FUNGAL ENZYMES AS ANIMAL FEED ADDITIVES

BY

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Thesis presented in partial fulfillment of the requirements for the degree of

Master of Science at the University of Stellenbosch

MARCH 2001

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DECLARATION

I, the undersigned hereby declare that the work contained in this thesis is my original work and has not previously in its entirety or in part been submitted at any university for a degree

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SUMMARY

The use of fungal enzymes as ruminant feed digestibility enhancers was investigated. Currently, ruminants may not digest 38 to 80 % of fibrous forages' content. A renewed interest in the potential of feed enzymes for ruminants was prompted by the high costs of livestock production, together with the availability of newer enzyme preparations. Direct application of enzyme preparations can improve *in vitro* dry matter (DM) and neutral detergent fibre (NDF) degradation, indicating that direct-fed fibrolytic enzymes may be effective in enhancing *in vivo* digestion of forages.

Two commercial enzyme products, Fibrozyme and Celluclast, and fungal extracellular enzyme extracts from *Aureobasidium pullulans*, *Trichoderma reesei*, *Aspergillus aculeatus*, and *Thermomyces lanuginosus* were evaluated for enhancing *in vitro* feed digestibility. Fibrozyme addition to both wheat straw and lucerne hay did not improve their *in vitro* digestibilities, even after a two hour pre-incubation period. The four fungal enzyme extracts did not enhance wheat straw's digestibility, but marginal increases were evident for lucerne hay. Celluclast addition resulted in marginal increases in the digestibility of both oat hay and oat silage, with no enhanced effect on lucerne hay and NaOH-treated wheat straw. No relationship could be found between the level of enzyme activity and the degree of feed digestion in the *in vitro* assay.

Enzyme hydrolysis with Celluclast, in the absence of rumen fluid, gave more conclusive results. All the feed samples tested showed a positive response to Celluclast addition, even the less digestible feeds, namely sugarcane bagasse and wheat straw.

In vitro results show that the assays were unsuccessful, because almost all of the experiments conducted showed inconclusive results. Alternative feed evaluation assays, which include the *in vivo*, *in sacco* and *in situ* methods of analysis, as well as gas production measurement and *in vitro* analysis with the DAISY^{II} system, should be evaluated. A more detailed study of feed digestibility should be motivated by determining which feeds are hydrolysable, their chemical composition, i.e. how accessible the feeds are, and also evaluation of feed mixtures. The enzyme supplements also need to be evaluated for optimum temperature and pH, as well as the compilation of enzyme cocktails.

OPSOMMING

Die gebruik van swamensieme om die verteerbaarheid van herkouervoere te verhoog, is ondersoek. Tussen 38 en 80 % van veselagtige voere se inhoud is tans onverteerbaar. 'n Hernieude belangstelling in die potensiaal van voerensieme vir herkouers word deur die hoë koste van veeproduksie, asook die beskikbaarheid van nuwe ensiempreparate gedryf. Direkte byvoeging van ensiempreparate kan die *in vitro* droëmateriaal (DM) en neutrale onoplosbare vesel (NOV) vertering verbeter, wat daarop dui dat fibrolitiese ensieme wat direk gevoer word, effektief mag wees tydens die *in vivo* vertering van voer.

Twee kommersiële ensiemprodukte, Fibrozyme en Celluclast, en die vier ekstrasellulêre ensieme van vier swamme, naamlik *Aureobasidium pullulans*, *Trichoderma reesei*, *Aspergillus aculeatus*, en *Thermomyces lanuginosus* is vir hul vermoë om die *in vitro* verteerbaarheid van voere te verbeter getoets. Byvoeging van Fibrozyme by beide koringstrooi en lusernhooi het geen verbetering in hul onderskeie *in vitro* verteerbaarheid tot gevolg gehad nie, selfs nie eens na 'n twee uur vooraf inkubasieperiode nie. Koringstrooi se verteerbaarheid is nie verbeter deur die byvoeging van die vier swam-ensiempreparate nie, maar 'n minimale verbetering is wel waargeneem in die verteerbaarheid van lusernhooi. Byvoeging van Celluclast het 'n minimale verbetering in beide hawerhooi en hawerkuilvoer se verteerbaarheid tot gevolg gehad, maar geen effek op lusernhooi of NaOH-behandelde koringstrooi se verteerbaarheid nie. Geen verwantskap is tussen die vlak van ensiemaktiwiteit en die mate van vertering tydens die *in vitro* toets gevind nie.

Ensiematiese afbraak met Celluclast, in die afwesigheid van rumenvloeistof, het meer konkrete resultate gelewer. Al die voermonsters het 'n positiewe respons op die byvoeging van Celluclast getoon, selfs ook die minder verteerbare voere, nl. suikerrietbagasse en koringstrooi.

In die wyer konteks was die resulate van die *in vitro* verteringstoetse egter onbeduidend as gevolg van groot variasie in die metings. Alternatiewe voerontledingstoetse, wat moontlik beter resultate mag lewer, sluit in *in vivo*, *in sacco* en *in situ* analises, asook die meting van gasproduksie en *in vitro* analise met die DAISY^{II} sisteem. 'n Meer uitgebreide studie van voerverteerbaarheid wat die bepaling van die afbraak van voere, hul chemiese samestelling, met ander woorde toeganklikheid van voere, en die ondersoek van voermengsels behels, behoort aandag te geniet. Die ensiemmengsels behoort ook ten opsigte van samestelling, optimum temperatuur en pH ondersoek te word.

This thesis is dedicated to my LORD and Saviour, Jesus Christ for many blessings, my fiancé Mugeleigh Bruiners, and also my family.

Hierdie tesis word aan my HEER en Verlosser, Jesus Christus vir vele seëninge, my verloofde Mugeleigh Bruiners, asook my familie opgedra.

BIOGRAPHICAL SKETCH

Francisco Martin Lakay was born in Cape Town, South Africa, on March 27, 1974. He attended Emil Weder Senior Secondary School (Genadendal) and matriculated at Genadendal in 1991. Francisco enrolled at the University of Stellenbosch and obtained a B. Sc. Degree in Microbiology and Biochemistry in 1998. He completed his B. Sc. Hons. degree in Microbiology in 1999 at the same university.

ACKNOWLEDGEMENTS

I wish to extend sincere thanks to the following persons and institutions for their contribution to the successful completion of the present study:

Prof. B. A. Prior, Department of Microbiology, University of Stellenbosch, for allowing me to conduct this study in his laboratory, for his encouragement, discipline, guidance, and devotion to his students and science;

Dr. C. W. Cruywagen, Department of Animal Science, University of Stellenbosch, for his guidance, knowledge and enthusiasm for the project;

Prof. W. H. van Zyl, Department of Microbiology, University of Stellenbosch, for his enthusiasm and insight;

Prof. P. Biely, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Czechoslovakia, for helping with calculations and optimisation of enzyme assays during his collaboration at the University of Stellenbosch in 2000.

Voermol and Tongaat-Hulletts, for initiating and supporting this project financially;

National Research Foundation (NRF), for financial support;

Lucia Holtshausen, Nicolas Brandt, for technical assistance during studies conducted at the Dept. of Animal Science, University of Stellenbosch;

Fellow colleagues in the lab and friends, for free information and camaraderie supplied during my studies at this university;

My fiancé Mugeleigh Bruiners and my family, for their love, encouragement and patience during this study;

Most of all, **Jesus Christ,** for making it all possible through HIM. Thank you!

CONTENTS

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. INTRODUCTION AND AIMS

Currently, ruminants may only digest between 38 and 80% of the cellulose in fibrous forages, depending on the degree of lignification. Therefore, it is obvious that if a greater amount of the total dietary energy from forages were available, lower cost diets could be formulated (Allen and Oba, 1998). The addition of fibrolytic enzymes produced by microorganisms as feed supplement to improve digestion and production efficiency in ruminants has recently gained renewed interest (Akin *et al.*, 1993, 1995; Annison, 1997). Some studies demonstrated a more than 10% increase in feed conversion ratio and average daily gain, particularly in lactating and growing cattle, upon the addition of fibrolytic enzymes to the diets. However, an important conclusion from these studies is that microbial additives in one diet formulation will not necessarily function optimally in a different diet formulation (Newbold, 1995; Howes *et al.*, 1998). These results demonstrate that the evaluation of fibrolytic enzyme addition to specific feed formulations can yield improved feed formulations with real cost benefits. Specific research is however needed to examine the effects of exogenous fibrolytic enzymes applied to forage harvested as dry hay, and as direct-fed additives to forage diets (Feng *et al.*, 1996).

Lignin, hemicellulose and cellulose account for between 30 and 80% of the organic matter in forage crops, and is known as neutral detergent fibre with the remaining organic matter almost completely digestible. In addition, the fibre concentration increases with maturing of plants, which is the most important factor affecting dry matter digestibility. Neutral detergent fibre separates the highly digestible fractions of feeds from the less digestible, non-uniform fractions and is apparently the best single chemical predictor of voluntary dry matter intake by ruminants (Buxton and Redfearn, 1997; Varga and Kolver, 1997).

Grasses and legumes have a hemicellulose content that range between 21 and 37% of total dry mass, depending on the maturity of the specific plant. Arabinoglucuronoxylan is a major polymer in the hemicellulose fraction that also increases with the maturity of the plant. This polymer is substituted with 4-*O*-methyl-α-D-glucuronyl residues linked (1→2) to xylose residues. The presence of these substituents presumably decreases the accessibility of microbial xylanases to xylan, thereby reducing forage digestibility (Smith and Forsberg, 1991).

Forage quality is a function of nutrient concentration in the herbage, intake potential, nutrient availability, and partitioning of metabolised products within animals. According to Buxton *et al.* (1995), plant characteristics are major determinants of forage quality, but animal variation can also influence its assessment (Buxton *et al.*, 1995). Plant cell walls limit intake and digestibility; therefore

intake of available energy is primarily a function of plant cell wall concentration. Animals are dependent on microbial fermentation in the rumen of cows, sheep, and goats to obtain the energy of complex carbohydrates contained in forage cell walls. Intake of roughage is mainly dependent on the rate of digestion and therefore feed utilisation by ruminants is greatly determined by the efficiency of microbial activity in the rumen (Jouany, 1994). The less digestible a feed is, the less dry matter a ruminant will consume (Ørskov, 1998).

