg/ml sugar released from the 8 hour enzymic digestion and 34.2 mg/ml from the 24 hour digestion. Protein release was 21.3 and 22.0 mg/ml respectively.

Waszczynskyj *et al.* (1981) used a hemicellulase, cellulase and pectinase (HCP) enzyme mixture on wheat bran in their work as a pretreatment step for a protein extraction process. They performed experiments to try to find optimum conditions of the mixture and varied the enzyme levels, pH and digestion time. Remarkably they did not investigate the effect of temperature. They found that the best yield was at pH 3.7 (the lowest tested), with maximum enzyme loadings and 7 hours digestion time (maximum tested). The results obtained here would appear to support their findings. Waszczynskyj *et al.* (1981) did not investigate the release of sugars.

Naturally if enzymic digestion of wheatfeed was to be commercialized using combinations of enzymes, commercial enzyme preparations would be employed. These would almost certainly be derived from sources different to the purified enzymes employed here. Moreover, commercial preparations often contain more than one type of enzyme activity, Sanjust *et al.* (2004) found that commercial cellulases often contained xylanase activity. Consequently, experiments such as were described here would need to be repeated using commercial preparations.

6.5 Conclusion

Conditions favouring the release of proteins and sugars from wheatfeed using a mixture of cellulase, hemicellulase and pectinase were identified. Not surprisingly these did not coincide. Most significantly long digestion times – certainly in excess of 8 hours, possibly as high as 24 hours were needed to solubilise sugar but low digestion times favoured protein release (probably substantially less than 8 hours). Strictly speaking it would be misleading to describe the maximum identified here as optimal. True maxima are revealed by regions of descent in all directions from the maxima. An extension of the experiments to regions of pH lower than 3.0 and greater than 5.8 and to temperatures greater than 55°C would therefore need to be conducted. Notwithstanding, pHs in the region 3.5 and temperatures between 35 and 40°C would appear to result in relatively high yields of both proteins and sugars.

7 Experiments on the Digestion of Wheatfeed in a Bioreactor

7.1 Introduction

Whereas the use of purified enzymes as reported in previous chapters provided useful information as to the best combination of enzymes and processing conditions to enhance the nutritive value of wheatfeed, a commercialized process would be reliant on industrially available enzymes. In this chapter therefore, experiments are described using a commercial cellulase preparation 'celluclast', produced by Novozymes (Bagsvaerd, Denmark).

One particular consideration during the scale-up of reacting systems is to determine whether mass transfer rates are significant compared to reaction rates. If they are, then there are implications for the design of the scaled-up reactors. In such cases, any design of reactor must ensure that access to the substrate must be readily granted to the enzyme. An indication as to whether mass transfer effects are significant can be gained by operating a continuously stirred tank bioreactor (CSTR) at two different stirrer speeds and comparing the rates of release of sugars and proteins at each set of conditions. The work conducted here examined the action of the celluclast enzyme at stirrer speeds of 50 and 150 rpm.

Also described here are the effects of particle size on the rate of release of sugars and proteins. Such information could help to determine whether particle size reduction of the wheatfeed is economically beneficial.

Finally, this chapter also includes work using celluclast to digest steam exploded wheatfeed in a CSTR.

7.2 Effect of Agitation Rate on Enzyme Reaction.

7.2.1 Introduction

Experiments were carried out in a stirred bioreactor. Both total reducing sugar and protein release were monitored during enzyme treatment. Stirrer speeds of 50 and 150 rpm were used.

7.2.2 Method

All the experiments were performed using a 2 l continuously stirred tank bioreactor. The method used was as described in section 3.7.2.1 with a few variations. Celluclast enzyme (2.5 ml) was added to 1.5 litres of wheatfeed suspension of 20% (weight/volume) at 50°C. Samples were taken periodically throughout a 5 hour digestion. A control experiment was also carried out using 2.5 ml distilled water in the place of the enzyme and a stirrer speed of 50 rpm.

All samples were frozen to preserve them for subsequent HPLC and Lowry-Peterson protein analysis.

7.2.3 Results

Figure 7.1 shows the release of sugars over time. After five hours digestion at 150 rpm, 15.7 mg/ml of reducing sugars were released, whereas at 50 rpm 14.0 mg/ml were obtained. This compares with a value of 10.6 mg/ml for the control experiment. Although operating at a higher agitation rate does result in a greater final yield of sugars, the effect of agitation rate appears to be slight.

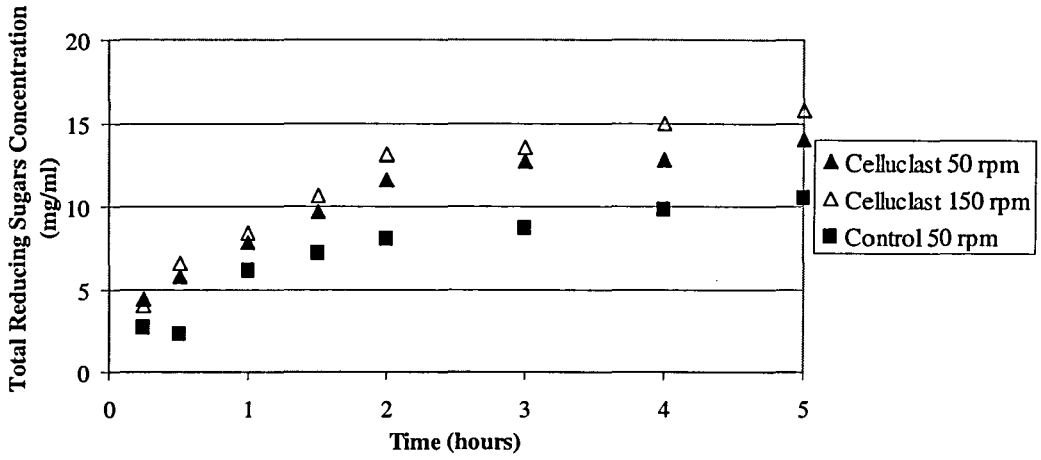


Figure 7.1 The Effect of Stirrer Speed on Sugar Release.

The results for the protein liberation are shown in Figure 7.2. The effect of stirrer speed and indeed the presence of the enzyme, was less marked than was observed for sugars.

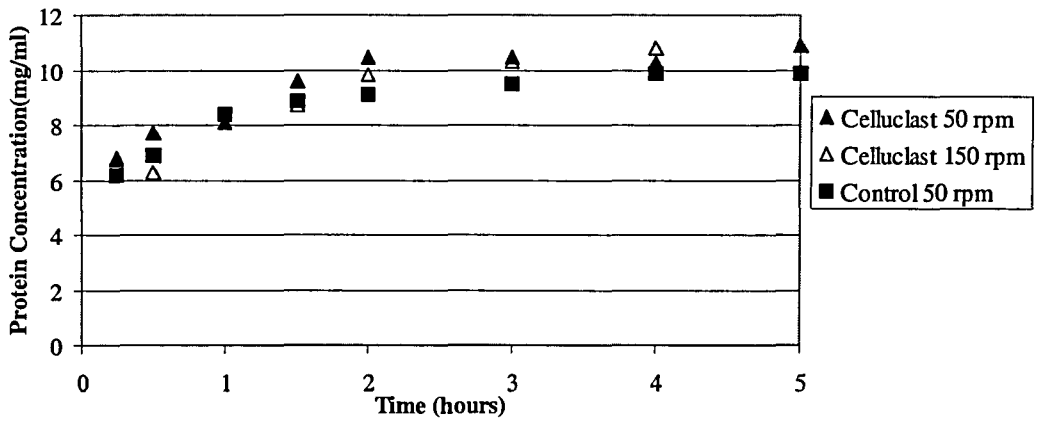


Figure 7.2 The Effect of Stirrer Speed on Protein Release.

7.2.4 Discussion

Enzyme treatment resulted in a higher release of reducing sugars than was obtained for the control (Figure 7.1). Although a higher final yield of sugars was obtained at the higher stirrer speed, the effect of agitation rate was slight. The

observed higher yield of sugars at 150 rpm might have resulted from a decrease in particle size owing to the more vigorous rate of agitation.

An appreciable amount of sugars (10.6 mg/ml) were released during the control experiment. The source of these sugars is likely to be starch which subsequent analysis (section 10) showed was present in wheatfeed at a level of 22.2% (weight/weight).

Protein release (Figure 7.2) seems to be independent of enzyme concentration or stirrer speed, this suggests that it is being solubilised at a rate which is probably only temperature dependent.

These results indicate that the system is not external mass transfer limited, but rather limited by the rate at which the enzyme attacks its substrate.

7.2.5 Conclusion

The finding that the system is not external mass transfer limited has considerable implications for scale-up, provided that the enzyme can be evenly distributed throughout the reaction mixture the precise reactor configuration used should not significantly affect the rate of either protein, or sugar release.

7.3 Effect of Particle Size on the Rate of Sugar and Protein Release.

7.3.1 Introduction

The particle size experiments were designed to investigate the effect of particle size of the substrate on the rate of enzymic digestion. Particle size experiments were carried out both in the 2 l continuously stirred tank bioreactor and at a smaller scale using 50 ml centrifuge tubes after it was discovered that the bioreactor agitator was not powerful enough to stir larger particle size experiments. Wheatfeed was sieved into various size fractions for both

experiments. The fractions were as shown in Table 7.1. For the stationary centrifuge tube experiments an additional fraction was used. This was obtained by milling large particles. Experiments were also carried out using 'as delivered' wheatfeed, i.e. wheatfeed that had not been separated into size fractions, in this report it is referred to as 'un-fractionated wheatfeed'.

Table 7.1 Table of Wheatfeed Fractions Used for Particle Size Experiments.

Particle Size of Fraction, x (μm)
x>839
553<x<839
348<x<553
348<x<197
x<197
x< 197 'milled'*

* This fraction comprised oversized particles (>839μm) milled to <197μm

7.3.2 Bioreactor Method

The bioreactor was used following the basic experimental method as described in section 3.7.2.1. but with the following variations. Riffled wheatfeed and various size fractions obtained from it as described in Table 7.1, were used in different experiments. The enzyme used was 2.5 ml celluclast. The experiment ran for 5 hours with samples taken periodically. The samples were frozen and later subjected to HPLC and Lowry-Peterson protein analysis. Two control experiments were carried out using the fines and un-fractionated wheatfeed respectively and used 2.5 ml buffer in the place of the enzyme.

7.3.3 Bioreactor Results

The results for the release of total reducing sugar with time are shown in Figure 7.3. The control composed of fines released the lowest amount of total reducing sugars achieving a final concentration of 5.0 mg/ml after 5 hours. A control

comprised of un-fractionated wheatfeed released rather more reducing sugars (10.6 mg/ml). The fines fraction with enzyme released 9.2 mg/ml, after 5 hours, which is less than the un-fractionated control. Treatment of a medium sized fraction (348<x<553μm) with enzyme lead to the release of 12.0 mg/ml after 5 hours whereas with un-fractionated wheatfeed a final sugar concentration of 14.0 mg/ml was obtained. Attempts to use particles of size greater than 348μm at a concentration of 200 g/l were unsuccessful owing to the fact the agitator was not powerful enough to stir the buffer-enzyme and wheatfeed mixture. The results obtained here show that higher yields and higher rates of sugar release were obtained from the larger size fractions, with the highest yields obtained from the unfractionated wheatfeed.

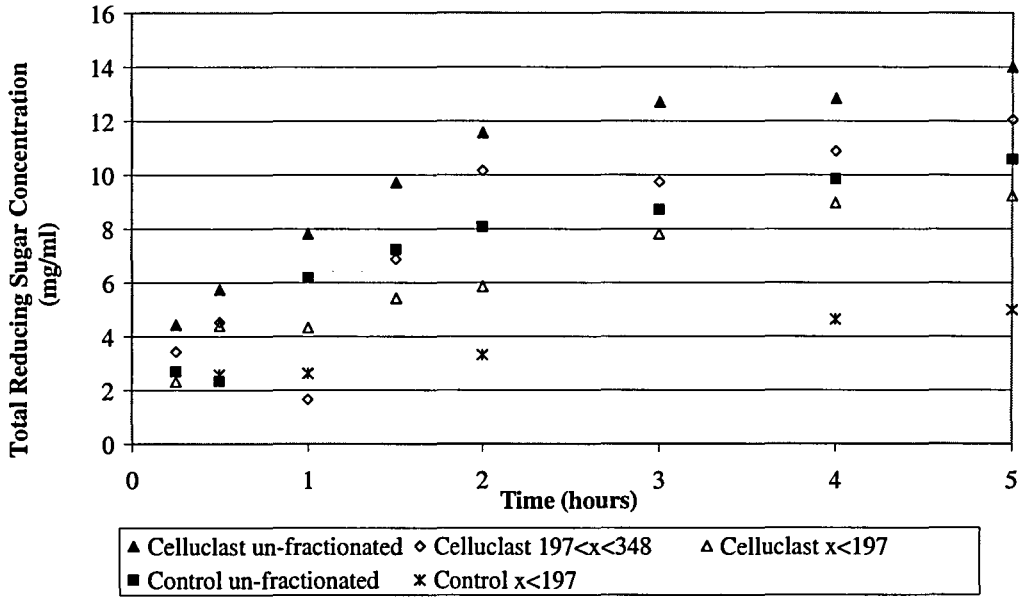


Figure 7.3 The Effect of Particle Size on Reducing Sugar Release.

The protein results are shown in Figure 7.4. The control composed of fines gave the lowest amount of protein liberation, with a maximum of 6.4 mg/ml after 5 hours. The un-fractionated control gave 9.9 mg/ml in the same time period. The fines fraction with enzyme released 7.9 mg/ml after 5 hours, the medium sized

fraction (348<x<553μm) released 10.6 mg/ml and the un-fractionated wheatfeed released 10.9 mg/ml. As for the release of sugars, the highest protein yields were obtained from the larger particle size fractions.

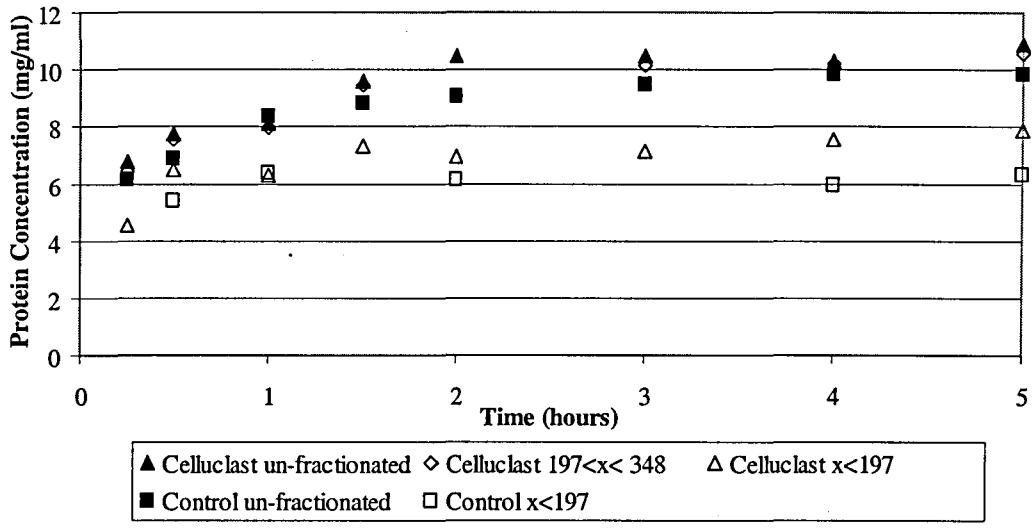


Figure 7.4 Effect of Particle Size on Protein Release.

7.3.4 Stationary Centrifuge Tube Method

In order to investigate the effects of particle size greater than 553 μm, a series of experiments were conducted using 50 ml centrifuge tubes. There was no mixing to ensure that the problems encountered with the continuously stirred tank bioreactor would not be repeated. The results are described in section 7.3.5.

Smaller scale experiments were performed using all the fractions of wheatfeed listed in Table 7.1. The fines milled fraction was also included, this comprised particles greater than 839 μm that had been milled to a size of less than 197 μm. Experiments were executed in duplicate. Centrifuge tubes (50 ml) were loaded with 5 g of the relevant wheatfeed fraction. Buffer-enzyme solution (25 ml

including 6.66 µl enzyme) was heated for 30 minutes in a water bath at 50°C until thermal equilibrium was reached. Next, the 25 ml buffer–enzyme solution was added to each of the centrifuge tubes, capped and mixed. The centrifuge tubes were placed in the water bath and allowed to digest for 24 hours with no shaking.

After 24 hours the tubes were removed and centrifuged for 10 minutes at 10,000 rpm and 10°C. The supernatants were taken and frozen for subsequent analysis for sugars and protein.

7.3.5 Stationary Centrifuge Tube Results

Figure 7.5 shows the results obtained for sugar release. It can be seen that the fines obtained by milling large particles liberated less of any of the sugars (except glucose) than any other sample including the fines fraction. If the results from the other fractions are compared, the amount of xylose released was in the range of 4.3 to 10.7 mg/ml. The maximum amount of xylose was released from the largest particle fraction, the least is released from the fines fraction with the sugar yield decreasing as the particle size decreases. Arabinose is released in the range of 1.8 mg/ml to 3.1 mg/ml. The greatest release was achieved using the $553<x<839$ particle size fraction. However, the four largest fractions gave results within a fairly narrow range (2.9 to 3.1 mg/ml). The lowest yield was obtained from the fines fraction.

The fructose liberation results show that the amount of this sugar released from the four larger particle size fractions lay within the range of 4.4 to 5.2 mg/ml. The fines fraction again gave a lower result at 3.5 mg/ml. Glucose was liberated in the range of 14.6 to 18.4 mg/ml.

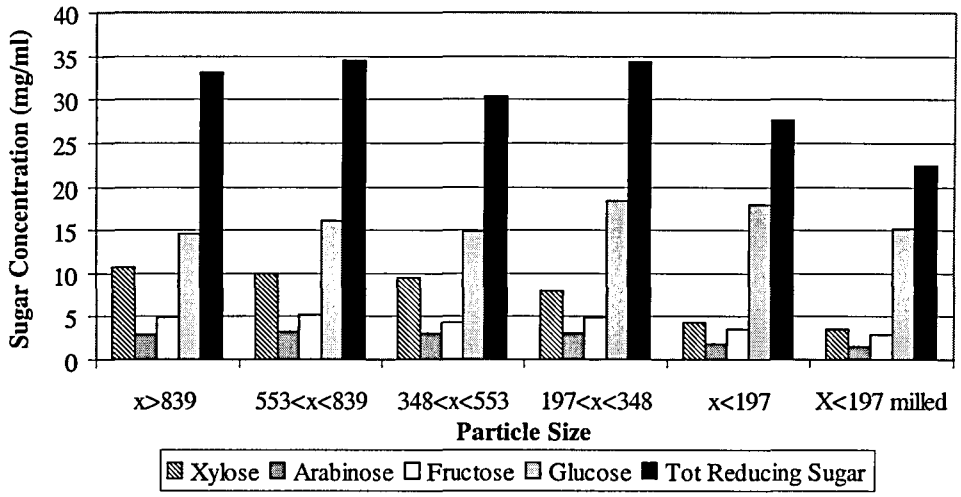


Figure 7.5 The Effect of Particle Size on Sugar Release.

The results for protein release (Figure 7.6) reveal similar yields for all particle size ranges, but somewhat higher yields were achieved for the larger end of the range for particle sizes. The milled fines again gave the lowest result.

