PRODUCTION OF ENZYMES FOR APPLICATION ON ANIMAL FEEDS

Busiswa Godana

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Supervisors: **Prof. S. Singh (Ph.D)**

Dr R. Mitra (Ph.D)

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DECLARATION

I hereby declare that this is my own, unaided work. It is being submitted for the degree of Master of Technology (Biotechnology) to Durban University of Technology, Durban. It has not been submitted before, for any degree or exam, at any other institution. Where use was made of the work of others, it has been duly acknowledged in the text.

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Busiswa Godana

March 2007

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Prof. S. Singh

This work is dedicated to my loving mother Neliswa Veronica Nokubonga Godana; I couldn’t have done this without your support.

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1.1 INTRODUCTION

Enzymes are naturally occurring and are produced by all living organisms and, as nature's catalysts; they speed up the chemical reactions that enable all living things to function. Enzymes, as with all proteins, are made from chains of amino acids. They catalyse reactions by binding to their substrate and stabilise the entire reaction process through to product formation, so that far less energy is required to move the reaction forward (Sheppy, 2001).

Feed enzyme products for ruminants are concentrated fermentation products that have specific enzyme activities, although usually the main activities are controlled. Enzyme preparations for ruminants are evaluated based on their capacity to degrade plant cell walls (Pendleton, 1998). These enzymes fall into general classification of cellulases and xylanases. Each of these categories can consist of numerous specific enzymes having activity against cellulose or xylan. Enzymes with cellulase activity include numerous types of endoglucanases that cleave internal β-1,4 and β-1,3 linkages, cellobiohydrolases that attack cellulose chains from the non- reducing end, and β-glucanases that cleave within the main chain of mixed linkage β-glucan. Thus, the specific types of cellulases and xylanases can differ substantially among products, and differences in the relative proportions and activities of these individual enzymes will have an impact on the efficacy of cell wall degradation by these products (Beauchemin *et al*., 2001).

Slow or incomplete digestion of fibrous substrates often limits the overall digestive process in the rumen and can significantly influence animal performance in livestock production systems that use forages as a major component of the diet. Poor nutrient utilisation, by young animals in particular, has been blamed on the compromising of the neonatal digestive system by cereal structural carbohydrates. These non-starch polysaccharides and especially the arabinoxylans and mixed linkage β-glucans present in the endosperm of cereal grains have been implicated as major causative factors in this response (Graham and Balnave, 1995). As a result, many strategies have been developed to stimulate the digestion of the fibrous components in ruminant feeds. These have included the use of specific nutrients that stimulate fibre digestion, processing feeds to increase the rate and extent of fibre digestion, and the use of direct-fed microbials and yeast culture to stimulate the natural ability of the microbial population in the rumen to digest fibrous substrates (Dawson and Tricarico, 1999).

Advances in fermentation technology have allowed for the economic production of large quantities of biologically active enzymes that can also be used as livestock feed supplements. These technologies provide new possibilities for altering digestive processes in a wide variety of animals. Specific enzyme preparation can be used to drive specific metabolic and digestive processes to increase nutrient availability and intake. Strategies that use supplemental enzyme activity in the rumen may be important since the digestibility of organic matter in the rumen does not reach 100% and even small changes in digestibility can influence the efficiency of ruminal fermentations (Lewis *et al*., 1996; Annison, 1997).

Many feedstuffs contain anti-nutritional factors that interfere with the digestibility, adsorption, or utilisation of nutrients, and thereby adversely affect animal performance. Anti-nutritional factors can also be expected to disturb the digestive process of farm animals, as they lack the appropriate enzymes in their gastrointestinal tracts to render them ineffective (Walsh *et al*., 1997). The nutritional availability of fibre to livestock varies greatly, depending on its composition and structure. Fibre is slowly and incompletely digested and therefore the proportion of fibre to cell solubles is the major determinant of energy available. Lignin interferes with microbial degradation of fibre polysaccharides by acting as a physical barrier and by being cross–linked to polysaccharides by ferulate bridges (Buxton and Redfearn, 1997).

Supplementing cattle feed with enzymes containing amylolytic, proteolytic, and cellulolytic activities showed significant improvements in average daily gain and feed conversion ratio (Beauchemin and Rode, 1996). However, exogenous enzymes can only be beneficial when the feed composition and the enzyme preparation are complementary, and maximal effects are obtained when the enzyme additives are applied to the feed in aqueous form. Increasing the interval between the enzyme application to the feed and feeding creates a stable complex that increases enzyme effectiveness, and enzyme application to the feed before feeding is more beneficial than direct application into the ruminal environment (Beauchemin and Rode, 1996).

Feed digestion rate and the absorption of digestion products depend on the formation of a complex between the digestive enzyme and its substrate, and subsequent release of its products. Fibre digestion is a complex process that is not only affected

by the forage, but also by the retention of forage particles in different segments of the gastrointestinal tract, amount and activity of enzymes secreted by fibrolytic organisms in the rumen (Bedford, 1995). Forage fibre consists of complex carbohydrates that include cellulose, hemicellulose, pectins and lignin. The use of crude enzyme preparations enhance feed digestibility by hydrolysing fibre not usually degraded by natural enzymes, enhancing bio-availability of polysaccharides and proteins and complementing the enzymes in young animals to enhance digestive systems not fully developed at weaning (Teller and Vanbelle, 1990).

1.2 Sources of differences in plant digestibility

One of the differences in fibre digestibility and utilization among forage species is between legumes and grasses. Legumes are typically more digestible than grasses because they contain less fibre, not because legume fibre is more digestible. In fact, legume fibre is more lignified and less digestible than that of grasses (Buxton and Redfearn, 1997). Not all plant fibre is digestible, even if it remains in the rumen for a long time. In mature forage stems, up to two-thirds of the NDF and more than half of the structural polysaccharides may be completely indigestible (Buxton and Casler, 1993). Lignin concentration is closely related to the proportion of indigestible dry matter (Buxton *et al*., 1996). Depending on maturity, ruminants digest 40-50% of NDF in legumes and 60-70% in grasses. The proportion of digestible energy obtained from NDF varies from 20-40% for legumes and 50-80% for grasses. Thus, most of the digestible energy in legumes comes from cell solubles not from fibre (Buxton *et al*., 1995).

Lignin is thought to interfere with microbial degradation of fibre polysaccharides by acting as a physical barrier. The importance of cross-linking of lignin to polysaccharides by ferulate bridges has been implicated as an additional factor inhibiting digestion of grass fibre. Similar cross-linking of lignin to fibre polysaccharides has not yet been identified in legumes (Jung and Allen, 1995). Physical and structural barriers may limit fibre digestibility beyond the effect of lignin. Waxes and the cuticle of the epidermis covering plants restrict microbes and enzyme access to forage tissues (Wilson and Kennedy, 1996) and plant anatomy at cellular level also influences fibre digestibility (Table 1.1). Both grass and legume stems have a ring of thick-walled lignified cells resistant to digestion (Akin, 1989).

Table 1.1 Summary of plant tissues and their relative digestibility (Buxton and Redfearn, 1997).

Tissue	Function	Digestibility	Comments
Mesophyll	Contain chloroplast	High	Thin wall, no lignin. Loosely arranged in legumes and grasses
Parenchyma	Metabolic	Moderate to high	In midrib of grass and main vein of legume leaves, leaf sheath and stem of grasses and petiole and stem of legumes. Highly digestible when immature.
Collenchyma	Structural	Moderate to high	In legume leaves and stems. Thick wall not lignified.
Parenchyma bundle sheath	Contain chloroplast	Moderate to high	Surrounds vascular tissue in leaf blades. Wall moderately thick and weakly lignified.
Phloem fibre	Structural	Moderate	In legume petioles and stems. Often does not lignify.
Epidermis	Dermal	Low to high	Outer wall thickened and lignified, and covered with cuticle and waxy layer.
Vascular tissue	Vascular	None to moderate	Comprises phloem and xylem. Major contributor to indigestible fraction.
Sclerenchyma	Structural	None to low	Up to 1200 μm long and 5-20 μm in diameter, thick lignified wall.

Several grass cell types become lignified during maturation, whereas in legumes, xylem and tracheary cells are the only major tissues lignified (Wilson, 1993). Lignified secondary walls of grass cells are digestible when microbes have access t these walls. Lignified primary walls and middle lamella, however, are not digestible. In contrast, lignified secondary walls of xylem and tracheary cells of legumes are not digestible (Wilson and Mertens, 1995). Lignification in these legume cells is much higher than those in grasses, which might be the reason secondary walls in legumes, cannot be digested (Buxton and Redfearn, 1997).

1.3 Cellulose and hemicellulose

Cellulose and hemicellulose are the major plant structural polysaccharides and account for approximately 70% of plant biomass. In addition to the pivotal role in maintaining the structural integrity of plants, cellulose and hemicellulose, serve as major source of nutrients for herbivores and as renewable substrate for the production of food, animal feed as well as textiles (Gilbert and Hazlewood, 1993; Beguin and Aubert, 1994). Cellulose is a linear polymer of glucose linked by β-1,4-glycosidic bonds, having a simple primary and complex tertiary structures and its repeating unit is cellobiose (Bhat and Hazlewood, 2001). Its chain length can vary between 100 and 14000 glucose residues, which form microfibrils (figure 1.1). The microfibrils consist of highly ordered crystalline regions interspersed by more disordered amorphous regions. The crystalline regions of cellulose are rigid and not easily accessible to endo-acting cellulases while the amorphous regions are easily attacked by dilute acid, endoglucanases or exoglucanases (Sinitsyn *et al*., 1990). Thus, for the complete hydrolysis of cellulose, either concentrated acid or a complete

cellulase system capable of attacking both amorphous and crystalline regions is necessary.

It has been established that cellulose can be converted to soluble sugars by enzymes of mainly microbial origin collectively termed cellulases and hemicellulases (Viikari *et al*., 1993). Microorganisms including fungi, bacteria and actinomycetes produce mainly three types of cellulase components: endoglucanase, exoglucanase and β-glucosidase, either as separate entities or in the form of aggregated complex, for cellulose hydrolysis (Wood, 1985; Bhat and Bhat, 1997).

The term hemicellulose refers to a group of homo- and heteropolymers consisting largely of anhydro-β-(1,4)-D-xylopyranose, mannopyranose, glucopyranose, and galactopyranose main chains with a number of substituents. The occurrence, abundance, distribution and structure of hemicelluloses, as well as their relationship with other cell wall components are dependent on the species, tissue, and growth conditions of the plant (Thompson, 1993; Wong and Saddler, 1993). Hemicelluloses are usually named according to the main sugar residues in the backbone, e.g., xylans, glucomannans, galactans and glucans (Viikari *et al*., 1993). Xylan is the most common hemicellulosic polysaccharide, representing more than 30% of the wood dry weight (Joseleau *et al*., 1992). Xylans are composed of 1,4-linked β-D–xylopyranosyl residues (Wong and Saddler, 1993).

The hemicellulose fraction of the cell walls of most species of land plants contains mainly xylan and mannan and requires a more extensive repertoire of enzymes to affect complete hydrolysis to soluble sugars (Biely *et al*., 1992; Hazlewood and

Gilbert, 1998). The two main enzymes involved are endoxylanases (xylanases) and endomannanases (mannanases), which attack the backbone structure (Viikari *et al*., 1993).

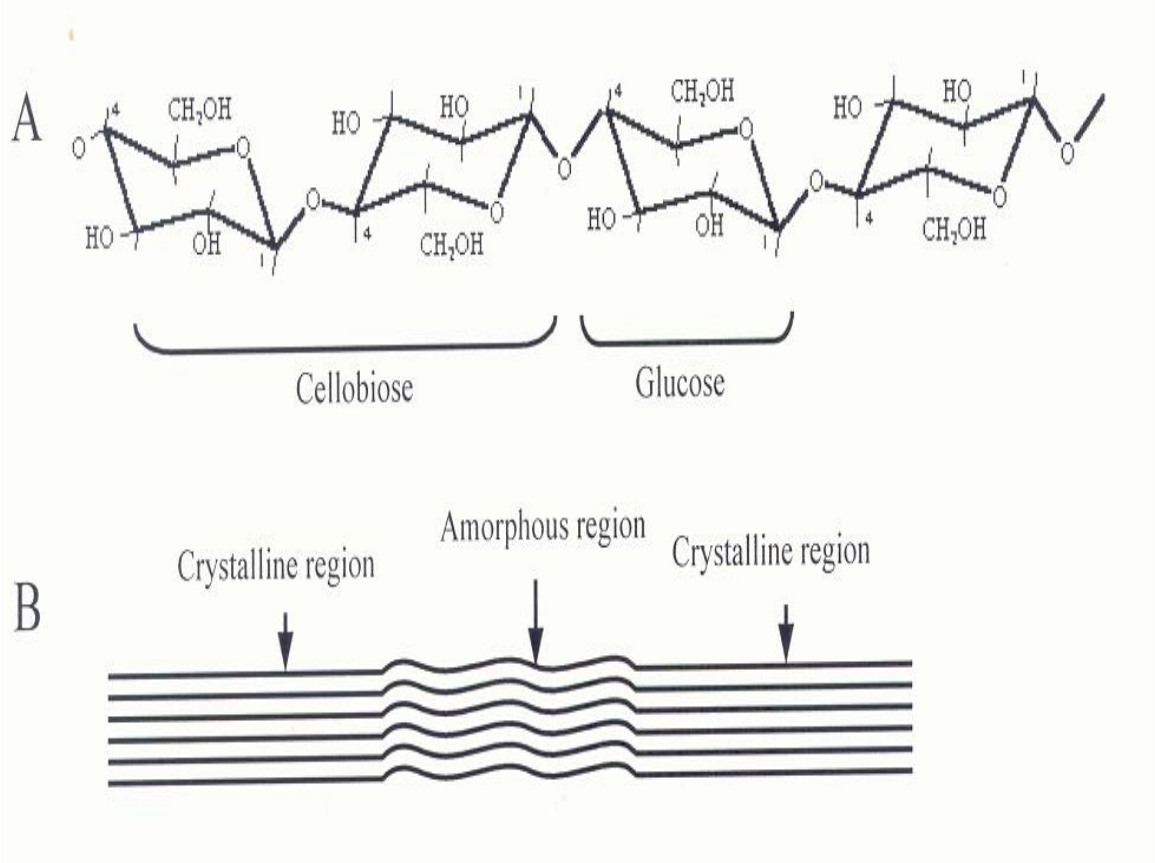

Figure 1.1 The structure of cellulose showing: A. Cellobiose and glucose sub-units, B. Cellulose microfibrils showing crystalline and amorphous regions (Beguin and Aubert, 1994).

1.4 Xylanases

Xylan is a complex heteropolysaccharide and its complete hydrolysis requires the action of different enzymes. β-1,4-endoxylanase are important enzymes that hydrolyse the backbone of xylan but for these enzymes to hydrolyse xylan backbone efficiently, other enzymes are required for the removal of side chains which can prevent xylanases from completely degrading branched xylan (figure 1.2). Several microorganisms involved in xylan degradation produce a suite of auxiliary enzymes such as α-L-arabinofuranosidase, α-D-glucuronidase, α-galactosidase, acetyl xylan esterase, and feruloyl esterase that are needed to make the backbone accessible (Shallom and Shoham, 2003).

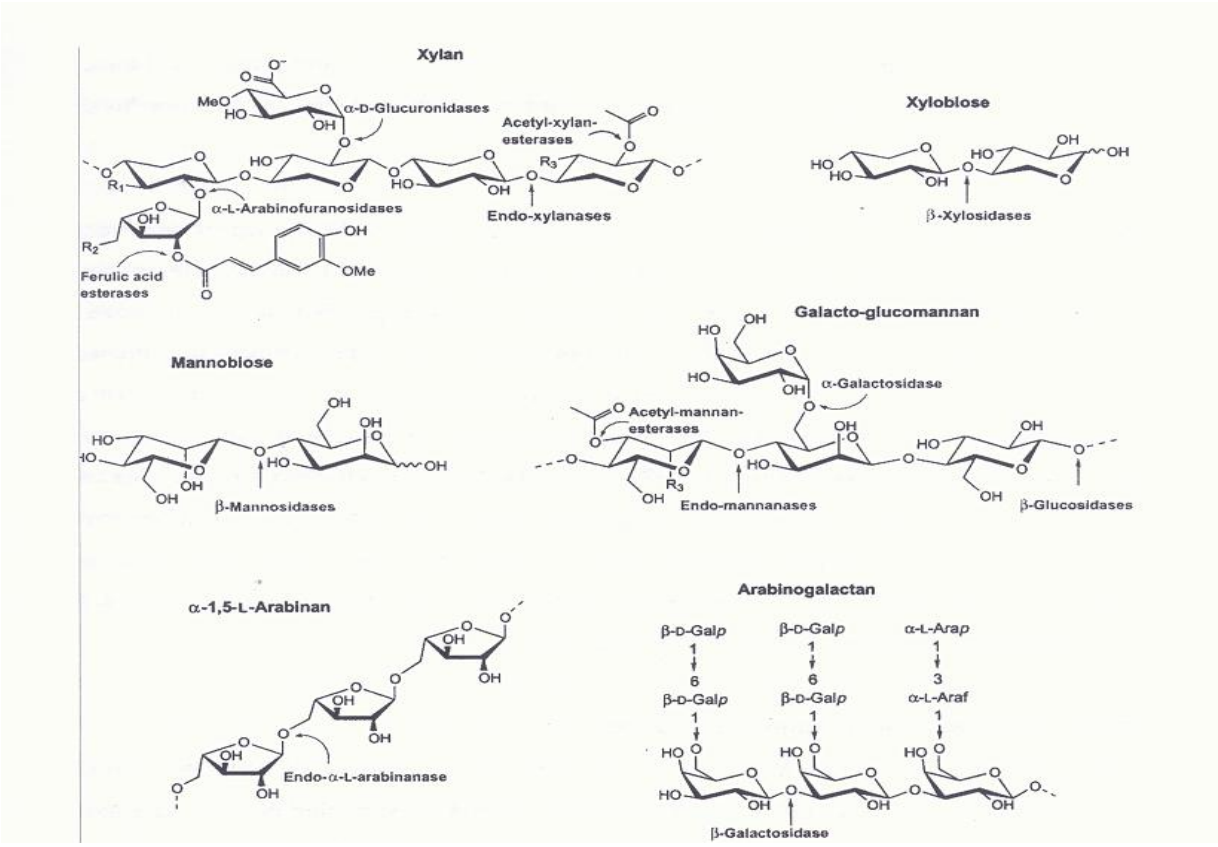

Figure 1.2. Schematic representation of hemicelluloses and enzymes responsible for attacking specific bonds in hemicelluloses (Shallom and Shoham, 2003).

Xylanases are produced by a wide range of bacteria and fungi, including aerobes, anaerobes, mesophiles, thermophiles and extremophiles. Aerobic bacteria and fungi generally produce extracellular xylanases. β-1,4-endoxylanase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the heteroxylan backbone, resulting in a decrease in the degree of polymerisation (DP) of the substrate. The attack of the substrates is random and the bonds to be hydrolysed depend on the nature of substrate. During hydrolysis of xylan, the main products formed are xylooligosaccharides (Wong *et al*., 1988).

1.4.1 Substrate specificity

Xylanases are specific for the internal β-1,4 linkages of the polymeric xylan and are designated as endoxylanases. Based on their action on different polysaccharides, endoxylanases have been classified as either specific or non-specific (Coughlan *et al*., 1993). The specific endoxylanases are specific on xylans with only β-1,4 linkages whereas non-specific endoxylanases hydrolyse β-1,4-linked xylans, β-1,4linkages of mixed xylans and other β-1,4-linked polymers such as carboxymethylcellulose (Bhat and Hazlewood, 2001).

Generally, endoxylanase activity and affinity for xylo-oligosaccharides decrease with decreasing degree of polymerisation (Coughlan *et al*., 1993). Most endoxylanases are specific for unsubstituted xylosidic linkages of xylans and release both substituted and unsubstituted xylo-oligosaccharides. Most endoxylanases, active on mixed xylan (rhodymenan with β-1,3 and β-1,4 linkages) are specific for the β-1,4 linkages (Coughlan, 1992). Endoxylanases have also been grouped as either debranching or

non-branching based on their ability to release arabinose in addition to hydrolysing the main chain (Coughlan *et al*., 1993).