Fibrolytic activity in the rumen is normally very high, and therefore not easily increased by a simple addition of exogenous enzyme products. However, a renewed interest in the potential of feed enzymes for ruminants was prompted by the high costs of livestock production together with the availability of newer enzyme preparations (Rode and Beauchemin, 1998). Fibrolytic enzymes effectively hydrolyse structural carbohydrates and yield more substrate for lactic acid-producing microbes (Beauchemin and Rode, 1996). Direct application of enzyme preparations can improve *in vitro* dry matter and neutral detergent fibre degradation, indicating that direct-fed fibrolytic enzymes may be effective in enhancing *in vivo* digestion of forage (Lewis *et al.*, 1996).

Microbial xylanolytic enzymes in admixture with cellulases can be used efficiently for improvement of animal feed, resulting in increased feed efficiency (Biely, 1993). A wide range of microbial feed additives, containing microorganisms, their products, or spent growth medium containing metabolic end products, is currently available commercially to livestock producers (Beharka and Nagaraja, 1998). Many of the current enzyme products are fermentation extracts obtained from the growth of bacteria such as *Lactobacillus, Enterococcus, Streptococcus, Bifidobacterium,* and *Bacillus* spp. as well as fungal species such as *Aspergillus niger, Aspergillus oryzae, Saccharomyces cerevisiae,* and *Trichoderma longibrachiatum*. These preparations primarily contain amylases, pectinases, cellulases, and proteases. In order to be commercially successful, enzymes for ruminants should be compatible, or complement the variety of feed additives (Martin and Nisbet, 1992; Newman and Jacques, 1995; Howes *et al.*, 1998).

The aims of this study were to investigate the following:

1. Evaluation of commercial fibrolytic enzyme preparations, such as Fibrozyme and Celluclast, when added to forages or roughages (including sugarcane bagasse) to enhance the fibre digestibility in rumen fluids from sheep.
2. Production and evaluation of suitable crude enzyme preparations from the fungi *Thermomyces lanuginosus, Aureobasidium pullulans, Aspergillus aculeatus,* and *Trichoderma reesei*, for use in feed formulations to enhance the fibre digestibility in rumen fluids from sheep.
3. Preliminary characterisation of the enzymes and formulation in appropriate mixtures.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

Lignocelluloses are the most abundant organic compounds in the biosphere. Biomass in the form of wastes accumulates in large quantities, causing a deterioration of the environment and loss of potentially valuable resources. Lignocellulose has attracted considerable attention as an alternate feedstock and energy resource, because of the large quantities available and of its renewable nature. Potential uses are in pulp and paper industries, production of fuel alcohol and chemicals, protein for food, and feed using biotechnological means (Kuhad and Singh, 1993). However, the limits to biodegradation of lignocellulose are a major problem in the commercial use of this material. For example, lignin reduces the availability of structural carbohydrates in forage cell walls to rumen microorganisms (Akin, 1995). The carbohydrate composition of some lignocellulosic substrates is indicated in Table 2.1.

TABLE 2.1. Cell wall carbohydrate composition of lignocellulosic substrates (adapted from Op den Camp *et al.*, 1988, Dijkerman *et al.*, 1997).

	Cellulose	**Hemicellulose**	**Lignin**
Substrate		**% Dry matter**	
Hay	26.0	26.8	4.7
Alfalfa	21.8	12.4	9.7
Wheat straw	44.0	29.6	10.4
Bagasse	39.3	27.2	12.2
Saw dust	45.0	15.1	25.3
Coconut fibre	17.7	2.2	34.0

The potential of lignocellulose as a renewable raw material is immense. It is produced and wasted annually in huge amounts (Table 2.2) and has several likely uses if suitable technology can be developed. An integrated approach has been proposed for biomass production in feed and biocatalyst applications (Fig. 2.1). The main possibilities of lignocellulose bioconversion are production of single-cell protein (SCP), enzymes, and sugar syrup, which can be used as feedstock (Kuhad and Singh, 1993).

Hemicellulases can be used for the bioconversion of lignocellulosic materials to produce products of higher value, such as SCP, fuel, and other chemicals. However, the characteristics and effects of hemicelluloses in the feedstock need to be assessed before the relevant hemicellulases can be selected and used effectively. Addition of hemicellulases can increase the digestibility of animal

feed and thereby widen the variety of its ingredients and increase its energy value (Viikari *et al.*, 1993; Wong and Saddler, 1993).

TABLE 2.2. Estimated Global Production of Lignocellulosic Wastes (Kuhad and Singh, 1993).

	Wastes (million tons)			
Continent / Country	**Cereal crop**	**Pulse crop**	**Oilseed crop**	**Plantation crop**
Africa	165	9	11	34
Asia	1135	51	61	174
Australia	35	1	2	12
Europe	550	10	8	1
Central America	500	49	21	84
South America	153	37	10	147
U.S.	440	44	19	15
Canada	60	2	<1	NA[a]
World	2946	166	142	548

[a] NA = Not available

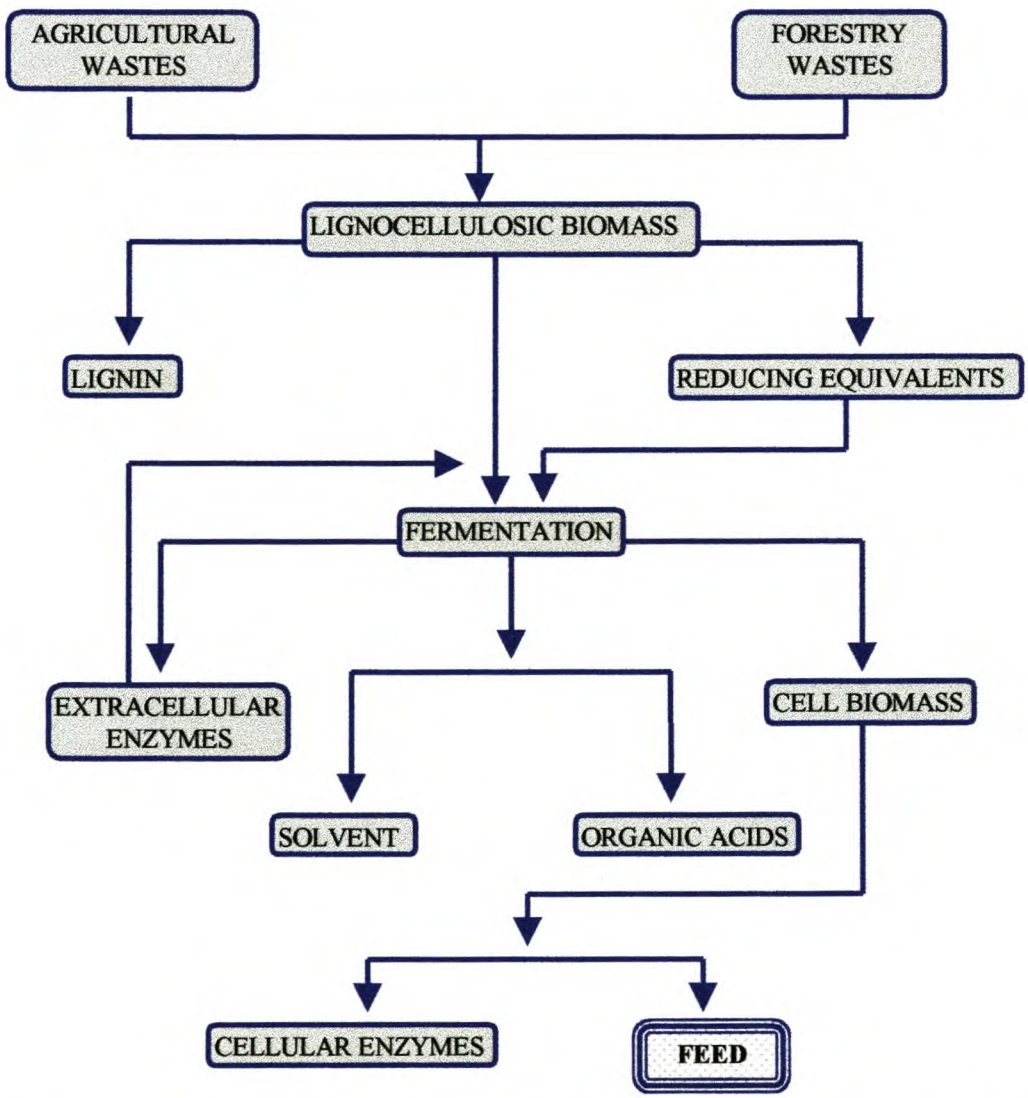


FIGURE 2.1. Integrated approach for bioconversion of lignocellulosic wastes into valuable products (adapted from Kuhad and Singh, 1993).