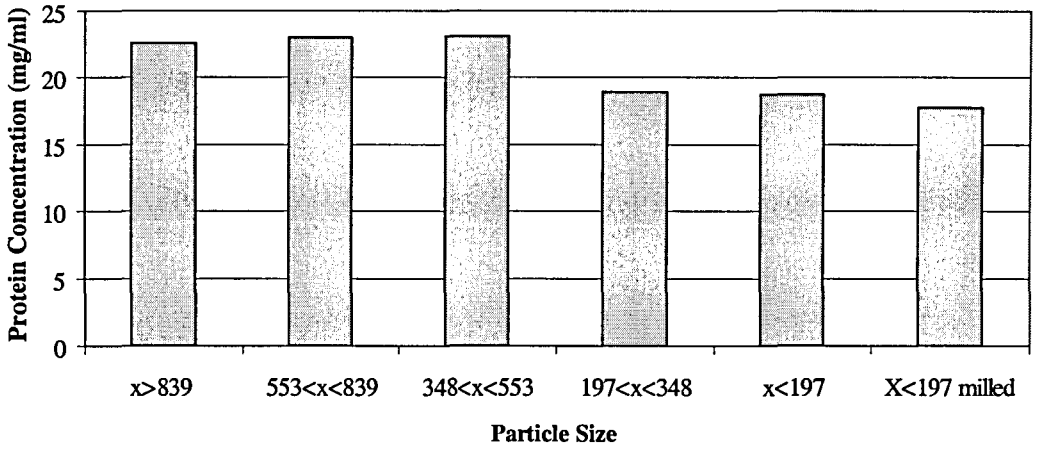


Figure 7.6 The Effect of Particle Size on Protein Release.

7.3.6 Particle Size Experiments Discussion

Overall the particle size experiments show a general trend that the smaller particle size fractions released less sugar and protein than the larger particles. The agitator of the large bioreactor was not powerful enough to stir the two largest particle size fractions although the experiments which were carried out on the smaller particles and fines, suggest that larger particles resulted in greater sugar release. This was an unexpected result; in normal circumstances reducing the particle size should result in an enhanced rate of release of sugars and proteins. One possible explanation for these findings is that various size fractions of the wheatfeed are obtained from different parts of the wheat during milling and therefore have different compositions. To investigate this possibility the largest particles (>839 μm) were milled to a size comparable to the fines fraction (<197 μm) and then digested under stationary conditions. The results obtained did not show significantly different patterns of sugar release. Unlike the bioreactor, samples could not be taken during the experiment so there are only final values available and no kinetic data was available. However, the possibility still remains that the fines were composed of those elements of the grain that are intrinsically less susceptible to attack by cellulase enzymes.

The protein results showed that the release of protein decreased as the particle size decreased, this could be due to larger particles containing more protein to release than smaller particles, or it could be due to mechanical shear breaking the proteins down during upstream processing. These smaller proteins may not be detected by the Lowry-Peterson assay.

More revealing results might have been obtained if a more powerful motor could have been fitted to the stirrer in the bioreactor so that kinetic data could have been obtained.

7.3.7 Particle Size Experiments Conclusion

The experimental results were inconclusive. The agitator of the bioreactor was not powerful enough to perform a digestion of all the particle size fractions for comparison and the following stationary centrifuge tube digestion results gave insufficient data for a conclusion to be achieved.

7.4 Effect of Steam Explosion On the Rate of Sugar and Protein Release.

7.4.1 Introduction

Initially the effects of pretreating the wheatfeed substrate by steam explosion were evaluated using substrate that had been pretreated and dried and at a much smaller scale than here (Appendix 4). If steam pretreatment were to be used in an industrial process, it is likely that freshly pretreated wheatfeed would be used.

Enzymic digestion (24 hour) of freshly steam exploded wheatfeed in the 2 litre bioreactor was carried out to evaluate how such treatment would effect the results in an industrial process. The steam exploded wheatfeed was not dried prior to use in the digestion as in an industrial environment this step would be omitted.

7.4.2 Method

The steam explosion was carried out following the procedure detailed in section 3.4.2. The steam exploded wheatfeed that was required for this experiment was produced in 30 g batches that were subsequently pooled. The pretreated wheatfeed was then digested in the 2 l bioreactor following the method described in section 3.7.2.1. The enzyme used was 25 ml celluclast and 1200 ml citrate buffer was used.

7.4.3 Results

The results for the steam exploded wheatfeed run (Figure 7.7) showed that there was a small increase in the initial rate of reducing sugars released for the steam exploded wheatfeed compared to that of the un-pretreated wheatfeed. However after 15 hours there are is no significant difference between the yield of sugars obtained.

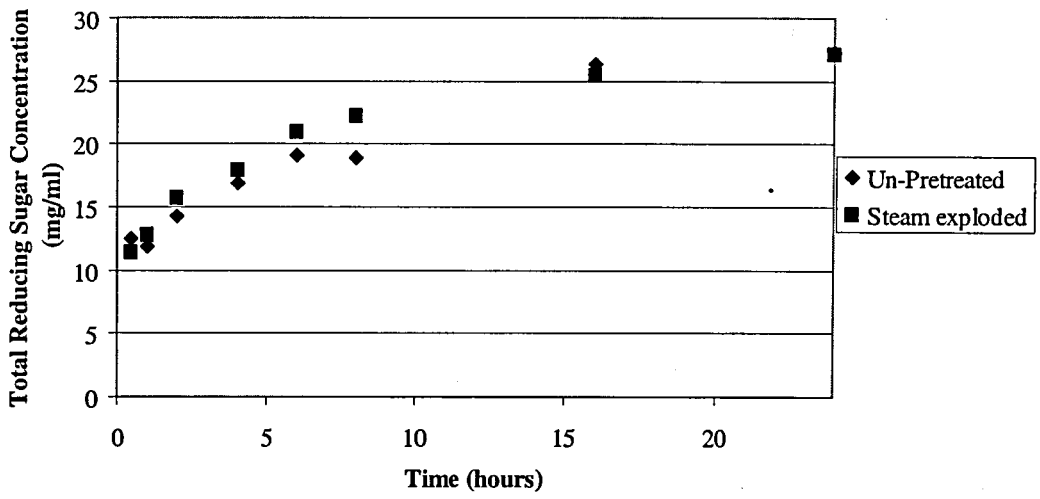


Figure 7.7. Total Reducing Sugar Released for Steam Exploded and Un-pretreated Wheatfeed After 24 Hours Digestion with Celluclast.

Figure 7.8 shows the results for individual sugars released during the digestion. The un-pretreated results are shown as solid symbols and the steam exploded results are shown as open symbols. The results show that there was more xylose released from the steam exploded wheatfeed than the un-pretreated wheatfeed during the earlier part of the experiment although after 16 hours very similar results were obtained from both experiments. After 24 hours the xylose released from the un-pretreated wheatfeed had marginally exceeded that of the steam exploded wheatfeed to reach a value of 27.3 mg/ml.

The arabinose results were very low for the un-pretreated wheatfeed with values less than 0.6 mg/ml. No arabinose was liberated from the steam exploded

wheatfeed. Less fructose was liberated from steam exploded wheatfeed than from un-pretreated wheatfeed with a final difference of 3.5 mg/ml after 24 hours.

More glucose was released from the steam exploded wheatfeed than from the un-pretreated wheatfeed. At first the release from the un-pretreated wheatfeed was higher than that of the steam exploded wheatfeed but with time the steam exploded sample released more glucose leading to a difference in final yield of 4.8 mg/ml.

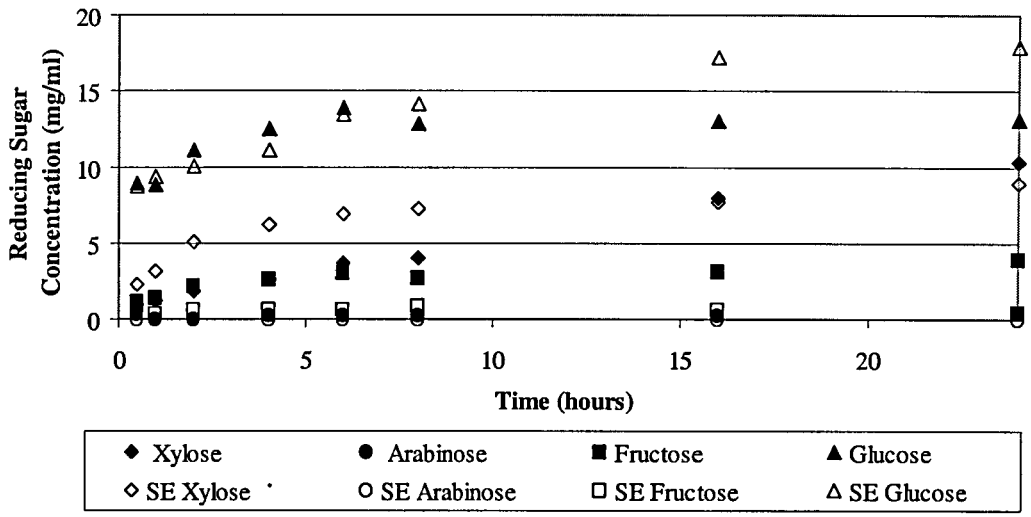


Figure 7.8 Individual Sugars Released During Steam Exploded Wheatfeed and Un-pretreated Wheatfeed Enzymic Digestion.

Figure 7.9 shows the final yields of sugars liberated from the wheatfeed and steam exploded wheatfeed after 24 hours digestion. The graph shows that the concentration of xylose and fructose present in the sample is less for the steam explosion samples than for the un-pretreated sample. Arabinose present in low amounts from un-pretreated wheatfeed is absent from the steam exploded wheatfeed. The figure shows that steam explosion led to an increase in glucose release from 11.9 mg/ml to 17.9 mg/ml.

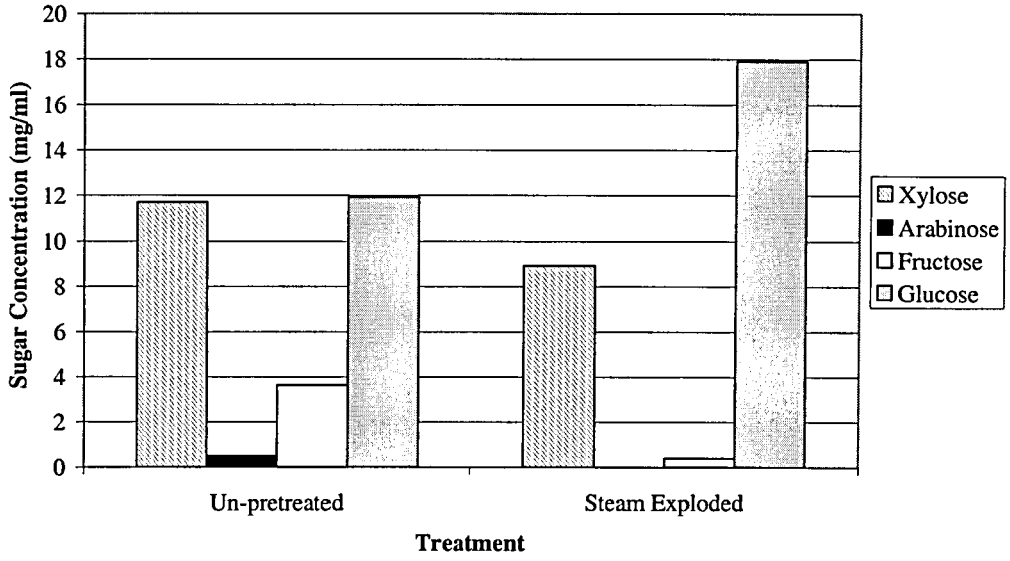


Figure 7.9 The Composition of Sugars Released after 24 Hours Digestion with Celluclast for Steam Exploded and Un-pretreated Wheatfeed.

The protein results (Figure 7.10) show that the yields of protein released from the steam exploded wheatfeed was lower than that from the un-pretreated wheatfeed with final results after 24 hours digestion of 9.6 and 18.6 mg/ml respectively.

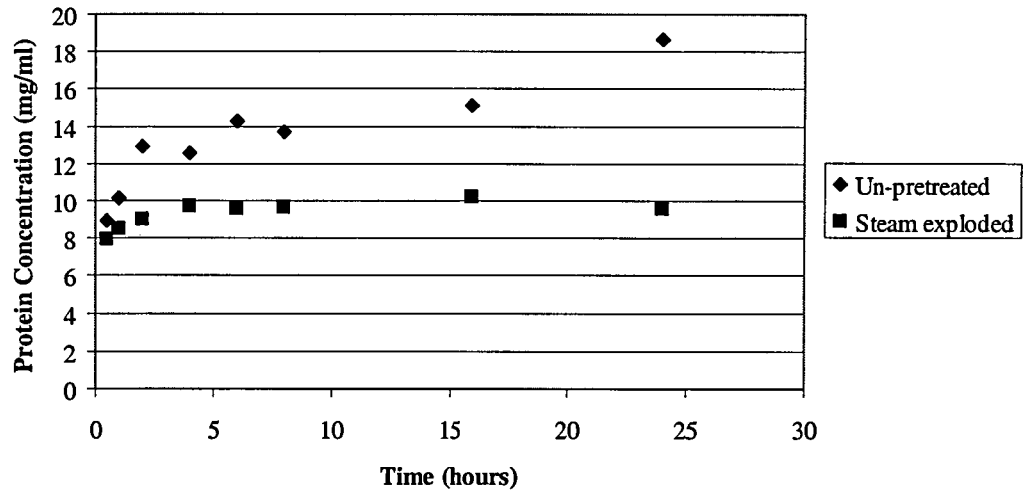


Figure 7.10 Protein Released for Steam Exploded and Un-pretreated Wheatfeed After 24 Hour Digestion with Celluclast.

7.4.4 Discussion

Steam explosion results in an altered pattern of sugar release. Arabinose and fructose are liberated in smaller quantities from steam exploded wheatfeed than un-pretreated wheatfeed, this could be because these sugars are degraded in the steam explosion process. In contrast, more xylose is liberated from steam exploded wheatfeed than from the un-pretreated wheatfeed during the early stages of digestion. Thereafter the rate of release slows and the final result after 24 hours is less than that of un-pretreated wheatfeed. Under severe steam explosion conditions sugars may degrade during the pretreatment step causing a loss of substrate (Soderstrom *et al.*, 2003) this could explain the reduced arabinose and fructose release from steam exploded wheatfeed. Hemicellulose comprises a xylose backbone with arabinose side chains (Edwards *et al.*, 2003), if the side chains are degraded by the steam explosion, better access to the xylose and the components closely associated with it might occur and consequently more xylose is released in the early stages of the digestion. All the available xylose might therefore be released early. In the case of the steam exploded sample the value of available xylose could be less than for the un-pretreated sample as some of the xylose may also have been degraded during the steam explosion process.

Despite modest differences in the patterns of individual sugar release the overall total quantity of reducing sugar released during the digestion of steam exploded wheatfeed (27.2 mg/ml) is approximately the same as for un-pretreated wheatfeed (27.3 mg/ml)

The amount of protein released is unambiguously lower for the steam exploded wheatfeed. This supports findings from the initial steam exploding experiments discussed in Appendix 4. The low yields are probably due to degradation of protein during the steam explosion process. However, the degradation products of protein are likely to be amino acids and oligopeptides which will still form a digestible part of the product.

The results show that steam explosion does not seem to enhance the final amount of sugar release as suggested by results from the initial steam explosion pretreatment experiments (Appendix 4) however this may be because these experiments use a longer digestion time (24 hours rather than 4) or due to differences in batches of wheatfeed. Whether steam explosion pretreatment is worth considering as a pretreatment step, may depend on the enzymic digestion time required.

7.4.5 Conclusion

Steam exploding the wheatfeed with no drying, prior to enzymic digestion does increase the initial rate of digestion of wheatfeed. However steam explosion resulted in the degradation of some sugars and the final yields after 24 hours were lower for steam exploded wheatfeed than for un-pretreated wheatfeed.

7.5 Overall Conclusion

The results from the effects of agitation rate suggest the system is not external mass transfer limited which means that provided that the enzyme can be evenly distributed throughout the reaction mixture the type of reactor employed for scale-up will not significantly affect the rate of either protein or sugar release.

The results for the particle size experiments were inconclusive. The experiment could not be performed in the manner required as the agitator was not powerful enough to mix slurries using larger particle sizes. The experiments set up to compensate for these inadequacies, namely stationary centrifuge tube experiments, did not give sufficient data for a conclusion to be reached.

The steam explosion results showed that steam exploding the wheatfeed immediately prior to digestion elevates the amount of sugar released for the early stages of digestion, however the final sugar liberation after 24 hours is lower for steam exploded wheatfeed than for un-pretreated wheatfeed.

8 Comparison of the Performance of Commercial Enzymes

8.1 Introduction

Any commercialization of the enzymic treatment of wheatfeed would be totally reliant on sources of enzymes which were commercially available. It is important to evaluate the ability of different enzymes to digest the wheatfeed in order that an informed decision can be made as to which enzyme will be the most economical to use taking into considerations both yields and costs. In this chapter a comparison of such enzymes is described. From these comparative experiments a single enzyme was chosen with which to conduct a number of further experiments, aimed at investigating the relationship between the amount of enzyme used and the release of sugars. These studies were conducted using a stirred bioreactor.

In evaluating the feasibility of a particular process, it is useful to be able to model the progress of any reaction stages so that the effects of changes can be predicted and used to arrive at an overall optimization of the process. The experiments described above yielded data of the sort that was amenable to modelling mathematically. Two approaches were taken; the first involved a kinetic expression whilst the second made use of a purely empirical relationship. The results obtained from both methods are discussed here.

8.2 Comparison of Enzymes Method

Experiments were carried out in the bioreactor following the basic method detailed in section 3.7.2.1. The enzyme used (25 ml) was varied for each experiment, viscozyme, celluclast, ultraflow, ronozyme and roxozyme were considered. Samples were taken periodically over the 24 hour digestion and frozen for further analysis.

Experiments were also carried out using different amounts of celluclast 25 ml, as previously discussed, then 12.5 ml. These results were then used in a model to predict the amount of enzyme needed to release all the sugar from the wheatfeed, after which it was predicted that no additional enzyme would increase the yield. The experiment with the calculated value of enzyme was performed, as was one with an excess of enzyme and another experiment with less than the required amount of enzyme. In all these experiments the total liquid volume was kept constant.

Prior to analysis, samples were defrosted, mixed, placed in micro-centrifuge tubes and centrifuged for 5 minutes at 10°C. The supernatants were taken and filtered into clean centrifuge tubes before being centrifuged for another 5 minutes. The samples underwent HPLC sugar and Lowry-Peterson protein analysis.

8.3 Comparison of Enzymes Results and Discussion

The results gained for the different sugars against time are shown in Figure 8.1. The use of viscozyme led to the release of the highest concentration of reducing sugars (42.1 mg/ml) after 24 hours digestion, this was followed by ultraflow (29.1 mg/ml), celluclast (27.7 mg/ml), roxozyme (26.4 mg/ml) and finally ronozyme (8.9 mg/ml). The results for ronozyme show a final yield lower than that obtained after 16 hours digestion, with a decrease from 16.2 mg/ml to 8.9 mg/ml sugar liberation. The control liberated 10.7 mg/ml.

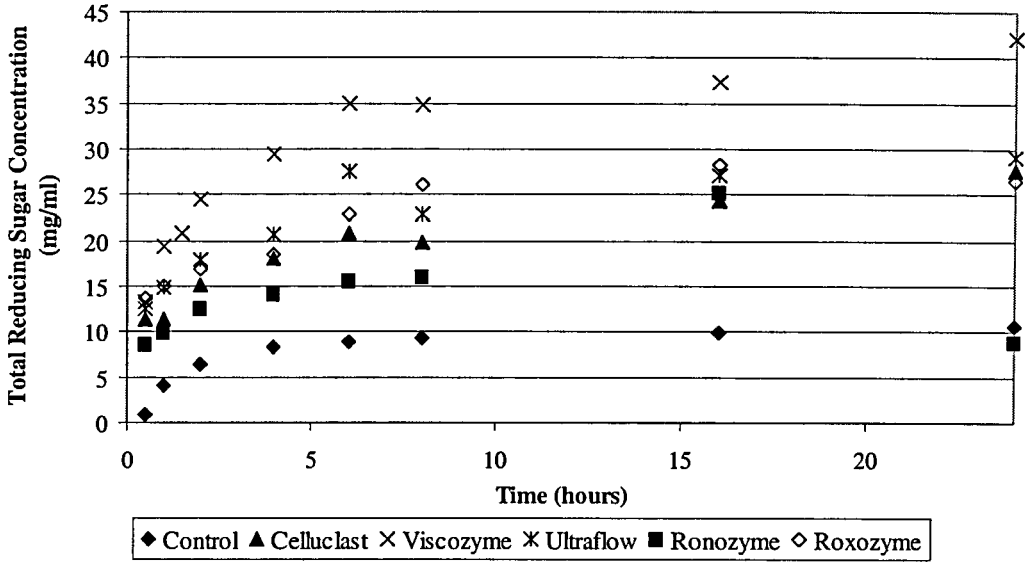


Figure 8.1 Total Reducing Sugars Released from Wheatfeed During 24 hours Digestion with Various Commercial Enzymes.