1.4.2 Mode of action

Fungal and bacterial endoxylanases cleave the internal β-1,4 linkages of the xylan backbone and release mainly xylobiose, xylotriose and substituted oligomers having two to four residues (Table 1.3). It has been reported that most endoxylanases cleave the xylan backbone leaving the substituent at the non-reducing end of the xylosyl chain or the oligosacchiride (Dekker, 1985). Moreover, the presence of the glucopyranosyl uronic acid sterically hinders the hydrolysis of the second and third xylosidic linkages to the right of the branch points by xylanases from *Trametes hirsute* (Comtat and Joseleau, 1981). Nevertheless, the xylanases from *Aspergillus niger* and *C. sacchari* were found to cleave the substituent on the reducing end and in the middle of the oligosaccharide chain, respectively (Dekker, 1985).

Based on amino acid sequence similarities and hydrophobic cluster analysis, endoxylanases can be grouped into two major families of glycosyl hydrolases, family 10 and 11 (Henrissat and Bairoch, 1996). Family 10 includes mainly acidic, high molecular mass endoxylanases while basic and low molecular mass endoxylanases belong to family 11. Endoxylanases of family 10 hydrolyse heteroxylans and homoxylans to a higher degree than those of family 11 and family 10 are the only endoxylanases that are capable of cleaving the glycosidic linkages of xylan either closer or adjacent to substituents, such as methylglucuronic acid and acetic acid (Biely *et al*., 1997). In addition, it was reported that the endoxylanases of family 10

require two unsubstituted xylose residues between the branch points, whereas family 11 endoxylanases require at least three unsubstituted xylose residues in sequence. The isolation and identification of methylglucuronic acid and arabinose-linked oligosaccharides released from methylglucuronoxylan and arabinoxylan by endoxylanases of family 10 revealed that the substitution at position 3 could cause greater steric hindrance of enzyme attack than at position 2. Furthermore, only endoxylanases of family 10 released monoacetylated xylobiose from acetylxylan, with the exception of a family 11 endoxylanase from *Trichoderma reesei* (Biely *et al*., 1997).

1.4.3 Synergism between xylan degrading enzymes

Efficient and complete hydrolysis of xylan requires the synergistic action of the main- and side-chain-cleaving enzymes with different specificities (Coughlan and Hazlewood, 1993). For xylan degrading enzymes, it is difficult to demonstrate the synergism by measuring the produced reducing sugars because of the heterogeneous nature of the substrate. Hence, the hydrolysis products must be separated, identified and quantified in order to obtain a detailed picture of the synergistic action of xylan-degrading enzymes. Three types of synergy between such enzymes have been described: homeosynergy, heterosynergy and antisynergy (Coughlan *et al*., 1993). Homeosynergy is the synergistic interaction between two or more different types of side-chain-cleaving enzymes (Coughlan *et al*., 1993) and is best demonstrated for ferulic acid esterase from *A. oryzae* and α-L-arabinofuranosidase from *P. capsulatum*, where the former enzyme facilitates the

release of arabinose from feruloylated arabinoxylan by the latter (Coughlan *et al*., 1993).

Heterosynergy is the synergistic interaction between side-chain- and main-chain-cleaving enzymes (Coughlan *et al*., 1993). Heterosynergy is uniproduct if the action of the main-chain-cleaving enzyme facilitates the release of a substituent by the side-chain-cleaving enzyme or vice-versa. Heterosynergy is biproduct if the extent of liberation of substituent, and the hydrolysis of the main chain, by the action of combined enzymes exceed the sum of the actions of the individual enzymes (Bhat and Hazlewood, 2001). Heterosynergy has been report between ferulic acid esterases and endoxylanases (Faulds and Williamson, 1991; Tenkanen *et al*., 1991).

Antisynergy occurs when the action of the one type of enzyme prevent the action of a second enzyme (Coughlan *et al*., 1993). This can be observed in the case either of arabinoxylan xylanohydrolase or glucuronoxylan glucuronohydrolase (Frederick *et al*., 1985), an enzyme that cleaves the main chain linkages only in the vicinity of a particular substituent. For example in the case of glucuronoxylan glucuronohydrolase, the presence of substituents is essential for its action on the main-chain and the removal of the substituent by the relevant side-chain-cleaving enzyme would preclude its action on the main chain. Although antisynergy is unlikely to occur *in vivo*, it may be observed in *invitro* experiments where only one or two enzymes are used in a specific application (Bhat and Hazlewood, 2001).

Table 1.2 Mode of action of xylanase and cellulases

Enzyme	Mode of action
Endoglucanase	-G-G-G-G-G-G-G-G-G-G-G- Cleaves 1,4-β-limkages at random (Wood and Bhat, 1988)
Cellobiohydrolases	-G-G-G-G-G-G-G-G-G-G-G-G- (type I) (type II) Releases cellobiose from both reducing type II and non-reducing ends (type I) (Barr *et al.,* 1996; Gilkes *et al.,* 1997)
β-Glucosidase or cellobiase	-G-G; G-G-G-G; G-G-G-G Releases glucose from cellobiose and hydrolyse short-chain cello-oligasaccharides from both reducing and non-reducing ends by releasing one glucose unit at a time (Christakopulos *et al*., 1994)
Xylanase or endoxylanase	S S X-X-X-X-X-X-X-X-X-X-X-X-X- S Cleaves 1,4-β-linkages of xylan at random with preference to unsubstituted regions (Coughlan *et al*., 1993; Coughlan and Hazlewood, 1993)

1.5 Cellulases

Cellulases are modular enzymes that are composed of independently folding, structurally and functionally discrete units, referred to as either domains or modules (Henrissat *et al*., 1998). These enzymes are responsible for the hydrolysis of the β-1,4 glucosidic bonds in cellulose. They are members of the glycoside hydrolase family of enzymes, which hydrolyse oligosaccharides and/or polysaccharides (Henrissat and Davies, 1997). Although cellulases cleave a single type of bond in a chemically simplistic substrate, the extensive intermolecular bonding pattern of cellulose generates a fascinating crystalline substrate that is particularly resistant to microbial degradation. Thus, multiple enzyme systems are required to effectively degrade cellulose (Bayer *et al*., 1998).

Cellulases are synthesized by a number of microorganisms, most notable fungi. Some bacterial species also exhibit cellulose-degrading ability. Many cellulases produced by bacteria appear to be bound to the cell wall and are unable to hydrolyse native lignocellulose preparations to any significant extent (Viikari and Teeri, 1997). Many fungi capable of degrading cellulose synthesize large quantities of extracellular cellulases that are more efficient in depolymerising the cellulose substrate. The cellulase system in fungi is considered to comprise three hydrolytic enzymes (Table 1.3). Any fungal species capable of degrading cellulose may produce multiple forms of each of these three enzymatic activities.

Table 1.3 Enzymes of the cellulase complex (Bhat and Bhat, 1997).

Systematic name	Enzyme no.	Trivial names	Substrate and product
1,4-β-D-glucan cellobiohydrolase	EC 3.2.1.91	Exoglucanase, cellobiohydrolase	Crystalline cellulose, cellobiose
Endo-1,4-β-D-glucan 4-glucanohydrolase	EC 3.2.1.4	endoglucanase	Amorphous cellulose, cello-oligosaccharides
β-D-glucoside glucohydralase	EC 3.2.1.21	Cellobiase, β-glucosidase	Cellobiose, triose, glucose

1.5.1 Substrate specificity

Endoglucanases specifically cleave the internal β-1,4 glycosidic bonds of amorphous, swollen and substituted celluloses as well as cello-oligosaccharides. These enzymes are generally inactive towards crystalline cellulose and cellobiose. Some endoglucanases attack barley glucan with mixed β-1,3 and β-1,4 linkages (Petre *et al*., 1986). Bhat *et al.* (1990) demonstrated a marked difference in the substrate specificities of endoglucanases purified from *P. pinophilum*, using substituted, unsubstituted and reduced cello-oligosaccharides. Thus, endoglucanases III and IV were active on cellotriose and higher cello-oligosaccharides, whereas, endoglucanases II, V, and required at least four, five and six glucose residues respectively. Although such variation in substrate specificity of endoglucanases was unexpected, it is speculated that microorganisms secrete multiple endoglucanases with a wide range of substrate specificities to effect efficient hydrolysis of complex cellulosic substrates (Bhat and Hazlewood, 2001).

Cellobiohydrolases (CBHs) are highly active on amorphous and swollen celluloses and cello-oligosaccharides rather poorly (Wood and Bhat, 1988). These enzymes are specific for β-1,4 linkages of the cellulose chain, but are inactive on cellobiose, carboxymethyl- and hydroxyethyl-celluloses. In general, CBHs attack cellulose chains from the non-reducing end and release cellobiose. Barr *et al.* (1996) confirmed two classes of CBHs based on kinetics studies and high-resolution structural data. The class one enzymes (e.g. CBH I from *T. reesei* and two endoglucanases E4 and E6 from *T. fusca*) hydrolyse the cellulose chains preferentially from the reducing end,

while class two CHBs (e.g. CBH II from *T. reesei* and E3 from *T. fusca*) release cellobiose specifically from the non-reducing end (Barr *et al*., 1996; Teeri, 1997).

β-Glucosidases can be classified as either aryl β-D-glucosidases (hydrolysing exclusively aryl-β-D-glycosides), cellobiases (hydrolysing diglucosides and cello-oligosaccharides) or β-glucosidases with broad substrate specificities. Most β-glucosidases show broad substrate specificities and hydrolyse aryl and alkyl β-D-glycosides and β-1,1-, β-1,2-, β-1,3-, β-1,4- and β-1,6-linked diglucosides, as well as substituted and unsubstituted cello-oligosaccharides (Bhat *et al*., 1993). Interestingly, an intracellular β-glucosidase from *S thermophile* was found to be an aryl-β-glucosidase, whereas two extracellular β-glucosidases from the same organism hydrolysed only cellobiose (Bhat *et al*., 1993).

1.5.2 Mode of action

All endoglucanases attack swollen and substituted celluloses and amorphous of regions of cellulose randomly and release glucose, cellobiose and cello-oligosaccharides. The cellobiohydrolases hydrolyse phosphoric acid-swollen cellulose and Avicel sequentially by removing cellobiose units from either reducing or non-reducing ends of cellulose chain (Wood *et al*., 1988; Vrsanska and Biely, 1992; Barr *et al*., 1996). Endoglucanase and CBH act synergistically to effect extensive hydrolysis of crystalline cellulose. Subsequently β-glucosidase completes the hydrolysis by converting the resultant cello-oligosaccharides and cellobiose to glucose (Wood, 1985).

Most endoglucanases attack internal glycosidic bonds of cello-oligosaccharides and release mainly cellobiose and cellotriose, while CBHs hydrolyse the second glycosidic bond from either the reducing or the non-reducing end of cello-oligosaccharides. However, β-glucosidase sequentially removes one glucose unit from either the reducing end, the non-reducing end or both ends (Bhat and Hazlewood, 2001). The viscosity of cellulose solution is directly related to the degree of polymerisation of individual cellulose chains. An endoglucanase that attacks the cellulose chain randomly produces the largest decrease in the viscosity of a cellulose solution per unit increase in reducing power (Bhat *et al*., 1989).

Bacterial cellulases are known to adopt different mechanism for hydrolysis of cellulose. For example, the aerobes *Cellulomonas, Pseudomonas, Thermoactinomycetes* and *Microsbispora* and the anaerobe *C. stercorarium* produce a cellulase system similar to that of fungi, and degrade cellulose by the cooperative interaction of different cellulase components (Beguin *et al*., 1992; Gilbert and Hazlewood, 1993). In contrast, the anaerobic thermophilic bacterium *C. thermocellum* degrades crystalline cellulose very effectively by means of a high molecular mass multi-enzyme complex called cellulosome (Beguin and Lemaire, 1996). The cellulosome is not restricted to *C. thermocellum* alone, various other anaerobic bacteria produce cellulosome on their cell surface, which possess similar properties to the cellulosome from *C. thermocellum* (Miron *et al*., 1989; Beguin and Lemaire, 1996). The exact mechanism by which the cellulosome achieves cellulolysis is unclear, but it is evident that the efficiency of the multi-enzyme complex is a function of its quaternary structure, and is dependent on endoglucanase /cellobiohydrolase

synergism and clustering of the complex on the substrate surface (Beguin and Lemaire, 1996).

1.5.3 Synergism between cellulases

Synergism is an enhanced effect of two or more enzymes when acting cooperatively, compared with their additive effect. Synergism between bacterial and fungal cellulases as well as between the subunits of *C. thermocellum* cellulosome, has been reported (Bhat *et al*., 1994; Wood *et al*., 1994). The most interesting types of synergism between fungal cellulase components are between: (i) endoglucanase/exoglucanase (CBH); (ii) exoglucanase (CBH)/exoglucanase (CBH) and (iii) endoglucanase/endoglucanase. An early model suggested that cellulose chains cleaved by endoglucanase become the substrate for exoglucanase, and these two enzymes cooperatively degrade cellulose (Wood *et al*., 1989). However, this model did not explain synergism between two different CBHs, or the inability of CBH to synergise with endoglucanases from different organisms. Subsequently, using highly purified endoglucanases and CBHs from *P. pinophilum*, it was demonstrated that only two endoglucanases (EG III and EG V), which were strongly absorbed on to cellulose, best synergised CBHs I and II; and the observed synergism was explained in terms of the different stereospecificities of these enzymes (Bhat and Hazlewood, 2001).

The attack of cellulose chain by a stereospecific endoglucanase will generate only one of two possible types of non-reducing ends, which will be hydrolysed by a stereospecific CBH. The successive removal of cellobiose by CBH will expose

another chain-end of a different configuration, which will be attacked by the other stereospecific CBH. Hydrolysis of the two chain-ends by two CBHs acting randomly, together with attack of the cellulose chains by another stereospecific endoglucanase to generate a reducing end of a different configuration, would facilitate the synergism between these enzymes (Wood *et al*., 1989). Nevertheless, it was argued that this synergism could be due to strong adsorption of CHBs and endoglucanases onto cellulose (Klyosov, 1990). Furthermore, Klyosov (1990) reported that two endoglucanases that adsorbed strongly on to cellulose degraded the cellulose synergistically.

Barr *et al.* (1996) and Teeri (1997) revealed that there are two classes of CBHs (exoglucanases), which attack cellulose chains from both reducing and non-reducing ends. Based on these results it was observed that the synergism between CBH/CBH was due to their ability to expose new hydrolysis sites to each other, as well as their ability to act from reducing and non-reducing ends (Barr *et al*., 1996). Thus, two CBHs acting from reducing and non-reducing ends and an endoglucanase appeared to be essential for effective hydrolysis of crystalline cellulose. The presence of another endoglucanase with different substrate specificity would increase the synergistic efficiency further, as observed in the case of the *P. pinophilum* cellulase system (Wood *et al*., 1989).

Studies of the *C. thermocellum* cellulase system revealed that two cellulosomal exoglucanases (S_5 and S_8 subunits) and an endoglucanase (S_{11} subunit), together with the S_1 (scaffoldin) subunit, are essential for maximum synergism during the hydrolysis of crystalline cellulose (Bhat *et al*., 1994). These results indicate that in the

case of *C. thermocellum* cellulase system, assembly of an enzyme complex is crucial for the maximum synergistic interaction of subunits during the solubilisation of crystalline cellulose.

1.6 Laccase

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the small group of enzymes called the blue copper proteins or the blue copper oxidases. These proteins contain multinuclear moieties that result in the characteristic blue colour (Thurston, 1994). Laccase is widely distributed in plants and fungi (Messerschmidt and Huber, 1990). In fungi, laccase is present in Ascomycetes, Basidiomycetes and is particularly abundant in many white-rot fungi that degrade lignin (Bourbonnais *et al*., 1995). Laccase, like all the other blue oxidases is a glycoprotein (Yarolopov *et al*., 1994). Fungal laccases are to be involved in the degradation of lignin or in the removal of potentially toxic phenols arising during lignin degradation (Thurston, 1994). In addition, fungal laccases are hypothesized to take part in the synthesis of dihydroxynaphthalene melanins, darkly pigmented polymers that protect organisms against environmental stress or in fungal morphogenesis by catalysing the formation of extracellular pigments (Zhao and Kwan, 1999).

The optimal pH of laccases is highly dependable on the substrate. When using ABTS (2,2'-azino-*bis*-3-ethylbenz-thiozoline-6-sulphonic acid) as substrate the pH optima are more acidic and are found in the range between pH3 and pH5 (Heinzkill *et al*., 1998). In general, laccase activity has a bell shaped profile with optimal pH that varies considerably. This variation may be due to changes to the reaction caused by

the substrate, oxygen or the enzyme (Xu, 1997). The optimal temperature of laccase can differ greatly from one strain to another. The laccase isolated from *Marasmius quercophilus* was found to be stable at 60°C for one hour (Farnet *et al*., 2000). The laccase from *Pleurotus ostreatus* is almost fully active in the temperature range of 40-60°C, with maximum activity at 50°C. The activity remains unaltered after prolonged incubation at 40°C for over 4 hours (Plamieri *et al*., 1993). Recent studies indicated that laccase plays an important role in degrading lignin in wood pulp, and could help in decolorizing and detoxifying the vast amount of effluents generated by the pulp and paper (Lee *et al*., 1999).

1.6.1 Substrate specificity

Laccases are remarkably non-specific as to their reducing substrates, and the range of substrates oxidised varies from one laccase to another. These enzymes catalyse the one-electron oxidation of a wide variety of organic and inorganic substrates, including polyphenols, methoxy-substituted phenols, aromatic amines and ascorbate with the concomitant four-electron reduction of oxygen to water (Thurston, 1994). Laccase has broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups, and as such, the ability to react with the phenolic hydroxyl groups found in lignin (Youn *et al*., 1995).

1.6.2 Mode of action

Laccase only attacks the phenolic subunits of lignin, leading to Cα oxidation, Cα-Cβ cleavage and aryl-alkyl cleavage. Laccases are able to reduce one molecule of

dioxygen to two molecules of water while performing one-electron oxidation of a wide range of aromatic compounds (Thurston, 1994), which includes polyphenols methoxy-substituted monophenols and aromatic amines (Archibald *et al*., 1997; Bourbonnais *et al*., 1995). This oxidation results in an oxygen-centred free radical, which can then be converted in a second enzyme-catalysed reaction to quinone. The quinone and the free radical product under-go a non-enzymatic coupling reactions leading to polymerisation (Thurston, 1994).

Laccases are similar to other phenol-oxidising enzymes, which preferably polymerise lignin by coupling of the phenoxy radicals produced from oxidation of lignin phenolic groups (Bourbonnais *et al*., 1995). It has been generally accepted that laccase cannot degrade non-phenolic lignin model compounds. Nonetheless, it is thought that laccase is capable of oxidizing non-phenolic compounds in the co-presence of a primary laccase substrate such as 2,2'azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (Youn *et al*., 1995).

1.7 Influence of cultivation techniques: SmF versus SSF

Enzyme production is a growing field of biotechnology. Most enzyme manufacturers produce enzymes using submerged fermentation (SmF) techniques with enzyme titres in the range of grams per litre. Such levels are a prerequisite if specific compounds are to be considered as commodities because product recovery costs are inversely proportional to concentration in fermentation broth. There is a significant interest in using solid-state fermentation (SSF) techniques to produce a wide variety of enzymes, mainly from fungal origin. Fungi play a key role in SSF, for their hyphal

development allows them to effectively colonise and penetrate the solid substrate (Viniegra-González *et al.,* 2003).

Production of enzymes by solid-state fermentation has potential advantages over submerged fermentation with respect to simplicity in operation, high productivity fermentation, less favourable for growth of contaminants and concentrated product formation (Ashokkumar *et al*., 2001). Studies have repeatedly shown that SSF, in comparison with SmF, provides higher volumetric productivities, is less prone to problems with substrate inhibition and yields enzymes with higher temperature or pH stability. Also, the fermentation time may be shorter and the degradation of the enzymes by undesirable proteases is minimised SSF offers numerous opportunities in processing of agro-industrial residues ((Hölker *et al*., 2004). This is partly because solid-state processes have lower energy requirements, produce lesser wastewater and are environmental friendly as they resolve the problem of wastes disposal (Pandey *et al.*, 2003).