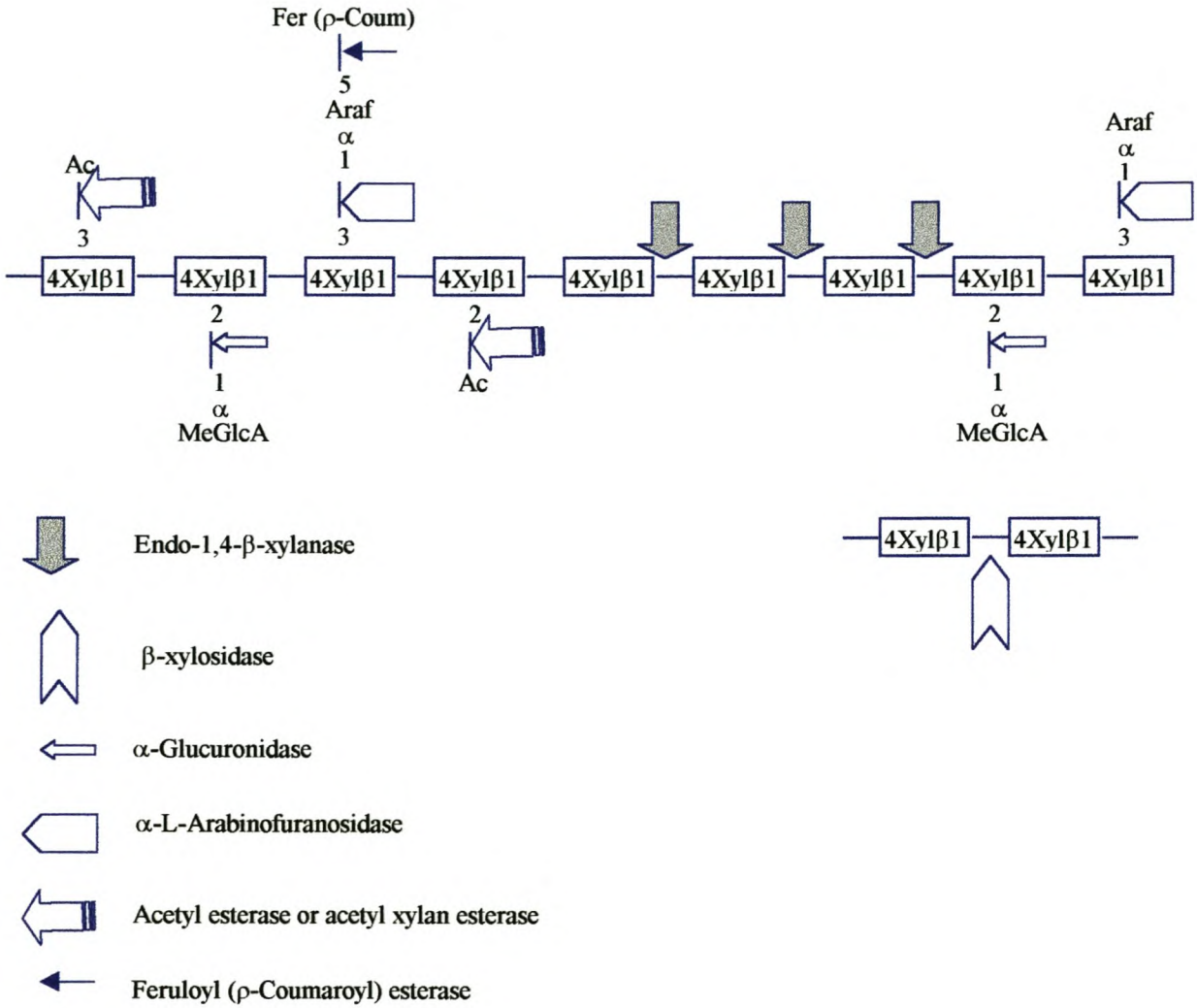
2.1. HEMICELLULASES

2.1.1. Hemicellulases involved in lignocellulose degradation

Plant cell wall polysaccharides consist mainly of celluloses and hemicelluloses. Hemicelluloses are usually classified according to the main sugar residues in the backbone, e.g. xylans, glucomannans, galactans and glucans. Xylan is the main component of hemicellulose, especially in hemicellulose from hardwood and grass species and is complexed with cellulose, pectin, and lignin (Buchert *et al.*, 1994; Jeffries, 1996). Hemicellulose mainly consists of a main chain of highly branched β-1,4-linked xylanopyranosyl residues, some of which are substituted by glycosidically linked α-L-arabinofuranosyl and 4-*O*-methyl α-D-glucopyranosyl uronic acid, and acetyl residues linked through ester bonds. A few of the L-arabinosyl residues are substituted with feruloyl and/or ρ-coumaroyl groups (Fig. 2.2). The presence of these various substituents therefore has a pronounced influence on the chemical and structural properties, and also on the enzymatic degradability of xylan in lignocellulose. The degradation of xylan requires a number of different esterases and glycosidases, with the endo-β-1,4-xylanases the most important amongst them, which cleave the backbone itself (Mendicuti Castro *et al.*, 1997; Garcia-Campayo *et al.*, 1994; McCrae *et al.*, 1994; and Ziser *et al.*, 1995).

According to Buchert *et al.* (1994), the structure of various types of hemicelluloses depends on the plant type, and may even vary between different parts of the same plant (Buchert *et al.*, 1994). Due to the complex structure of hemicelluloses, several different enzymes are needed for their enzymatic degradation and modification (Viikari *et al.*, 1993) (Fig. 2.2). There are two different types of side-group-cleaving enzymes. Some of them hydrolyse only short substituted oligomers that have been formed by the backbone-depolymerising enzymes. Other enzymes attack the intact polymeric substrates (Gilbert and Hazlewood, 1993). Microbial enzymes act synergistically to convert xylan to its constituent simple sugars. These enzymes include: (a) endo-1, 4-β-xylanases (EC 3.2.1.8), which cleave internal glycosidic bonds within the xylan backbone; (b) β-xylosidases (β-D-xyloside xylohydrolase) (EC 3.2.1.37), which hydrolyses xylobiose to xylose; (c) α-glucuronidase (EC 3.2.1.139), which removes glucuronic acid side-chains from the xylose units; (d) α-L-arabinofuranosidases (EC 3.2.1.55), which hydrolyses arabinose side-chains; (e) acetylxylan esterases (EC 3.1.1.6), which rupture the ester linkages (esterases) between xylose units of the xylan polymer and acetic acid; and (f) feruloyl (EC number not designated) and ρ-coumaroyl (EC number not designated) esterases hydrolyse between arabinose side-chain residues and phenolic acids. Therefore, side-chains must be cleaved before the xylan backbone can be completely hydrolysed (Sunna *et al.*,

1997). Where endo-xylanase and β-xylosidase are the primary enzymes involved in xylan cleaving, its complete degradation is dependent on the synergistic action of additional enzymes to remove the substituents (Christov and Prior, 1993).



Abbreviations: Ac, acetyl group; Araf, L-arabinofuranose; ρ-Coum, ρ-coumaric acid; Fer, ferulic acid; MeGlcA, 4-O-methyl-D-glucuronic acid; Xyl, xylose.

FIGURE 2.2. A hypothetical plant xylan fragment showing the sites of action of the enzymes involved in its hydrolysis (adapted from Biely, 1993)

2.1.2. Organisms producing hemicellulases

2.1.2.1. Endo-1,4-β-D-xylanase

Numerous bacteria, yeasts and fungi produce these enzymes. Endoxylanases show the highest activity against polymeric xylan, and their rate of hydrolysis normally decreases with a decrease in chain length of the oligomeric substrates (Buchert *et al.*, 1994). Xylobiose, xylotriose, and substituted oligomers of two to four xylosyl residues are the main products formed from the hydrolysis of xylan. However, the length and type of the substituted products depends on the mode of action of the individual xylanases. Also, interaction of xylanases with their substrates depends upon the substitution of the xylan moiety. Substrate attack is not random, and the bonds to be hydrolysed depend on the nature of the substrate; e.g. length and degree of branching of the substrate, or the presence of substituents. It has been reported that at least xylotriose, one of the end products, inhibits the action of xylanases. In addition, in addition to their hydrolytic activity, several xylanases have transferase activity (Sunna *et al.*, 1997).

Endoxylanases are classified into two major families of glycosyl hydrolases; namely 10 (previously F) and 11 (previously G). Xylanases from Family 10 are larger, more complex and produce smaller oligosaccharides, whereas Family 11 xylanases are more specific for xylan. Substrate-binding domains are more commonly found in Family 10 xylanases, than in Family 11 xylanases. Only Family 11 xylanases with substrate-binding domains are those encoded by *Thermomonospora fusca* TfxA and *Streptomyces lividans* XylB genes. One study on glucanase families reported 23 endo-β-1,4-xylanases (EXs) in Family 10 and 17 in Family 11 (Biely *et al.*, 1997), whereas BLAST searches conducted in January 1996 revealed 77 Family 10 and 88 Family 11 xylanases. These differences may reflect published information and sequences deposited in databases. Enzymes belonging to Family 10 exhibit greater catalytic versatility or lower substrate specificity than enzymes of Family 11 (Jeffries, 1996).

The fungal xylanases of *Aspergillus* and *Trichoderma* spp., and bacterial xylanases of *Bacillus* spp., *Streptomyces* spp. and *Clostridium* spp. have been intensively studied (Tables 2.3 and 2.4). The optimum pH for xylan hydrolysis is ca. 5 for most fungal xylanases and they are normally stable between pH 2 and 9. Fungal and bacterial endoxylanases show a strong relationship between their molecular weight (MW) and isoelectric point (pI) values. Those with a low MW generally have a high pI value and are members of Family 11, whereas high MW members have a low pI value and belong to Family 10 (Biely *et al.*, 1997). Most endoxylanases are optimally active at temperature ranges between 45 and 75 °C. However, the purified endoxylanases from various species of

thermophilic bacteria such as *Thermotoga* are optimally active at temperatures between 80 and 105 °C. The above-mentioned tables also summarise a wide variety of other fungal xylanases (Viikari *et al.*, 1993; Sunna *et al.*, 1997).

TABLE 2.3. Physicochemical properties of purified β-1,4-xylanases from bacteria (adapted from Sunna *et al.*, 1997).