Figure 8.2 shows that the enzymes differed in their pattern of digestion as manifested by the different proportion of individual sugars released. Viscozyme released predominantly glucose, whereas celluclast released a larger percentage xylose than any of the other enzymes (37%).

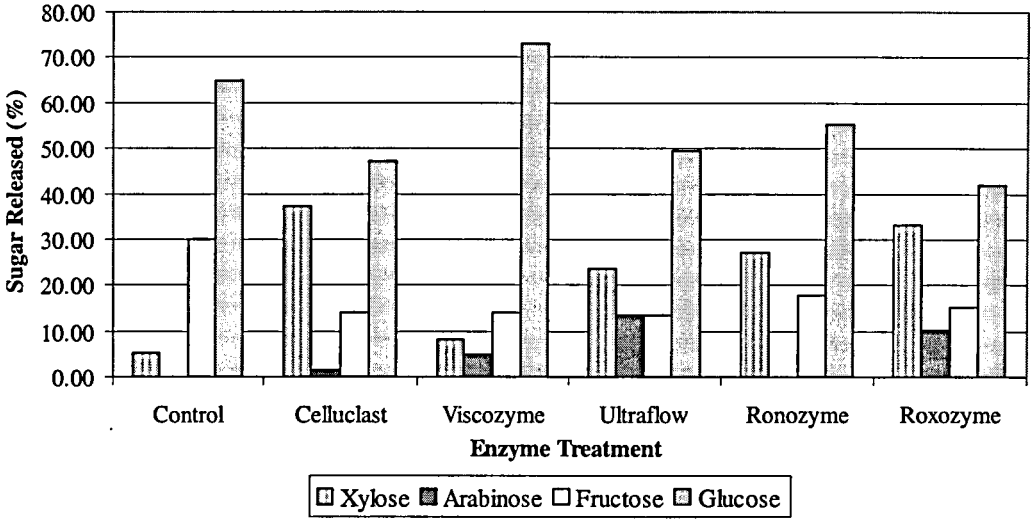


Figure 8.2 The Percentage of Different Reducing Sugars Released after 24 Hours Digestion with Various Enzymes.

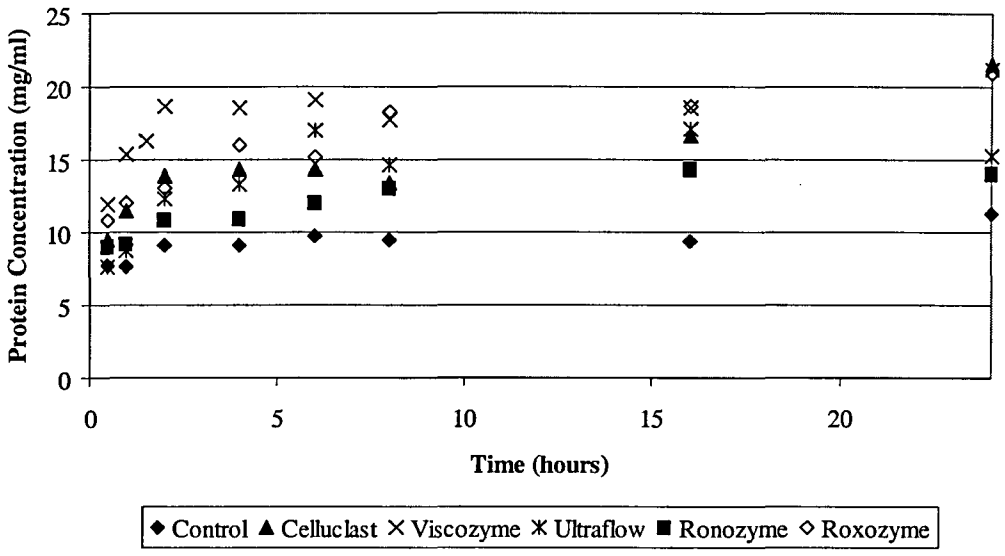


Figure 8.3 Protein Release from Wheatfeed during 24 Hour Digestion with Various Commercial Enzymes.

Figure 8.3 shows the protein released by the various enzymes over 24 hours. These results reveal less discrepancy between the different enzymes than was observed for the release of sugars (Figure 8.1). Celluclast released the most protein giving a protein liberation of 21.5 mg/ml, this was followed by viscozyme (21.1 mg/ml), roxozyme (20.9 mg/ml), ultraflow (15.3 mg/ml) and finally ronozyme (14.1 mg/ml). The control released 11.3 mg/ml.

8.4 Comparison of Enzymes Conclusion

Although the use of viscozyme resulted in the highest yield of reducing sugars (Figure 8.1), the pattern of digestion (Figure 8.2) suggests that the enzyme celluclast appears to attack the xylans and other hemicellulosic components of wheatfeed more effectively. Coupled to this is the high yield of protein obtained (Figure 8.3) using celluclast. It was therefore decided to conduct further experiments with the latter.

8.5 Kinetic Modelling

The protein release was measured after various celluclast loadings were added to the bioreactor and digestions carried out for 24 hours. The results are shown in Figure 8.4. Generally the greater the enzyme loading the more protein is released. This is an expected result, however there is a drop in protein concentration at long digestion times that appears to be significant. One explanation could be that the temperature of 50°C denatures the protein slowly over a period of time. Alternatively it could be that there may be natural proteases in the wheat bran which are released as the fibre is broken down by the enzyme. Once released, the proteases are free to break down the released protein.

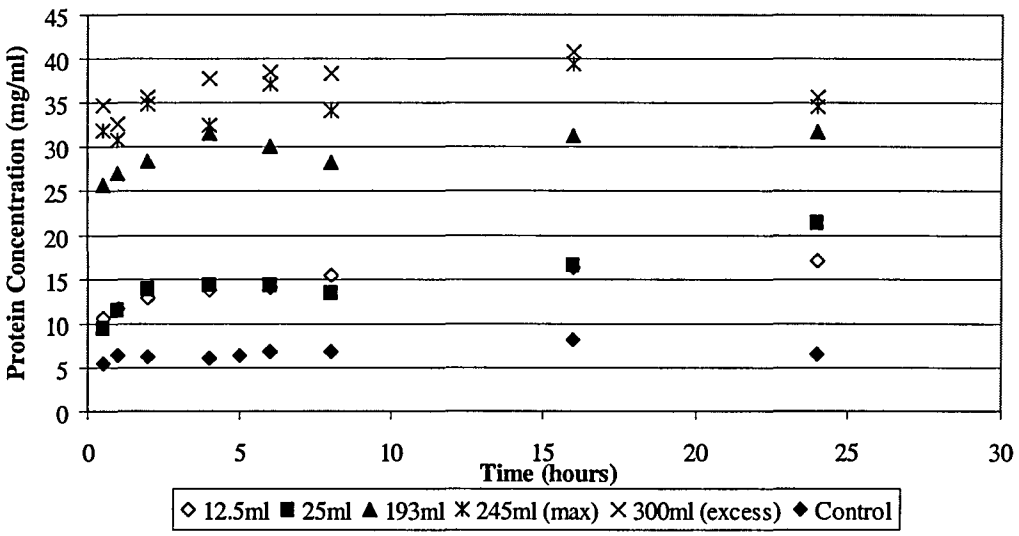


Figure 8.4 Protein Released During Wheatfeed Digestion with Different Enzyme Loadings.

An attempt was made to use Michaelis-Menten kinetics to describe the sugar release into solution during digestion over 24 hours with various celluclast loadings, but there was a clear deviation from Michaelis-Menten predictions and

the experimental results achieved. This is demonstrated by plotting the rate against the product concentration as predicted by Michaelis-Menten (Equation 8.1) with the experimental results.

$$R_P = K_1\left(\frac{C_{E0}(C_{A0}-C_P)}{K_M+(C_{A0}-C_P)}\right) \qquad (8.1)$$

Where

K_1 = constant (mg/ml)/h.

K_m = Michaelis constant (mg/ml).

C_{E0} = The enzyme loading (mg/ml).

C_{A0} = The total sugar available in the fibre (mg/m).

C_P = The concentration of sugar (mg/ml).

R_P = Rate of production of sugar (mg/ml)/h.

The Michaelis-Menten model takes into account the amount of substrate used and the initial rate of the reaction (Lehninger, 1975).

The results for *Rp* against *Cp* were plotted and a curve fitting program used to fit the Michaelis-Menten equation to the data, the resulting graph is shown below for the case of 25 ml enzyme loading in the bioreactor. The graph (Figure 8.5) shows the data points and the fitted line. It can be clearly seen that the model does not give a good fit.

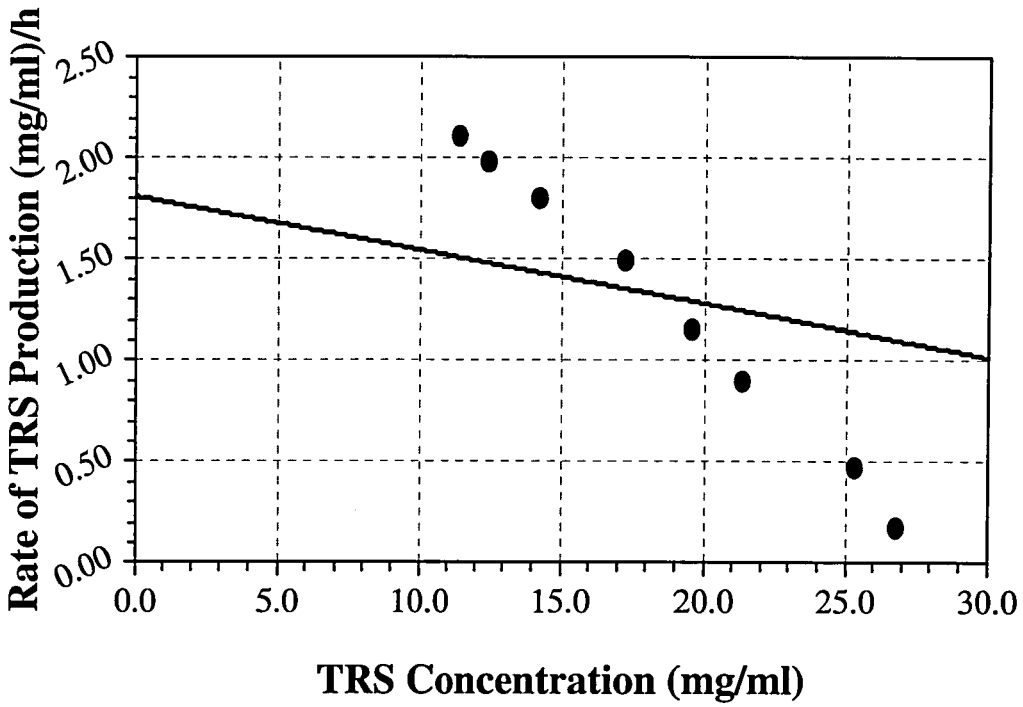


Figure 8.5 The Michaelis-Menten Model Fitted to the Data.

Where TRS means total reducing sugar.

The results appear to show that the measured rate was greater than that predicted by the Michaelis-Menten kinetics (seen more clearly in Figure 8.5).

Hellgardt (2002), proposed an expression that accounted both for enzyme concentration and the possible effects of product inhibition on the maximum concentration of sugar released during digestion by an enzyme. The expression was as follows

$$C_{P\max} = C_{A0} = C_{P0} + KC_{E0}^{n} \qquad (8.2)$$

Where

C_{pmax} = the maximum concentration of product (sugar) that can be obtained at a given enzyme loading (mg/ml).

C_{E0} = The enzyme loading (mg/ml).

C_{A0} = The total sugar available in the wheatfeed (mg/ml).

C_{P0} = The initial concentration of sugar estimated from experimental data (mg/ml).

K = A constant $(ml/mg)^n$.

n = A constant.

C_{A0} was taken as 68.3 mg/ml. This was calculated from values given for the weight percentage of hemicellulose and cellulose in bran from literature (Fisher, 1985).

In this model it is assumed that the final total reducing sugar concentration would increase as the amount of enzyme added was increased and that there existed a maximum level of enzyme which would release all the sugar bound in the wheatfeed. Enzyme loadings above this level would, according to the model, not result in additional sugar liberation.

An experiment was carried out in the 2 l bioreactor using 300 g wheatfeed and 1525 ml enzyme buffer solution following the procedure described in section 3.7.2.1. The amounts of celluclast used were 12.5 ml and 25 ml (concentrations in the bioreactor of 9.84 and 19.67 mg/ml respectively).

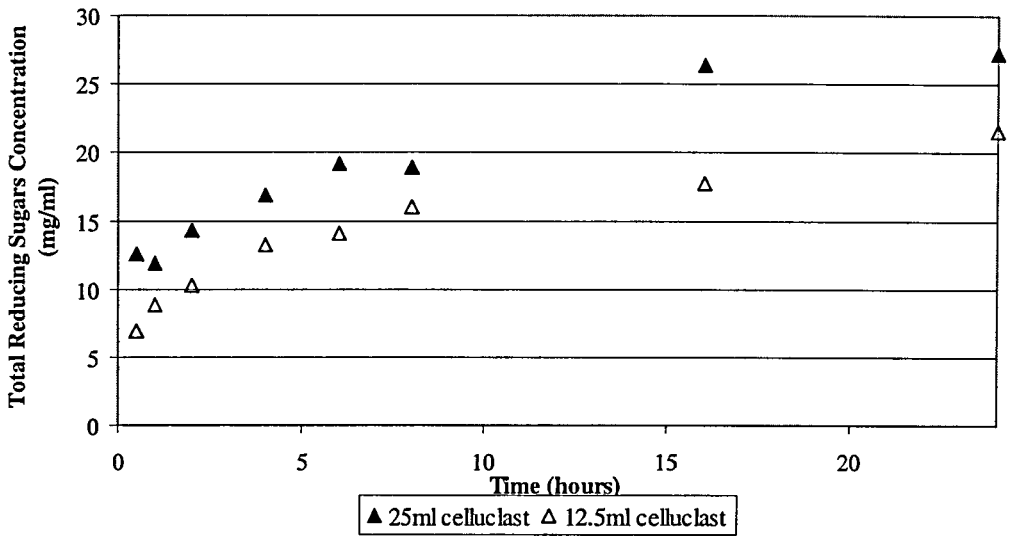


Figure 8.6 Total Reducing Sugar Release for Two Enzyme Loadings.

Figure 8.6 shows that at the higher level of celluclast a greater yield of reducing sugars was released.

By using the experimental data for the two enzyme levels at 12.5 ml and 25 ml and a C_{P0} of 7.5 mg/ml in Equation 8.2, simultaneous equations were set up to find the constants K and n. These were used in the equation with the maximum total reducing sugar available (68.3 mg/ml) to find the concentration of enzyme which will result in the release of all of the available sugar.

The results were as follows;

K= 4.552

n = 0.493

Therefore

$$C_{P\max} = C_{P0} + 4.552C_{E0}^{0.493} \qquad (8.3)$$

It was calculated that to release all the reducing sugar in the cellulose and hemicellulose, 245 ml of enzyme would be required.

An experiment was carried out using this enzyme loading along with an excess loading (300 ml enzyme) to evaluate if all the total reducing sugar was released and if adding more enzyme had an effect. In addition, a value of 193 ml enzyme was used to determine whether the maximum total reducing sugar could be achieved with this enzyme loading even though it was less than the predicted maximum, it was however, much higher than the loadings used in previous work. The results are shown in Figure 8.7.

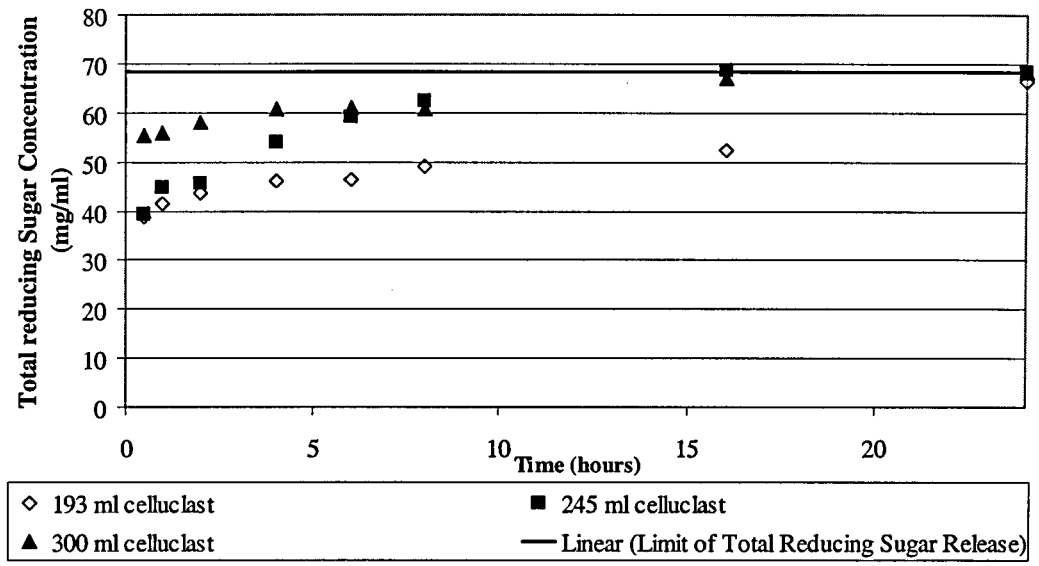


Figure 8.7 The Effect of using the Maximum and Excess Enzyme Loading on Concentration of Total Reducing Sugar Released.

It can be seen that the calculated enzyme loading of 245 ml does just release all the total reducing sugar available, the excess amount of enzyme (300 ml) resulted in no further increase to the concentration of reducing sugars. From this data, it can be concluded that a higher enzyme loading will release a higher concentration of sugar into solution until a maximum concentration has been reached, after which additional enzyme addition will have no further effect. The result for the

193 ml enzyme loading also releases the maximum amount of total reducing sugar available but the final sample point deviates from the curve and so is deemed to be incorrect.

A model proposed by Hellgardt (2002) was differentiated and used in an attempt to model the rate of sugar release, but the model did not fit the experimental data. However, it would be useful to be able to model the concentration-time curve for the results gained so that predictions can be made for the concentration of sugar released at a given time, for any enzyme loading below the maximum.

A new kinetic model was formed by fitting a curve to the experimental data plotted against time and using a curve fitting program to find an expression which describes the curve, the data points for the concentration of sugars were recalculated using the expression. This was carried out for the 12.5 ml, 25 ml, 193 ml and 245 ml enzyme loadings. The curve fitted to the experimental data for the 12.5 ml loading is shown below (Figure 8.8). The graphs for the 25, 193 and 245 ml enzyme experiments can be found in Appendix 6. The expression was differentiated and applied to the recalculated concentrations to find the rate at a given time. The rates were plotted against the concentrations. This gave a series of straight lines (Figure 8.9). The gradients were all negative which suggests that at a given concentration of product the rate of sugar production falls to zero, the original concentration time graphs (Figures 8.6 and 8.7) would also suggest this. This suggests that the enzyme is subject to product inhibition, although an alternative explanation is that of enzyme denaturation, or inactivation occurring.