In the employment of mixed cultures, SSF offers an option that cannot be achieved by SmF. This is due to the fact that during growth, fungal consortia secrete a broad spectrum of enzymes (Yang, *et al.,* 2004; Hölker and Lenz, 2005). During co-cultivation of different fungi, some of the individual enzyme activities show synergistic increase whereas others remained unchanged (Guiterrez-Correa *et al*.,1999). In addition, SSF offers the possibility to explore and to use interactions between fungi by properly regulating the water activity as a selection parameter depending on the different water demands of the individual cocultured fungi.

Sterility is very often required in SmF process because many contaminants may out compete the process organism under the conditions of high water availability provided. Often SSF processes involve an organism which grows rapidly under the low water conditions and if an active inoculum is added to the substrate, the process organism is able to out compete the contaminating organisms, meaning that strict aseptic operation of the bioreactor may not be essential in SSF, although operation should be carried out in a clean manner (Pandey *et al*., 2000).

Calculation of the costs of crude enzyme production by SSF showed that in the case of cellulase production, the economical efficiency is higher by a factor of 100 than in the case of SmF (Tengerdy, 1996). In addition to the cheaper growth substrates, the minimised requirements for sterility and low requirements for instrumentation and equipment can make downstream processes in the production of enzymes unnecessary (Hölker and Lenz, 2005). This holds for those cases when the fermented substrate can be used directly in a follow up process such as the saccharification of renewable raw materials employed in ensiling, bio-ethanol or biogas production or in the production of animal fodder supplements. This is made possible by the high concentrations of enzymes in the solid substrates which makes further concentrating processes unnecessary (Viniegra-González *et al.,* 2003).

1.7.1 General aspects of SSF

There are several factors that affect SSF processes; these include selection of a suitable strain, substrate and selection of process parameters. The selection of substrate for SSF process depends upon several factors mainly related with cost and

availability and thus may involve screening of several agro-industrial residues. In SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it, but also serves as an anchorage for the cells (Pandey *et al*., 1999). The substrate that provides all the needed nutrients to the microorganisms growing in it should be considered as the ideal substrate.

Among several factors, which are important for microbial growth and activity in a particular substrate, particle size and moisture level or water activity are the most critical (Sarrette *et al*., 1992; Pandey and Soccol, 1998). In general, smaller substrate particles would provide larger surface area for microbial attack and thus should be considered as a desirable factor. However, too small substrate particles may result in substrate agglomeration in most of the cases, which may interfere with microbial aeration and thus may result in poor growth. At the same time, larger particles provide better aeration efficiency but provide limited surface for microbial attack (Pandey *et al*., 2000).

Currently industrial demand of most enzymes is met by production using submerged fermentation (SmF) employing genetically modified strains. The cost of production in SmF is high and it is uneconomical to use many enzymes in several processes. This necessitates reduction in cost by alternative methods. Due to the lack of free-water, smaller fermenters are required for SSF and therefore less effort is required for downstream processing. Wild-type strains of bacteria and fungi perform better in SSF conditions than genetically modified microorganisms, reducing energy and cost requirements even further (Barrios-Gonzalez *et al*., 1993). The industrial applications of enzymes produced under SSF are outlined in Table 1.4.

Table 1.4 Industrial applications of enzymes produced under SSF (Tengerdy, 1998)

Industrial applications	Enzymes
Enzyme assisted ensiling	Fungal cellulases and hemicellulases
Bioprocessing and crops residues	Fungal cellulases and hemicellulases
Fibre processing (retting)	Fungal pectinases, cellulases and hemicellulases
Feed supplement	Amylases, proteases, lipases, cellulases and hemicellulases
Biopulping	Xylanases
Directed composting	Hydrolytic enzymes
Soil bioremediation	Laccases, ligninases
Post harvest residue decomposition	*Trichoderma harzianum* cellulases
Biopesticide	*Trichoderma harzianum* cellulases

1.8 Scope of the present study

Ruminants diets in most developing countries are based on fibrous feeds, mainly mature pastures and crop residues. These feeds are unbalanced and particularly deficient in protein, minerals and vitamins and are highly lignified with low digestibility. These characteristics result in low intake and productivity and only approximately 40% of energy in forage becomes available to the animal. Enzymes can be used as biological tool to enhance digestion through the action of cellulase, hemicellulase and lignase enzymes resulting in improved meat, milk and wool production. The development of feed additives holds great promise for the

improvement of livestock growth and yield for both large commercial and smaller subsistence farmers.

1.8.1 Hypothesis

It is hypothesised that the enzymes that degrade components of feed (cellulose, hemicellulose or lignin) will increase the digestibility of feeds, thereby increasing the yield, improving the milk and wool production.

1.8.2 Aim

The aim of this study was to develop optimal media for the production of lignocellulolytic enzymes (laccases, xylanases, and cellulases) and to evaluate the application of these enzymes to improve the nutritional digestibility of high fibre feedstocks, such as veld grass.

1.8.3 Objectives

The following objectives were established to achieve the above aim:

i) to evaluate the inductive effect of wheat straw and oulandsgrass as carbon sources,

ii) to compare solid state fermentation and submerged fermentation for production of xylanase, cellulase and laccase,

iii) to evaluate the effect of different types of CSL on the production of xylanase and cellulase in shake flasks and in bioreactor and

iv) to evaluate the hydrolytic effect of the produced enzymes in forage pre-treatment studies.

CHAPTER 2 PRODUCTION OF XYLANASE, CELLULASE AND LACCASE FROM FUNGAL ISOLATES IN SHAKE FLASKS AND SOLID STATE FERMENTATION

2.1 ABSTRACT

Enzymes that degrade components of feed (cellulose, hemicellulose and lignin) have generated interest as these enzymes are considered to increase digestibility of feeds. Seven fungal strains were evaluated for their ability to produce xylanase, cellulase and laccase using wheat straw and *Eragrostis curvula* (oulandsgrass) as carbon sources in shake flasks. Oulandsgrass induced high levels of xylanase and cellulase while none of the carbon sources appeared to induce laccase production. *T. aureoviride* (FS2-2) was found to be the best producer of xylanase both on oulandsgrass and wheat straw (5418 and 4162 nkat / ml, respectively) while Abo 67 (unidentified) was the poor producer (less than 50 nkat / ml) of both xylanase and cellulase. Four fungal strains that showed high enzyme levels in shake flasks were evaluated in SSF for enzyme production using oulandsgrass as a substrate. Enzyme levels produced in SSF were approximately 6 to 10 fold higher than the levels achieved in SmF depending on the strain used. Maximum xylanase activity achieved in SSF was 14842 nkat / ml produced by *A. fumigatus* followed by *A. terreus var carneus* producing 14368 nkat / ml. Supplementing the production media with 0.5% of yeast extract had a positive effect on growth and xylanase production in all strains with *A. fumigatus* resulting in a maximum activity of 17662 nkat / ml after 5 days.

2.2 INTRODUCTION

A general opinion about the choice of fermentation method for the production of any microbial product would normally be submerged fermentation (SmF) unless there appears a particular reason why other methods should be chosen because SmF is intrinsically less problematic, heat transfer is better and homogeneity is much better (Pandey, 2000).

Currently, industrial demand of most enzymes is met by production using submerged fermentation (SmF), generally employing genetically modified strains. The cost of enzyme production in SmF is high and it uneconomical to use many enzymes in several processes. This necessitates reduction in cost by alternative methods and SSF should be considered an alternative method. Due to the lack of free-water, smaller fermenters are required for SSF and therefore less effort is required for downstream processing. Wild-type strains of bacteria and fungi perform better in SSF conditions than do genetically modified microorganisms, reducing energy and cost requirements even further (Barrios-Gonzalez *et al*., 1993).

SSF offers numerous opportunities in processing of agro-industrial residues. This is partly because SSF processes have lower energy requirements, produce lesser wastewater and are environmentally friendly as they resolve the problem of solid waste disposal (Pandey, 2003). Among the advantages of SSF, often is cited that enzyme titers are higher than in SmF, when comparing the same strain and fermentation broth (Viniegra-González *et al.,* 2003). In

general, production of enzymes in SSF has potential advantages over SmF with respect to simplicity in operations, high productivity, minimized risk of microbial contamination and product concentration (Ashokkumar *et al.,* 2001).

In industry enzymes are mostly produced under submerged conditions, mainly because the processes associated with scale-up are much simplified compared to those required for scale-up in SSF. Liquid fermentation also allows greater control of parameters, such as pH, heat transfer and nutrient conditions (Robinson *et al.,* 2001). The cost of carbon source is the major contribution to the enzyme production costs for large scale processes.

Lignocellulosic material such as wheat straw, sugar cane bagasse are less expensive sources of carbon, are readily available and require minimal or no treatment (Liming and Xueliang, 2004). In this study wheat straw and oulandsgrass (*Eragrostis curvula*) were used as carbon sources for production of xylanase, cellulase and laccase in submerged fermentation and solid state fermentation by selected fungal strains and to determine how these enzyme activities compare.

2.3 MATERIALS AND METHODS

2.3.1 Fungal cultures

Fungal strains (Table 2.1) were isolated from soil at the University of the North and identified at the University of Stellenbosch using morphological descriptions (Domsch *et al*., 1980). Sequence analyses of the ITS region of the ribosomal RNA genes (White *et al*., 1990) confirmed the identification. The cultures were screened for enzyme activities and production of xylanase, carboxymethylcellulase (CMCase) and laccase as described below.

Table 2.1 Identification of fungal strains and their sources

Fungal strains	Identification	Source
Abo 67	Unidentified	University of the North
Abo 374	*Aspergillus terreus var carneus*	Fish river (Eastern Cape)
AZ7a_2	*Basidiomycete isolate*	University of the North
AZ17xyl	*Aspergillus fumigatus*	University of the North
$FS2_2$	*Trichoderma aureoviride*	University of the North
$FS4_1$	*Trichoderma virens*	University of the North
Rut C30	*Trichoderma ressei*	University of the North

2.3.2 Media and growth conditions

2.3.2.1 Growth of pre-inoculum on YPD broth

Fungal isolates were grown on MEA (Malt Extract Agar, Biolab) medium until the mycelia covered the whole surface of the agar plate. An agar block (approximately 5 X 10mm) of each culture was fragmented and transferred to a test tube containing 1 ml of sterile YPD (yeast-peptone-dextrose) broth (pH 5). The test tubes were incubated at 30°C for 24 h with shaking at 100 rpm.

2.3.2.2 Enzyme productions in shake flasks

Eragrostis curvula (Oulandsgrass) and wheat straw were obtained from Meadow Feeds (Paarl) and ground to a fine texture and thereafter used as carbon sources. Shake cultures were performed in duplicate in 500 ml Erlenmeyer flasks containing 100 ml of growth media which comprised of the following: oulandsgrass or wheat straw (2%), yeast nitrogen base (YNB), L-asparagine at pH 5. After autoclaving at 121°C for 20 min, flasks were inoculated using the pre-inoculum (section 2.3.2.1). The inoculated flasks were incubated at 30°C with shaking on an orbital shaker at 120 rpm for 5 days with sampling every 24 hours. Broth from each flask was centrifuged for 20 minutes at 10 000 rpm. Supernatants were decanted and re-centrifuged for 12 minutes at 13 000 rpm. Clear supernatants were stored at 4ºC before enzyme assays (section 2.4).

2.3.2.3 Enzyme production in SSF

Carbon source (2g oulandsgrass) was autoclaved in 100 ml Erlenmeyer flasks, and then 5 ml of sterile complex growth medium comprising of yeast nitrogen base with amino acids, L-asparagine, KH_2PO_4 and yeast extract; was added to the substrate to ensure a 75% moisture content. Each flask was then inoculated with an overnight grown culture and then incubated at 30°C for five days in a sealed plastic container containing wet paper towel. The cultures were harvested by adding 25 ml of distilled water into each flask and shaking the flasks on an orbital shaker at 160 rpm for two hours, followed by centrifuging for 20 minutes at 10 000 rpm. Supernatants were decanted and re-centrifuged for 12 minutes at 13 000 rpm. Clear supernatants were stored at 4ºC before enzyme assays.

2.3.2.4 Large scale SSF

Abo 374 was used for large scale production of xylanase and cellulase in SSF. 50g of milled oulandsgrass was weighed into 2800 ml Erlenmeyer flasks and autoclaved for 20 minutes. An agar block (approximately 5 x 10mm) was fragmented and transferred to 150 ml of growth medium, which was then added to the flasks containing sterile oulandsgrass as the carbon source. The duplicate flasks were incubated at 30°C for 8 days, and samples were taken starting from day 4 to day 8. The flasks contents were harvested on nylon filter. The filter was then placed in an autoclavable bag and the substrate was squeezed using a vice and the supernatant was collected. The supernatant

was centrifuged for 12 minutes at 13 000 rpm and then assayed for xylanase and cellulase activity.

2.4 Enzyme assays

2.4.1 Xylanase and cellulase

Xylanase activity was assayed according to the method of Bailey *et al.* (1992) using a 1% (w/v) solution of birchwood xylan (Fluka) incubated with appropriately diluted enzyme in 0.05M citrate buffer for 5 minutes at 50°C. The released reducing sugars were determined spectrophotometrically at 540 nm after adding 3,5-dinitrosalicylic acid (DNS) reagent to the reaction mixture, followed by boiling for 15 minutes and cooling. Cellulase activity was determined in a similar manner with the exception that 1% carboxymethylcellulose (CMC, Sigma) was used as the substrate. The enzyme activity was expressed in nkat. An enzyme unit (nkat) was defined as the release of one nmol of product per second.

2.4.2 Laccase

Laccase activity was assayed according to the method of Jönsson *et al*. (1997) using 2,2 azino bis 3 ethylbenzthiazolinesulfonate (ABTS) as a reducing substrate. The assay mixture consisted of 500 μl distilled water, 500 μl culture supernatant and 500 μl sodium acetate (200 mM, pH 5.2). The

reaction was initiated by the addition of 500 μl 1.6 mM ABTS and monitored at 420 nm and 25°C for 10 minutes.

2.5 RESULTS

2.5.1 Xylanase production on oulandsgrass and wheat straw using SmF

The different isolates showed varying xylanase levels on both wheat straw and oulandsgrass. All the organisms screened appeared to prefer oulandsgrass (Figure 2.1) as a carbon source over wheat straw with exception of the *Basidiomycete* isolate ($AZ7a_2$) and the positive control organism (*T. reesei* Rut C30), which showed more xylanase activity on wheat straw (Figure 2.2). The highest activity (5819 nkat / ml), was produced on oulandsgrass by *T. aureoviride* (FS2-2) and the lowest activity was produced both on oulandsgrass and wheat straw by Abo 67.

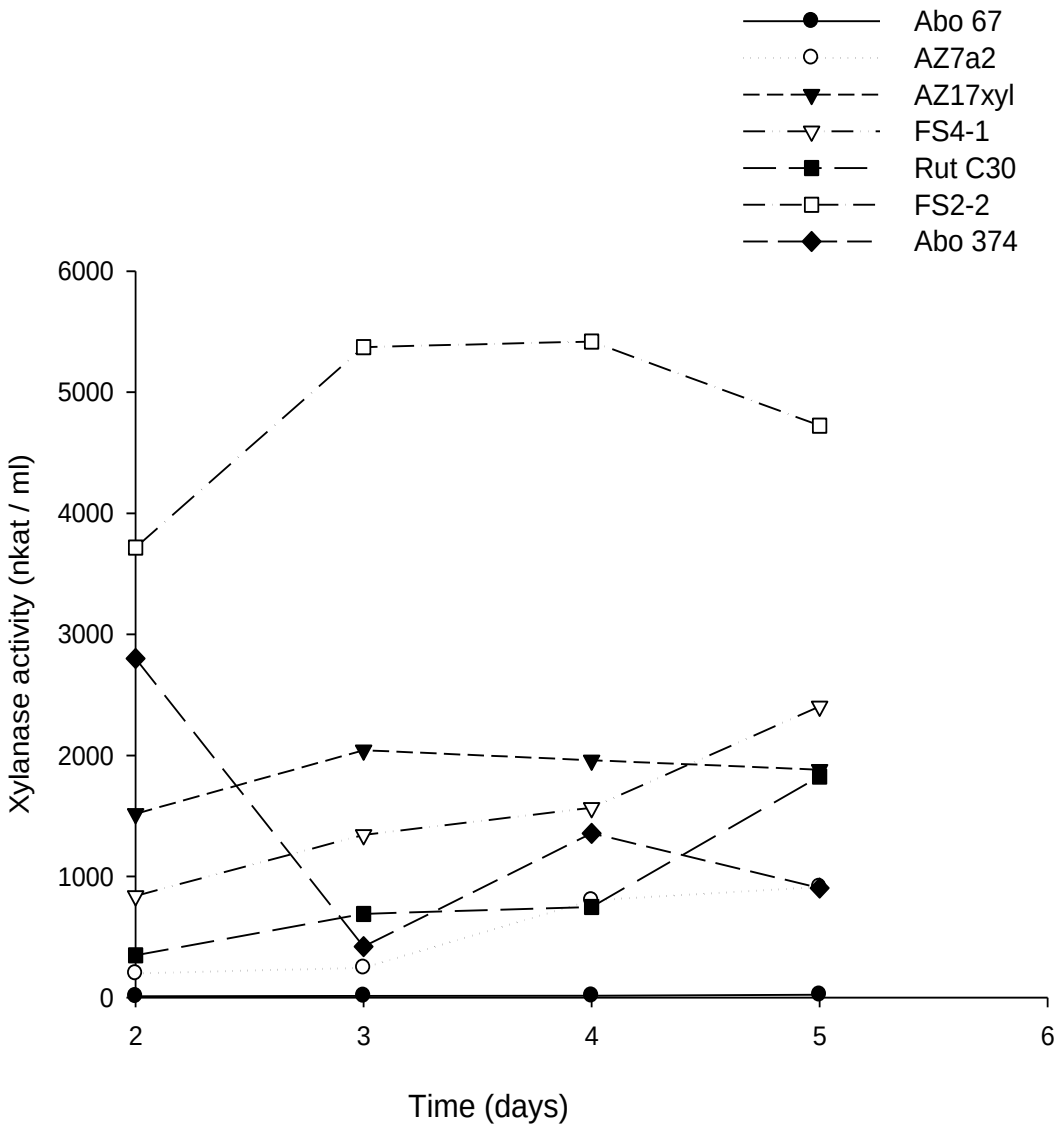

Figure 2.1 Xylanase production on oulandsgrass after growth for 5 days at 30°C and pH 5 in shake flasks. Each point represents the mean of duplicate determinations.