Organism	Xylanase	Mol. Wt.[a] (kDa)	pI	Optimal pH	Optimal Temp. (°C)	Hydrolysis products
Bacillus sp. C-125	A	43.0	n.d.	6.0 - 10.0	70	X_2, X_3, X_4, X_n
	N	16.0	n.d.	6.0 - 7.0	70	X_2, X_3, X_4, X_n
Bacillus sp. NCIM 59	I	35.0	4.0	6.0	55 - 60	X_2, X_3, X_4, X_n
	II	15.8	8.0	6.0	50	X_2, X_3, X_4, X_n
Bacillus sp. 41 M-1	J	36.0	5.5	9.0	50	X_2, X_3, X_n
Bacillus sp. XE		22.0	7.8	6.0	75	X_2, X_3, X_4
Bacillus polymyxa CECT 153	$X_{34}C$	34.0	9.3	6.0 - 7.0	45	X_1, X_2, X_3
	$X_{34}E$	34.0	>9.3	4.0 - 6.0	50 - 62	X_1, X_2, X_3
	X_{22}	22.0	9.0	6.0 - 7.0	55	X_2, X_3, X_4
	X_{61}	61.0	4.7	6.5	50	X_2, X_3, X_4
Bacillus pumilus IPO		24.0	n.d.	6.5	45 - 60	X_2, X_3, X_4, X_n
Bacillus stearothermophilus 21		39.5	4.8	7.0	60	X_2, X_3
Bacillus stearothermophilus T-6	T-6	43.0	7.0	6.5	75	X_1, X_2
Baciilus subtilis PAP 115		32.0	n.d.	5.0	50	X_1, X_2, X_3
Cellulomonas fimi	A	13.2	8.5	5.0	45	n.d.
	B	22.0	8.0	6.0	40	n.d.
	C	150.0	4.5	5.5 - 6.5	40	n.d.
Clostridium sp. SAIV	A	30.0	n.d.	5.5 - 6.5	50	n.d.
Clostridium acetobutylicum ATCC 824	A	65.0	4.4	5.0	50	X_2, X_3, X_4, X_5
	B	29.0	8.5	5.5 - 6.0	60	X_2, X_3
Clostridium stercorarium	A	44.0	4.5	5.5 - 7.0	75	X_1, X_2, X_3, X_4
	B	72.0	4.4	5.5 - 7.0	75	X_1, X_2, X_3, X_4
	C	62.0	4.3	5.5 - 7.0	75	X_1, X_2, X_3, X_4
Clostridium stercorarium HX-1	D	53.0	4.5	6.5	75	X_2, X_3
Clostridium thermolacticum TC 21	A	39.0	4.4	6.5	80	X_1, X_2, X_3
	B	55.0	4.5	6.5	80	X_1, X_2, X_3
	C	65.0	4.6	6.5	80	X_1, X_2, X_3
Fibrobacter succinogenes S85	1	53.7	8.9	7.0	39	X_1, X_2, X_3, X_4, X_n, A
	2	66.0	8.0	6.3	55	X_1, X_2, X_3, X_4
Streptomyces sp. T_7		21.8	7.8	4.5 - 5.5	60	X_1, X_2, X_3, X_4, X_5
Streptomyces sp. 3137	X-I	50.0	7.1	5.5 - 6.5	60 - 65	X_1, X_2
	X-II-A	25.0	10.0	5.0 - 6.0	60 - 65	X_1, X_2
	X-II-B	25.0	10.2	5.0 - 6.0	60 - 65	X_1, X_2
Streptomyces sp. EC10	X_{IA}	32.0	6.8	7.0 - 8.0	60	X_1, X_2, X_n
	X_{IB}	22.0	8.9	7.0 - 8.0	60	X_1, X_2, X_n
	X'_{II}	21.0	5.2	7.0 - 8.0	60	X_1, X_2, X_n
Streptomyces sp. A451	I	22.8	8.6	5.6	50	X_2, X_3, X_4, X_5
	II	33.1	8.9	5.4	50	X_2, X_3, X_4, X_5
Streptomyces sp. B-12-2	1a	26.4	7.5	6.0	55	X_2, X_3, X_n
	1b	23.8	8.3	6.0	60	X_2, X_3, X_n
	2	36.2	5.4	7.0	60	X_2, X_3, X_4, X_n
	3	36.2	5.0	7.0	60	X_2, X_3, X_4, X_n
	4	40.5	4.8	6.0	60	X_2, X_3, X_4, X_n
Streptomyces cyaneus MT 813	I	37.5	5.1	8.0	72	n.d.
	II	34.0	5.2	6.5	65	n.d.
Streptomyces thermoviolaceus OPC-520	I	54.0	4.2	7.0	70	X_1, X_2
	II	33.0	8.0	7.0	60	X_1, X_2, X_3
Thermoanaerobacterium sp. JW/SL-YS485		350.0[b]	4.37	6.2	80	n.d.
Thermonospora curvata	1	36.0[b]	4.2	7.8	75	X_2, X_3, X_n
	2	19.0[b]	7.1	7.2	75	X_3, X_4, X_n
	3	15.0[b]	8.4	6.8	75	X_3, X_4, X_5, X_n
Thermotoga sp. Fj SS3-B.1		31.0	n.d.	5.3	105	X_2, X_3
Thermotoga maritima MSB8	XynA	120.0	n.d.	6.2	92	X_2, X_3
	XynB	40.0	5.6	5.4	105	X_2, X_3
Thermotoga thermarum	1	266.0[b]	n.d.	6.0	80	X_2, X_4, X_5, X_n
	2	35.0	n.d.	7.0	90 - 100	X_2

[a]SDS-PAGE; [b]size exclusion chromatography; n.d., not determined; X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; X_n, xylooligosaccharides.

TABLE 2.4. Physicochemical properties of purified β-1,4-xylanases from fungi (adapted from Sunna *et al.*, 1997).

Organism	Xylanase	Mol. Wt.[a] (kDa)	pI	Optimal pH	Optimal Temp. (°C)	Hydrolysis products
Aspergillus awamori CMI 142717	I	39.0	5.7 – 6.7	5.5 - 6.0	55	X_1, X_2, X_3
	II	23.0	3.7	5.0	50	X_2, X_3, A
	III	26.0	3.3 – 3.5	4.0	45 - 50	X_2, X_3
Aspergillus flavipes		45.0	n.d.	5.0	55	X_2, X_3, X_4, X_5
Aspergillus kawachii IFO 4308	A	35.0	n.d.	5.5	60	X_1, X_2, X_3, X_n
	B	26.0	n.d.	4.5	55	X_1, X_2, X_3, X_n
	C	29.0	n.d.	2.0	50	X_1, X_2, X_3, X_n
Aspergillus nidulans CECT 2544	X_{22}	22.0	6.4	5.5	62	X_2, X_3, X_4, X_n
	X_{34}	34.0	3.4	6.0	56	X_1, X_2, X_3, X_n
Aspergillus oryzae D5		46.5	3.6	5.0	55	n.d.
Aureobasidium sp. NRRL-Y-2311-1		20.0	8.5	4.5	45	n.d.
Aureobasidium pullulans Y-2311-1	II	25.0	9.4	4.8	54	X_1, X_2
Bipolaris sorokiniana H83		30.0	9.5	5.5	70	n.d.
Cryptococcus flavus IFO 0407		25.0	10.0	4.5	55	X_2, X_3, X_4, X_n
Fusarium oxysporium f. sp. melonis		80.0	n.d.	5.0	50	n.d.
Gloeophyllum trabeum BAM Ebw 109		39.0	5.0	4.0	80	X_2, X_3
Humicola grisea var. *thermoidea*	I	95.0	n.d.	6.0 - 7.0	60	X_1, X_2, X_3, X_4
	II	13.0	n.d.	7.0	60	X_1, X_2, X_3, X_4
Humicola grisea var. *thermoidea*	2	25.5	n.d.	5.5	70	X_n
Myrothecium verrucaria CMI 45541		15.9	4.3	5.5	45	X_3
Neocallimastix frontalis MCH3	I	45.0	n.d.	6.0	55	X_n
	II	70.0	n.d.	5.5	55	X_n
Neurospora crassa 870	I	33.0	4.5	4.8	50	X_1, X_2, X_3, X_4, X_5, A
	II	30.0	4.8	4.8	50	X_1, X_2, X_3, X_4, X_5, A
Penicillium chrysogenum Q176		35.0	4.2	6.0	40	X_1, X_2
Penicillium purpurogenum	B	23.0	5.9	5.0	50	n.d.
Pichia stipitis CBS 5775		43.0	n.d.	5.0	30	n.d.
Robillarda sp. Y-20	I	17.6	9.5	4.5 - 6.0	50	X_1, X_2, X_3
	II	59.0	3.5	4.5 - 6.0	50	X_1, X_2, X_3
Schizophyllum commune	A	21.0	4.5	5.0	50	X_1, X_2
Schizophyllum radiatum CMI 90347		27.7	n.d.	4.9	55	X_2, X_3, X_4, X_5
Talaromyces byssochlamydoides YH-50	X-a	76.0	4.3	5.5	75	X_1, X_2, X_n, A
	X-b-I	54.0	3.8	4.5	70	X_1, X_2
	X-b-II	45.0	4.0	5.0	70	X_1, X_2
Talaromyces emersonii CBS 814.70	II	74.8	5.3	4.2	78	X_2, X_3, X_4, X_n
	III	54.2	4.2	3.5	67	X_2, X_3, X_4, X_n
Thermoascus aurantiacus C436		32.0	7.1	5.1	80	X_2, X_n
Thielavia terrestris 255B	II	25.7	6.1	4.0	60 - 65	n.d.
Trichoderma harzianum E58	20 kDa	20.0	9.4	5.0	50	X_2, X_n
	22 kDa	22.0	8.5	4.5 - 5.0	45 - 50	n.d.
	29 kDa	29.0	9.5	5.0	60	X_2, X_n
Trichoderma koningii IMI 73022	1	29.0	7.2	4.9 - 5.8	60	X_1, X_2, X_3, A
	2	18.0	7.3	4.9 - 5.5	50	X_2, X_3, X_4
Trichoderma lignorum	A	21.0	5.1	3.5	45	X_1, X_2, X_3, X_n
	B	20.0	8.7	6.5	45	X_1, X_2, X_3, X_n
Trichoderma reesei Rut C30	pI 5.5	19.0	5.5	4.0 - 4.5	n.d.	X_1, X_2, X_3, X_n
	pI 9.0	20.0	9.0	5.0 - 5.5	n.d.	X_1, X_2, X_3, X_n
Trichoderma reesei VTT-D-80133	I	32.0	4.1 – 4.2	4.0 - 5.0	n.d.	X_1, X_2, X_n
	II	23.0	6.4 – 6.5	4.0 - 5.0	n.d.	X_1, X_2, X_n

[a]SDS-PAGE; n.d., not determined; X_1, xylose; X_2, xylobiose; X_3, xylotriose; X_4, xylotetraose; X_5, xylopentaose; X_n, xylooligosaccharides; A, arabinose.