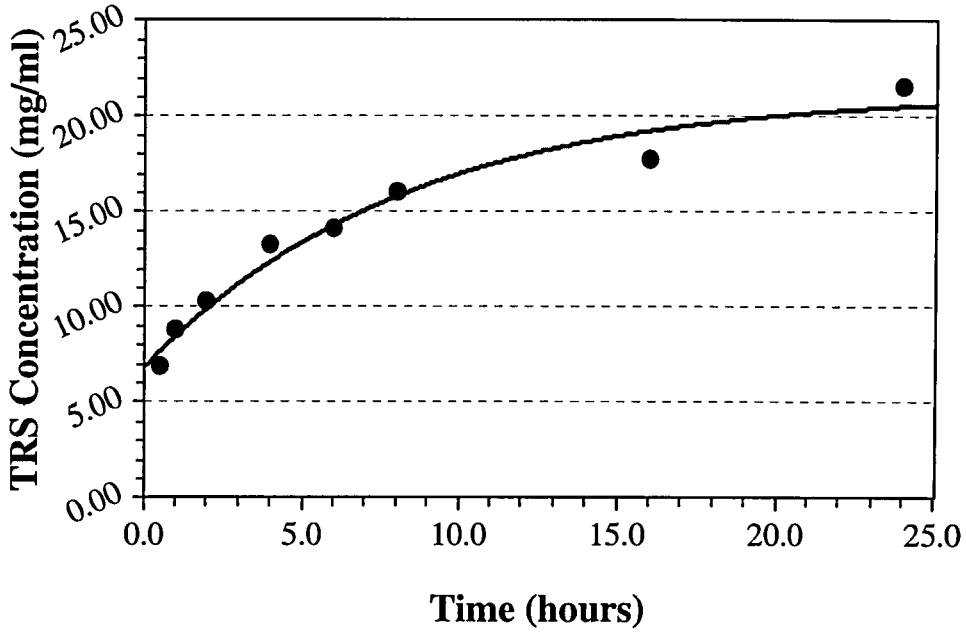


Figure 8.8 Total Reducing Sugar Concentration Plotted against Time for the 12.5 ml Enzyme Loading.

a=14.5 (mg/ml), b=1.47, c=0.121 (ml/mg). where TRS means Total Reducing Sugar

An exponential association was used to describe the data (Equation 8.4).

$$C_P = a\left(b - e^{-cC_P}\right) \tag{8.4}$$

The expression was differentiated to give the rate of production of sugar.

$$R_p = ac\left(e^{-cC_P}\right) \tag{8.5}$$

This was then plotted against the concentration (shown in Figure 8.9) resulting in a straight line.

$$R_P = mC_P + A \tag{8.6}$$

where m=gradient and A=intercept.

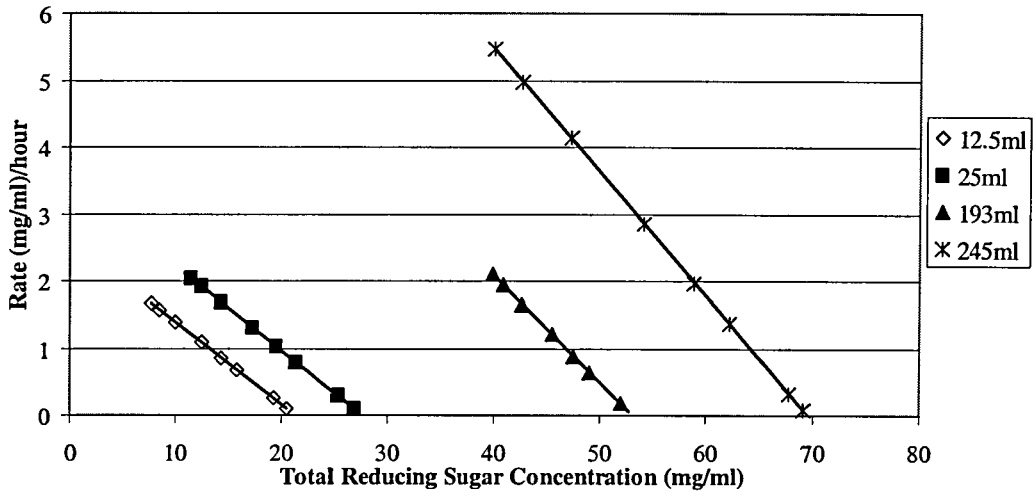


Figure 8.9 The Calculated Rate of Sugar Production Plotted against the Free Sugar Concentration for all the Enzyme Loadings.

The trendlines gave the following equations.

12.5ml; y =-0.121x+2.59, 25ml; y = -0.126x+3.50, 193ml; y = -0.159x+8.43

245ml; y = -0.185x+12.9

As the intercept and gradients changed for each enzyme loading an expression was sought to describe the relationship between the intercepts, gradients and enzyme loading. Graphs were plotted of enzyme loading against the rate-sugar concentration expression intercepts and gradients (Figures 8.10 and 8.11) and subsequently found to be straight lines. The expression for each was substituted for m and A into the original rate expression (Equation 8.6) to predict an expression to describe the rate in terms of the concentration of sugar dependant on the enzyme loading.

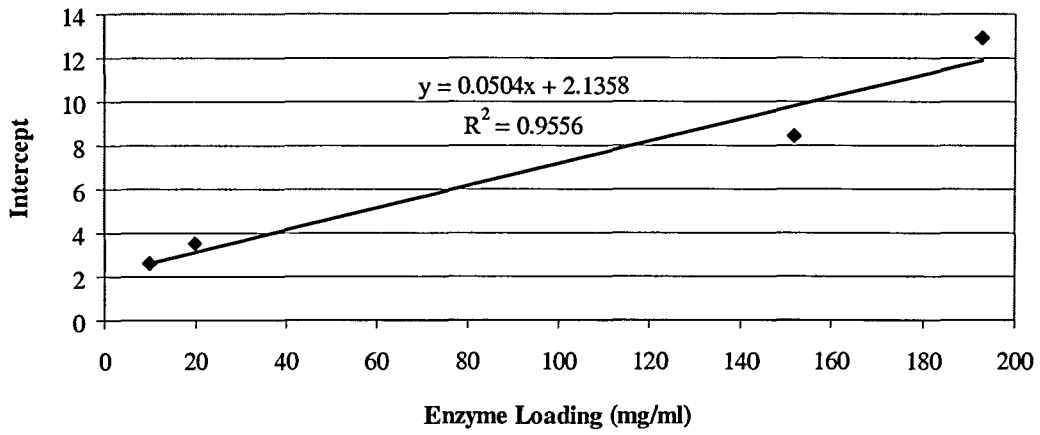


Figure 8.10 Intercepts Plotted Against Enzyme Loadings.

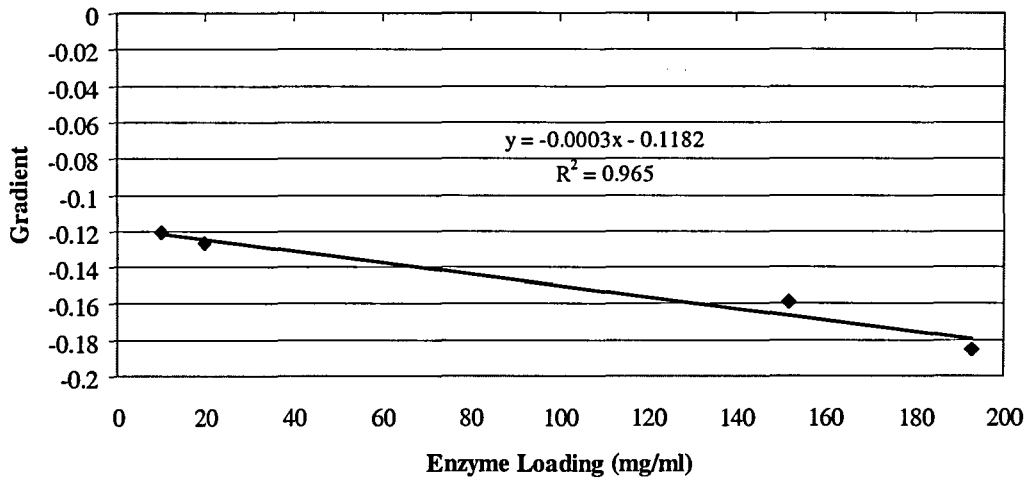


Figure 8.11 Gradients Plotted Against Enzyme Loadings.

The expression was

$$R_P = \left((-0.0003C_{E0} - 0.118)C_P\right) + \left(0.0504C_{E0} + 2.14\right) \qquad (8.7)$$

This expression was then integrated between C_P and C_{P0} and t=t and t=0 (as $dC_P/dt = R_p$). to give an expression for C_P as a function of time.

$$t = \frac{-1}{A}\ln\left[\frac{(AC_P + B)}{(AC_{P0} + B)}\right] \tag{8.8}$$

where

t=time (hours)

and

$A = -0.0003C_{E0} - 0.118$

$B = 0.0504C_{E0} + 2.14$

This was performed using a value of 7.5 mg/ml for C_{P0} as both the 25 ml and 12.5 ml enzyme loadings had initial values close to this, however it was observed that the curve did not fit the experimental values well at the higher enzyme loadings. The model predicted the curves to start from the 7.5 mg/ml value and as the initial part of the experiment happened so quickly this was not the case. It was decided to use the value of sugar concentration at 0.5 hours to describe the initial value, it can be seen from the graph (Figure 8.7) that this is not far from the initial value gained if the curve is traced back to zero time. The initial value changed with the enzyme loading, a graph of the sugar released after 0.5 hours against the enzyme loading (Figure 8.12) was used to fit a curve and gain an expression to describe the relationship between the two. This was then substituted into the equation (Equation 8.8) in the place of C_{P0}.

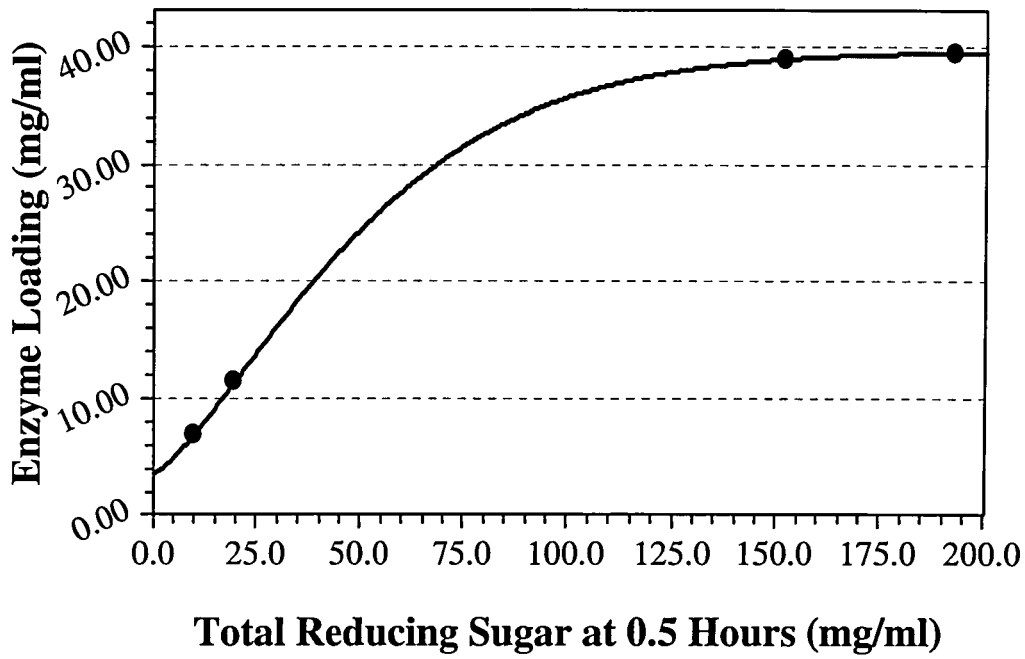


Figure 8.12 Released Sugar Concentration after 0.5 Hours for Differing Enzyme Loadings.

The final expression was as follows;

$$t = \frac{-1}{A} \ln \left[\frac{(AC_P + B)}{\left(A\left(39.6 - 36.0e^{0.00429 C_{E0}{}^{1.36}}\right) + B \right)} \right] \tag{8.9}$$

The modelled curves (denoted by lines) were fitted for the enzyme levels for which experiments had been carried out (see Figure 8.13) and compared to the original smoothed curves. The model was not able to return an answer for some of the results, this occurs when:

$$AC_p + B = 0 \tag{8.10}$$

and the rate is effectively 0. This feature however makes it possible to use the equation to calculate the highest concentration of total reducing sugar which will

be achieved by an enzyme loading. A concentration fractionally smaller than this can be put into the equation to determine a time at which that concentration will be achieved and therefore the curve modelled. A curve was generated for an enzyme loading of 100 ml, also seen in Figure 8.13.

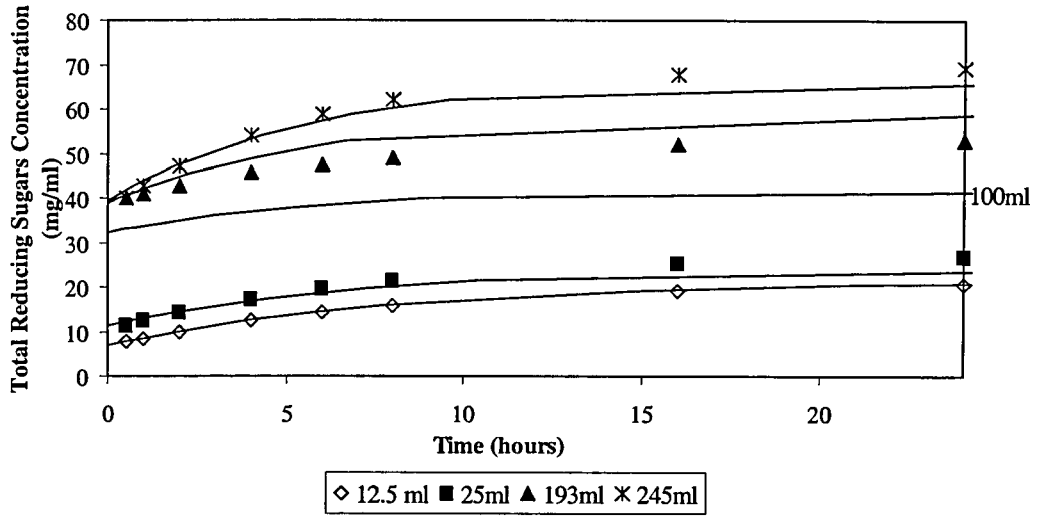


Figure 8.13 Comparison of Smoothed Experimental Results and Modelled Curves for Different Enzyme Loadings.

The treatment described above was purely empirical and not based on a mechanistic model. Movagharnejad *et al.* (2000) considered a shrinking particle, however in this model there are no terms to account for this. In the case of wheatfeed, the substrate is a mixture of a range of sizes so including a term to express the particle size specifically would be difficult. This may be appropriate for the situation described here but a mechanistic model would need to account for variations in particle size. The expression gained is simple and works for celluclast on wheatfeed. It is possible that a variation of the expression may apply to celluclast working on other substrates although this has not been studied in this project.

8.6 Overall Conclusion

All the commercial enzymes resulted in the release of sugars and proteins. Different enzymes attack different parts of the wheatfeed and the resulting sugar solution composition varied depending on the enzyme used.

A model was used to predict the maximum amount of celluclast necessary to release the maximum amount of sugar and experiments were performed to validate the predictions.

An empirical model was formulated and used to fit the experimental data.

9 Solid State Digestion of Wheatfeed and Enzyme Mode of Action

9.1 Introduction

The intended ultimate use of the enzymically digested wheatfeed is as an ingredient in dry pet food formulations (e.g. as biscuits), therefore there are obvious benefits in conducting the enzymic process in the presence of as little water as possible. This would reduce both the time and costs of a subsequent drying operation. Reducing the amount of free water would have implications not only for the enzyme reaction itself and the products liberated, but also for the mixing of the wheatfeed mixture during enzyme processing. This chapter describes experiments performed at reduced water levels to investigate the performance of a commercially available cellulase (celluclast) on both un-pretreated and steam exploded wheatfeed. Different enzyme levels were added to un-pretreated wheatfeed in order to determine the effect on product yield. Operation of microbial or enzymic processes under such conditions is referred to in the literature as 'solid state' and the origins of this form of processing go back hundreds of years to the so-called 'Koji' processing of rice starch to produce saké and other alcoholic beverages developed in the Far East.

Also described in this Chapter is a second series of experiments designed to gain some insight into the mode of action of the cellulase. Previous workers have claimed that cellulases bind to their substrate as a pre-requisite to hydrolysis (Medve *et al.*, 1998). Experiments are described here in which cellulase was contacted with pure cellulose fibres and an assay was performed to estimate the fraction of cellulase remaining unbound to its substrate.

9.2 Solid State Digestion Introduction

Two sets of experiments are described here. In the first the effects of the level of cellulase were investigated. These experiments were carried out using 2, 10 and 20% enzyme loadings (weight percent of wheatfeed). The second experiment was designed to evaluate how the quantity of free liquid in the wheatfeed-enzyme system affected the digestion and to investigate if high yields could be achieved at low liquid levels. Enzyme-buffer solution volumes of 5, 12.5 and 25 ml were used, whilst keeping the amount of wheatfeed constant. Buffer is mainly distilled water and so in reducing the buffer level, the water level is consequently reduced.

9.2.1 Enzyme Loading Experimental Method

The basic method using petri dishes as detailed in section 3.7.2.2 was used, with a few variations and was always executed in duplicate. The enzyme-buffer solutions (10 ml) contained different levels of celluclast (a commercial enzyme), the composition of these are shown in Table 9.1. Riffled wheatfeed (10 g) was used. Digestion was carried out for 24 hours with manual stirring at 4, 8, and 16 hours. The glucose release was measured using the Accu-check meter and the protein release by the Lowry-Peterson assay.

Table 9.1 Composition of Enzyme-Buffer Solution for Various Enzyme Loadings.

Enzyme level (% weight of wheatfeed)	**Citrate buffer at pH 5.0 added (ml)**	**Celluclast added (ml)**
20	8.33	1.67
10	9.17	0.83
2	9.833	0.167
Control	10	0

9.2.2 Enzyme Loading Results

Figure 9.1. shows the results obtained for glucose release. Enzymic digestion of wheatfeed clearly released more glucose than the control, liberating approximately an additional 6 mg/ml. All the enzymic digestions released similar amounts of glucose, giving results within the range of 16.0 to 16.7 mg/ml.

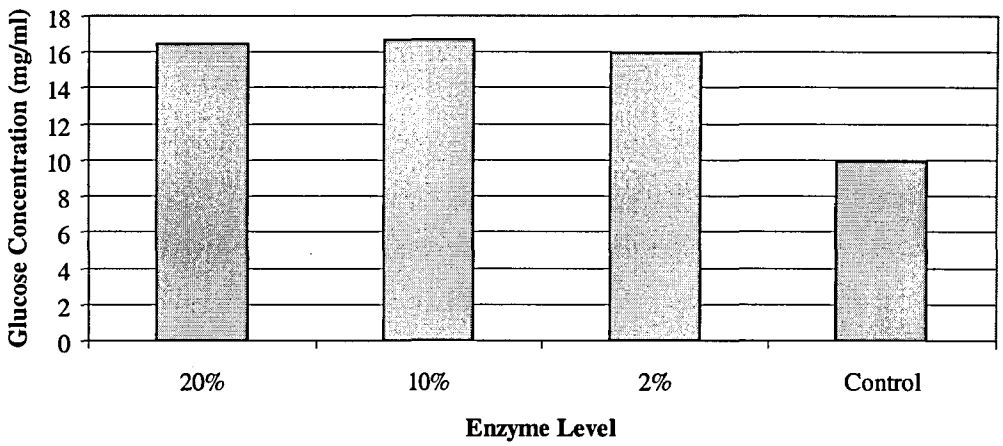


Figure 9.1 Glucose Released after Solid State Digestion for 24 Hours at 50°C.

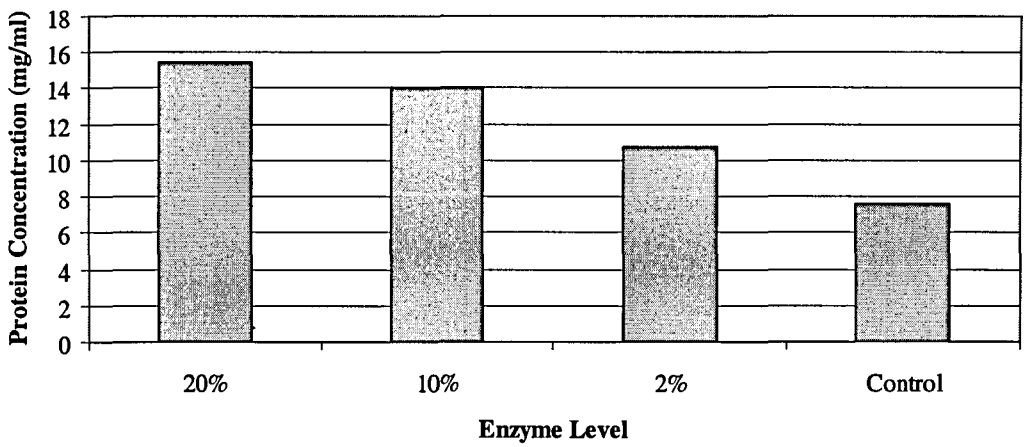


Figure 9.2 Protein Released after Solid State Digestion for 24 Hours at 50°C.