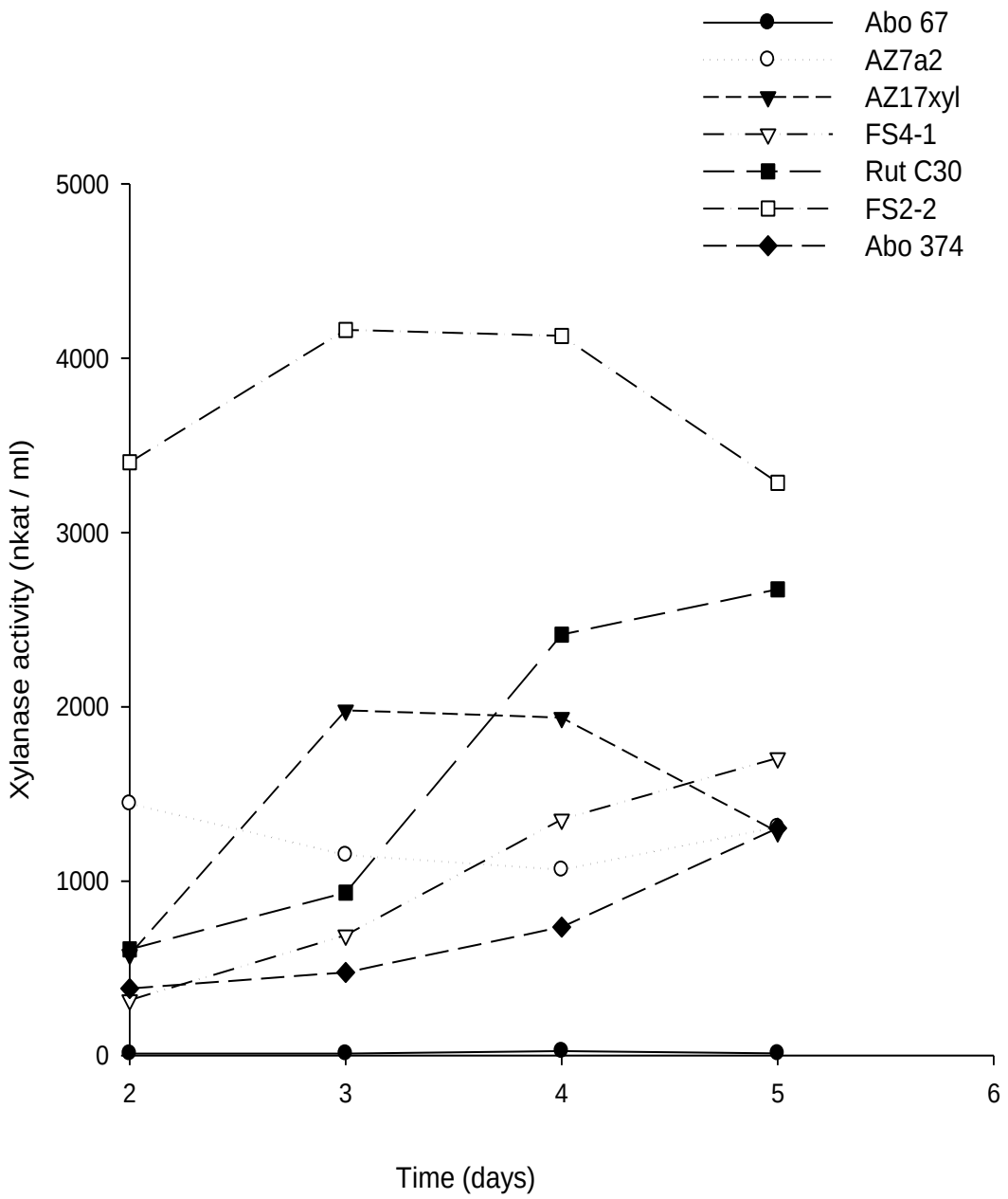

Figure 2.2 Xylanase production on wheat straw after growth for 5 days at 30°C and pH 5 in shake flasks. Each point represents the mean of duplicate determinations.

2.5.2 Cellulase production on oulandsgrass and wheat straw using SmF

The cellulase levels were 10-fold less than xylanase levels produced by same isolates under similar conditions when using wheat straw or oulandsgrass as substrates. The highest cellulase titre of 215 nkat/ml (Figure 2.3) was produced by FS2-2 (*T. aureoviride*) on oulandsgrass while the highest activity on wheat straw (174 nkat/ml) was produced by *Basidiomycete* isolate (AZ7a). All the other isolates produced cellulase levels below 100 nkat/ml cellulase activity on wheat straw (Figure 2.4).

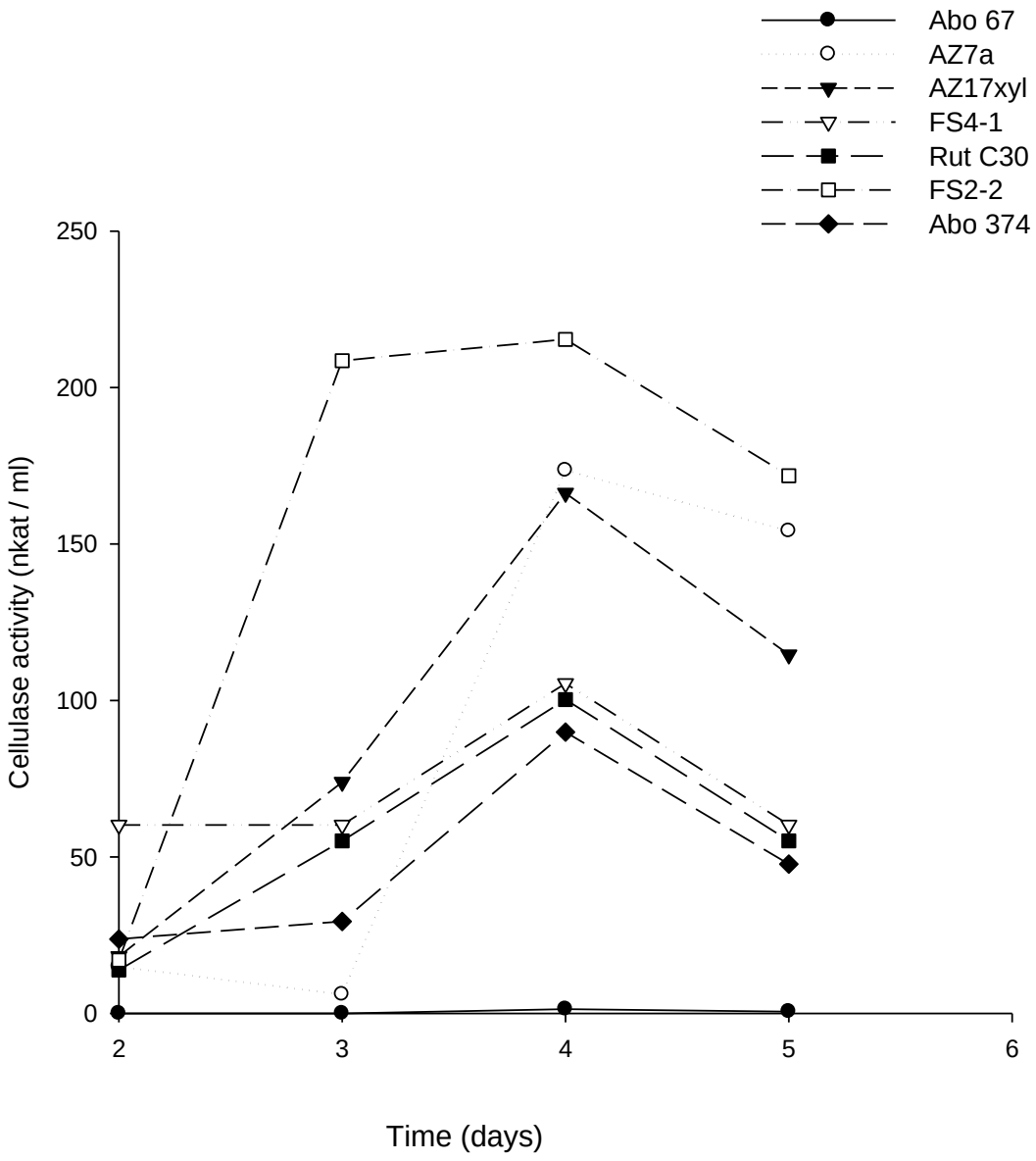

Figure 2.3 Cellulase production on oulandsgrass after growth for 5 days at 30°C and pH 5 in shake flasks. Each point represents the mean of duplicate determinations.

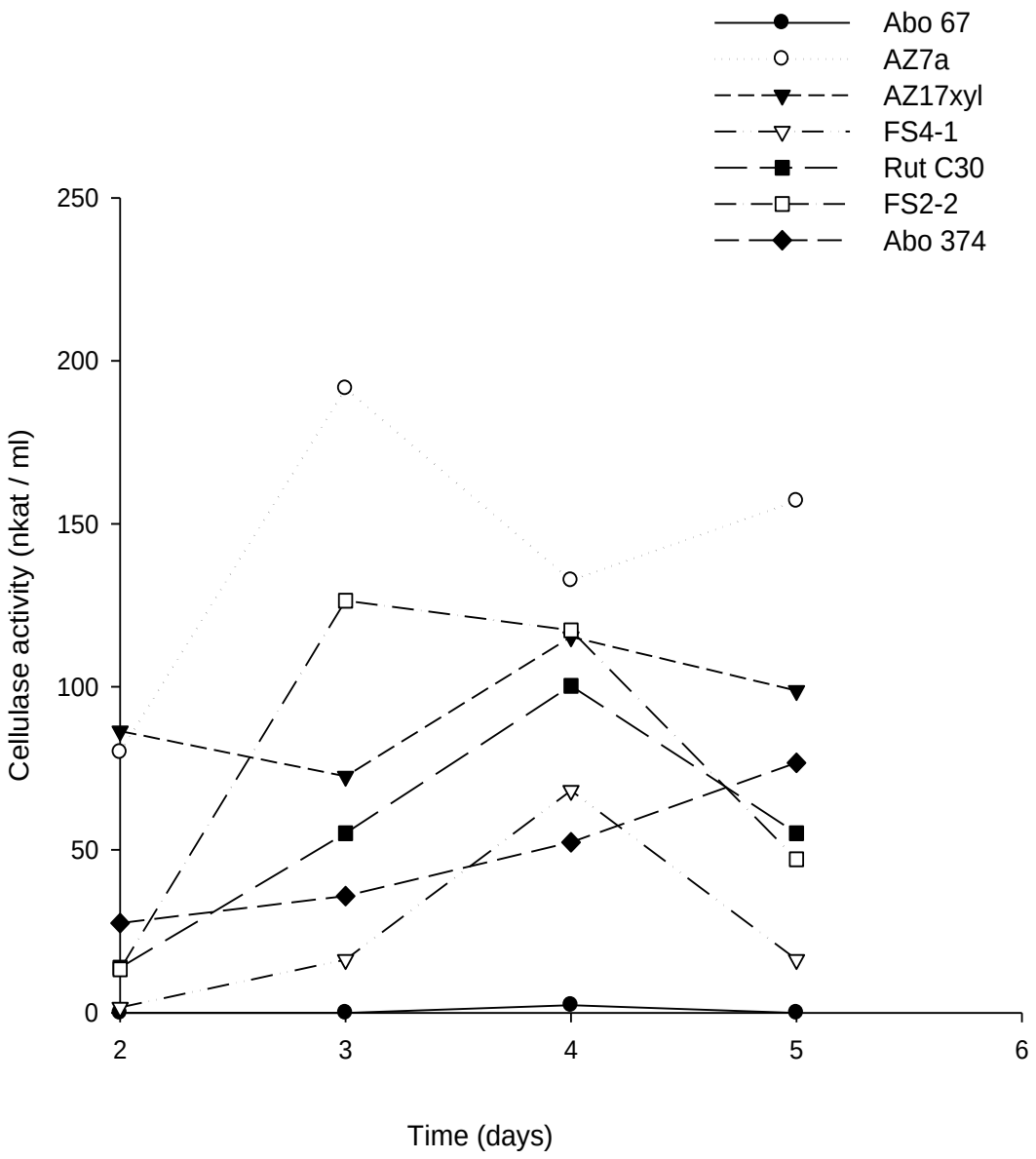

Figure 2.4 Cellulase production on wheat straw after growth for 5 days at 30°C and pH 5 in shake flasks. Each point represents the mean of duplicate determinations.

2.5.3 Laccase production in SmF and SSF

The laccase levels produced by all the strains were insignificant both in SmF and SSF ($<$ 0.1 U / l) irrespective of the substrate used (data not shown).

2.5.4 Xylanase production on oulandsgrass in SSF

Based on the results of submerged fermentation and trial run using wheat straw and oulandsgrass as carbon sources where oulandsgrass resulted in higher enzyme levels compared to wheat straw, SSF experiments were carried out using oulandsgrass as a carbon source. Although fungal growth was similar, FS2-2 resulted in low levels of enzyme activity reaching a maximum of 8219 nkat / ml on day three and dropping to about 3586 nkat / ml after 5 days (Figure 2.5). Abo 374 and AZ17 xyl displayed similar xylanase production profile and these strains reached a maximum of 14842 and 14368 nkat / ml, respectively after 3 days (Table 2.2).

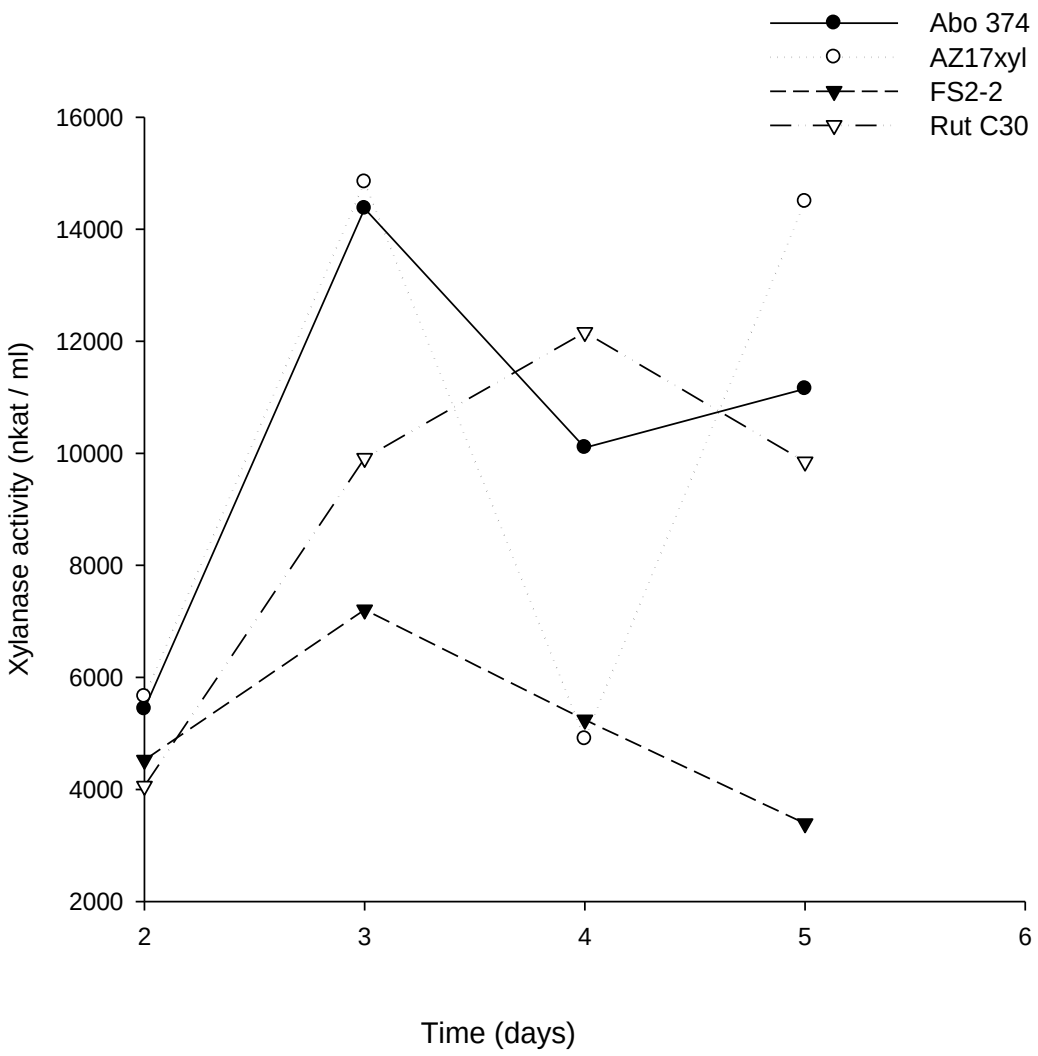

Figure 2.5 Xylanase production on oulandsgrass in SSF over 5 days at 30°C. Each point represents the mean of duplicate determinations.

Table 2.2 The xylanase and cellulase activities (nkat / ml) produced both in SmF and SSF by four fungal strains using oulandsgrass as a carbon source

		SmF				SSF			
Fungal strains		**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 2**	**Day 3**	**Day 4**	**Day 5**
FS22	Xyl	3716	5372	5418	4724	4843	3316	8219	3586
	Cel	170	209	215	178	418	679	741	715
AZ17xyl	Xyl	1516	2043	1959	1850	5657	14842	4903	14494
	Cel	18	74	166	115	289	698	1043	1282
Abo 374	Xyl	280	421	1355	912	5435	14368	10099	11150
	Cel	24	29	90	48	160	327	548	806
Rut C30	Xyl	349	691	748	1825	4055	9912	12160	9848
	Cel	21	39	189	39	318	575	518	1410

Xyl = xylanase
Cel = cellulase
Each value represents the mean of duplicate determinations.

2.5.5 Cellulase production on oulandsgrass in SSF

Cellulase levels were relatively low for all the organisms with AZ 17 xyl being the highest producer (Figure 2.6) whereas Abo 374 resulted in lower cellulase levels. After 48 h FS2-2 produced 795 nkat / ml and thereafter an activity level of 700 nkat / ml was maintained throughout the fermentation.

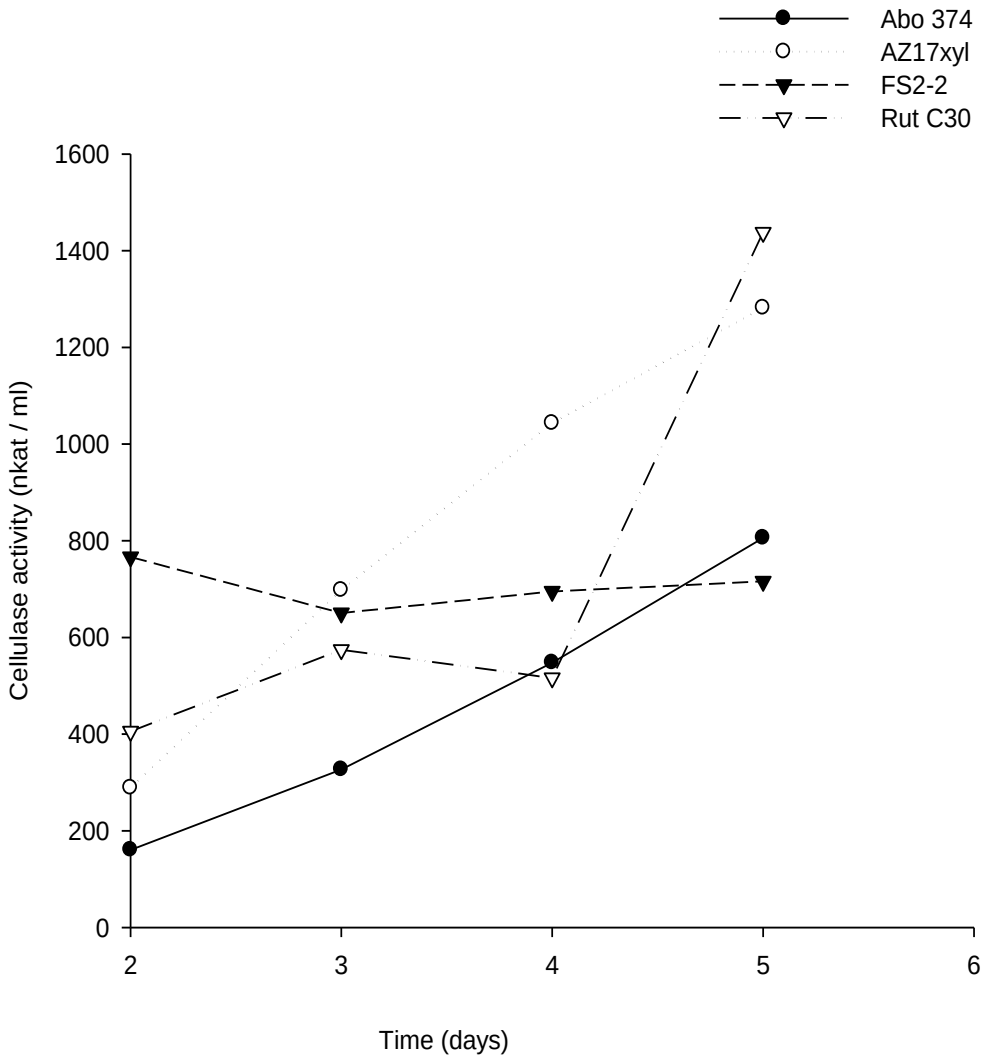

Figure 2.6 Cellulase production on oulandsgrass in SSF over 5 days at 30°C. Each point represents the mean of duplicate determinations.