2.1.2.2. 1,4-β-D-Xylosidase

These enzymes are produced by a variety of fungi and bacteria, with molecular weight values ranging from 34 to 240 kDa (Table 2.5). They may be monomeric, dimeric, or trimeric (Coughlan *et al.*, 1993). Many have transferase activity, in addition to direct hydrolase action, and exhibit specificity for both the sugar and the linkage. They generally exhibit little or no action against polymeric xylans. True β-xylosidases cleaves artificial β-xylosides, like ρ-nitrophenyl β-D-xyloside, and unsubstituted β-1,4-linked xylo-oligosaccharides, including xylobiose (Sunna *et al.*, 1997).

TABLE 2.5. Properties of purified bacterial and fungal β-xylosidases (adapted from Eriksson *et al.*, 1990; Sunna *et al.*, 1997).

Organism	Mol. Wt. (kDa)	Subunit (kDa)	Form	pI	Optimum pH
Bacteria					
Bacillus pumilus	130.0	70.0	Dimeric	4.4	
Bacillus stearothermophilus	150.0	75.0	Dimeric	4.2	
Clostridium acetobutylicum	224.0	85.0	Dimeric	5.8	
		63.0			
Thermoanaerobacter ethanolicus	165.0	85.0	Dimeric	4.6	
Thermotoga sp. FjSS3-B.1	174.0	92.0	Dimeric	4.1	
Fungi					
Aspergillus awamori	110.0		Monomeric	4.2	
Aspergillus niger	78.0		Monomeric	n.d.	6.7 – 7.0
Aspergillus oryzae	168.0	82.0	Dimeric	4.1	
Aspergillus pulverulentus	180.0	65.0	Trimeric	4.7	
	190.0	100.0	Dimeric	3.5	
Aureobasidium pullulans	240.0	121.0	Dimeric	<3.0	
Emericalla nidulans	240.0	116.0	Dimeric	3.2	
Neurospora crassa	83.0		Monomeric	4.3	
Penicillium wortmanni	100.0		Monomeric	5.0	
Pichia stipitis	34.0		Monomeric	n.d.	
Talaromyces emersonii	181.0	97.5	Dimeric	8.9	
Trichoderma reesei	100.0		Monomeric	4.7	
Trichoderma viride	101.0		Monomeric	4.4	3.5

The activity towards xylo-oligosaccharides generally decreases rapidly with increasing chain length. The main hydrolysis product of β-xylosidase is xylose, which has been reported to inhibit the action of β-xylosidase in a competitive manner. This may affect the sugar yield in hydrolysis experiments significantly, as β-xylosidase is the key enzyme for the production of monomeric xylose. Although some extracellular β-xylosidase activity is found in the culture fluids of organisms, the enzyme is mainly cell associated in bacteria and yeast (Viikari *et al.*, 1993; Sunna *et al.*, 1997).

2.1.2.3. α-D-Glucuronidase

This is the least studied of the enzymes participating in degradation of xylans; partly due to a lack of a suitable activity assay method. Many fungal hemicellulase preparations lack α-glucuronidase activity; therefore this enzyme was not described until 1986. Most fungi apparently secrete too little α-1,2-glucuronidase for a complete cleavage of the uronic acid side chains that protect the neighbouring β-1,4-linkages of the xylan backbone from cleavage by xylanases and/or xylosidases (Eriksson *et al.*, 1990). The enzyme source mainly determines the substrate specificities of α-glucuronidases. The enzymes from *Streptomyces olivochromogenes* and *Agaricus bisporus* require a low-molecular weight glucuronoxylan substrate and release 4-*O*-methylglucuronic acid from 4-*O*-methyl-glucuronose-substituted xylooligomers, but not from the polymer. The presence of acetyl groups next to the glucuronosyl substituents hinders the action of α-glucuronidase from *A. bisporus*. The enzyme from the ruminal bacterium *Flavobacter succinogenes* is unable to release 4-*O*-methylglucuronic acid from intact xylan, whereas that of *Aspergillus niger* and *Schizophyllum commune* are able to liberate 4-*O*-methylglucuronic acid from methyl-glucuronoxylan. A few microbial α-glucuronidases have been purified and characterised (Table 2.6) (Sunna *et al.*, 1997).

TABLE 2.6. Biochemical properties of some bacterial and fungal α-glucuronidases

Organism	Mol. Wt.[a] (kDa)	pI	Optimum pH	Optimum Temp.(°C)	Reference
Bacteria					
Clostridium stercorarium	72.0, 76.0	4.3	5.5 - 6.5	60	Bronnenmeier *et al.* (1995)
Piromonas communis	103[b]		5.5	50	Wood and Wilson (1995)
Thermoanaerobacterium saccharolyticum	71.0	4.3	5.5 - 6.5	60	Bronnenmeier *et al.* (1995)
Fungi					
Aspergillus tubingensis	107.0	5.2	4.5 - 6.0	70	De Vries *et al.* (1998)
Phanerochaete chrysosporium	112.0	4.6	3.0 - 5.0	50	Castanares *et al.* (1995)
Schizophyllum commune	125.0	3.6	4.5 - 5.5	40	Tenkanen and Siika-aho (2000)
Trichoderma reesei RUT C30	91.0	5.0 - 6.2	4.5 – 6.0	40	Siika-aho *et al.* (1994)

[a]SDS-PAGE; [b]Gel filtration.

2.1.2.4. α-L-Arabinofuranosidase

Only a few of these enzymes have been isolated and characterised. Two types of arabinases occur, the exo-acting α-L-arabinofuranosidase (EC 3.2.1.55), which is active against ρ-nitrophenol- α-L-arabinofuranosidases and on branched arabinans; and the endo-1,5-α-L-arabinase (EC 3.2.1.99), which is active only toward linear arabinans. Exo-acting arabinan-degrading enzymes are the most common enzymes (Viikari *et al.*, 1993). Production of α-arabinosidases is often associated with the production of pectolytic enzymes. Arabinosidase activity has been detected in various plant, fungal

and bacterial sources, but only a few have been isolated and characterised (Table 2.7), partly due to a lack of defined 'natural substrates' (Coughlan *et al.*, 1993). Several rumen bacterial isolates showed high levels of arabinofuranosidase activity when grown in the presence of arabinose or arabinose containing polysaccharides. However, only low arabinofuranosidase levels were detected when the isolates were grown on a substrate containing glucose or cellobiose. Native enzymes are found in mono-, di-, tetra-, hexa-, and octameric forms. The molecular weight values of the native enzymes range from 53 to 495 kDa; pI values range from 3.6 to 9.3, and optimum pH values from 2.5 to 6.9 (Sunna *et al.*, 1997).

TABLE 2.7. Occurrence and properties of α-arabinofuranosidases (adapted from Viikari *et al.*, 1993; Sunna *et al.*, 1997).

Organism	Mol. Wt. (kDa)	Subunit (kDa)	Form	pI	Optimum pH
Bacteria					
Bacillus polymyxa	166.0	65.0	Dimeric	4.7	
		33.0			
Bacillus subtilis	65.0		Monomeric	5.3	
Bacillus stearothermophilus	256.0	64.0	Tetrameric	6.5	
Bacteroides xylanolyticus	364.0	61.0	Hexameric	n.d.	
Butyrivibrio fibrisolvens	240.0	31.0	Octameric	6.0	
Clostridium acetobutylicum	94.0		Monomeric	8.2	
Ruminococcus albus	310.0	75.0	Tetrameric	3.8	6.8
Streptomyces sp. 17-1	92.0		Monomeric	4.4	
Streptomyces diastaticus	38.0		Monomeric	8.8	
	60.0		Monomeric	8.3	
Streptomyces purpurascens	495.0	62.0	Octameric	3.9	
Thermonospora fusca	92.0	46.0	Dimeric	n.d.	
Fungi					
Aspergillus awamori	32.0			n.d.	5.0
Aspergillus niger	53.0		Monomeric	3.6	3.8
Aspergillus niger 5-16	67.0		Monomeric	3.5	
Dichomitus squalens	60.0			5.1	3.5
Phanerochaete chrysosporium	55.0		Monomeric	7.3	
Talaromyces emersonii	210.0	105.0	Dimeric	3.5	
Trichoderma reesei	53.0		Monomeric	7.5	4.0

N.d., not determined.

2.1.2.5. Acetylxylan esterase

Esterases have been localised in plant and animal tissues, and microorganisms. The degradation of acetylated polysaccharides, like pectins and xylans, are achieved by means of acetyl esterases. Acetylxylan esterase production by fungi and bacteria was only reported in 1985, probably due to a lack of suitable substrates to evaluate their activity (Kormelink *et al.*, 1993). Several xylanolytic or cellulolytic fungi, e.g. *Aspergillus* sp., *Trichoderma reesei*, *Rhodoturula mucilaginosa*,

Thermoanaerobacter ethanolicus showed acetyl xylan esterase activity; as well as several bacteria including *Pseudomonas fluorescens*, *Butyrivibrio fibrisolvens*, *Streptomyces* spp., *Thermoanaerobacterium saccharolyticum*, *Caldocellum saccharolyticum*, *Thermonospora fusca*, and *Fibrobacter succinogenes*. Acetylxylan esterases have been purified from *Trichoderma reesei*, *Aspergillus awamori*, *A. oryzae*, *Thermomonospora fusca*, and from the rumen bacterium *Fibrobacter succinogenes* S85 (Shoa and Wiegel, 1995). According to Kormelink *et al.* (1993), a cooperative action between combinations of fungal acetylxylan esterase and endo-xylanase occur in hydrolysing acetyl xylan (Kormelink *et al.*, 1993). It seems however, that a xylanolytic system is not always necessary to initiate the deacetylation of acetyl xylan (Sunna *et al.*, 1997).