The protein release is shown by Figure 9.2. As with the sugars, the control gives the lowest result (7.6 mg/ml). However, in this case, protein yields decrease as the enzyme loading decreases. A difference of 4.7 mg/ml was obtained between the highest (20%) and the lowest (2%) loadings.

9.2.3 Enzyme Loading Discussion and Conclusion

The results showed that there was essentially no difference between the glucose liberation for the different enzyme loadings tested. This would suggest that the 2% loading was sufficient and that no substantial benefit was gained by adding additional enzyme.

The enzyme loading did appear to effect the protein release from the wheatfeed, however, enzymes are proteins and extra enzyme would be present in the solution tested. Therefore the increase in protein release with enzyme loading was probably a feature of the experiment. This is supported by work discussed later in this chapter (section 9.2.8) where 1 µg/ml of enzyme was used, (which is less than used here) and protein was detected using the Lowry-Peterson assay. Therefore further experiments were carried out using the 2% enzyme loading.

9.2.4 Enzyme Digestion at a Reduced Water Content Method

The basic method using petri dishes is detailed in section 3.7.2.2. This method was used with a few variations. Experiments were carried out on both un-pretreated wheatfeed and steam exploded wheatfeed. Wheatfeed/steam exploded wheatfeed (5 g) was used in the petri dishes with varying amounts of buffer-enzyme solution (5, 12.5, or 25 ml). For each liquid level, experiments were carried out in duplicate, with a celluclast loading of 2% (2% of the weight of wheatfeed), controls with no enzyme present were also performed.

After 24 hours digestion time, each of the samples were removed from the petri dish and placed individually into a stomacher bag and more buffer added. The amount of buffer added was dependant on the amount initially used for the

digestion. For example if 5 ml of buffer-enzyme solution was used for the digestion then 20 ml buffer was added, the total amount of buffer used for each experiment was 25 ml. The samples were then subjected to stomaching for 1 minute. The liquor was frozen for subsequent HPLC sugar and Lowry-Peterson protein analysis.

The experimental set up is shown more clearly in Table 9.2 where E denotes enzyme and S that steam exploded wheatfeed was used. The number refers to the amount of buffer used (ml) for the digestion.

Table 9.2. Experimental Set up for Enzyme Digestion at a Reduced Water Content.

Sample	**Enzyme present**	**Steam exploded**	**Buffer-enzyme soln (ml)**	**Buffer added (ml)**
5	-	-	5	20
5E	+	-	5	20
5S	-	+	5	20
5ES	+	+	5	20
12.5	-	-	12.5	12.5
12.5E	+	-	12.5	12.5
12.5S	-	+	12.5	12.5
12.5ES	+	+	12.5	12.5
25	-	-	25	0
25E	+	-	25	0
25S	-	+	25	0
25ES	+	+	25	0

9.2.5 Enzyme Digestion at a Reduced Water Content Results

The results obtained are shown in Figure 9.3 where the same sample nomenclature is as described earlier for Table 9.2. The results show that for steam exploded wheatfeed treated with enzyme the amounts of reducing sugars released increased with the quantity of water present. This was also the trend observed for each of the sugars detected. Arabinose if released at all was only present in small quantities.

The un-pretreated wheatfeed results show the same trends as the steam exploded wheatfeed, with increased total reducing sugar liberation as the liquid level of the digestion increases. An exception was the un-pretreated 25ml enzymic results where the total reducing sugar released at the 25 ml level was less than at the 12.5 ml level. This is due to a decrease in both glucose and fructose release. For the un-pretreated wheatfeed experiments the results show (with the exception of 25E) that glucose and xylose liberation increased as the level of buffer was increased. Arabinose was released in small quantities over the range of liquid levels and the amount of fructose detected was fairly consistent across the samples.

Both sets of control experiments (i.e. for steam exploded and un-pretreated wheatfeed) released less total reducing sugars than their respective enzymically digested counterparts. Enzymic digestion increased the amount of xylose and glucose released, the arabinose release was also enhanced in the un-pretreated samples.

The levels of sugars released from the enzyme treated steam exploded wheatfeed was less than for the enzyme un-pretreated samples, except at the 25 ml level where both results were close together (18.5 mg/ml for un-pretreated and 19.1 mg/ml steam exploded). For the first two enzyme-buffer solution levels the sugar yield was greater for the unpretreated control than the enzyme treated steam exploded wheatfeed. It can be seen that the increase in sugar yields were large as the buffer level increased for the enzyme treated steam exploded samples.

The levels of sugar liberated from the steam exploded wheatfeed controls were the lowest for all buffer levels.

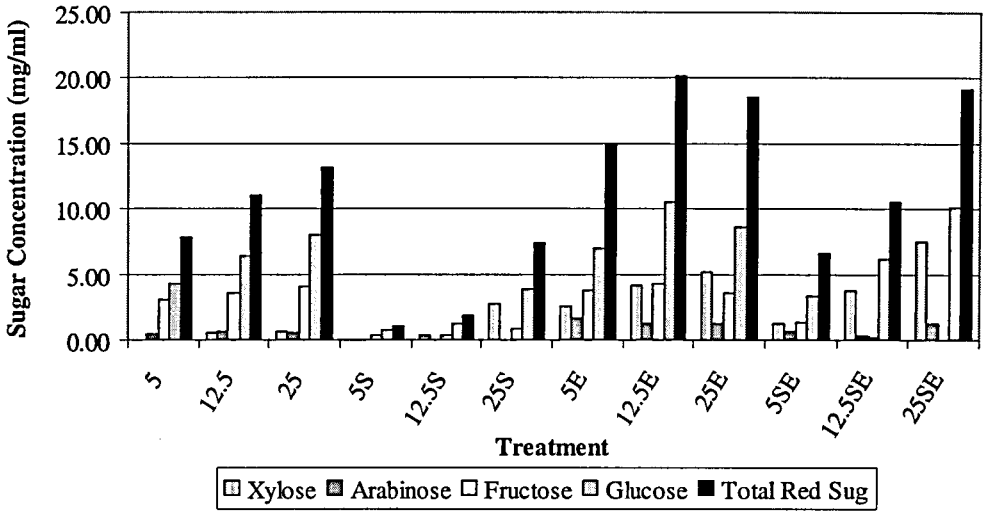


Figure 9.3 Reducing Sugars Released at Different Water Contents for Steam Exploded and Un-pretreated Wheatfeed.

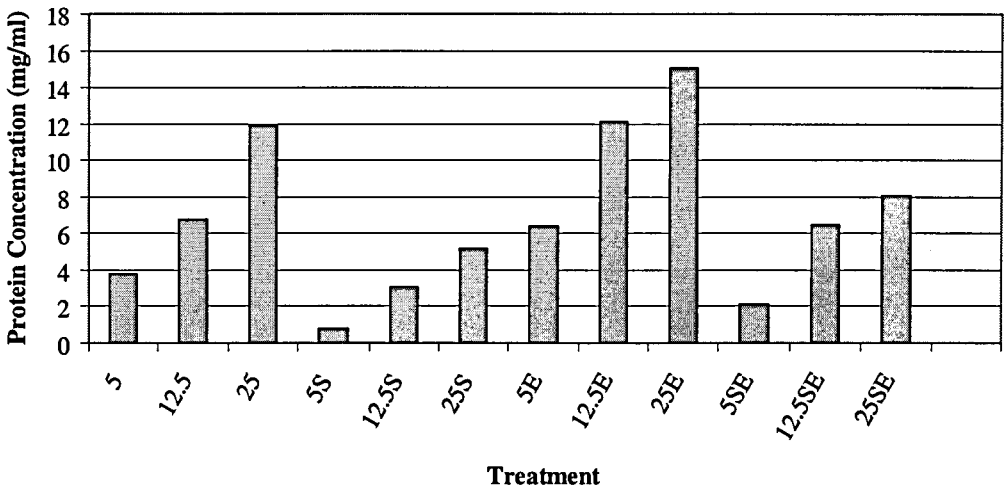


Figure 9.4 Protein Released at Different Water Contents for Steam Exploded and Un-pretreated Wheatfeed.

The results for protein release (Figure 9.4) clearly show that as the level of water increases protein liberation also increases. The steam exploded control gave the lowest results, with the maximum result at 5.1 mg/ml. This can be compared to the enzymically digested steam exploded wheatfeed samples which gave a maximum result of 8.0 mg/ml. The un-pretreated sample gave maximum results of 11.9 mg/ml and 15.0 mg/ml, for the control and enzymic digestions respectively.

9.2.6 Enzyme Digestion at a Reduced Water Content Discussion

The results show that reducing the amount of water led to a decrease in the yields of sugar liberated.

The controls released less sugars for both steam exploded and un-pretreated samples than the equivalent samples enzymically digested with celluclast. The steam exploded controls released less sugar than the un-pretreated wheatfeed controls, this follows the same trend discovered in the initial steam explosion experiments discussed (Appendix 4). The steam exploded enzymically digested samples gave an unexpected result, in that the sugars released were lower than the amount released by the un-pretreated samples (Figure 9.3). This is in contrast to the findings from the initial steam explosion experiments, where the reverse was the case (Appendix 4)

The protein results confirm findings from the sequential treatment experiments (section 5.3) which suggested that steam exploding reduced the amount of protein released.

The liquid level used in the bioreactor and conical flask experiments uses a ratio of 1 g wheatfeed to 5 ml water, this is equivalent to the 25 ml experiments here. Whilst the lower level of water gives reduced sugar and protein yields it may be worth considering, given the cost and time savings of eliminating the need to dry the product. The amount of enzyme could also be reduced in this case as it has be

shown in this chapter that the enzyme loading need not be higher than 2% with regard to sugar release. Previous experiments with high liquid levels have been carried out using a 10% celluclast loading

9.2.7 Enzyme Digestion at a Reduced Water Content Conclusion

Reducing the amount of water added for the digestion of wheatfeed with a commercial cellulase generally resulted in lower yields of sugars and protein. The results obtained using un-pretreated wheatfeed showed that halving the liquid volume from 25 ml to 12.5 ml did not significantly affect sugar liberation but did result in lower yields of protein. A reduction in liquid volume of this magnitude would almost certainly be desirable. Reducing the liquid volume to 5 ml reduced the amount of sugar liberated by approximately 3 mg/ml compared to that released at the 12.5 ml liquid level (for all the experiments except steam pretreated control). In the un-pretreated wheatfeed digested with enzyme case, these conditions still give a good sugar liberation and the reduction of liquid by one quarter would be worth it. However in this case the protein release is almost halved.

9.2.8 Enzyme Mode of Action Introduction

The enzyme binding experiments were designed to evaluate the way which the cellulase acts on the substrate - that is whether it binds to the surface of the substrate and remains bound, or whether it binds and is then released. The amount of protein in the aqueous phase was assayed using Lowry-Peterson following contact with cellulose fibres.

9.2.9 Enzyme Mode of Action Method

The experimental method used is outlined in section 3.7.2.3. The Lowry-Peterson assay was used to analyse the concentration of protein (from the addition of enzyme), in the aqueous phase, after celluclast was contacted with cellulose fibre in a liquid medium.

9.2.10 Enzyme Mode of Action Results

The results for the enzyme binding experiment are shown in Figure 9.5. The protein level drops sharply, from 0.1 mg/ml to 0.03 mg/ml within the first 10 minutes, then remains fairly constant.

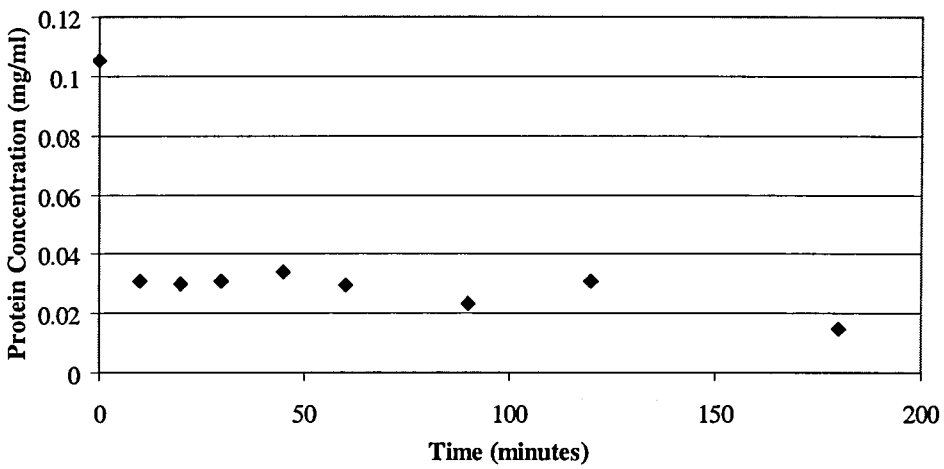


Figure 9.5 Assay of Free Protein Following Addition of Celluclast Enzyme to Cellulose Fibres.

9.2.11 Enzyme Mode of Action Discussion

The concentration of protein in solution drops quickly at first, this is probably because initially there are many unoccupied enzyme binding sites on the cellulose, As the enzyme attaches it is removed from the solution. The protein concentration after this initial attachment remains substantially constant and the variation observed in Figure 9.5 is probably the result of experimental errors. It is possible the enzyme binds to the substrate reacts and is then released into the solution. However once in solution it is rapidly bound up again. This evidence agrees with results from the commercial enzyme experiments discussed in section 7.2 where the stirrer speed of the bioreactor was varied and the rate of digestion observed. Changing the stirrer speed did not make much difference to the rate of digestion. The rate of enzyme attachment or reattachment to the substrate may be increased

by external mixing, but it seems likely that any such increase would be relatively small given the rapid attachment under conditions of mild agitation.

9.2.12 Enzyme Mode of Action Conclusion

Rapid removal of protein from solution was observed which is compatible with a mechanism in which the cellulase binds to its substrate. Subsequent release and binding are probably rapid.

9.3 Overall Conclusion

These experiments have shown that an enzymic digestion of wheatfeed can be successfully carried out in a reduced liquid system using a 2% loading of celluclast, however the more liquid available during the digestion step the greater the liberation of sugars and protein. Steam pretreating the wheatfeed prior to solid state digestion does not enhance the amount of sugars, or protein liberated.

The enzyme binds to the substrate. The rate of binding is initially fast and then subsequent release and reattachment is also probably very rapid.

10 Digestibility of Wheatfeed After Treatment.

10.1 Introduction

All of the treatments of wheatfeed described in earlier chapters, were carried out in order to make a final product that is more digestible and could be incorporated into dog food. To evaluate how the treatments affect digestibility, wheatfeed which had undergone selected treatments was produced and then tested for the percentage of fibre left and also for starch and gel starch. Digestions were carried out either in excess liquid, or in solid state form. Wheatfeed subjected to both kinds of treatment was prepared for digestibility testing, but was handled in different ways. The samples resulting from the liquid digestion were added to poultry meal prior to drying, this step was omitted for the solid state digestion samples.

10.2 Methods

10.2.1 Reduced Liquid Samples

The samples shown in Table 10.1 were analysed for both fibre and starch.

Table 10.1 Treatment of Wheatfeed for Starch and Fibre Analysis.

Sample	Designation in Figures	Treatment
Wheatfeed	wheatfeed	Riffled wheatfeed with no further treatment
Solid State Wheatfeed Control	SS wheatfeed cont	Riffled wheatfeed (10 g), added to 10 ml citrate buffer at pH 5.0, digested in a sealed petri dish, in an incubator at 50°C for 24 hours. After this, it was dried in a vacuum oven before being ground in a pestle and mortar to a particle size of 355 μm<1000 μm.
Solid State Wheatfeed Digested with Celluclast.	SS wheatfeed cell	Carried out as for the solid state control, except in place of the buffer, 10 ml buffer-enzyme solution (9.167 ml buffer, 0.83 ml enzyme) was used.
Steam Exploded Wheatfeed	SE wheatfeed	Steam exploded wheatfeed which was then dried in the vacuum oven before being ground in a pestle and mortar to a particle size of 355 μm<1000 μm.
Steam Exploded Control	SE cont	This was to serve as a control for steam exploded wheatfeed. 10 ml distilled water was added to 10 g riffled wheatfeed and mixed, then dried in a vacuum oven overnight. It was then ground to a particle size of between 355 μm and 1000 μm.

10.2.2 Enzyme Digestions Carried Out With Excess Liquid

It was important that experiments using an excess of liquid should be included in the production of samples for digestibility testing, in order to establish whether more liquid improves the final digestibility of the wheatfeed. These experiments were carried out in 50 ml conical flasks following the basic procedure set out in section 3.7.2.4. Celluclast was the enzyme used for the digestion. The buffer enzyme solution was made as follows. Citrate buffer pH 5.0 (8.33 ml) was mixed with 1.67 ml celluclast and mixed, 1 ml of this was added to a further 9 ml of buffer and mixed to make 10 ml enzyme-buffer solution.

After digestion, the digested fibre would be present as oligomers of carbohydrates and proteins of various chain lengths in the liquor. Therefore the liquor could not be disregarded as this would leave more resistant fibre giving inaccurately high fibre results. The sample however, was too wet to dry in the vacuum oven without further processing. Therefore, the samples were added to a readily digestible component of pet food (poultry meal) in the ratio of 1 g of wheatfeed to 3 g, (or 1 conical flask to 6g poultry meal) before being dried. After the samples were dried they were ground in a pestle and mortar, to particle sizes between 355 μm and 1000 μm.

These experiments were carried out for wheatfeed digested with a 10% celluclast loading (10% weight of wheatfeed), wheatfeed control (no enzyme present) and the same, but using steam exploded wheatfeed in place of wheatfeed as described in Table 10.2.

Table 10.2 Treatment of Wheatfeed for Fibre Analysis.

Sample	Designation in Figures	Treatment
Wheatfeed 4 hour Digestion Control	wheatfeed 4hr cont	Wheatfeed which has undergone a liquid 4 hour digestion with no enzymes present.
Wheatfeed 4 hour Digestion with Celluclast	wheatfeed 4hr cell	Wheatfeed which has undergone a liquid 4 hour digestion with celluclast.
Steam Exploded Wheatfeed 4 hour Digestion Control	SE wheatfeed 4hr cont	Steam exploded wheatfeed which has undergone a liquid 4 hour digestion with no enzyme present.
Steam Exploded Wheatfeed 4 hour Digestion with Celluclast	SE wheatfeed 4hr cell	Steam exploded wheatfeed which has undergone a liquid 4 hour digestion with celluclast.

10.3 Results

Figure 10.1 shows the percentage starch in wheatfeed and treated wheatfeed. The samples have a starch composition in the range of 29.2% to 33.5%. The lowest result is given by the solid state celluclast sample and the highest is given by the steam explosion control (SE cont). There is not a large difference between the various treatments.

The results for the percentage of starch gelatinisation were in the range of 10.5 % (wheatfeed) to 31.5 %. The highest result was clearly given by the steam exploded wheatfeed (SE wheatfeed), with 16.7 % more gelatinised starch than any other sample. There was slightly more starch gelatinisation for both of the solid state experiments (SS wheatfeed cont/cell) compared to the other results (excluding steam exploded wheatfeed).

There are no starch results for samples from the enzyme digestion with excess liquid because once the digested wheatfeed was mixed with poultry meal the overall level of starch in the mixture was too low to detect.