2.5.6 Xylanase and cellulase production on oulandsgrass supplemented with yeast extract (0.5 %) in SSF

Supplementing the production media with 0.5% of yeast extract had a positive effect on growth and xylanase production in all strains with *A. fumigatus* starting off at approximately 9500 nkat/ml, increasing daily to a maximum of approximately 17600 nkat/ml by day five. *A. terreus* and *T. ressei* only showed improvement in xylanase activity by day five (Figure 2.7). Cellulase activity produced by the *Aspergillus* species dropped by approximately 40% when the production media was supplemented with yeast extract while *T. aureoviride* increased to almost twice the value obtained in media without yeast extract in day five (Figure 2.8). In large scale SSF (50 g of substrate), xylanase activity dropped by 50% compared to the levels achieved on a small scale (2 g of substrate) whereas the cellulase activity improved by approximately 50% even though the growth period was increased to 8 days (Figure 2.9). The drop in xylanase activity maybe due to the fact that in this experiment a fragmented agar block covered with spores of the Abo 374 instead of a pre-grown inoculum that was used in previous experiment.

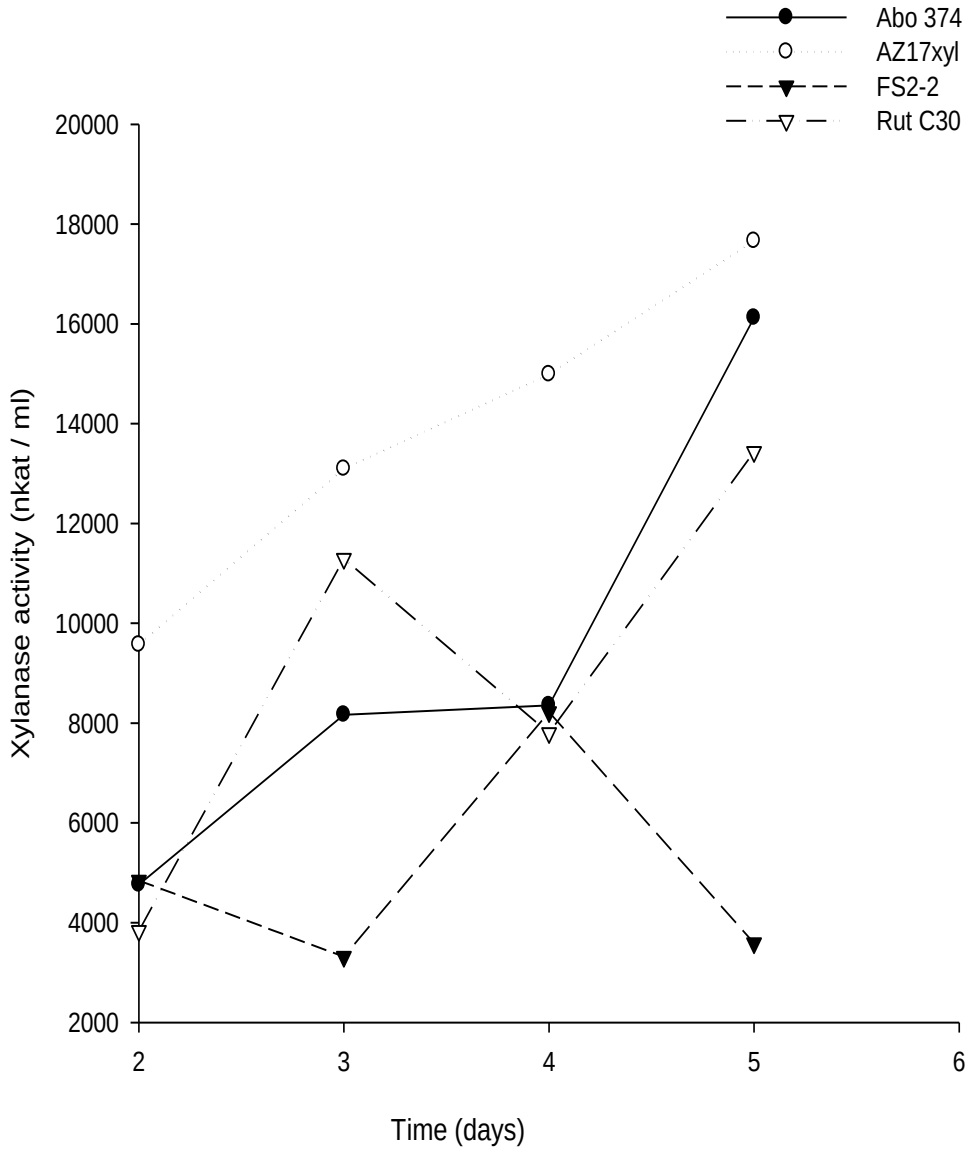

Figure 2.7 Xylanase production on oulandsgraas supplemented with 0.5% Yeast extract in SSF over 5 days at 30°C. Each point represents the mean of duplicate determinations.

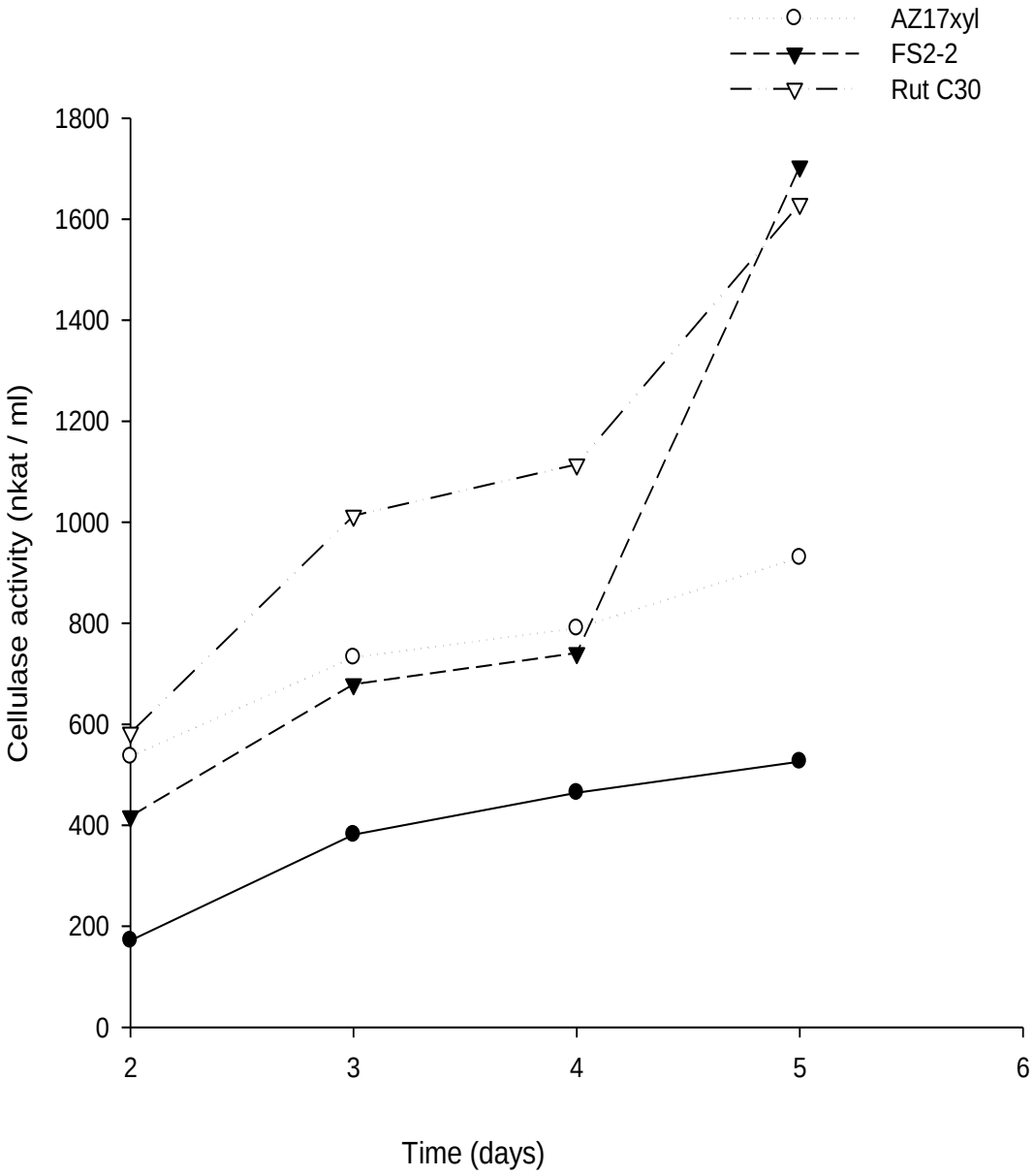

Figure 2.8 Cellulase production on oulandsgrass supplemented with 0.5% yeast extract in SSF over 5 days at 30°C. Each point represents the mean of duplicate determinations.

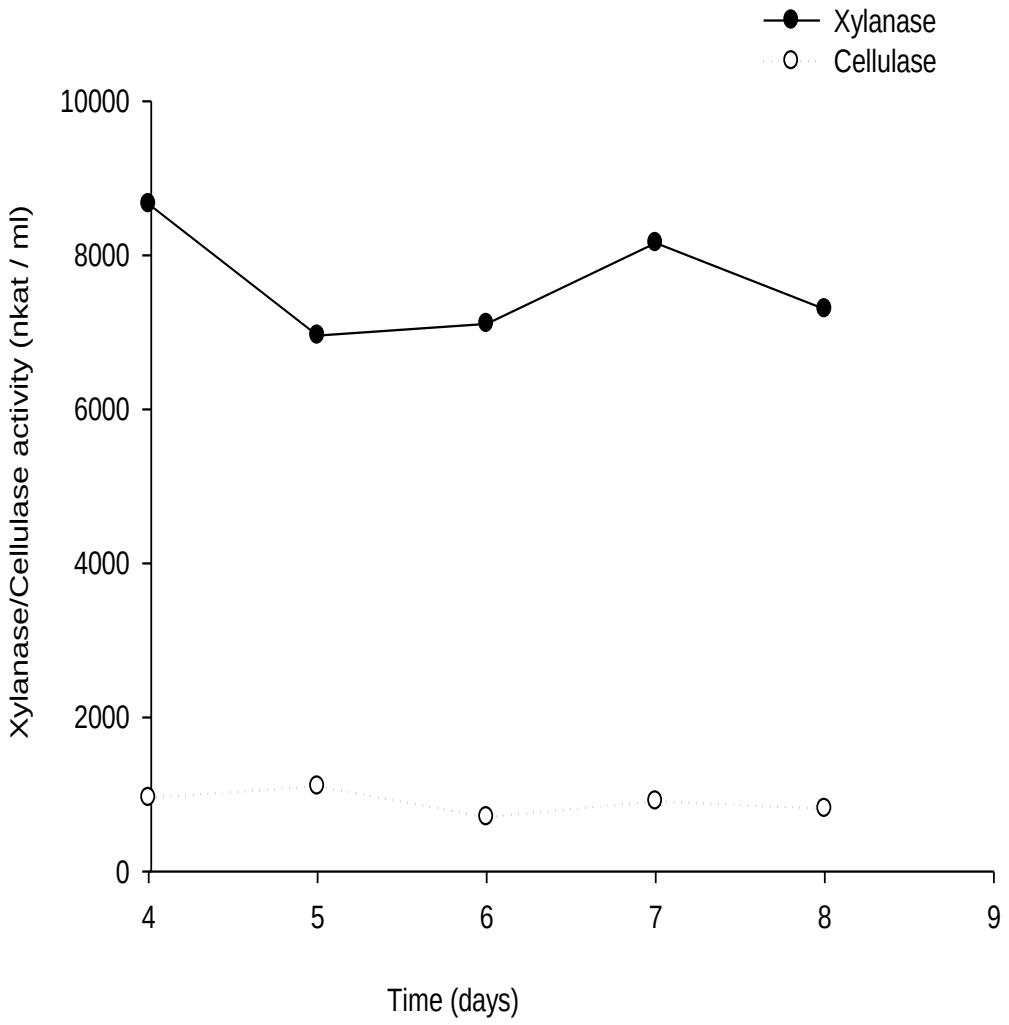

Figure 2.9 Xylanase and cellulase production by Abo 374 on oulandsgrass supplemented with 0.5% yeast extract in large scale SSF over 8 days at 30°C. Each point represents the mean of duplicate determinations.

2.6 DISCUSSION

Many hydrolytic enzymes are inducible by certain low molecular weight substances structurally related to the natural substrate. The efficiency of induction by certain compound can be determined by both its binding affinity to regulatory macromolecules and its actual concentration inside the cell (Hrmova *et al*., 1986). To evaluate the inductive effects of effects of various carbon sources, growth experiments were carried out using media containing the compounds tested at the same initial concentration.

2.6.1 Submerged fermentation

Although fungal growth was abundant and appeared almost similar, the fungal strains showed differences in enzyme activity and daily levels as well as changing ratios of enzymes during the five day growth period. The use of wheat straw as a substrate resulted in lower levels of enzyme activity. *A. terreus var carneus* (Abo 374), *A. fumigatus* (AZ17xyl), *T. aureoviride* (FS2-2) and *T. virens* (FS4-1) showed higher enzyme levels compared to *T. reesei* Rut C30.

Xylanase levels were optimal on days three and four and mainly dropped by day five and the xylanase was produced earlier than cellulase, considering the complex structure of the substrates this maybe due to the fact that some parts of the carbon sources were more accessible to the fungus than others. Olsson *et al*. (2004) reported similar findings for xylanase and cellulase production by

T. reesei Rut C30 using sugar beet pulp as a substrate. The xylanase levels (2405 nkat/ml) reported by Colina *et al*. (2003) for *T. reesei* Rut C30 on rice straw were slightly higher than those obtained in this study for the same organism under similar cultivation conditions.

All isolates with the exception of AZ7a produced high levels cellulase when cultivated on oulandsgrass and FS2-2 was found to be the best producer (215 nkat/ml) and this result was about 3 fold higher than that reported for *T. reesei* ZU-02 on corn cob residue which is also a cheap substrate (Liming and Xueliang, 2004). The cellulase levels produced by *T. reesei* Rut C30 were ten fold higher than those reported by Olsson *et al.* 2004 for the same organism cultivated on cellulose and sugar beet pulp.

The production trends were similar on both substrates used in this work as there was an increase in cellulase levels from day two up to day four and on day five there was a slight decline of activity. The tendency of the enzymes to adsorb to the substrates may complicate the observation because the enzyme levels in the free solution may provide true reflection of the real enzyme production (Domingues *et al*., 2000).

Laccase production levels were insignificant for all the isolates with Abo 67 (unidentified strain) and AZ7a2 (*Basidiomycete* isolate) resulting in slightly significant levels (0.05 and 0.06 nkat/ml, respectively) on oulandsgrass. *Aspergillus niger* DM-15 has been reported not to contain a laccase gene and

this might hold true for the other *Aspergillus* and *Trichoderma* strains in this study.

2.6.2 Solid state fermentation

In SSF four fungal strains (*A. terreus, A. fumigatus*, *T. aureoviride* and *T. ressei* Rut C30 were evaluated for xylanase, cellulase and laccase activity using oulandsgrass as a carbon source. Fungal biomass was abundant and was almost similar for all the strains but the enzyme levels were significantly different with *A.terreus* and *A. fumigatus* showing similar xylanase production trends and almost similar enzyme activity on day two and three (Figure 2.5).

The initial lag for xylanase and cellulase production was shorter in SSF than that of SmF. The shorter fermentation time for the production of higher enzyme levels in SSF process is an advantage in terms of process cost of enzyme production. A similar finding was observed in the production of β-fructofuranosidase by *A. niger* (Ashokkumar *et al*., 2001). The fermentation time required to reach maximum xylanase in SSF was three days whereas the cellulase production was increasing with fermentation time and did not stabilise even after five days for most strains. In SmF the maximal xylanase and cellulase levels were reached by day three to four and mainly dropped by day five.

In SSF different fungal strains displayed differing enzyme production trends and this maybe attributed to the structure of the substrate, with the fungus

adapting the enzymes produced to correspond to currently exposed substructures of the substrate. When the production media in SSF was supplemented with yeast extract both the cellulase and xylanase levels improved and in some cases (*T. aureoviride* and *T. reesei* Rut C30) to almost double the initial levels achieved in the absence of the yeast extract. These results were in agreement with those reported in literature where fungi were found to produce higher xylanase and cellulase activities on organic nitrogen sources (Lemos *et al.,* 2001; Bakri *et al.,* 2003; Yang *et al*., 2006).

In this work SSF enzyme levels were approximately 6 to10 fold higher than the levels achieved in SmF per ml of culture liquid. Similar results were reported in literature when SSF and SmF were investigated for the production of enzymes by fungi using agro-industrial residues for example Ashokkumar *et al.* 2001reported the productivity of β-fructofuranosidase to be 5-fold higher in SSF, Patil and Dayanand, 2006 reported a similar finding for production of pectinase by *A. niger*, pectinase levels were higher in SSF than in SmF.

Ratios of xylanase to cellulase changed significantly during the five day growth period for different fungal strains in both SSF and SmF. The significant difference in enzyme levels when wheat straw and oulandsgrass were used as carbon sources maybe attributed to the hemicellulose nature of the substrates, their degradability by the fungal strains and the presence of some nutrients in the carbon source (Sonia *et al*., 2005). Various factors affect enzyme expression on complex substrates, these include substrate accessibility, rate and amount of release of the xylo-oligosaccharide, chemical

structure of xylan and quantity of xylose released which act a carbon source (Kulkarni *et al*., 1999).

CHAPTER 3 Evaluation of wheat straw as a carbon source and CSL as a nitrogen source for the production of xylanase, cellulase in shake flask and lab scale fermentation

3.1 ABSTRACT

Xylanases and cellulases are group of industrial enzymes with applications particularly in the animal feed industry because of their ability to enhance the digestibility of feed. These enzymes can be produced using agro-industrial residues in fermentation thereby reducing the cost of enzyme production. Different concentrations of wheat straw and straw teas produced by alkaline and acidic extraction were evaluated for the induction of xylanase and cellulase by *A. terreus var carneus* in shake flasks. Maximum xylanase and cellulase activities of 3935 and 319 nkat / ml (respectively) were induced by 30g/l of wheat straw. Straw teas had showed no significant inductive effect on enzyme production as levels lower than 250 nkat / ml were observed for xylanase and cellulase levels were lower than 90 nkat / ml. Among the three types of CSL evaluated, CSL^{PT} resulted in higher xylanase and cellulase activity (5355 and 144 nkat / ml, respectively) while lower levels of both enzymes were observed in medium containing CSL^{PTUF}. CSL^{PT} was evaluated in 15 L bioreactor with stirrer speed regulated automatically to maintain 30% dissolved oxygen tension. Highest xylanase activity of 2113 nkat / ml was observed which was 60% lower than the levels obtained in shake flasks while the cellulase activity improved by approximately 50%.

3.2 INTRODUCTION

The members of the fungal genus *Aspergillus* are commonly used for the production of polysaccharide degrading enzymes. Enzymes such as xylanase, cellulase and laccase have been identified as enzymes that contribute to the digestibility of animal feed. These enzymes have gained various applications because of their ability to convert complex and environmentally destructive processes to more feasible and environmentally friendly ones (Polizeli *et al*., 2005). In order to achieve high productivity and lower production cost of the enzyme cocktail an economical viable substrate has to be identified; wheat straw has been reported to be a suitable substrate for induction of the enzymes of interest (Sonia *et al*., 2005).