Acetylxylan esterases remove the *O*-acetyl substituents at the C2 and C3 positions of xylose residues in acetylxylan. They liberate acetic acid from partially acetylated 4-*O*-methyl-D-glucuronoxylan and this deacetylation makes the xylopyranosyl units of the main xylan chain more accessible to degradation by endo-β-1,4-xylanases (Shao and Wiegel, 1995). Acetylxylan esterase activity increases the rate of hydrolysis of xylan by xylanases, but the extent of the synergy is dependent on the degree of acetylation. The highest specific activity of acetylxylan esterase has been reported in *A. niger*. However, little is known about their physicochemical properties (some are given in Table 2.8). The two monomeric isoenzymes being produced by *T. reesei* are glycosylated and have a molecular weight of 34 kDa (Sunna *et al.*, 1997). Acetylated xylooligosaccharides are the preferred substrate of the *S. commune* enzyme (Biely *et al.*, 1996a, 1996b).

TABLE 2.8. Properties of some purified bacterial and fungal acetyl xylan esterases

Organism	Mol. Wt. (kDa)	pI	Optimum pH	Optimum Temp.(°C)	Reference
Bacteria					
Bacillus pumilus PS 213	40.0	4.8	8.0	55	Degrassi *et al.* (1998)
Fibrobacter succinogenes S85	55.0[a]	4.0[c]	7.0	45	McDermid *et al.* (1990)
Streptomyces lividans	34.0[a]	9.0[d]	7.5	70	Dupont *et al.* (1996)
Thermoanaerobacterium sp.	195.0[b]	4.2[d]	7.0	80	Shao and Wiegel (1995)
strain JW/SL-YS485	106.0[b]	4.3[d]	7.5	84	Shao and Wiegel (1995)
Fungi					
Aspergillus niger	30.48[a]	3.0 – 3.2[d]	5.5 – 6.0	50	Kormelink *et al.* (1993)
Penicillium purpurogenum	(I) 48.0[b]	7.5	5.3	50	Egana *et al.* (1996)
	(II) 23.0[b]	7.8	6.0	60	Egana *et al.* (1996)
Schizophyllum commune	31.0		7.7	30 - 45	Halgasova *et al.* (1994)
Trichoderma reesei RUT C30	34.0[a]	7.0, 6.8[c]	5.0 – 6.0	60 - 65	Christov and Prior (1993)

[a]SDS-PAGE, [B]Gel filtration, [c]Chromatofocusing, [d]Iso-electrofocusing.

2.1.2.6. Cinnamoyl esterase

Cinnamoyl esterases, a specific class of carboxylic ester hydrolases, were first detected by Deobald and Crawford in 1987 (Kroon *et al.*, 1997). Their specificity is defined mainly by recognition of molecular structures adjacent to the ester bond (Williamson *et al.*, 1998). There are two types of cinnamoyl esterases, those acting on 4-hydroxy-3-methoxycinnamic (ferulic) acid (feruloyl esterases), and those acting on 4-hydroxycinnamic (ρ-coumaric) acid (ρ-coumaroyl esterases). They work on widely different substrates, although they catalyse a similar chemical reaction while acting on plant cell walls. They have been detected in the culture supernatants of various microorganisms (Kroon *et al.*, 1997). These esterases have been isolated, purified, and characterised from both bacteria and fungi (Table 2.9).

Phenolic acids influence the biodegradability of cell wall polysaccharides and also restrict forage digestibility by ruminants. This is probably due to the inhibition of ruminal bacteria by phenolic components or the limitation of hydrolysis by phenolic acid substituents in hemicellulose (Tenkanen *et al.*, 1991).

Ferulic acid is esterified with arabinose in the arabinoxylans of wheat bran, wheat flour, sugarcane bagasse, barley straw, and maize. Some other polysaccharides are also ferulated, e.g. in spinach and the pectins in sugar beet. Therefore, ferulic acid may crosslink lignin and carbohydrates and also different carbohydrates in annual plants (Tenkanen, *et al.*, 1991). Cross-linking of the feruloyl groups to form diferulic acid, which covalently cross-links polysaccharide chains, strengthen hemicellulose and thereby restrict enzyme digestibility. Ferulic acid release from plant cell wall polysaccharides by feruloyl esterase is dependent upon the interaction with other carbohydrates, and accessibility of the feruloyl group on the polymer side-chain. The activities of all of the feruloyl esterases tested to date are dramatically increased in the presence of a xylanase (Faulds *et al.*, 1992).

Despite the potential importance of cinnamoyl esterases, definite information on their properties is still very sparse. This is partly due to the relatively few cinnamoyl esterases that have actually been isolated and characterised (McCrae *et al.*, 1994). In addition, it is difficult to obtain suitable substrates to study their enzyme action (Castanares *et al.*, 1992).

TABLE 2.9. Biochemical properties of some cinnamoyl esterase activities

Organism	Enzyme	Mol. Wt.[a] (kDa)	pI	Optimum pH	Optimum Temp. (°C)	Reference
Bacteria						
Clostridium stercorarium	FE	33.0		8.0	65	Donaghy *et al.* (2000)
Streptomyces avermitilis CECT 3339	FE			6.0	50	García *et al.* (1998)
Streptomyces olivochromogenes NRCC 2258	FE	29.0	7.9 - 8.5	5.5	30	Faulds and Williamson (1991)
Streptomyces viridosporus T7A	CE			9.0	45 - 50	Donnelly and Crawford (1988)
Fungi						
Aspergillus awamori IMI 142717	CE	75.0	4.2	5.0	50	McCrae *et al.* (1994)
	FE	112.0	3.7	5.0	50	McCrae *et al.* (1994)
Aspergillus awamori	FE	35.0	3.8			Koseki *et al.* (1998)
Aspergillus niger	FE-I	132.0	3.0			Faulds and Williamson (1993)
	FE-II	29.0	3.6			Faulds and Williamson (1993)
Aspergillus niger CBS 120.49	FE-III	36.0	3.3	5.0	55 - 60	Faulds and Williamson (1994)
Aspergillus niger CS 180	FE/CE	75.8	4.8	6.0	50	Kroon *et al.* (1996)
Aspergillus oryzae VTT-D-85248	FE	30.0	3.6	4.5 - 6.0	45	Tenkanen *et al.* (1991)
Aspergillus sp.	FE	42.0	3.7	4.0 - 6.0	40 - 60	Dzedzyulya *et al.* (1999)
Neocallimastix MC-2	CE	5.8	4.7	7.2	40	Borneman *et al.* (1991)
	FE-I	69.0	4.2	6.2	40	Borneman *et al.* (1992)
	FE-II	24.0	5.7	7.0	40	Borneman *et al.* (1992)
Penicillium expansum	FE/CE	57.5		5.6	37	Donaghy and McKay (1997)
Penicillium pinophilum CMI 87160ii	FE/CE	57.0	4.6	6.0	55	Castanares *et al.* (1992)

[a]SDS-PAGE; CE, ρ-Coumaroyl esterase; FE, Feruloyl esterase; FE/CE, Cinnamoyl esterase.

2.2. CELLULASES

2.2.1. Cellulose structure

Cellulose is the major carbohydrate synthesised by plants and the most abundant biopolymer on earth. It is a linear polymer that consists of glucose subunits linked via β-1,4 bonds and therefore, the basic repeating unit is cellobiose (Fig. 2.3A). Its chain length can vary between 100 and 14 000 glucose residues, which can form bundles or microfibrils where the molecules are orientated in parallel and held together by hydrogen bonds. These microfibrils consist of highly ordered crystalline regions interspersed by more disordered amorphous regions (Fig. 2.3B). Cellulose fibrils present in plant cell walls are mainly embedded in a matrix of hemicellulose and lignin (Béguin and Aubert, 1992; 1994).

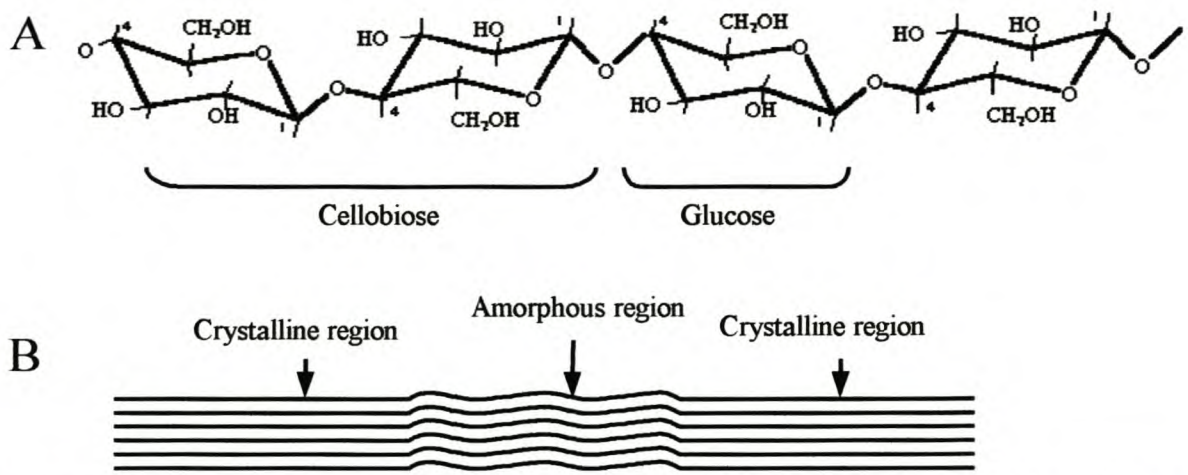


FIGURE 2.3. The basic structure of cellulose. (A) β-glucosidic bonds. (B) Schematic structure of a fibril (adapted from Béguin and Aubert, 1994)

The role of cellulose is exclusively structural. It enables plant cells to withstand osmotic pressure, due to its high tensile strength and is also responsible for plants' resistance to mechanical stress. Native cellulose is also completely insoluble in water (Béguin and Aubert, 1992; 1994).

The main natural agents of cellulose degradation are fungi and bacteria. Plants also synthesise cellulases, which play a role in morphogenesis and developmental processes e.g., in the ripening of fruits. Among the fungi and bacteria, a variety of aerobes and anaerobes, mesophiles and thermophiles have been described that can degrade crystalline cellulose and use it as a carbon and energy source. However, the conversion of cellulosic material into glucose is a process of considerable complexity. Cellulose fibres are embedded in a matrix of hemicellulose and lignin, which

severely restricts the access of cellulolytic enzymes to the substrate. Therefore, most cellulolytic organisms also produce hemicellulases (Bhat and Bhat, 1997; Béguin and Aubert, 1992).