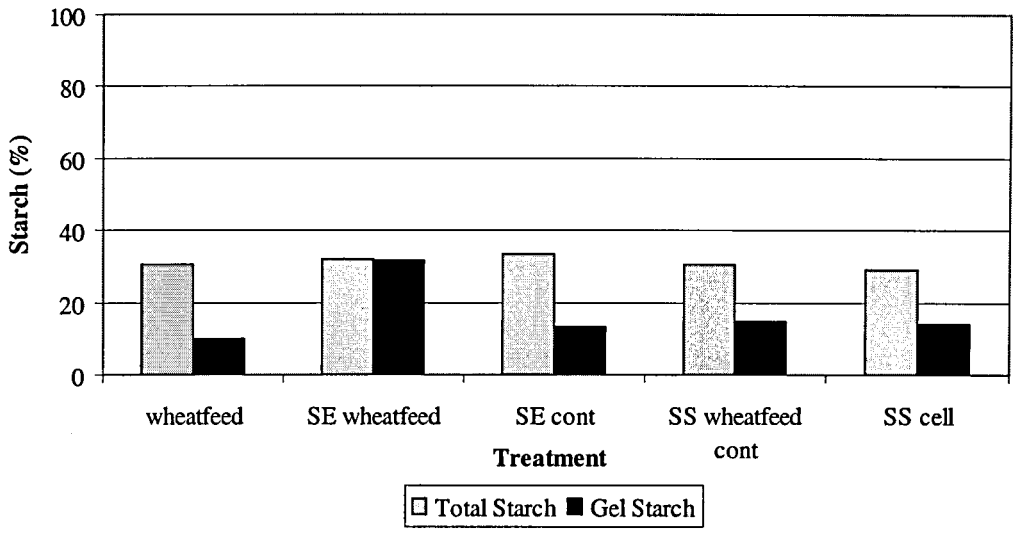


Figure 10.1 Starch in Wheatfeed Samples after Various Treatments

The results for the total fibre in the samples are shown in Figure 10.2. These results can be split into three groups. The highest results are given by wheatfeed (32.2%) and steam exploded wheatfeed control 4 hour digestion (SE wheatfeed 4 hr cont) (32.1%)

The lowest results are given by solid state celluclast digested (SS wheatfeed cell) (16.1%), wheatfeed 4 hour celluclast digested (wheatfeed 4hr cell) (17.2%) and steam exploded 4 hour celluclast digested (SE wheatfeed 4hr cell) (20.5%). These are the only three treatments which included enzyme digestion and would therefore suggest that using celluclast to digest the wheatfeed decreases the percentage of fibre in the sample. This is confirmed further by comparison of the enzyme treatments with their respective controls. The solid state wheatfeed control gave a result of 25.1% fibre compared to 16.1%, the wheatfeed 4 hour

control gave a result of 29.0% compared to 17.2% and the steam explosion 4 hour digest control gave 32.1% compared to 20.5%. In these comparisons it is the 4 hour digest wheatfeed treatment which shows the greatest difference with 11.8 % reduction in total fibre.

The other treatments are grouped closely together mainly giving fibre percentages between 28 % and 29.5 %. The solid state wheatfeed control gave a slightly lower percentage at 25.1 %.

The soluble fibre results range between 2.3 % and 7.4 % with the lowest result from the steam exploded wheatfeed 4 hour celluclast digestion and the highest result from steam exploded wheatfeed. The insoluble fibre results range between 13.0 % for the solid state celluclast digest sample and 28.6 % for wheatfeed. There is no significant difference in the total fibre results for the steam exploded wheatfeed and steam exploded wheatfeed control, however, the steam exploded wheatfeed shows a larger percentage of soluble fibre than the steam exploded control.

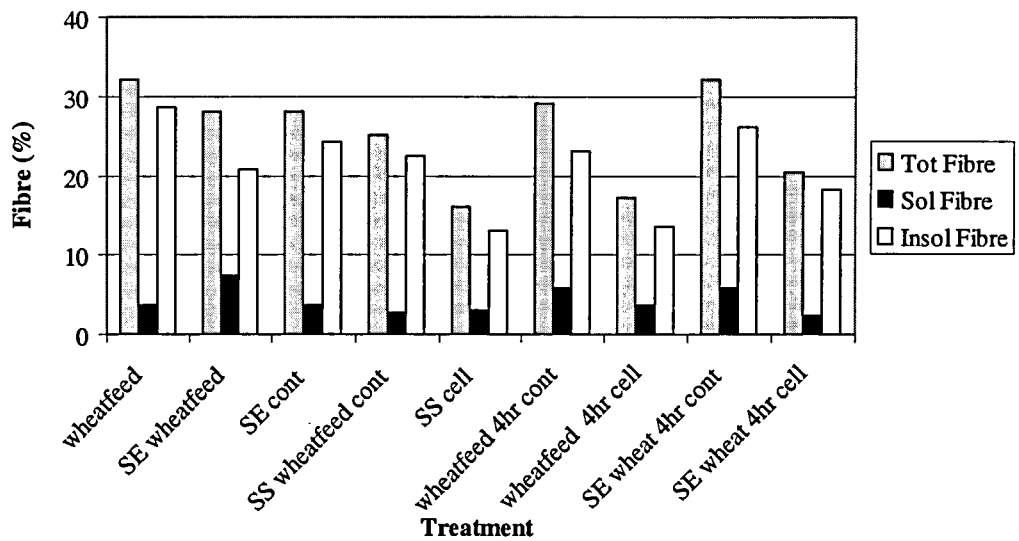


Figure 10.2 Fibre in Wheatfeed Samples after Various Treatments

10.4 Discussion

The results show that the level of starch in all the samples are within a small range and are probably due to variations in the composition of the raw material. However, starch gelatinisation was most pronounced in the steam exploded sample. This is to be expected, as the heat treatment performed during steam explosion would be sufficient to cause the gelatinisation of the starch. Comparing the results for the steam explosion control samples with those that were steam exploded, further demonstrate that it was the steam explosion which was the cause of the gelatinisation. The control had not been steam exploded, the wheatfeed had been added to the same amount of water as the steam exploded sample and dried, this sample gave low starch gelatinisation results. The samples which underwent the solid state digestion showed some starch gelatinisation. This could be due to long treatment at 50°C prior to being dried.

The samples containing the lowest amount of fibre were those treated with celluclast; this suggests that the enzyme does break down fibre contained within wheatfeed. The highest fibre contents were found in samples that had been steam exploded then subjected to a 4 hour control digestion. This somewhat anomalous result might have arisen because some of the more digestible components of the fibre were degraded during the steam explosion process, leaving the less digestible components and thus increasing the percentage fibre in the remaining material.

Both the steam exploded wheatfeed and the steam exploded wheatfeed control had a similar amount of fibre component, however, a difference can be seen in the amount of soluble and insoluble fibre in these samples. The amount of soluble fibre is greater in the steam exploded sample than the steam exploded control sample, thus suggesting that the fibre is broken down during the steam explosion process to smaller molecules which are soluble.

Samples taken from the other treatments showed that both the insoluble and soluble fibre is greater in the control experiments than the celluclast digested samples. This suggests that the enzyme breaks down both insoluble and soluble fibre without discrimination.

10.5 Conclusion

The digestibility of wheatfeed is improved by enzymic digestion of wheatfeed. Steam explosion was shown not to significantly enhance hydrolysis. This type of thermal treatment does however cause the starch to become gelatinised rendering it more digestible. It is likely that extruding the product would also have this effect.

Purely steam exploding the wheatfeed would produce material with a higher soluble fibre percentage than un-pretreated wheatfeed.

Although useful indicators of digestibility the tests reported here are not a substitute for animal feeding trials which would have to be undertaken if commercialization of the enzymic treatment of wheatfeed was ever to become a reality.

11 Conclusions and Suggestions for Further Work

11.1 Conclusions

Digestion of wheatfeed using enzymes led to the release of a variety of sugars and proteins.

A number of different enzyme mixtures were evaluated, but the combination of hemicellulose, cellulose and pectinase gave the most satisfactory yields of sugars and proteins.

Experiments were conducted to determine the optimum conditions for the digestion of wheatfeed using hemicellulose, cellulose and pectinase; these were a pH of 3.2 and a temperature of 35°C.

Sequential addition of enzymes revealed useful information on the pattern of attack of the components of wheatfeed by individual enzymes. Most significant was the finding that xylose is only released once cellulase has been added.

The performance of commercially available enzymes was evaluated. All products tested successfully liberated sugars and proteins. One enzyme preparation, 'celluclast' was selected for further study as it yielded appreciable amounts of sugars and proteins and appeared, uniquely, to attack xylans and other xylose-containing polymers present in wheatfeed.

In kinetic evaluations using celluclast in a stirred bioreactor, the stirrer speed did not affect the rate of sugar release and it was concluded that mass transfer effects were not limiting.

The effects of particle size of the wheatfeed were also investigated. Unexpectedly, smaller particles were not digested more rapidly than larger ones. Substrate mixtures comprising of the largest size fraction could not be digested in an agitated bioreactor owing to torque limitations of the motor fitted to the bioreactor.

Sugars and proteins were also shown to be liberated when wheatfeed was enzymically digested under conditions that are referred to as 'solid state'. However, the yields of sugars and proteins obtained were lower than those obtained at higher water content. The protein liberation was effected more than the sugar release.

A kinetic model was used to predict the amount of enzyme necessary to release all the available sugars. Experiments conducted to investigate this prediction proved positive. Limitations in the model reduced its usefulness but an empirical model was subsequently proposed and described the kinetic behaviour of the process of digestion well.

Pretreatment of the wheatfeed by steam explosion gave inconsistent results. The amounts of additional sugars released would not appear to warrant recommending steam explosion as a commercial process for wheatfeed, but further work would be needed to confirm this.

A variety of digestibility tests were performed and the results obtained showed that enzymically digesting wheatfeed does result in an increase in digestibility.

11.2 Suggestions for Further Work

The strategy for seeking to replace the more expensive components of animal feeds with cheaper ones (provided that the nutritional value of the feed is not thereby impaired) makes economic sense. The sources of any such replacements

need to be consistent and any further processing necessary to make these materials acceptable should not add excessively to the costs of the final product. The commercial enzyme used in this study is expensive at £70/kg, the cheapest enzyme from the range tested costs £15/kg and therefore these ensure the work here does not fall into the category described above. However, steps to convert what is essentially a waste material i.e. the wheatfeed, and improve its suitability for incorporation in feeds should be pursued further. Although commercial enzymes may be ruled out on economic terms, the use of crude preparations of enzyme mixtures might prove more attractive. One way of obtaining these would be to carry out a solid state fermentation on the wheatfeed, or some other substrate, using a potent cellulase producer. The most obvious candidate would be *Trichoderma reesei* but other cellulolytic organisms such as *Cellulomonas fimi* should also be considered. Batches of wheatfeed could be fermented in relatively small scale bioreactors and the partially digested material remaining at the end of the fermentation could be used as a source of crude enzymes for direct addition to wheatfeed. Alternatively, the wheatfeed could be fermented for long periods in a solid state fermentation to achieve the required increase in digestibility. This last option would require further work in devising low cost digesters. This should not present insuperable difficulties as the process approximates to a controlled composting and guidance would be available from the existing literature as to how this might be achieved.

Any incorporation of ingredients that contained enzymes or living micro-organisms would need to be treated to ensure that enzymes and cells were inactivated. This would probably need to be achieved using thermal methods. Fortunately, the intended use of the final material is as a dry feed product which is traditionally produced by extrusion and the conditions under which extrusion takes place might prove sufficient to prove the thermal energy input for inactivation. This is a further area where more work needs to be done. It can be seen from a trial run carried out in this study that enzyme treated wheatfeed can be added to the other ingredients of dog food and extruded (Appendix 7).

A number of processes in the food industry result in the production of generally dilute aqueous stream containing sugars and/or proteins. It may be possible to economically concentrate such materials for incorporation in feeds. Alternatively, other cheap sources of protein could should also be evaluated these would include soya, rapeseed and other meals. Both enzymic digestion of meals and other technologies such as protein extraction should be investigated.

The possibility of being able to dispense entirely with enzymes is another strategy worthy of further investigation. Steam explosion would certainly merit additional attention. Although the results obtained here were variable, different regimes of operation might exist that could either result in the partial break down of indigestible components or increase the susceptibility of such components to enzyme attack. Other pretreatment methods such as alkaline hydroxide, acid hydrolysis or milling would also warrant further investigation.

The digestibility tests employed here, though useful in indicating the extent of fibre breakdown, are really only poor substitutes for *in vivo* feeding trials. These would need to be conducted to provide evidence of acceptability to the animals and to provide assurance that there were no long term health risks to the animal.

12 Appendices

12.1 Appendix 1: Discussion of Methods of Analysis

Protein

There are several methods of protein and sugar analysis which could have been chosen for this study. The Lowry-Peterson assay was chosen as the method for most of the protein analysis as it works well on proteins in solution and is simple to carry out. The Kjeldahl method could also have been used but this method takes longer and less samples can be analysed together, however this method was used for solid samples such as evaluating the amount of protein initially in the wheat bran.

A BioRad kit was trialed as the reaction for this test takes 5 minutes compared to two reactions of a total of 40 minutes for the Lowry-Peterson assay. The samples also required less dilution than the Lowry-Peterson test thus eliminating some dilution error. The BioRad kit is made by BioRad Laboratories and is based on the method of Bradford. In this method Coomassie Brilliant Blue G-250 binds to protein in the samples and changes from a reddish to a blue colour. The absorption maximum of the dye changes from 465 to 595 nm. Therefore the change of the absorbance at 595nm on a UV photospectrometer is proportional to the protein concentration in the sample (Chang, 1994).

Chang (1994) reports that the Bradford method is more sensitive than the Lowry method, however the trials using this method of protein analysis gave much lower results than those obtained using the Lowry-Peterson method. The BioRad experimental procedure booklet showed a table of amino acids and gave a comparison of Lowry and BioRad measurements. For some of the amino acids the BioRad gave much lower readings than the Lowry method, this suggests that

these could have been the amino acids mainly present in protein from the wheat bran.

Sugars

In the analysis of sugars several methods were employed. The Accu-check method was very quick and simple to use and so gave a quick estimate as to the glucose present, however the results were generally higher than those obtained from HPLC. The glucose kit method gave results similar to those from HPLC analysis although there were some differences. HPLC was the most lengthy of the analysis methods used taking 25 minutes for each sample injected. The Accu-chek meter took 40 seconds per sample, the glucose kit method took 40 minutes for the reaction to occur although there was a longer preparation time than for the other methods of analysis. However in this method more than one sample could be analysed at once.

Initially the Nelson–Smogui method was trialed as this would be a fairly quick way of testing for all reducing sugars rather than just glucose, however the results obtained were very low. Low (1994) states that this method can be interfered with by biological molecules which probably explains why the solutions from the wheat digestion did not give good results. Another problem with this method is that different reducing sugars give different results (Low, 1994) and therefore a good indication of how much the fibre had been digested could not be obtained unless the exact make up of the sugars in the solution was known. As the different enzymes combinations tested would digest different parts of the wheat releasing different sugars it would be impossible to know from the Nelson-Smogui method which enzyme combinations had been most effective.

Fibre

There are two approaches to measuring dietary fibre:-gravimetrically, or chemically. In gravimetric methods, digestible carbohydrates, lipids, and proteins are solublised by chemicals and enzymes and the indigestible residues are collected by filtration. In chemical methods digestive carbohydrates are removed by enzymic digestion, the fibre components are hydrolysed with acid and the monosaccharides released are measured. In both approaches starch must be removed, otherwise it will increase the estimate for dietary fibre (Bennink, 1994).

The most widely used dietary fibre analysis methods are those of the Association of Official Analytical Chemists (AOAC), the Theander-Marlett and the Englyst-Cummings methods (Bennink, 1994).

The AOAC is a gravitational method of measuring fibre. Duplicates of dry, ground, fat-free samples are gelatinised with heat stable α-amylase, then enzymically digested with amyloglucosidase and protease. This removes the starch and proteins. The mixture is filtered and the insoluble fibre collected. Ethanol is added to the filtrate and the soluble fibre precipitates. This mixture is filtered and the soluble fibre collected. Both fibre residues are washed with ethanol and acetone, dried and weighed. One duplicate is incinerated and weighed to find the ash content and the other undergoes protein analysis (Bennink, 1994) to calculate the residual protein.

Total dietary fibre = insoluble fibre + soluble fibre – protein – ash. (James, 1995)

In the Theander-Marlett approach, free sugars and lipids are extracted with ethanol and hexane from duplicate samples of dry ground food. Starch is removed by enzymic digestion. The mixture is then filtered and centrifuged several times to remove the insoluble residue. Ethanol is then added to the filtrates and the soluble polysaccharides precipitated, both the fibre fractions are

hydrolysed with sulphuric acid separately and the concentration of the sugars in the two hydrolysates are determined. The residue (lignin) is washed, dried and weighed.

Fibre = monosaccharides +lignin (Bennink, 1994)

For the Englyst-Cummings procedure, dietary fibre is measured as non-starch polysaccharides (NSP). The food is defatted if necessary then the starch is gelatinised and enzymically digested. Sulphuric acid is added to hydrolyse remaining NSP (Bennink, 1994), which can then be measured colourimetrically. The procedure can be modified so that values can be obtained for dietary fibre (as total NSP), soluble fibre (soluble NSP), insoluble fibre (insoluble NSP) and resistant starch (James 1995).

The three methods explained give reasonably comparable estimates for dietary fibre. The Englyst–Cummings does not include lignin and resistant starch in the estimate, therefore this generally gives the lowest result. The AOAC method will tend to over-estimate the value for fibre if the food is rich in simple sugars. It is possible that some sugars are trapped and precipitated with ethanol. This issue is not encountered if the Englyst-Cummings method is used. The AOAC and Englyst-Cummings procedures use enzymes to digest protein, this allows some fibre to be solublised affecting the results (Bennink, 1994).

The differences in the methods and values must be bourne in mind when comparing results of fibre for different foodstuffs, although each are comparatively reliable. The Englyst-Cummings method requires the lowest level of skill and the simplest equipment (Bennink, 1994), and is therefore probably the easiest to conduct.

12.2 Appendix 2: Lowry-Peterson Reagents A and B

Materials

12.5g Sodium Carbonate

0.125g Copper Sulphate

0.25g Potassium Sodium Tartrate

4g Sodium Hydroxide

Folin–Ciocalteu Phenol Reagent

Distilled water

Copper Tartrate Carbonate

12.5g of Sodium Carbonate was dissolved in 500ml of distilled water.

0.125g Copper Sulphate and 0.25g of potassium sodium tartrate were dissolved in 62.5ml of distilled water.

The sodium carbonate was added to the copper sulphate and potassium sodium tartrate solution slowly with stirring.

0.8M Sodium Hydroxide

4g Sodium hydroxide was dissolved in 125ml of distilled water.

Reagent A and B

Reagent A: 125ml of copper tartrate carbonate and 125ml of 0.8M sodium hydroxide were mixed together along with 250ml distilled water.

The solution must be stored in the fridge and will only remain stable for about three weeks, after which it should be disposed.

Reagent B: Folin-Ciocalteu phenol reagent was diluted with distilled water in the ratio 1:5. It must be stored in the fridge in an amber bottle.

12.3 Appendix 3: Students Two Tailed T-Test

Method

The t-test experiments were carried out in 50 ml conical flasks and followed the basic method as detailed in section 3.7.1. Only experiments using the β-glucanase and cellulase (GC) mixture, the hemicellulase, cellulase and pectinase (HCP) mixture and xylanase (X) on it's own will be repeated along with a control (C). The first two mixtures are the ones which seem to work the best, whereas the xylanase did not seem to have much effect on the wheatfeed and so is repeated as a contrast.

The protein in the samples was measured using the Lowry-Peterson assay. The glucose was measured using the Sigma glucose kit method, and the reducing sugars were measured by HPLC. The glucose results from these last two tests can be compared.

For each enzyme mixture duplicates were carried out, these are given the numbers 1 and 2 in the nomenclature of the samples in the results. For each duplicate, for the Lowry-Peterson and glucose tests three samples were taken for analysis from the main sample. These are denoted by the letters A, B, and C.

For the samples which underwent HPLC analysis, where there was sufficient sample, three injections from each duplicate were analysed, these are denoted A, B, and C. Where only two samples were injected they were called A and B.

For example HCP1B was a sample where the wheatfeed was digested with an enzyme mixture of hemicellulase, cellulase and pectinase. The sample was taken from duplicate number one and was the second sample to be taken from the stock sample. The results achieved were used for a Student's two-tailed t-test to calculate if there were any significant differences between duplicates and different treatments.