Corn steep liquor (CSL) is a complex substrate produced in the corn-processing industry and is used as a fermentation feedstock for the production of enzymes, polysaccharides, secondary metabolites as it is an excellent source of nitrogen. CSL also contains lactic acid, a suitable carbon source for fermentative microorganisms. In addition, CSL contains amino acids, peptides, proteins, carbohydrates, vitamins, trace metals, minerals and several complex growth factors (Hakala *et al*., 2004). In this study different wheat straw concentrations and straw teas were evaluated for their inductive effects on xylanase and cellulase production in shake flasks; the effect of different types of CSL as nitrogen sources was also evaluated in shake flasks and bench scale batch fermentation.

3.3 MATERIALS AND METHODS

3.3.1 Inoculum

A. terreus var carneus (Abo 374) was grown on malt extract agar for 5 to 7 days at 30°C. The spores were harvested by adding 5ml of Tween 80 (0.1%) and the final spore concentration in production flask was 5 X 10^6.

3.3.2 Effect of different wheat straw and straw teas as inducers of xylanase and cellulase in shake flasks

Production media consisted of the following per 1000 ml water: corn steep liquor, 25g; $MgSO_4.7H_20$, 0.5g and KH_2PO_4, 5g. The production flasks had different concentrations of wheat straw (30, 10, and 2) or straw tea (30 g of wheat straw extracted at pH 12, 3 and 5.7, CSIR personal communication). Straw tea was made by adding the required amount of wheat straw to 100 ml of distilled water and adjusting pH to 12, 5.7 and 3 with 10 N KOH or 0.2 N H_2SO_4 respectively. The initial pH of production flasks was adjusted to pH 5 with the acid and base used in straw tea preparation. The straw solution was then autoclaved for 20 minutes, allowed to cool and tea was strained to use as a medium supplement. After inoculation the flasks were incubated at 30°C with shaking at 120 rpm for 7 days. Samples of 10 ml volume were removed after 2, 4 and 7 days and assayed for xylanase and cellulase activity (section 2.4.1). The supernatant used for enzyme activity was obtained by filtering broth through fibre glass filters (MN GF-1 47mm Rundfilters).

3.3.3 Effect of different CSL types on production of xylanase and cellulase in shake flasks

The inoculum was prepared as outlined in section 3.3.1 above. Shake flask cultivation was performed in triplicate in 500 ml Erlenmeyer flasks containing 200 ml of production medium which consisted of the following components per liter of distilled water: CSL (7.84 g), $MgSO_4.7H_2O$ (0.1 g), KH_2PO_4 (1 g), antifoam (0.2 ml), and wheat straw (2 g). Three types of CSL (Rouqette CSL ^{PT}) Roquette CSL^{SD} (spray-dried) and Afprod CSL^{PTUF} (phytase treated and ultra-filtered) were evaluated maintaining a final protein concentration of 3.92 g/l. The initial pH of the production flasks was adjusted to pH 5 and the flasks were autoclaved at 121°C for 20 min. The inoculated flasks were incubated at 30°C with shaking at 100 rpm for 7 days with 10 ml samples taken on day 2 to day 7. Samples were analyzed for xylanase and cellulase activity (Section 2.4.1).

3.3.4 Evaluation of the best CSL type in lab scale fermentation

Batch cultivations were carried out in a 15 L Biostat C bioreactor equipped with Mettler-Toledo pH and dissolved oxygen probes (Braun Biotech, Germany) using a working volume of 6.3 L. The vessel had four baffles with one propeller at the top and three disk turbine impellers fitted equidistantly on the stirrer shaft. Fermentation medium consisted of the following per liter: CSL (7.84 g), $MgSO_4.7H_2O$ (3.15 g), KH_2PO_4 (31.5 g), antifoam (2 ml), and wheat straw (26.27 g). The salts, CSL and wheat straw were weighed out and 2 L of

tap water was added. Thereafter this mixtue was added to the fermenter and made up to a final volume of 6.3 L with tap water, followed by in-situ sterilization for 45 min at 123°C. A spore suspension of 700 ml volume at a concentration of 5 x 10^6 spores / ml was used as an inoculum. The growth temperature was maintained at 30°C and pH controlled at 5 using 5N sodium hydroxide (NaOH) and 20% sulfuric acid (H_2SO_4). The dissolved oxygen was maintained at 30% by cascade control, the initial aeration was in head space until spore germination. After germination the bioreactor was aerated through the sparger at 1 vvm. The first sample was remove after 64 h and therafter samples were removed every 4-8 h until the fermentation process was terminated at 152 h. Samples were assayed for xylanase and cellulase activity as described in section 2.4.1.

3.4 RESULTS

3.4.1 Evaluation of different concentrations of wheat straw and straw teas as inducers of xylanase and cellulase activity in shake flasks

In previous experiments, 30g of wheat straw was found to induce xylanase and cellulase production in shake flasks (data not shown). When used at those levels in the fermenters there was a problem in sampling as the amount of solids in the broth blocked the sample ports. In an attempt to reduce the solid loading in fermentation broth different concentrations of wheat straw and straw teas were evaluated for xylanase and cellulase production; 30g/l wheat straw resulted in higher xylanase (Figure 3.1) and cellulase (Figure 3.2) activity compared to all the other concentrations that were evaluated. The enzyme levels obtained on 2 g/l wheat straw and straw tea were lower than 250 nkat/ml for xylanase and ranged between 30 and 90 nkat/ml for cellulase.

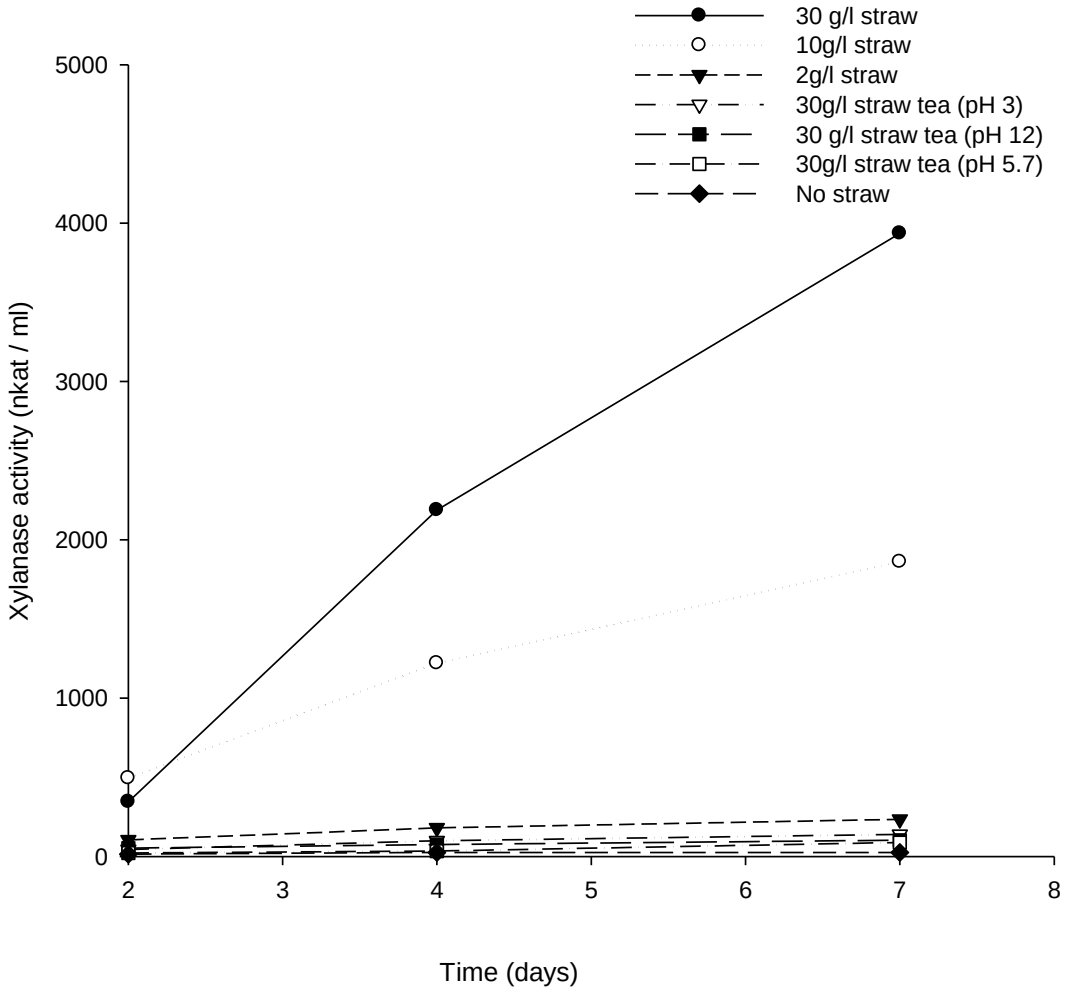

Figure 3.1 Xylanase activities produced on different wheat straw concentrations by *A. terreus var carneus* at 30°C in shake flasks grown for 7 days at 120 rpm. Each point represents the mean of duplicate determinations

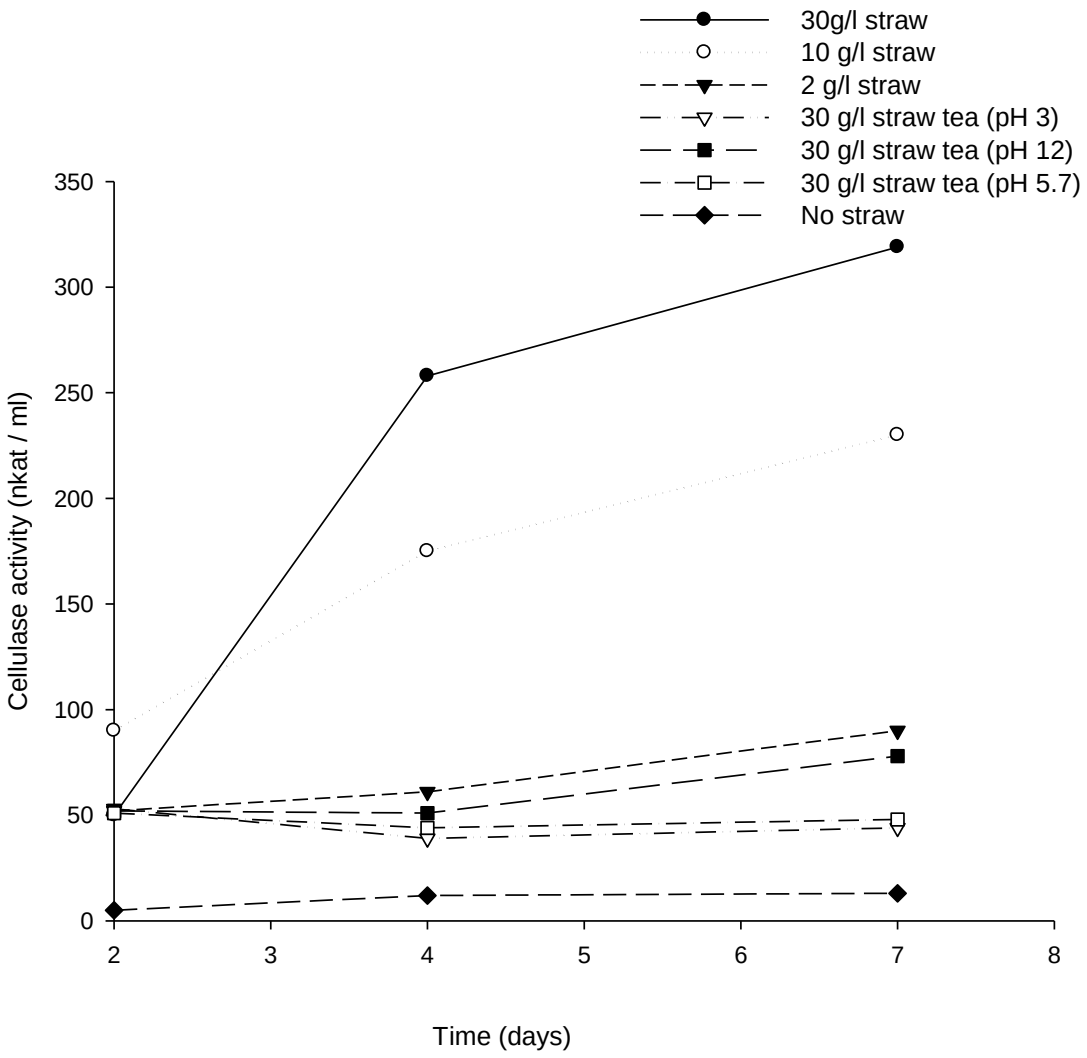

Figure 3.2 Cellulase activities produced on different wheat straw concentrations by *A. terreus var carneus* at 30°C in shake flasks grown for 7 days at 120 rpm. Each point represents the mean of duplicate determinations

3.4.2 Effect of different CSL types on xylanase and cellulase production in shake flasks

All three types of CSL tested supported fungal growth. High levels of xylanase (5355 nkat / ml) were achieved in medium containing phytase treated CSL after seven days of cultivation (Figure 3.3). The lowest xylanase levels (4425 nkat / ml) were observed in medium containing Afprod CSL^{PTUF}. Cellulase activity was also high on the medium containing CSL^{PT} (Figure 3.4).

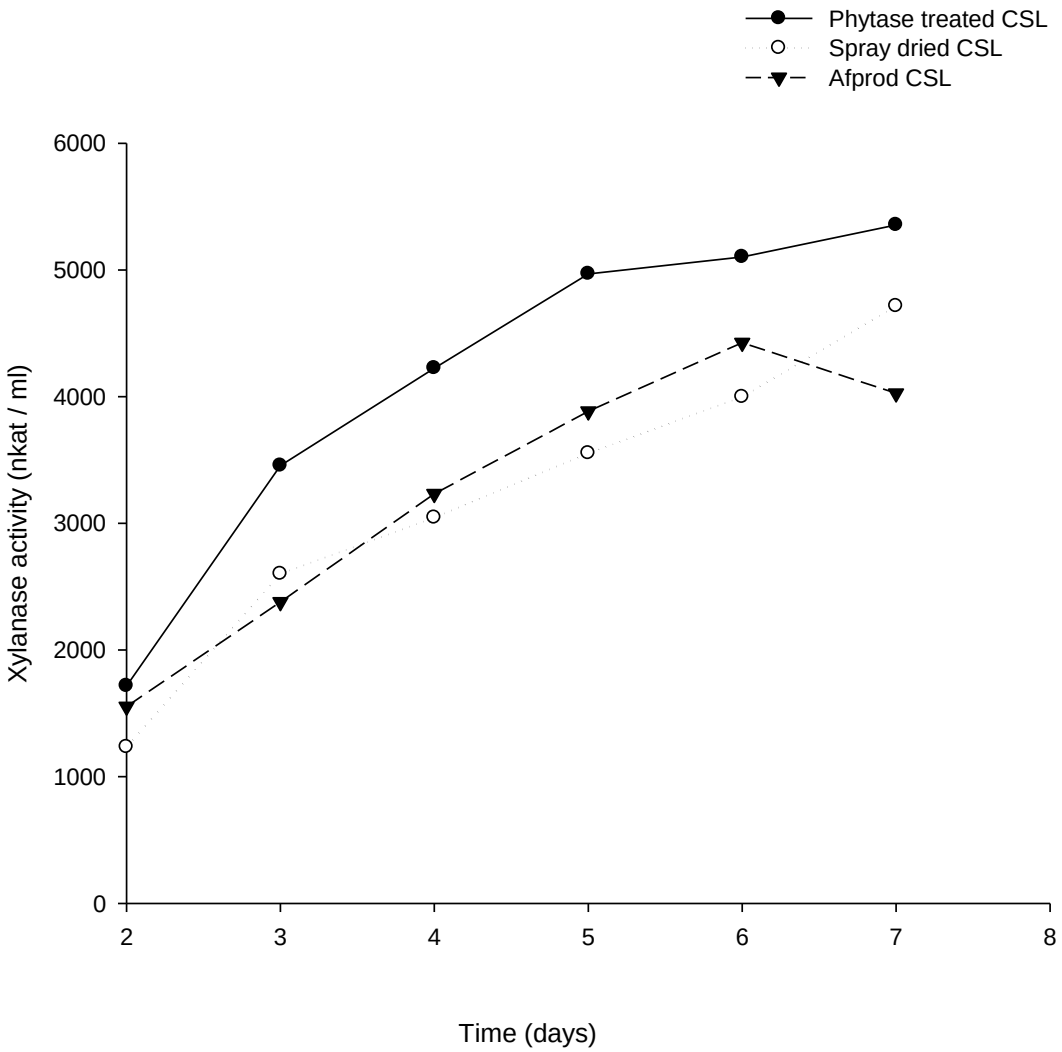

Figure 3.3 Xylanase activities produced by *A. terreus var carneus* at 30°C on different CSL types in shake flasks cultivated for 7 days at 120 rpm. Each point represents the mean of duplicate determinations

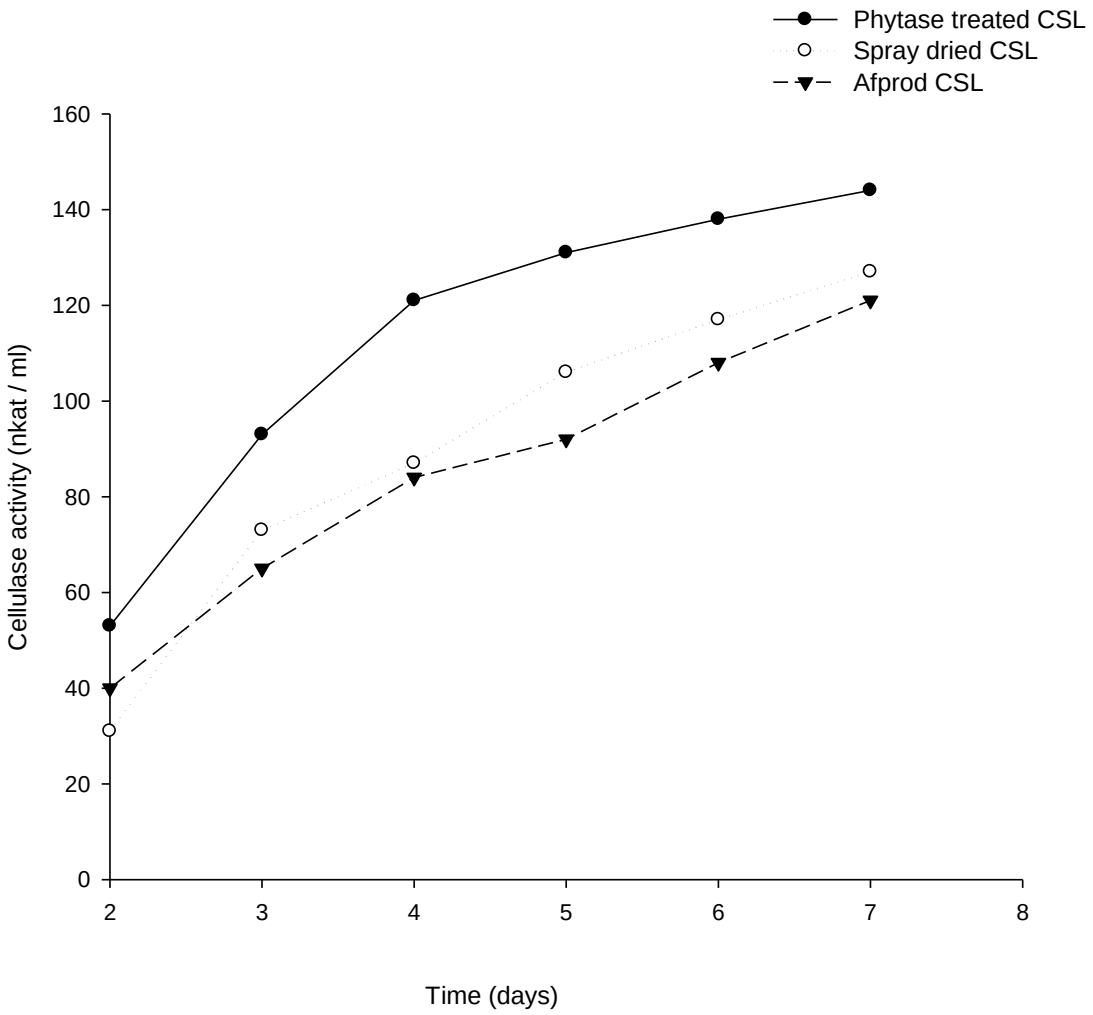

Figure 3.4 Cellulase activities produced by *A. terreus var carneus* at 30°C on different CSL types in shake flasks cultivated for 7 days at 120 rpm. Each point represents the mean of duplicate determinations

3.4.3 Evaluation of the best CSL type in lab scale fermentation

The fermentation was carried out in duplicate in 15 L Braun C vessels for seven days. In previous work conducted on a lab scale level, enzyme activity was detected after 50 h; the fermentation broth was assayed for xylanase and cellulase activity from 64 h. The highest levels of xylanase (2113 nkat / ml) and cellulase (289 nkat / ml) activity were observed at day 6 and the enzyme activities were increasing up to the end of the fermentation (Figure 3.5).