2.2.2. Organisms producing cellulases

2.2.2.1. Endo-glucanase

The recommended name for this specific enzyme (1,4-[1,3:1,4]-β-D-Glucan 4-glucanohydrolase, EC 3.2.1.4) is cellulase, according to International Union of Biochemistry standards, with endoglucanase as an alternative. However, the term cellulase is often applied to the complete complex of cellulolytic enzymes (Clarke, 1997).

Endoglucanases act almost exclusively on the noncrystalline regions of the substrate (Béguin and Aubert, 1992). Endoglucanase activity rapidly decreases the viscosity of cellulose solutions, and is in general confined to amorphous cellulose regions. Exceptions are enzymes from the fungi *Trichoderma viride* and *Trichoderma koningii*, because they can decrease the viscosity of both amorphous Avicel and untreated cotton linters (Clarke, 1997). Table 2.10 lists the physicochemical properties of some isolated and purified endoglucanases, produced by bacteria and fungi. The fungal enzymes range in size between 11 and over 100 kDa, with the majority ranging between 30 and 55 kDa. Bacterial enzymes tend to be a little larger in molecular weight; many with sizes greater than 65 kDa. Fungal enzymes are mostly glycoproteins, with carbohydrate chains linked via both asparagine and serine and threonine residues. Some bacterial enzymes are also glycosylated. The glycan chains apparently provide both protection against proteolytic attack and thermostability; it also aids in the adsorption of the enzymes to insoluble substrates (Clarke, 1997).

2.2.2.2. Cellobiohydrolase

Fungal cellobiohydrolases (1,4-β-D-Glucan cellobiohydrolase, EC 3.2.1.91) have a broad specificity, hydrolysing both crystalline and amorphous cellulose, but generally inactive toward substituted cellulose, such as carboxymethyl cellulose. These enzymes have slightly higher molecular weights than endoglucanases (41 - 85 kDa), but they also have acidic pI values. All characterised fungal enzymes appear to be glycosylated. Table 2.11 summarises the properties of some cellobiohydrolases purified from various bacteria and fungi (Clarke, 1997).

TABLE 2.10. Physicochemical properties of some bacterial and fungal endoglucanases (adapted from Clarke, 1997).

Organism	Mol. Wt. (kDa)	pI	Optimum pH	Optimum Temp. (°C)	Stability pH	Stability Temp (°C)
Bacteria						
Clostridium cellulolyticum	48.0		6.0	48		
Clostridium thermocellum	39.0	6.2	6.0 - 6.6	60		70
	56.0	6.2	6.0	60	5.0 - 7.0	65
	64.0		5.9	60	5.0 - 7.0	
	91.0 - 99.0		6.4	80	5.0 - 7.0	85
	94.0	6.72	5.2	62		
Fibrobacter succinogenes	50.1	4.25	5.9	39 - 44		
	58.0		6.4	30		<50
	65.0	4.75 - 4.9	6.4	39	5.9 - 7.1	<45
	94.0	9.18	5.8	39	5.4 - 6.2	<45
	118.0	9.4				
Pseudomonas fluorescens	40.0		8.0		7.0 - 8.0	
	100.0		8.0		7.0 - 8.0	
			7.0		7.0 - 8.0	
Ruminococcus albus	30.0	6.0 - 6.1				
Thermotoga maritima	27.0		6.0 - 7.5	95		80
Fungi						
Aspergillus aculeatus	25.0	4.8	4.5	50	2.0 - 9.0	45
	38.0	3.4	4.0	65	3.5 - 9.0	65
	66.0	4.0	5.0	70	3.0 - 8.0	70
	68.0	3.5	2.5	60	3.5 - 6.0	50
Aspergillus niger	23.6	4.47	4.8			
	26.0		3.8 - 4.0	45	1.0 - 9.0	
	31.0	3.67	4.0		5.0 - 8.0	70
	46.0	3.3				
Coriolus versicolor	29.5		5.0	55	4.0 - 6.0	55
Dichomitus squalens	42.0	4.8	4.8 - 5.0	55 - 60	4.0 - 8.0	65
	47.0	4.1	4.6 - 4.8	55 - 60	4.0 - 8.0	65
	56.0	4.3	4.8	55 - 60	4.0 - 8.0	65
Humicola insolens	57.0		5.0	50	3.5 - 9.5	65
Humicola grisea	63.0		5.0	50	3.5 - 9.5	65
Irpex lacteus	35.6		4.0 - 5.0	40	3.0 - 6.0	50
Phanerochaete chrysosporium	28.3	4.40				
	32.3	5.32				
	36.7	4.72				
	37.0	4.20				
	37.5	4.65				
Polyporus versicolor	11.4	4.5				
Schizophyllum commune	38.0	3.5	5.5	40	4.0 - 8.0	30 - 70
Sclerotium rolfsii	27.5	4.20	2.8 - 3.0	50	3.0 - 7.0	
	50	4.55	4.0	74	4.0 - 7.0	
	77.6	4.51	4.0	50	4.0 - 7.0	
Thermoascus auranticus	34.0	1.8	4.5 - 5.0	65	5.0 - 9.0	65
	49.0		4.5 - 5.0	68	2.0 - 12.0	65
	78.0		5.0	75	5.0 - 9.0	65
Trichoderma koningii	13.0	4.72				
	31.0	5.09				
	48.0	4.32				
	48.0	4.32				
Trichoderma reesei	20.0	7.5				
	43.0	4.0				
	48.0	5.5				
	55.0	4.5				
	56.0	5.0				
	67.0	6.5				

In contrast to most endoglucanases, soluble carboxymethylcellulose is hydrolysed quite poorly by cellobiohydrolases, probably because hydrolysis cannot proceed beyond substituted residues. Cellobiohydrolases are regarded as essential enzymes for degradation of the native cellulose (Schülein, 1988). They are often the most abundant protein in the culture filtrates of fungi, such as *T. reesei*. Most of the fungal enzyme produced is secreted, but species of *Trichoderma* apparently also produce a conidial bound enzyme (CBHII) (Clarke, 1997).

TABLE 2.11. Properties of some bacterial and fungal cellobiohydrolases (adapted from Clarke, 1997)

Organism	Mol. Wt. (kDa)	pI	Optimum pH	Optimum Temp. (°C)
Bacteria				
Fibrobacter succinogenes	75.0	6.7	6.2	39 - 45
	40.0	4.9	5.9 - 6.2	45 - 50
Clostridium thermocellum	75.0			<50
Ruminococcus albus	200.0		6.8	7.4
Ruminococcus flavefaciens	230.0		5.0	39 - 45
Thermotoga maritima	29.0		6.0 - 7.5	95
Fungi				
Humicola insolens	72.0		5.0	50
Irpex lacteus	56.0		4.0 - 5.0	55
	65.0		5.0	50
Penicillium pinophilum	46.0	4.36	2.5	
	50.7	5.0	4.5	
Sclerotium rolfsii	41.7	4.32	4.5	50
Sporotrichum pulverulentum	48.6	4.3		
Sporotrichum thermophile	63.8	4.52	3.5	80
Trichoderma koningii	62.0	3.8	2.5; 5.0	
Trichoderma reesei	64.0	3.9		
	53.0	5.9		
Trichoderma reesei CBHI	65.0	3.6 - 4.2		
Trichoderma reesei CBHII	85.3	6.3		

2.2.2.3. β-Glucosidase

These enzymes (β-D-Glucoside glucohydrolase, EC 3.2.1.21) bind to cellobiose and soluble cello-oligosaccharides and release glucosyl residues sequentially from the non-reducing end. They not only provide an energy and carbon source in the form of glucose, but also facilitate the efficient hydrolysis of cellulose by clearing cellobiose, a competitive inhibitor of endoglucanase and cellobiohydrolase (Clarke, 1997).

β-Glucosidase is the largest cellulolytic enzyme and can be di- or multimeric. Monomeric enzymes range between 41 and 170 kDa. Bacterial enzymes are mainly monomeric, but still relatively large with molecular weights of between 50 and 122 kDa (Table 2.12). All studied enzymes have

acidic pI values (pH 3.2 - 5.9), except that of *T. reesei*. Most β-glucosidases are glycosylated, except the 39.8 and 47 kDa enzymes from *T. koningii* and *T. viride*, respectively. Optimum pH activity for these enzymes ranges within the acidic region, 1.5 - 6.8; the majority being most active at pH 4 - 5. The optimum enzyme activity is generally found above 50 °C (Clarke, 1997).

Fungal β-glucosidases may be released extracellularly, retained by the cell, or both. *T. reesei, Talaromyces emersonii,* and *Schizophyllum commune* have both types of β-glucosidases. However, bacterial enzymes appear to predominantly be cell-associated, e.g. that of the rumen bacteria *Fibrobacter succinogenes, Ruminococcus albus, Ruminococcus flavefaciens,* and *Clostridium thermocellum* (Clarke, 1997).