Results and Discussion

Lowry-Peterson Assay

The samples for the different duplications for one enzyme treatment were compared using the Student's two-tailed t-test to find out if there was any significant difference between the two samples. The null hypothesis was that there was no difference between the means of the two samples. The results are shown in the table below. where the comparison is made at the 5% level.

Confidence Level for Duplicates for Lowry-Peterson Assay

Samples compared	Test of significance
C1 and C2	+
X1 and X2	-
HCP1 and HCP2	-
GC1 and GC2	-

+ = significance at the 5% level.

This identifies that there is no significant difference between X1 and X2, HCP1 and HCP2 and GC1 and GC2. Therefore the duplicates can now be treated as one as one group of samples, so the duplicates are merged. C1 and C2 however show that there is only a certainty of 0.12 % that there is no significant difference between the samples so these must still be treated as two separate samples and therefore are not merged and retain the duplicate number.

The samples can now be compared to each other using the t-test to determine if there is any significant difference in the results between the different enzyme treatments of the wheatfeed (table shown below).

Confidence Levels for Comparing Samples from the Lowry -Peterson Assay Which Has Undergone Different Treatments.

Enzymes compared	C1	C2	X	HCP	GC
C1	- - - - - - -	+	-	+	+
C2	+	- - - - - -	-	+	+
X	-	-	- - - - -	+	+
HCP	+	+	+	- - - - - -	+
GC	+	+	+	+	- - - - - - -

The results were calculated using an excel spreadsheet which returns a probability that the null hypothesis is true. From this, the percentage certainty, or confidence level is calculated. The lower the confidence level, the more likely the differences in the results have arisen by random error in the samples rather than due to the treatment. The samples are said to be significantly different, or to show no significant difference at the 5% level. That is, if the confidence level is over 5% then the differences in the means of the samples are real and are fairly likely to have come about due to different sample treatment rather than random error in the samples.

It can be seen that although the control samples must be treated separately, there is no significant difference between the xylose result and both control results, thus suggesting that the xylose treatment does not have much effect on releasing protein. There is however, significant difference between both controls and both the HCP and GC samples suggesting that these samples do have an effect.

Glucose Kit.

The same numerical analysis was carried out using the results from the glucose kit test. The table below shows the results gained from this analysis. It can be seen all of the samples show no significant difference between the two duplicates so the samples from both duplicates can be merged and treated as one population.

Confidence Level for Duplicates for the Glucose Kit Test

Samples compared	Confidence Level
C1 and C2	-
X1 and X2	-
HCP1 and HCP2	-
GC1 and GC2	-

Confidence Levels for Comparing Samples Which Have Undergone Different Treatments Using the Glucose Kit Test.

Enzymes compared	Con	X	HCP	GC
Con	------	-	+	+
X	-	------	+	+
HCP	+	+	------	-
GC	+	+	-	------

The glucose values determined for all the enzyme mixtures studied follow a similar trend to those found for protein; i.e. xylanase (X) does not have a large effect on glucose release, conversely HCP and GC mixtures do. There is no significant difference shown between these latter two enzymic treatments, so the amount of glucose released by both must be similar.

The results demonstrate that although the results of duplicates often agree, it is useful to carry out experiments in duplicate as sometimes erroneous results occur.

HPLC

For each of the three sugars the HPLC analysis from the duplicates were compared using the t-test to see if there was a significant difference between the means of the samples using the null hypothesis as before. The samples could then either be merged, or treated separately to compare the sugars released for different treatments. The sugars are treated separately.

Xylose

For xylose, the C1 and C2 results were not included in the calculations because there was a large difference in the C1 results and as only two samples underwent HPLC, it is difficult to say which is the unusual result.

Results from HPLC for Duplicates Comparing the Sugar Xylose

Samples compared	Confidence Level
X1 and X2	+
HCP1 and HCP2	+
GC1 and GC2	-

The table above shows that only the GC duplicates can be merged the others must be treated separately. Due to the differences in the duplicates of the same treatments, nothing would be gained from comparing the differences due to different treatments.

Fructose

The tables below show the results from comparing the duplicates of the treatments. The fructose results show that there is no significant difference between duplicates of the same treatment. The results from the duplicates can therefore be merged and treated as one population. The results from the different treatments can then be compared

Results from HPLC for Duplicates Comparing the Sugar Fructose

Samples compared	Confidence Level
Con1 and Con2	-
X1 and X2	-
HCP1 and HCP2	-
GC1 and GC2	-

Comparing Different Treatments for Fructose Results

Enzymes compared	C	X	HCP	BGC
C	- - - - - -	-	-	-
X	-	- - - - - -	-	-
HCP	-	-	- - - - - -	-
GC	-	-	-	- - - - - -

It can be seen that there is no significant difference in the amount of fructose released between any of the treatments. This suggests that an amount of fructose in the wheatfeed will dissolve into a liquid medium regardless of any enzymic treatment. This amount is not increased by enzymic degradation of the bran.

Glucose

The results comparing the duplicates of the treatments are shown below.

Results from HPLC for Duplicates Comparing the Sugar Glucose.

Samples compared	Confidence Level
Con1 and Con2	-
X1 and X2	-
HCP1 and HCP2	-
GC1 and GC2	-

As there is no significant difference between any of the duplicates all the duplicate samples can be merged and compared. The treatments compared. The results are shown in the table below.

Comparing Different Treatments for Glucose Results

Enzymes compared	Con	X	HCP	BGC
Con	- - - - - - -	+	+	+
X	+	- - - - - - -	+	+
HCP	+	+	- - - - - - -	+
GC	+	+	+	- - - - - - -

The results show that there is a significant difference between all of the treatments and also between the treatments and the control.

It can be concluded from the HPLC results, that the best sugar to evaluate the effect of different enzyme mixtures is glucose, as the xylose results are generally low and duplicates of the same treatment show significant difference. The fructose has been shown to be an ineffective way of evaluating how well different treatments have worked. The glucose results however show no significant difference in the duplicates of the same treatment, but the effect of changing the enzyme mixture can be actively quantified.

Sugar Comparison.

HPLC data can be converted into concentrations for each reducing sugar using an area generated by the HPLC for a known concentration of the sugar. These concentrations can be added together to give a total concentration of reducing sugars. This can be compared with results gained from the Glucose Kit test. The results are shown below.

Comparison of Glucose and Total Reducing Sugars from the Two Different Analysis Methods

Sample	Glucose concentration mg/ml		TRS mg/ml
	Glucose kit	HPLC	HPLC
C1	8.25	6.63	13.40
C2	9.55	6.53	11.93
X1	9.90	7.73	14.11
X2	9.29	6.49	11.46
HCP1	16.54	16.63	30.74
HCP2	16.67	16.44	29.65
GC1	17.78	11.51	25.68
GC2	17.79	12.89	27.71

Where TRS = total reducing sugars.

The glucose test only measured the glucose in the sample. The results for the HPLC only take into account xylose, fructose and glucose (not all the reducing sugars), but can also be shown just as a measure of glucose which can be directly compared with the results from the glucose test. It can be seen that the results from HPLC analysis are generally lower than the results from the glucose kit test, although not by a consistent value. This could account for the earlier findings by the glucose test that HP was the best enzyme mixture, a fact later contradicted by the results from the HPLC analysis. The glucose kit is however a fairly effective quick test.

12.4 Appendix 4: Processing Characteristics of Wheatfeed.

Moisture and Protein Content

It was important to measure the protein content of the wheatfeed to give an idea of the maximum amount of protein available for release by enzymes

The protein content of dried samples of wheatfeed were analysed using the Kjeldahl method explained in section 3.6.5. It was found that the average protein content was 16.8%.

The moisture content was analysed by taking multiple samples of 2 g of wheatfeed and drying it overnight in an oven at 105°C. The dry samples were then reweighed and the moisture content calculated, the average moisture content was 11%.

These results differ slightly from the values suggested by (Fisher, 1985) who gave percentages of various bran components at 14% moisture and also completely dry bran. This suggests the bran he tested composed of 14% moisture and not 11%. The wheatfeed used for this study had a protein content of 16.8%, or 18.9% if a dry sample was used, this value was higher than that suggested by (Fisher, 1985) which was 12.4%. The differences in the results could be due to bran tested in the literature being a different variety than that used in the study, or due to seasonal changes. There is also the possibility that the bran previously tested was subjected to different up-stream processing to that used in this study. The results would also be dependant on the analysis methods employed.

Steam Explosion

Introduction

Steam explosion was carried out to degrade part of the fibre, which would leave larger pores for the enzyme to access the remaining fibre and therefore enhance the enzymic treatment.

Method

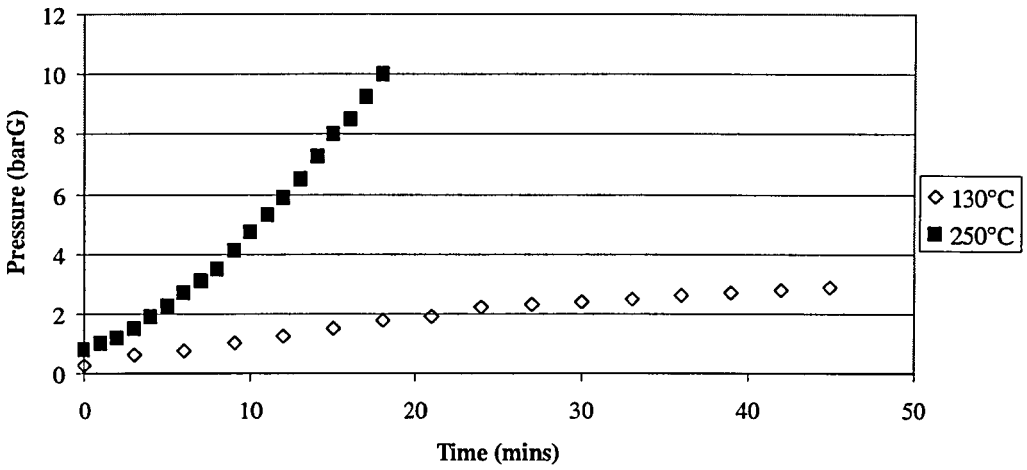
The initial steam explosion pretreatment experiments were performed using the basic method detailed in 3.4.2 however, some variations to that method were used. The explosion was carried out under two very different conditions, firstly at 250°C and secondly at 130 °C. In the first case, the pretreatment was allowed to continue until the pressure in the metal container reached 10 barG and in the second, the duration of pretreatment was restricted to 45 minutes.

After steam explosion the pretreated wheatfeed was dried and frozen for storage until later enzyme digestion. Before use the wheatfeed was defrosted.

The enzymic digestion was carried out in 50 ml conical flasks following the basic method detailed in section 3.7.1. A variation to this method was that celluclast was used. Celluclast (1.67 ml) was mixed into 10 ml citrate buffer at pH 5.0 and mixed. A further dilution was made by taking 1 ml of this mixture and adding to 9 ml of citrate buffer solution and mixing. The resulting 10 ml enzyme-buffer solution was used in the experiment.

Results and Discussion

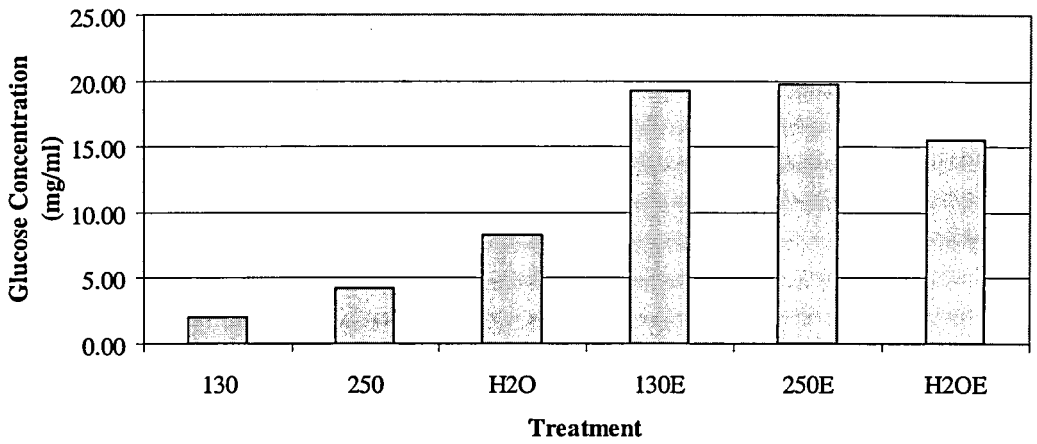
The steam explosion pressure profiles for the two runs can be seen in the figure below.



Steam Explosion Pressure Profile for Two Extreme Runs.

The wheatfeed pretreated under these two conditions underwent an enzymic digestion. The results shown in the figure below show that after enzymic digestion, the most glucose was released from the wheatfeed which had been subjected to 250°C, this was closely followed by the wheatfeed which had been subjected to 130°C. The un-pretreated wheatfeed, shown on the graph as H2OE gave the lowest release of glucose. However, the results show a different pattern for the non enzymically digested samples, seen on the left three columns of the graph. In this case, the un-pretreated sample (H2O) releases the most glucose.

This could be because some of the available glucose is lost by degradation of the fibre during the steam explosion process. The 130°C steam exploded wheatfeed (130) could release less glucose than the 250°C steam exploded wheatfeed (250) because the higher temperature may give a harsher treatment which opens the pores of the remaining fibre.



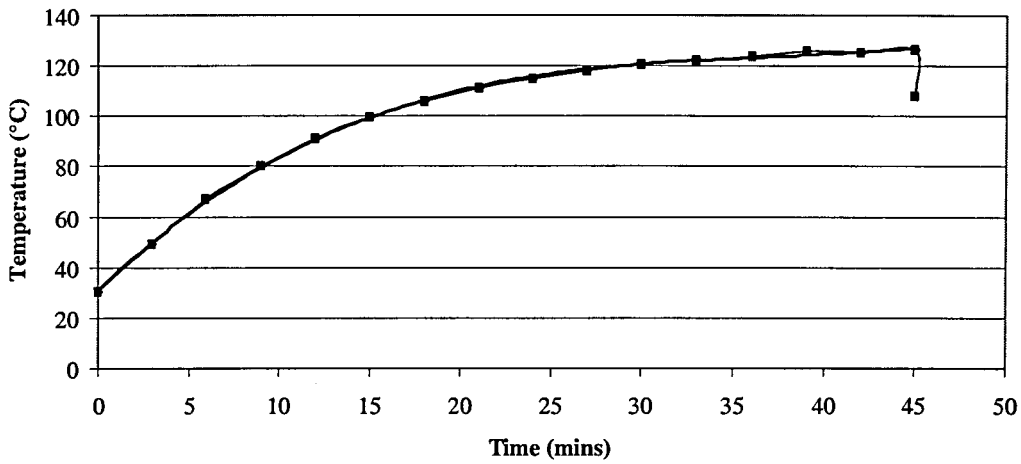
Comparison of the Sugar Released from Enzymically Digested Steam Pretreated and Un-pretreated Wheatfeed Measured using the Accu-check Meter.

Where, 130 denotes wheatfeed steam exploded at 130°C for 45 minutes, 250 denotes wheatfeed steam exploded at 250°C until the pressure reached 10 barG., H20 denotes wheatfeed mixed with the same amount of water as the steam exploded wheatfeed, but omits the steam explosion step. An E in the treatment name denotes that the sample was digested by enzymes.

The resulting pretreated wheatfeed from the 250°C looked and smelt burnt. It was consequently decided that treatment at this temperature would give the final product an undesirable taste and therefore experiments using these conditions were ceased regardless of the beneficial effects on glucose release. Further steam pretreatments were always carried out at 130°C for 45 minutes.

It can be seen from the figure below that during a typical 130°C run, the metal container never quite reaches 130°C. The highest temperature achieved is about 127°C. The sharp drop in temperature at the end of the graph was at a time of 45 minutes, when the steam release valve was opened. The severity of the steam pretreatment was calculated by splitting the graph into three sections (0 to 12 minutes, 12 to 24 minutes and 24 to 45 minutes) and assuming a linear gradient over each section of time. The severity of each section was calculated and then

the total severity of the process calculated. The severity of the three sections were 1.96, 18.07 and 99.05 respectively. The total severity was 119.1, or a $\log R_0$ of 2.07. This is a lower severity than used by other workers (Josefsson *et al.*, 2002; Soderstrom *et al.*, 2003), but to achieve a higher severity would involve a higher temperature and as previously stated this is undesirable.



Steam Explosion Temperature Profile at an Oven Temperature of 130°C.

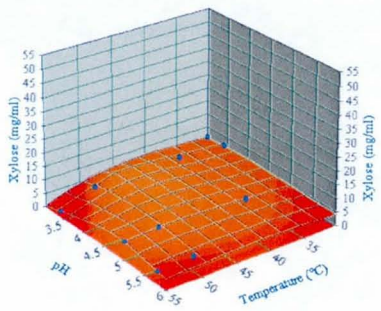
Conclusion

Steam explosion pretreatment of wheatfeed at both 130°C for 45 minutes and 250°C for 18 minutes enhanced the release of glucose during enzymic digestion. The 250°C treatment releases the most glucose however, the wheatfeed looks and smells burnt.

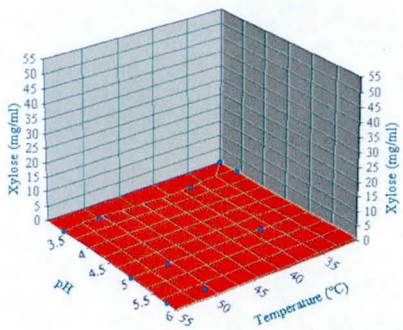
Steam explosion degrades some of the more easily available glucose, this is shown by the drop in glucose in the results for the control digest samples.

12.5 Appendix 5: Optimum Conditions Graphs

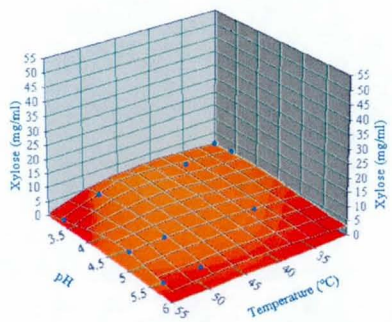
Surface Plot of Xylose Released from Wheatfeed after Enzymic Digestion for 8 Hours.



Surface Plot of Xylose Released from Wheatfeed in the Absence of Enzymes for 8 Hours.

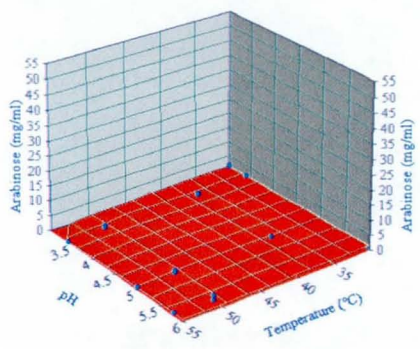


Surface Plot of the Corrected* Xylose Release from Wheatfeed after Enzymic Digestion for 8 Hours.

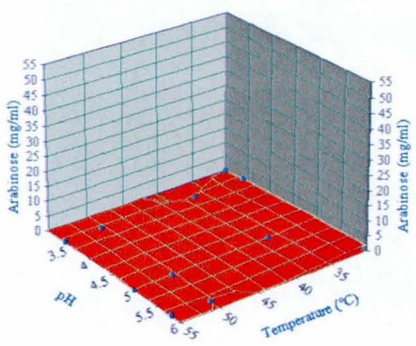


* These were obtained by subtracting the concentrations detected in the control experiments conducted at identical conditions.

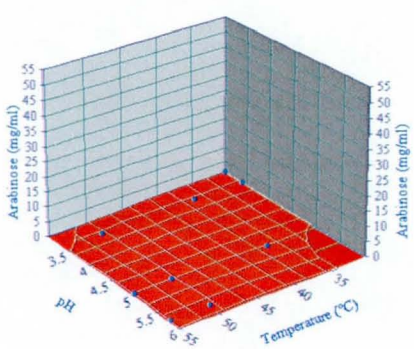
Surface Plot of Arabinose Released from Wheatfeed after Enzymic Digestion for 8 Hours.