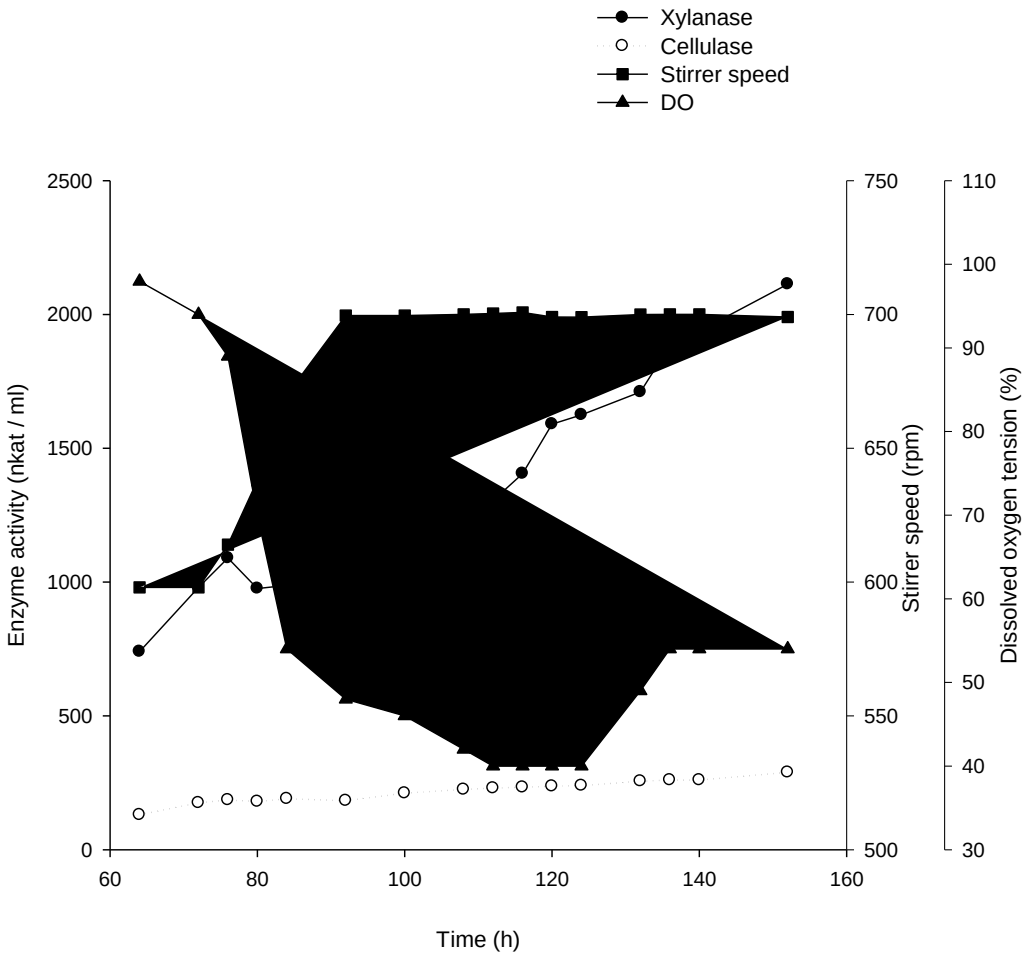

Figure 3.5 Xylanase and cellulase activity produced by *A. terreus var carneus* in a 15 L bioreactor over a 152 h cultivation period at 30°C. Each point represents the mean of duplicate determinations

3.5 DISCUSSION AND CONCLUSION

Agro-industrial residues are generally considered suitable carbon sources for the production of enzymes. *A. terreus var carneus* was has been found to produce xylanase and cellulase on wheat straw in shake flasks; however there is a great difficulty in using this substrate in fermentation processes. Some of the difficulties include compaction which causes non uniform growth, gradients of temperature, pH and oxygen concentration (Patil and Dayanand, 2006) and a difficulty in obtaining homogeneous representative samples. In an attempt to alleviate these problems different concentration of wheat straw were evaluated for their inductive effects on xylanase and cellulase production. Maximum xylanase and cellulase activities of 3935 and 319 nkat / ml (respectively) were obtained in medium containing 30 g/l of wheat straw. There was no significant enzyme production observed when the straw teas prepared by alkaline and acidic extraction of 30 g/l wheat straw were used as inducers. The inductive effect of a substrate maybe attributed to its hemicellulose/cellulose nature and the presence of some nutrients in the carbon (Sonia *et al.*, 2005) thus the failure of straw teas extracted from 30 g/l of wheat straw maybe due to the fact that important nutrients needed to induce enzyme production remained in the solids of wheat straw during extraction.

Among the different types of CSL evaluated as nitrogen sources, phytase treated CSL was more effective for xylanase and cellulase production (5355 and 144 nkat / ml, respectively). The xylanase levels produced in medium

containing CSL^{PT} were 12% higher than those obtained in CSL^{SD} and 25% higher than CSL^{PTUF}. Phytase was reported to improve nutritional quality of phytic acid rich feeds as well as fermentation substrates such as CSL. Phytase treatment of CSL increases the free phosphate content of the substrate while reducing the phytin content. CSL^{PTUF} resulted in low levels of xylanase and cellulase even though treated with phytase and this maybe due to essential molecules and trace elements being removed during ultra-filtration. CSL supplies nitrogen and carbon sources, improving fungal growth and thus enzyme production (Vicentim and Ferraz, 2006). The results obtained were in agreement with results reported in literature using *Sclerotium rolfsii* for xylanase and cellulase production, supplementing production medium with CSL improved production of both enzymes by 30% (Sadana *et al*., 1980). Rajoka, 2004 reported that organic nitrogen sources such as CSL were poor nitrogen sources for cellulase synthesis. The cellulase levels obtained in this experiment were higher than those produced by the same organism in media containing yeast nitrogen base (YNB) as a nitrogen source.

Lab-scale fermentations were conducted using the CSL^{PT} as a nitrogen source as it resulted in higher xylanase and cellulase production in shake flasks. The highest xylanase activity of 2113 nkat / ml was obtained at 152 hrs and was over 50% lower than the activity obtained in shake flasks. This result contradicted the findings of Singh *et al*. (2000) who reported higher xylanase activity in bioreactor than in shake flasks when using xylose as a substrate, however, other researchers also reported lower xylanase activity in bioreactor

than shake flasks when using xylan substrates for xylanase production by *Thermomyces lanuginosus* (Purkarthofer *et al*., 1993; Gomes *et al*., 1993; Hoq *et al*., 1994). Xylanase activity obtained in this study was 10-fold lower than that reported by Bailey *et al.* (1993) in batch *T. reesei* cultivations on beech wood xylan (30 g/l) and corn steep liquor (10 g/l).

Xylan is the key substrate for the induction of xylanase synthesis and during in-situ sterilization of the fermenters; wheat straw adhered to the walls of the vessels reducing the amount of carbon source available in the medium for fungal growth. Another reason for the decrease maybe shear stress inside the vessel caused by agitation as the stirrer speed reached 700 rpm after 4 days; Purkarthofer *et al*. (1993) reported that 120 rpm was optimal for *T. lanuginosus* strain DSM 5826 grown on corn cobs. Similarly, Hoq *et al*. (1994) found that *T. lanuginosus* strain RT9 had an optimum agitation rate of 200 rpm. Aeration rates have also been found to influence xylanase production in bioreactor. Palma *et al*. (1996) observed that an increase in aeration rate reduced the xylanase activity produced by *Penicillium janthinellum*, while Hoq *et al*. (1994) and Reddy *et al*. (2002) reported that an aeration rate of 1 vvm favoured xylanase production by *T. lanuginosus* RT9 and *T. lanuginosus* SSBP, respectively. Cellulase activity (289 nka/ml) at 152 h was approximately 50% higher than that obtained in shake flasks.

The data obtained in these experiments showed that straw teas were not effective as inducers of xylanase and cellulase production. Other attempts to reduce solid loading in bioreactors by milling straw to a soluble fine powder

still need to be investigated. Phytase treated CSL resulted in higher xylanase activity in shake flasks, however, a decrease of approximately 60 % in xylanase activity was observed in bioreactor cultivations. Further investigations with bioreactor cultivations must therefore focus on optimizing parameters *viz*; aeration, pH, agitation to establish best enzyme yields.

CHAPTER 4 PRE-TREATMENT OF FORAGE WITH XYLANASE, CELLULASE AND LACCASE

4.1 ABSTRACT

Enzymes are used extensively by the feed industry to increase the digestibility and absorption of nutrients, to remove anti-nutritional factors from feeds and research has demonstrated that supplementing animal diets with fibre degrading enzymes has significant potential to improve feed utilization and animal performance. This study was conducted to optimize the enzyme pre-treatment phase of forage with fungal cellulolytic and hemicellulolytic enzyme systems prior to feeding. Abo 374 (*A. terreus var carneus*) enzyme preparation caused a decrease in weight of oulandsgrass after 24 h incubation period even though the weight loss was approximately four percent lower than that caused by a commercial enzyme preparation used for comparison. Both the Abo 374 enzyme preparation and a Cellulase 3S (commercial enzyme preparation) seemed to cause more weight reduction when the ratio of cellulase was twice that of xylanase. Pretreatment of forage with laccase followed by treatment by an enzyme preparation with cellulolytic and hemicellulolytic activity resulted in a greater weight loss of forage compared to laccase alone.

4.2 INTRODUCTION

The chemical constituents of forages can be divided into cell wall constituents and cell contents. The cell contents are essentially completely digested *in vivo* and comprise of soluble carbohydrates. The cell wall constituents represented by cellulose, hemicellulose and lignin, vary in digestibility depending on their polymeric configuration, degree of crystallinity and degree of lignification. One of the key problems in the use of forages in feeds is the low susceptibility of lignocellulose to hydrolysis which is attributable to the crystalline structure of cellulose fibrils surrounded by hemicellulose and the presence of lignin seal which prevents penetration by degrading enzymes (Yang and Wyman, 2004; Taniguchi *et al*., 2005). Lignin is highly indigestible under anaearobic conditions and its presence exerts a profound negative effect on the digestibility of cell wall fractions (Jones and Theodorou, 2000).

Preparations of enzymes that degrade cell walls (cellulases and xylanases) have the potential to hydrolyze forage fiber. A number of studies have shown that the addition of such enzymes to grass before ensiling reduced the concentration of plant structural carbohydrates compared with untreated silages (van Vuuren *et al.,* 1989; Jacobs and McAllan, 1991; Kung *et al*., 2000; Stokes and Zheng, 1995; Feng *et al*., 1996). Such enzymatic action on silage may enhance *in vivo* digestion. Improved digestibility of organic matter by sheep, (Huhtanen *et al*., 1985), increased growth (Bolsen *et al*., 1980) and milk production (Stokes and Zheng, 1995; Chen *et al*., 1994) by cattle has been observed with enzyme treated silages. The nutritive value of

oulandsgrass is relatively low due to its high lignocellulosic content (personal communication). Use of fiber degrading enzymes hold potential in increasing the nutritive value of poor forages hence the purpose of the research in this chapter was to optimize the enzyme pretreatment phase of forage with cellulolytic and hemicellulolytic enzyme preparations of selected fungi and their effect was compared with that of commercial enzyme preparations.

4.3 MATERIALS AND METHODS

4.3.1 Enzyme sources

Table 4.1 Sources of commercial enzyme preparations

Enzymes	Source	Activity (U/g)
Enzeco cellulase	Enzyme international corporation	3000
Depol 670L	Amano	25 000
Cellulase 3S	Yakult pharmaceuticals	3 000

Cellulase and xylanase enzyme system of Abo 374 (*A. terreus var* carneus) were produced in large scale SSF as explained in Chapter 3. Commercial enzyme preparations used are listed in Table 4.1. Enzeco cellulase was used for comparative purposes. Laccase was produced in shake flasks using a recombinant strain *A. niger* D-lcc2#3 and *A. niger* DM-15 was used as control because this organism does not contain the laccase gene.

4.3.2 Pre-treatment of oulandsgrass

Milled oulandsgrass of approximately 250 mg was weighed onto a pre-weighed nylon filter (8 cm^2) with a porosity of 40 μm. The sachets were then dried at 100°C overnight and re-weighed to determine the weight of dried grass in each sachet. Individual sachets were placed in screw cap bottles (in duplicate) to which 10ml of either Enzeco cellulase, Abo 374 (concentrated) and Abo 374 (50% concentration diluted with citrate buffer - 0.05M, pH 5. Citrate buffer was used as a negative control for the enzyme pre-treatments. A 200 μl volume of sodium-azide (2mg/ml) was added to each bottle to

prevent bacterial growth. The bottles were then incubated at 39°C for 24h on an orbital shaker at 160 rpm. Duplicate sachets were removed at 3, 6, 9 and 24h, followed by rinsing in distilled water at 4°C for 1 h. The sachets were then dried at 100°C overnight and the final weight of the dried sachet was recorded.

4.3.3 Application of enzyme cocktails to oulandsgrass

Enzeco cellulase contains a 1:1 ratio of xylanase to cellulase; with activities of each enzyme being about 3000 nkat/ml. This commercial preparation was more effective compared to the Abo 374 enzyme preparation, which had a ratio of 10:1 xylanase to cellulase (approximately 7000 and 700 nkat/ml of xylanase and cellulase, respectively). From the above experiment (4.3.2) it was concluded that Enzeco cellulase displayed a greater degree of hydrolysis compared to Abo 374. Hence further applications focused on incorporating different ratios of xylanase and cellulase as follows: i) Cellulase 3S was added to Abo 374 to maintain a 1:1 ratio; ii) Cellulase 3S was added to Abo 374 to maintain a 1:2 ratio and iii) the effect of Cellulase 3S alone was also evaluate at a ratio 1:1 and 1:2 in separate experiments. The sachets were prepared and incubated as described in section 4.3.2. Duplicate sachets were removed at 4, 8, 24 and 48h.

4.3.4 Effect of laccase pre-treatment and commercial enzymes on oulandsgrass

To evaluate the effect of laccase pretreatment, sachets containing oulandsgrass were prepared as described in section 3.3.2. A laccase preparation (10.9 U / ml) produced in shake flasks by a recombinant fungal strain of *A. niger* D-lcc2#3 was applied to these sachets containing oulandsgrass for 24 h at 39°C. *A. niger* DM-15 strain that did not contain a laccase gene was used as negative control. Following the 24h incubation period the sachets containing the oulandsgrass was thoroughly rinsed with distilled water. Commercial enzyme preparations containing cellulase and hemicellulases (Depol 670L and Enzeco cellulase) were hereafter applied for a further 24 h 39°C. Samples (individual sachets) were removed at 4, 8 and 24h. Sachets were then rinsed for 1h, dried and weighed to determine weight loss.

4.3 RESULTS

4.3.1 Digestion of oulandsgrass by xylanase and cellulase preparations

During the first nine hours of incubation there was no significant difference in weight reduction of oulandsgrass after application of Enzeco cellulase (positive control) and the Abo 374 enzyme preparations. Approximately 12 % reduction in weight of oulandsgrass was observed after incubation for 24 h in Enzeco cellulase while concentrated Abo 374 enzyme preparation resulted in approximately eight percent weight reduction in comparison to the buffer control (Figure 4.1).

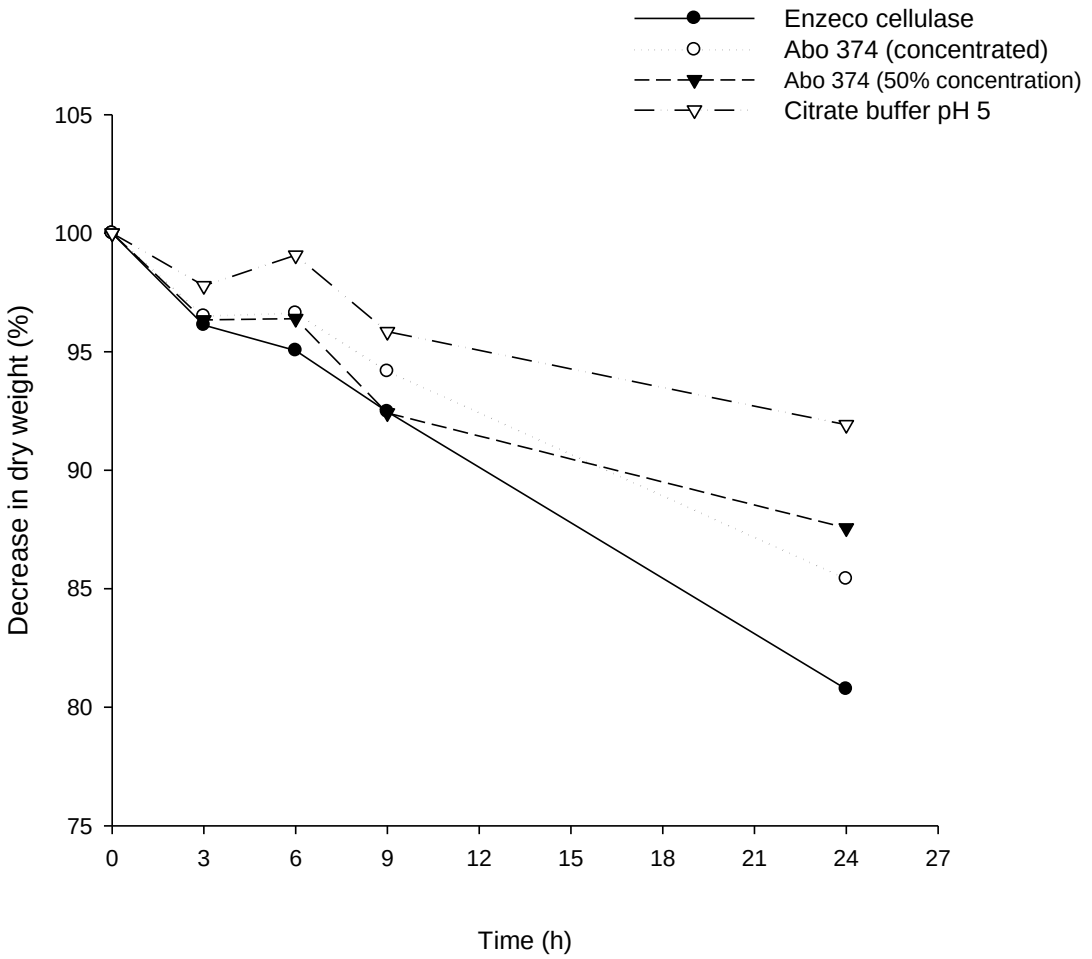

Figure 4.1 Loss in dry weight of oulandsgrass during treatment with Abo 374 enzyme preparation at 39°C for 24 h.

4.3.2 Enzyme pre-treatment using different ratios of xylanase to cellulase

Application of different ratios of xylanase and cellulase on grass resulted in a greater weight reduction compared to the control (citrate buffer, pH 5). Enzeco cellulase and Abo 374 enzyme preparation supplemented with Cellulase 3S to maintain a xylanase: cellulase ratio of 1:2 (7000: 14000 nkat/ml) resulted in approximately eight percent weight reduction while Cellulase 3S with a 1:1 ratio of xylanase and cellulase resulted in slightly higher weight reduction compared to Enzeco cellulase (Figure 4.2).