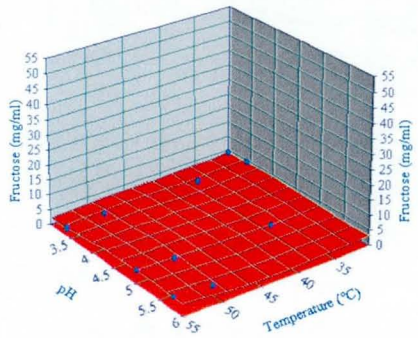
Surface Plot of Arabinose Released from Wheatfeed in the Absence of Enzymes for 8 Hours.



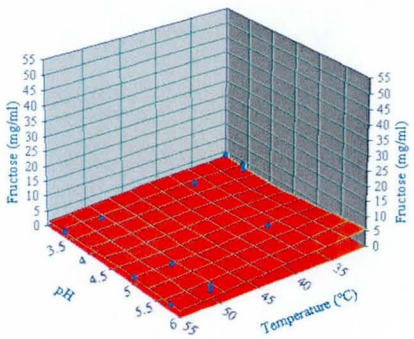
Surface Plot of the Corrected* Arabinose Release from Wheatfeed after Enzymic Digestion for 8 Hours.



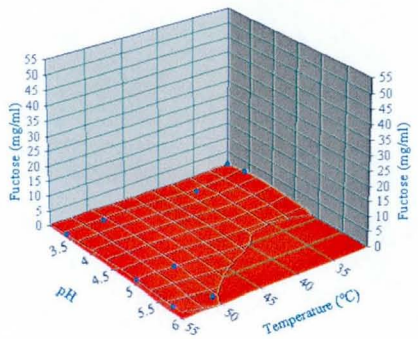
Surface Plot of Fructose Released from Wheatfeed after Enzymic Digestion for 8 Hours.



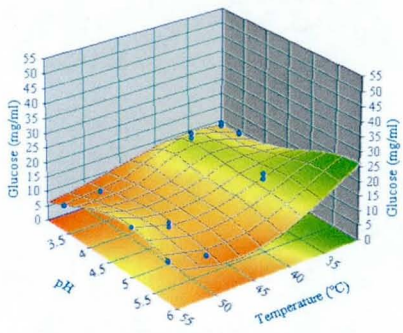
Surface Plot of Fructose Released from Wheatfeed in the Absence of Enzymes for 8 Hours.



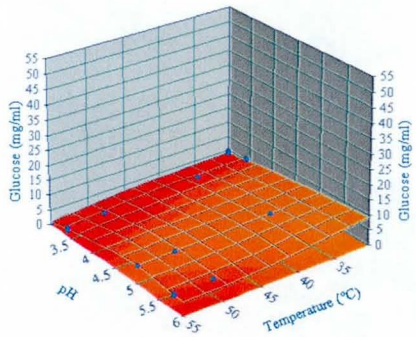
Surface Plot of the Corrected* Fructose Release from Wheatfeed after Enzymic Digestion for 8 Hours.



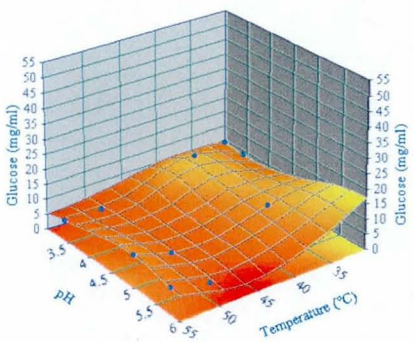
Surface Plot of Glucose Released from Wheatfeed after Enzymic Digestion for 8 Hours.



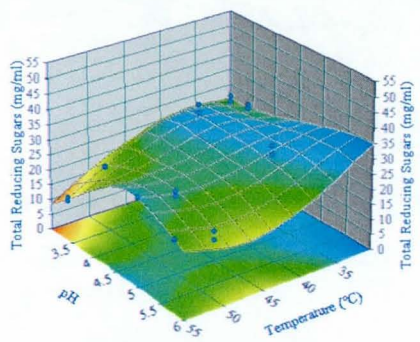
Surface Plot of Glucose Released from Wheatfeed in the Absence of Enzymes for 8 Hours.



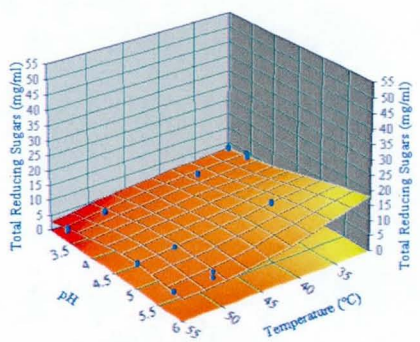
Surface Plot of the Corrected* Glucose Release from Wheatfeed after Enzymic Digestion for 8 Hours.



Surface Plot of Total Reducing Sugars Released from Wheatfeed after Enzymic Digestion for 8 Hours.



Surface Plot of Total Reducing Sugars Released from Wheatfeed in the Absence of Enzymes for 8 Hours.



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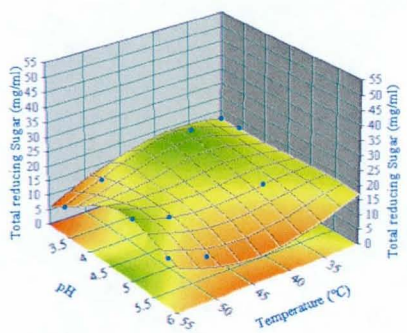
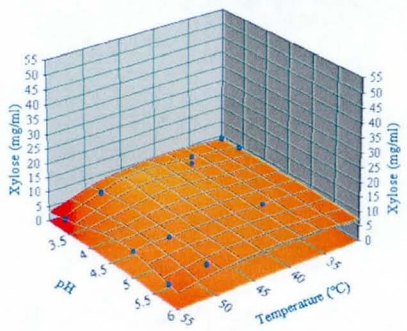
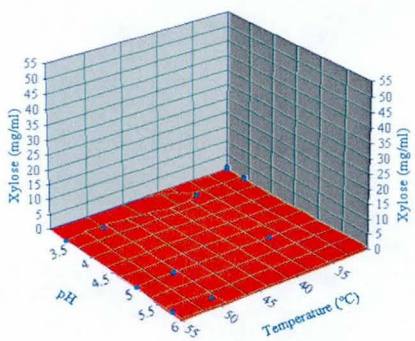


Surface Plot of Xylose Released from Wheatfeed after Enzymic Digestion for 24 Hours.



Surface Plot of Xylose Released from Wheatfeed in the Absence of Enzymes for 24 Hours.



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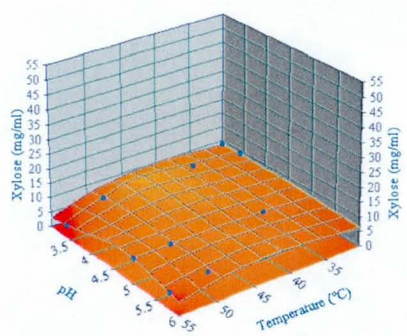
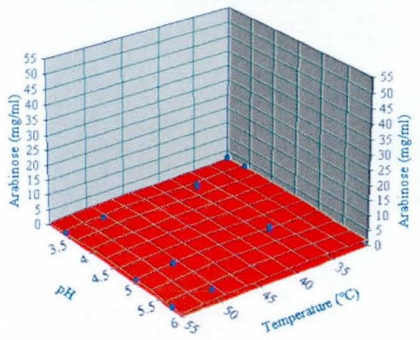
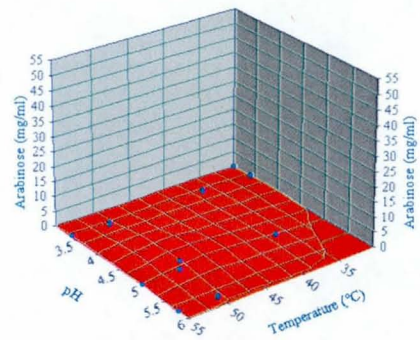


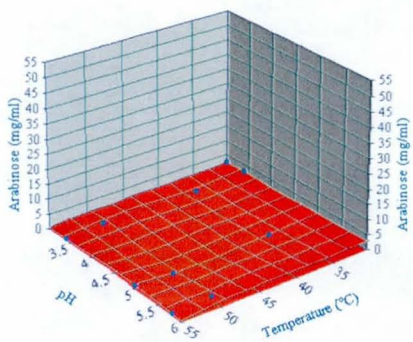
Surface Plot of Arabinose Released from Wheatfeed after Enzymic Digestion for 24 Hours.



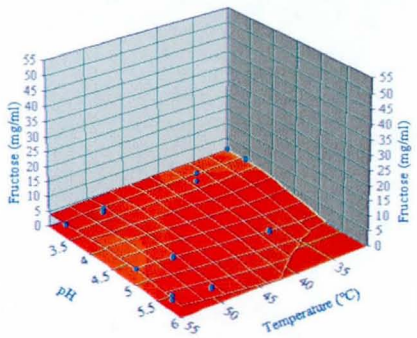
Surface Plot of Arabinose Released from Wheatfeed in the Absence of Enzymes for 24 Hours.



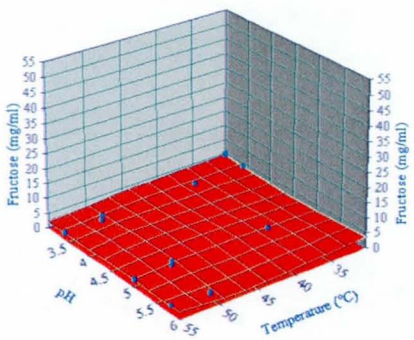
Surface Plot of the Corrected* Arabinose Release from Wheatfeed after Enzymic Digestion for 24 Hours.



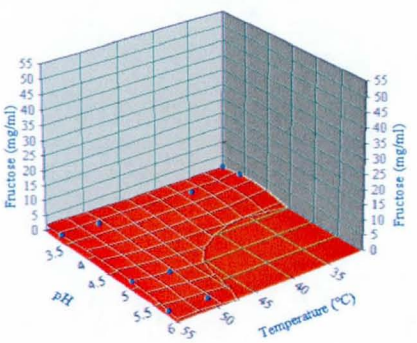
Surface Plot of Fructose Released from Wheatfeed after Enzymic Digestion for 24 Hours.



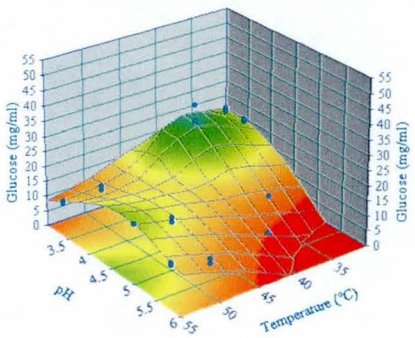
Surface Plot of Fructose Released from Wheatfeed in the Absence of Enzymes for 24 Hours.



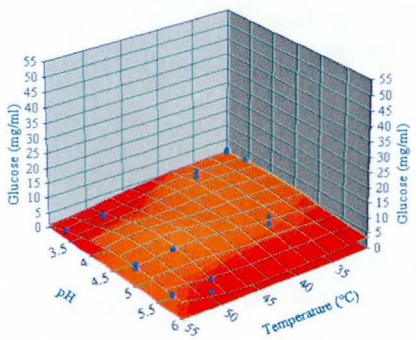
Surface Plot of the Corrected* Fructose Release from Wheatfeed after Enzymic Digestion for 24 Hours.



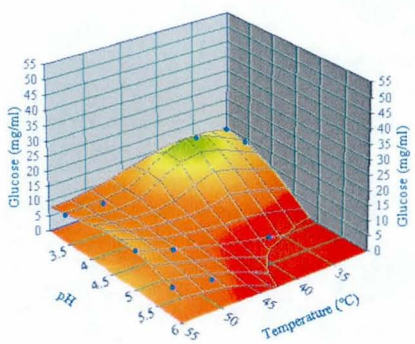
Surface Plot of Glucose Released from Wheatfeed after Enzymic Digestion for 24 Hours.



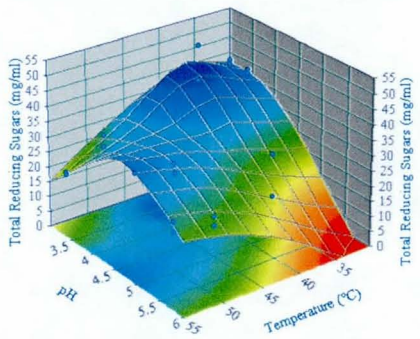
Surface Plot of Glucose Released from Wheatfeed in the Absence of Enzymes for 24 Hours.



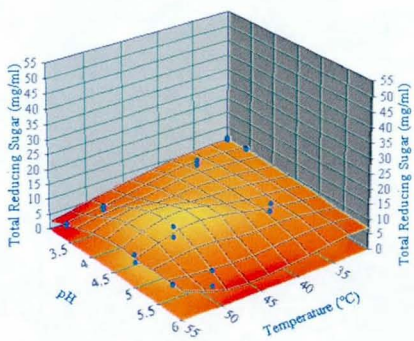
Surface Plot of the Corrected* Glucose Release from Wheatfeed after Enzymic Digestion for 24 Hours.



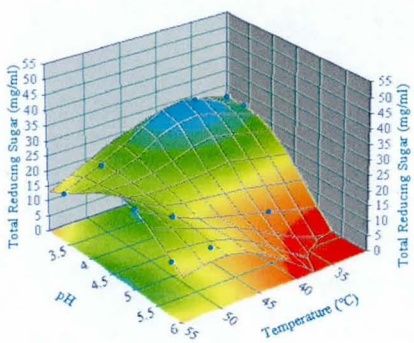
Surface Plot of Total Reducing Sugars Released from Wheatfeed after Enzymic Digestion for 24 Hours.



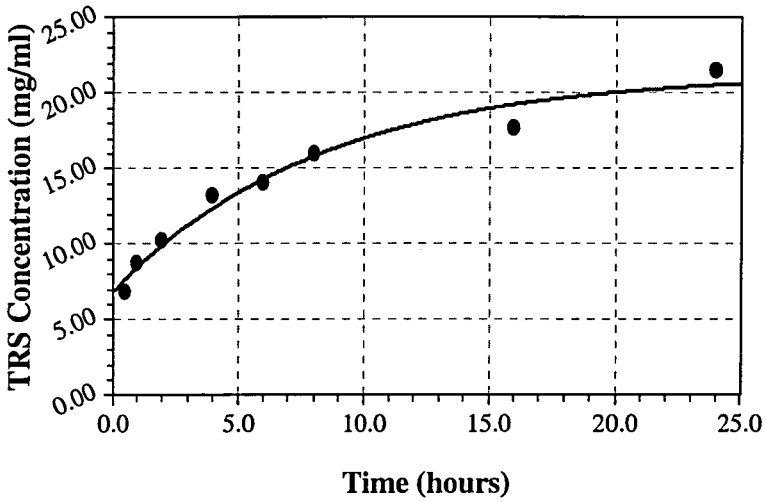
Surface Plot of Total Reducing Sugars Released from Wheatfeed in the Absence of Enzymes for 24 Hours.



Surface Plot of the Corrected* Total Reducing Sugars Released from Wheatfeed after Enzymic Digestion for 24 Hours.

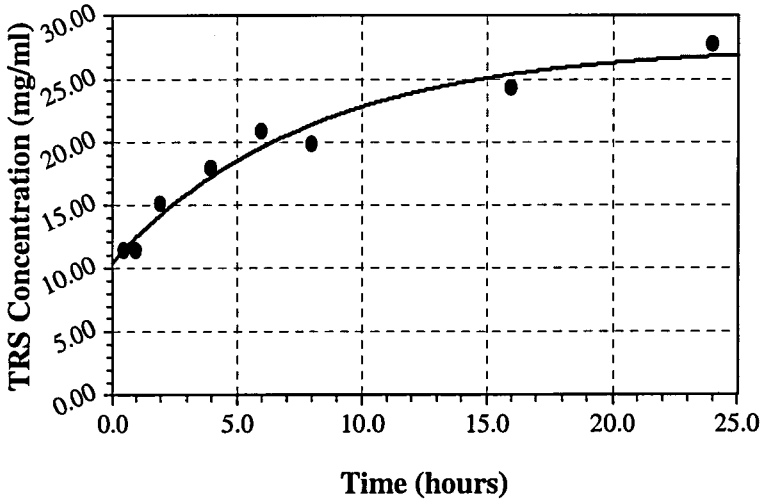


12.6 Appendix 6: Enzyme Loadings Kinetic Graphs



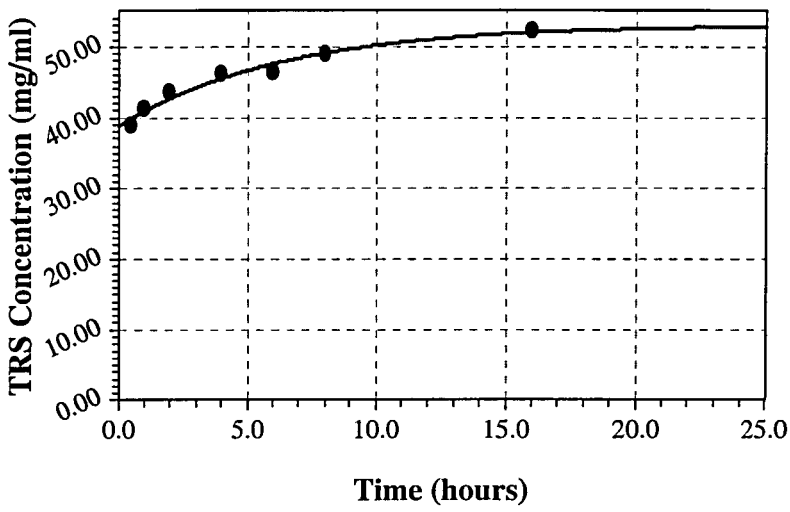
The Total Reducing Sugar Concentration Plotted Against Time for the 12.5 ml Enzyme Loading.

a=14.5 (mg/ml), b=1.47, c=0.121 (ml/mg)



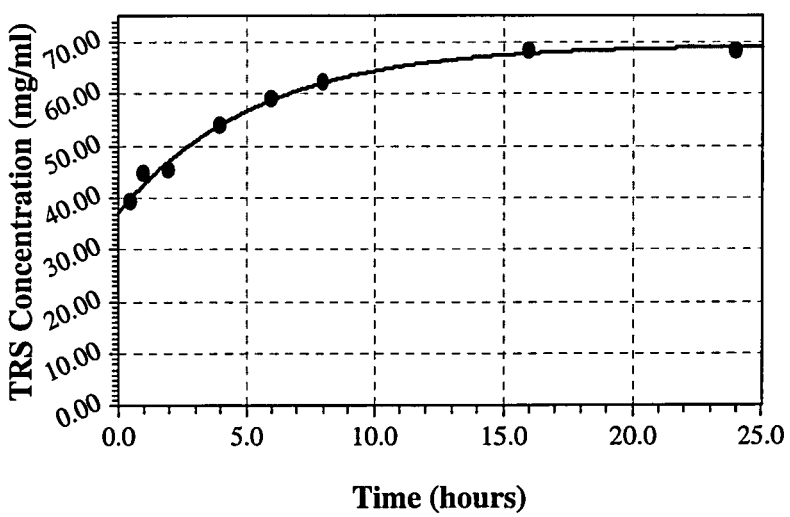
The Total Reducing Sugar Concentration Plotted Against Time for the 25 ml Enzyme Loading.

a=17.2 (mg/ml), b=1.60, c=0.126 (ml/mg)



The Total Reducing Sugar Concentration Plotted Against Time for the 193 ml Enzyme Loading.

a=14.3 (mg/ml), b=3.72, c=0.160 (ml/mg)



The Total Reducing Sugar Concentration Plotted Against Time for the 245 ml Enzyme Loading.

a=32.3 (mg/ml), b=2.15, c=0.185 (ml/mg)

12.7 Appendix 7: Extrusion

The wheatfeed was incorporated into a typical kibble recipe and extruded through a single screw extruder. The ingredients representative of a dog biscuit (kibble) recipe (see table below) and extruding the mixture. The final product can be seen in the figure below. The digested wheatfeed caused no problem with extrusion.

Typical Kibble Recipe.

Ingredient	**Weight % of Mixture**
Wheat	65
Poultry Meal	15
Wheatfeed	10
Maize	7
Sunflower Oil	3
Total	**100**

Extruded Dog Food Using Enzymically Treated Wheatfeed

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