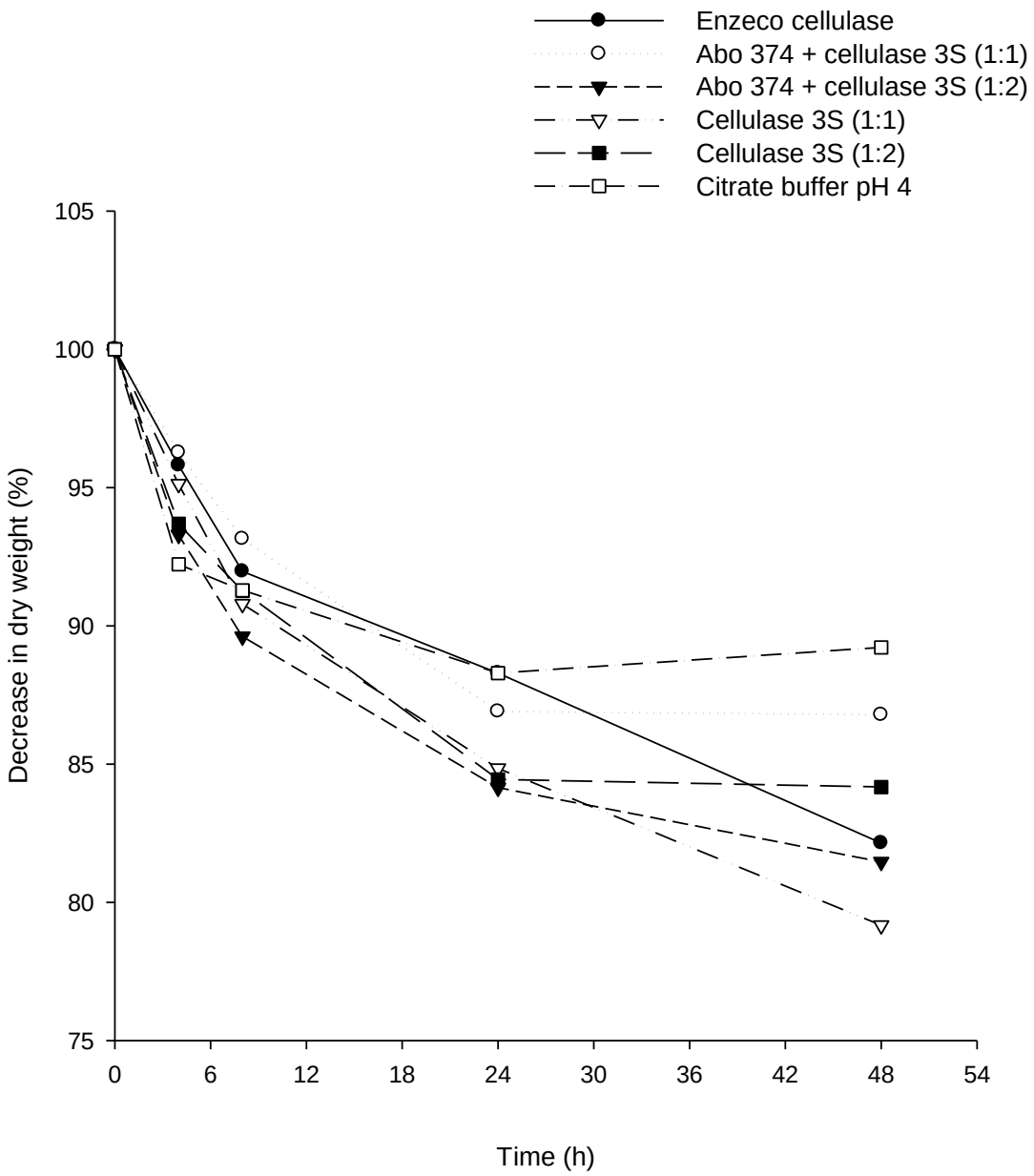

Figure 4.2 Loss in weight reduction of oulandsgrass during treatment with enzyme preparations containing different ratios of xylanase to cellulase at 39°C for 48 h

4.3.3 Laccase pre-treatment followed by incubation with commercial enzymes

Application of the *A. niger* DM-15 enzyme preparation did not yield any change in weight of the oulandsgrass. However, a 2% weight reduction was observed with *A. niger* D-lcc2#3. Interestingly, incubation in citrate buffer for 24 h following application of *A. niger* D-lcc2#3 for a similar period resulted in a significant weight reduction. Pre-treatment in laccase followed by incubation in Depol 670L containing xylanase and cellulase activity resulted in approximately 30% weight reduction while approximately 22% was observed with the Enzeco cellulase application (Figure 4.3).

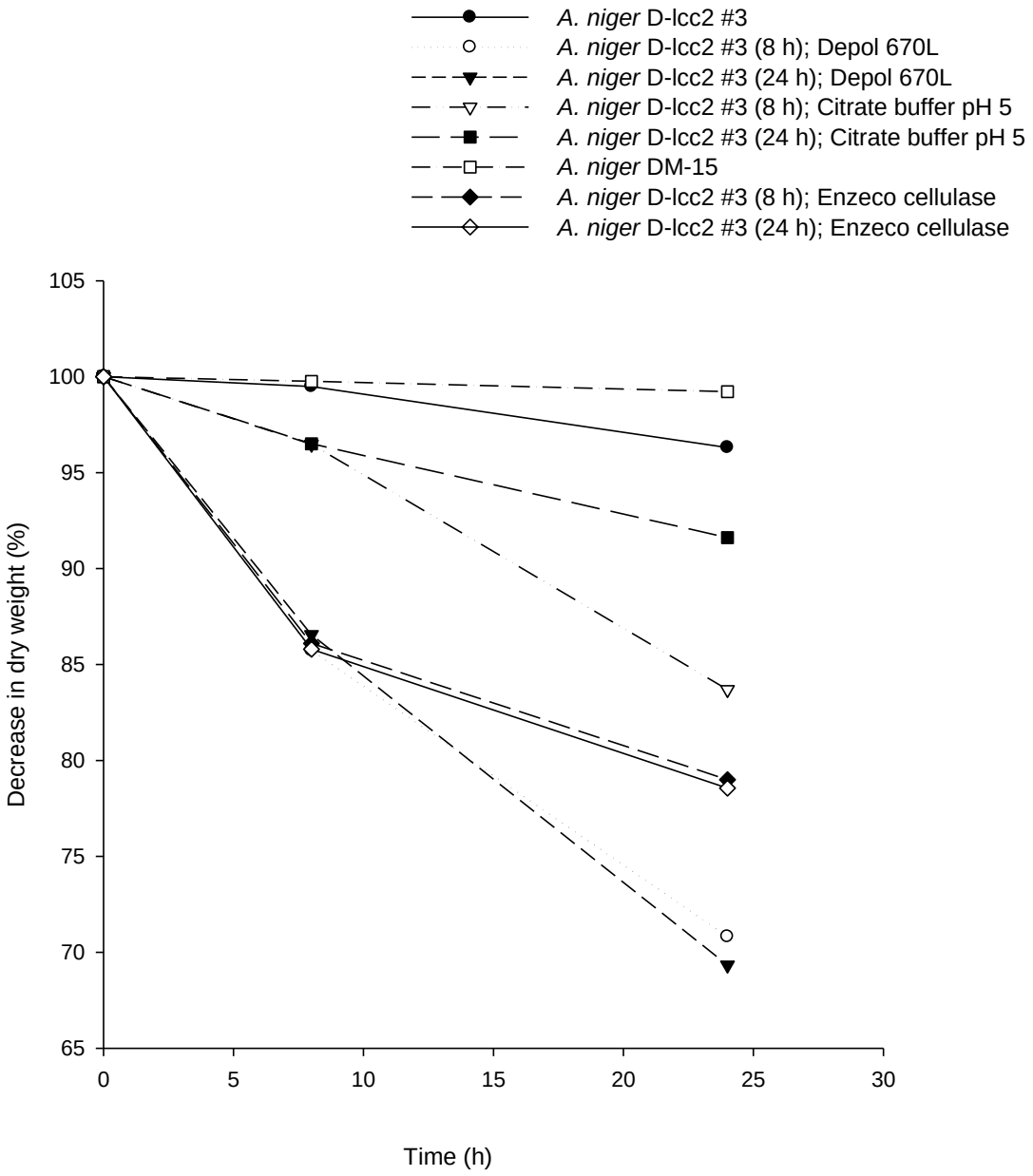

Figure 4.3 Loss in dry weight of oulandsgrass during pre-treatment with laccase at 39°C for 24 h followed by treatment with commercial enzymes for 24 h

4.4 DISCUSSION AND CONCLUSION

An increase in the digestibility and nutritive value of feeds can make a significant difference in the growth of livestock. Since only about 40% of the energy in forages becomes available to the animal, use of enzymes as biological tool to enhance digestion through the action of cellulase, hemicellulase and lignase enzymes resulting in improved meat, milk and wool production. Howes *et al.* (1998) reported that it was not clear whether the major benefit of enzyme application occurred in pre-feeding treatment or after the feed enters the rumen. In this study oulandsgrass known to be forage of poor quality was used for the optimization of the pre-treatment of forage prior to feeding.

Treatment of oulandsgrass with different concentrations of Abo 374 enzyme preparations containing cellulase and xylanase activity resulted in hydrolysis of oulandsgrass thus evident in the reduction of the final oulandsgrass weight. According to Howes *et al.* (1998) treatment of feed or forage well ahead of feeding time such as in silage inoculant applications, allows the potential for increased rumen activity and an increase of up to 10% digestibility of straw was reported by Nakashima and Orskov (1989).

A buffer control of pH 4 exhibited about five percent more weight reduction compared to the weight reduction caused by the same buffer at pH 5, meaning that forage is more digestible in acidic pH. Dilute acid pretreatment has been reported to have an advantage of not only solubilizing hemicellulose

but also converting solubilized hemicellulose to fermentable sugars (Saha, 2003; Saha *et al*., 2005). Both the supplemented fungal preparation and commercial enzyme preparations showed better performance when the concentration of cellulase was double that of xylanase. A related finding has been reported although a direct comparison might not be possible as that study was done using ruminal fluid; Wallace *et al.* (2001) reported that in a wider range of fibrolytic enzyme preparations those with higher exo- or endo-xylanase activity did not increased rates of fermentation of corn silage when glucanase activity was low. In contrast, preparations with higher cellulase activity gave a great improvement in gas production irrespective of xylanase activity.

Forage treatment with laccase alone was not too effective as it only gave approximately three percent weight reduction whereas pretreatment in laccase followed by treatment with a commercial preparation containing cellulolytic and hemicellulolytic enzyme activities exhibited approximately 30% loss in dry weight of oulandsgrass. This may be due to laccase aiding in lignin degradation thereby making it easier for the other enzymes to access and digest the cellulose and hemicellulose in oulandsgrass. Commercial enzyme preparations used in animal feed are evaluated in their capacity to degrade plant cell walls and each of these enzymes can consist of numerous specific enzymes having activity against cellulose and xylan for example, enzymes with cellulase activity include numerous types of endoglucanases, cellobiohydrolases and glucanases that cleave different bonds of the cellulase chain (Beauchemin *et al*., 2001). In addition to fibre degrading enzymes,

these products contain secondary enzymes such as amylases, proteases and pectinases and this may present difficulties in terms of comparison of fungal enzyme preparations with known enzyme activities with commercial enzyme preparations.

In this study it was observed that enzyme cocktails with higher cellulase activity than xylanase result in higher hydrolysis of forage. Oulandsgrass pretreatment in laccase resulted in higher weight loss and a lower pH aided higher hydrolysis of oulandsgrass. Since there were few reports found in literature dealing with forage pretreatment prior to feeding, this study provided bases for future research. Further optimization of the pretreatment step may result in improvement in animal growth rates and production.

CHAPTER 5 GENERAL DISCUSSION

Xylanases and cellulases are industrially important enzymes that are used in different industrial applications to hydrolyse plant polysaccharides such as xylan and cellulose. These enzymes have found use in the animal feed industry because of their ability to enhance digestibility of animal feed hence enhancing animal growth rates. However, the cost of production process, cost of carbon and nitrogen sources and low yields of these enzymes presents major problems for their industrial application (Virupakshi *et al*., 2005; Yang *et al*., 2006).

According to South African animal feed manufactures the available commercial enzyme preparations manufactured abroad are not suitable for SA feed due to uniqueness of the feed formulations. The composition of South African animal feeds is different to those for which the enzyme preparations have been developed. Thus this study was conducted to evaluate commercial viable carbon sources and nitrogen source to maximize the production of xylanase, cellulase and laccase. The produced enzymes were applied to veld grass to evaluate their potential to be incorporated in South African feed formulations.

The investigations on the ability of cellulose and hemicellulose hydrolyzing microbial strains to utilize inexpensive substrate have been conducted in SmF and SSF. Wheat straw and oulandsgrass were chosen as carbon sources because of their affordability and their huge potential availability. Most of the

fungal strains except *T. ressei* Rut C30 adapted well in oulandsgrass and resulted in higher xylanase and cellulase activities when grown on this substrate in SSF and SmF. Higher enzyme activities on oulandsgrass maybe attributed to certain molecules that are essential for enzyme induction being readily accessible to the fungus (Olsson *et al*., 2004). The laccase levels produced by all the evaluated strains were lower than 0.1 nkat / ml and in literature there were no reports found of laccase production by the strains used in this study.

The enzyme activities obtained in SmF were lower than those in SSF. This finding is no different from reports in literature; other researchers have reported high enzyme activities in SSF compared to SmF (Ashokkumar *et al.,* 2001; Patil and Dayanand, 2006). The hyphal development of fungi allows them to effectively colonize and penetrate the solid substrate thereby gaining full access to the substrate (Pandey *et al.*, 2000). SSF is known for high product concentration but has a relatively low energy requirement. There was a huge variation in the ratio of xylanase to cellulase produced throughout the fermentation with xylanase levels higher than the cellulase in all the evaluated strains. This variation could be associated with certain parts of the substrate being more available to the organism and may also be result of heterogeneity of hemicellulose, with its branches hydrolysed by different enzymes at different times (Olsson *et al*., 2004; Kang *et al*., 2004).

In an attempt to reduce solid loadings that created sampling and heat transfer problems in bench scale submerged fermentation, straw teas were prepared

by acidic or alkaline extraction of wheat straw and evaluated for induction of xylanase and cellulase synthesis. These teas had no significant effect as inducers of xylanase and cellulase in this study. This might mean that an interaction with substrate is required for induction or essential molecules that were required for induction were not efficiently extracted from the wheat straw. Olsson *et al*. (2004) reported that *T. reesei* Rut C30 grown on alkaline extracted sugar beet pulp resulted in lower cellulase and hemicellulase activities compared to that obtained on sugar beet pulp. Singh *et al*. (2000) reported high levels of xylanase production by *T. lanuginosus* SSBP grown on corn cobs than when utilizing expensive carbon source. When Liming and Xueliang (2004) compared cellulase production in pure cellulose and corn cobs residue, the cellulase concentration and yield produced with corn cobs residue were similar to that with purified cellulose based on the same cellulose content.

In this study three types of corn steep liquor; phytase treated (CSL^{PT}), phytase treated and ultrafiltered (CSL^{PTUF}) and spray dried (CSL^{SD}), were evaluated as a nitrogen source for xylanase and cellulase production. CSL^{PT} resulted in higher xylanase and cellulase activities (5355 and 144 nkat / ml, respectively) compared to the other two types and lower enzyme levels were observed in medium containing CSL^{PTUF}. Phytase has been reported to improve nutritional quality of phytic acid rich feeds as well as fermentation substrates such as CSL. Phytase treatment of CSL increases the free phosphate content of the substrate while reducing the phytin content (Hakala *et al*., 2004). The low enzyme levels in medium containing CSL^{PTUF} even though treated with

phytase maybe due to essential molecules and trace elements being removed during ultra-filtration. Rajoka (2004) reported that organic nitrogen sources such as CSL were poor nitrogen sources for the production of cellulase but in this study cellulase activity was higher than that observed in medium containing yeast nitrogen base as a nitrogen source. Fungi have been found to produce higher xylanase activities on organic nitrogen sources (Purkarthofer *et al*., 1993; Singh *et al*., 2000; Bakri *et al*., 2003; Yang *et al.,* 2006).

Many researchers reported a decrease in fungal xylanase activity when scaled up from shake flasks to a bioreactor utilizing xylan substrates (Purkarthofer *et al*., 1993; Gomes *et al*., 1993; Hoq *et al*., 1994). A similar finding was observed in this study when CSL^{PT} medium which resulted in better xylanase and cellulase activity in shake flasks was scaled up to a bioreactor, approximately 60% decrease was observed in xylanase activity. This decrease in activity maybe caused by several factors such as shear stress caused by agitation which may change fungal morphology and the aeration rates inside the vessel. Shear stress has been found to cause morphological and physiological changes to some filamentous fungi (Mitard and Riba, 1988). The agitation speed used in this study was higher than that reported in literature for other fungi. An optimum agitation rate of 200 rpm has been reported by Hoq *et al.* (1994) for *T. lanuginosus* strain RT9 while Purkarthofer *et al.* (1993) reported that 120 rpm was optimal for *T. lanuginosus* strain DSM 5826 grown on corn cobs. Aeration rates have also been found to influence enzyme production in bioreactor. Reddy *et al*.

reported that an aeration rate of 1 vvm favoured xylanase production by *T. lanuginosus* SSBP while Palma *et al*. (1996) observed that an increase in aeration rate reduced xylanase production by *P. janthinellum*.

A. *terreus var carneus* (Abo 374) enzyme preparation produced in this study in combination with commercial enzyme preparations were applied to oulandsgrass to evaluate their ability to hydrolyze veld grass. Enzeco cellulase was used as a control in all the pretreatment studies and it resulted in approximately 40% oulandsgrass hydrolysis compared to Abo 374 enzyme preparation. It was observed that the ratio of xylanase to cellulase of *A. terreus var carneus* enzyme preparation was 10:1 whereas that of Enzeco cellulase was 1:1. Based on this observation, the Abo 374 enzyme preparation was supplemented with a commercial preparation to maintain a 1:1 and 1:2 ratio of xylanase to cellulase. Cellulase 3S, a commercial enzyme preparation used to supplement Abo 374 enzyme preparation was also evaluated at the same ratios. Both the supplemented Abo 374 and Cellulase 3S resulted in approximately 30% more hydrolysis of oulandsgrass when the cellulase ratio was higher than that of xylanase. Öhgren *et al.* (2007) reported that supplementing the cellulase preparation with xylanases improved hydrolysis because xylanases hydrolyze the hemicelluloses thereby increasing accessibility of cellulose to cellulase. Accessory enzymes *viz*., glucosidase, mannanase and have been reported to have a significant effect on cellulase performance (Berlin *et al*., 2005).

A buffer control at pH 4 resulted in more reduction in oulandsgrass weight compared to the reduction caused by the same buffer at pH 5. Saha *et al.* (2005) reported that dilute acid pretreatment aids in hydrolysis of hemicellulose. There was no significant weight reduction observed when laccase alone was applied to oulandsgrass but when the grass was pretreated with laccase prior to treatment with commercial preparation resulted in approximately 30% weight reduction. This was approximately 10-15% improvement compared to using the commercial preparation alone.

In conclusion, from this study it was observed that SSF results in higher enzyme levels compared to higher SmF. Most of the organisms employed were capable of utilizing oulandsgrass and wheat straw as carbon sources and CSL as a sole nitrogen source. The ability of these organisms to grow on inexpensive substrates appeared as an attractive feature as it would result in reduced production costs. Weight reduction was observed when the enzymes produced in this study were applied to oulandsgrass which indicated that they were able to hydrolyze oulandsgrass which is known to be forage of poor quality. Further optimization of the pretreatment step is still required focusing on investigation of steam, acid and alkaline pretreatment prior to enzyme hydrolysis; screening for accessory enzyme and supplementing the xylanase and cellulase activities with accessory enzymes during the enzyme pretreatment stage. The development of enzyme formulations for use as feed additives may become extremely attractive in the South African context. It is hoped that with the elucidation of new technologies such as directed evolution

new enzymes in the current market will provide greater flexibility in ensuring a higher degree of success in the food and feed industry.

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