TABLE 2.12. Properties of some bacterial and fungal β-glucosidases (adapted from Clarke, 1997)

Organism	Mol. Wt. (kDa)	pI	Optimum pH	Optimum Temp. (°C)
Bacteria				
Alcaligenes faecalis	122.0		6.0 - 7.0	
Clostridium thermocellum	50.0		6.0	65
Ruminococcus albus	82.0		6.5	30 - 35
Streptomyces lividans	66.0			
Fungi				
Aspergillus fumigatus	340.0	4.5	4.5	65
	41.0		5.0	
Aspergillus niger	150.0			
	137.0	3.8		
Aspergillus oryzae	218.0	4.3	4.0 - 5.0	
Aspergillus wentii	170.0		1.5 - 5.0	
Aureobasidium pullulans	340.0		4.5	75
Candida guilliermondii	48.0		6.8	
Humicola insolens	250.0	4.23	5.0	50
Neocallimastix frontalis	153.0	3.9	6.0	50
Saccharomyces cerevisiae	313.0		6.8	
	300.0		6.4 - 6.8	45
Schizophyllum commune	96.0		5.4	52
	110.0		5.4	52
Sclerotium rolfsii	95.0		4.2 - 4.5	68
	95.5		4.2 - 4.5	68
	95.5		4.2 - 4.5	68
Talaromyces emersonii	45.7	3.6	5.7	70
	57.6	4.41 - 4.50		35
	100.0			
	135.0	3.4 - 4.17	4.1	70
Thermoascus aurantiacus	87.0		4.5 - 5.0	70
Trichoderma koningii	39.8	5.53		
	39.8	5.85		
Trichoderma reesei	70.0	8.2	6.0	
	73.0			
	81.6	8.5	4.5 - 5.0	
	98.0		6.5	

2.2.3. Mechanism of cellulase degradation

It is traditionally believed that the hydrolysis of (semi) crystalline cellulose by fungal cellulase systems requires the cooperative action of a range of cellulolytic enzymes. Two types of synergism have been proposed for fungal cellulases. Endo-exo synergism generally functions via a sequential enzymatic action: endoglucanases attack the amorphous regions of cellulose initially; thereby providing new chain ends for cellobiohydrolase (CBH) action. Exo-exo synergism, on the other hand, is based on the formation of a loose complex between the enzymes from *T. reesei* (CBHI and CBHII) in solution; therefore adsorption of the individual components to cellulose will be maximal in optimal synergistic admixtures. However, results in literature are sometimes contradictory and inconclusive; therefore plausible mechanistic concepts for cellulase synergistic action cannot be advanced. There are some significant findings, namely that synergism between cellulases is dependent on: (1) the ratio of individual enzymes, (2) the importance of the degree of substrate saturation, and (3) the influence of the physicochemical properties of the substrate itself (Nidetzsky *et al.*, 1994).

According to the results of Nidetzky *et al.* (1994) obtained for exo-exo synergism, CBH I prepares a more readily hydrolysable substrate for CBH II and vice versa. This indicates that a simultaneous action of both enzymes is not required to observe 'synergism'. It does not however rule out the possibility that CBH I-CBH II complexes may exist, but their formation does not appear to be required for synergistic action on filter paper. For endo-exo synergism a sequential mechanism of enzyme action may apply: endoglucanase (EG) III prepares a more easily hydrolysable substrate for CBH I and CBH II. However, EG III activity is not influenced after substrate pre-treatment with CBHs. EG I act synergistically with CBH I and very little with CBH II, and only shows higher activity on filter paper pre-treated with CBH I and not by CBH II (Nidetzsky *et al.*, 1994).

The filamentous fungus *T. reesei* is probably the most efficient producer of cellulases. It produces the three major types of enzyme, namely: endoglucanases, cellobiohydrolases, and β-glucosidases. Their synergistic action is indicated in Fig. 2.4 (Montenecourt, 1983).

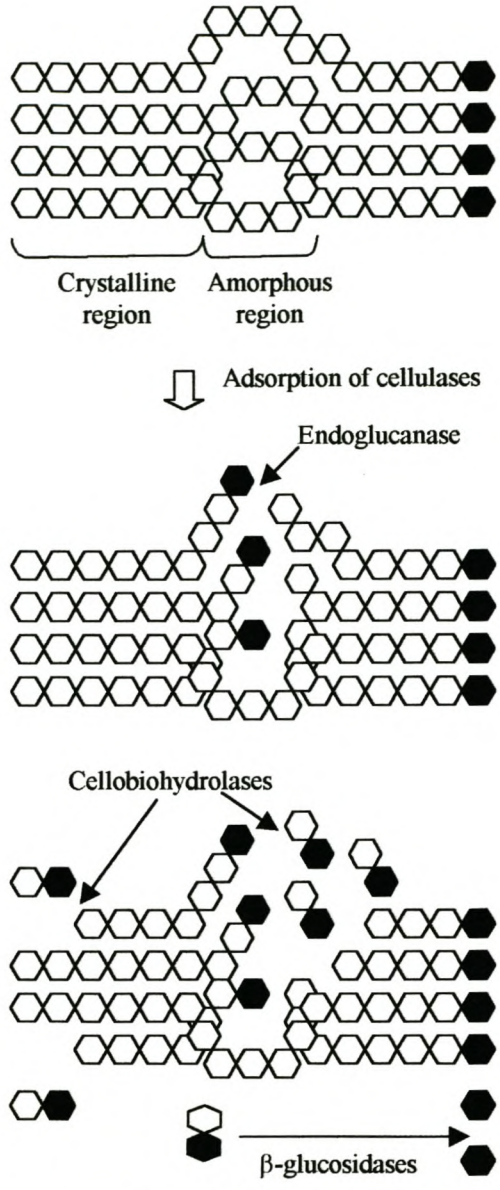


FIGURE 2.4. Schematic representation of the synergistic action of *T. reesei* cellulases. Glucose residues are indicated by hexagons; glucose residues with reducing ends are shown in black (Béguin and Aubert, 1992).

2.3. APPLICATION OF FIBROLYTIC ENZYMES AND MICROORGANISMS IN THE ANIMAL FEED INDUSTRY

2.3.1. Structure of the plant cell wall

Maximised energy gain from forage cell walls is important for high and efficient animal production. Forages of high cell wall content have a low digestibility and thus result in low intake by ruminants. Forage cell wall content can vary in digestibility within the range of 30 to 60 %, and in individual cell types from 0 to 100 %. It is lower in legumes than in grasses, lower in temperate (C3) than tropical (C4) grasses, and lower in young than old material (Wilson, 1994).

The content and digestibility of forage cell walls is significantly influenced by the growth environment; the major factor being temperature. This is mainly due to a decline in cell wall digestibility because of greater lignification and faster tissue maturation, which are greater for grasses than legumes. Water stress, applied at mild to moderate levels, usually results in herbage of lower cell wall content and higher dry matter digestibility (Wilson, 1994).

Growth and development of the cell wall in plants consists of a primary and secondary growth phase. The cell wall is composed of polysaccharides, proteins, and phenolic acids during primary growth (Table 2.13). No lignin is deposited during primary growth, but only pectins, xylans, and cellulose. Ferulic acid and a small amount of ρ-coumaric acid are esterified to the arabinoxylan polymers laid down in the primary wall of grasses. During secondary wall growth the additional polysaccharide material that is deposited, is richer in cellulose than xylans, and pectins are no longer being added to the wall. In addition, ferulic acid is not incorporated into the secondary wall. Secondary wall thickening initiates the deposition of the lignin polymer, in addition to an apparent incorporation of some of the arabinoxylan ferulate esters of the primary wall into cross-linkages of the xylans to lignin. ρ-Coumaric acid is also incorporated into the secondary wall with ether linkages to the lignin polymer, but these ρ-coumarate ether molecules probably do not have the additional cross-linkage to arabinoxylan via an ester bond, as is the case for ferulate ethers. Phenolic acids crosslink lignin to the structural carbohydrates of plant cell walls, and lignin is known to depress cell wall digestibility, presumably by reducing access to the structural carbohydrates by anaerobic bacteria. As lignification of the cell wall proceeds, the lignin that is deposited shifts from guaicyl-type lignin to lignin richer in syringyl units. According to Jung and Allen (1995), all forage species contain phenolic acids in the cell wall (Jung and Allen, 1995). Dry season tropical grasses have a relatively high content of hydroxycinnamic acids covalently bound in the cell wall, which may in part account for their lower digestibility (Jung *et al.*, 1983). Liberation of phenolic acids from the cell wall is unlikely to have an adverse effect on the rumen microbial metabolism (Lowry *et al.*, 1993).

TABLE 2.13. Composition of primary and secondary wall regions of mature lignified cells in grasses and legumes (adapted from Jung and Allen, 1995).

Cell wall region	Wall polymer component			
	Polysaccharides	Lignin	Phenolic acids	Protein
	Grasses			
Middle lamella /primary wall	Cellulose, glucuronarabinoxylans, mixed linkage β-glucans, heteroglucans, pectic polysaccharides (minor)	Guaiacyl (major), syringyl (minor), ρ-hydroxyphenol (middle lamella only)	Ferulic acid esters and ethers, ρ-coumaric acid esters (minor)	Proteins with low or no hydroxyproline, extensin (minor)
	Legumes			
	Pectic polysaccharides, cellulose, heteroglucans, heteroxylans (minor)	Guaiacyl (major), syringyl (minor)	Ferulic acid ethers and esters (minor)[a], ρ-coumaric acid esters (minor)[a]	Extensins, other proteins
	Grasses			
Secondary wall	Cellulose, glucuronarabinoxylans, heteroglucans, mixed linkage β-glucans (minor)	Syringyl (major), guaiacyl (minor)	ρ-Coumaric acid esters and ethers	None
	Legumes			
	Cellulose, 4-O-methyl-glucuronxylans, glucomannans (minor)	Syringyl (major), guaiacyl (minor)	ρ-Coumaric acid esters and ethers (minor)[a]	None

[a]Localisation of phenolic acids in legumes cell walls is assumed based on data from other plant species.

2.3.2. Factors limiting fibre degradation by farm animals

Four major factors regulate ruminant fibre digestion, namely: (1) plant structure and composition, which regulate bacterial access to nutrients; (2) nature of the population densities of the predominant fibre-digesting microbes; (3) microbial factors that control adhesion and hydrolysis of complexes by hydrolytic enzymes of the adherent microbial populations; and (4) animal factors that increase the availability of nutrients through mastication, salivation and digestion kinetics. The presence of silica and tannins in forages present additional layers of recalcitrant material to be penetrated by microorganisms. Bacteria usually access readily digestible inner tissues through stomata, lenticels, or damaged areas, and digestion proceeds from the inside out. Rumen fungi also degrade the more vulnerable areas of plant tissue, but in addition they also have the ability to penetrate the plant cuticle; which aids in reducing the tensile strength of the tissue and thereby provide additional sites of access for bacteria (Varga and Kolver, 1997). Fig. 2.5 illustrates the potential interactions influencing the rate of ruminal fibre digestion and passage.

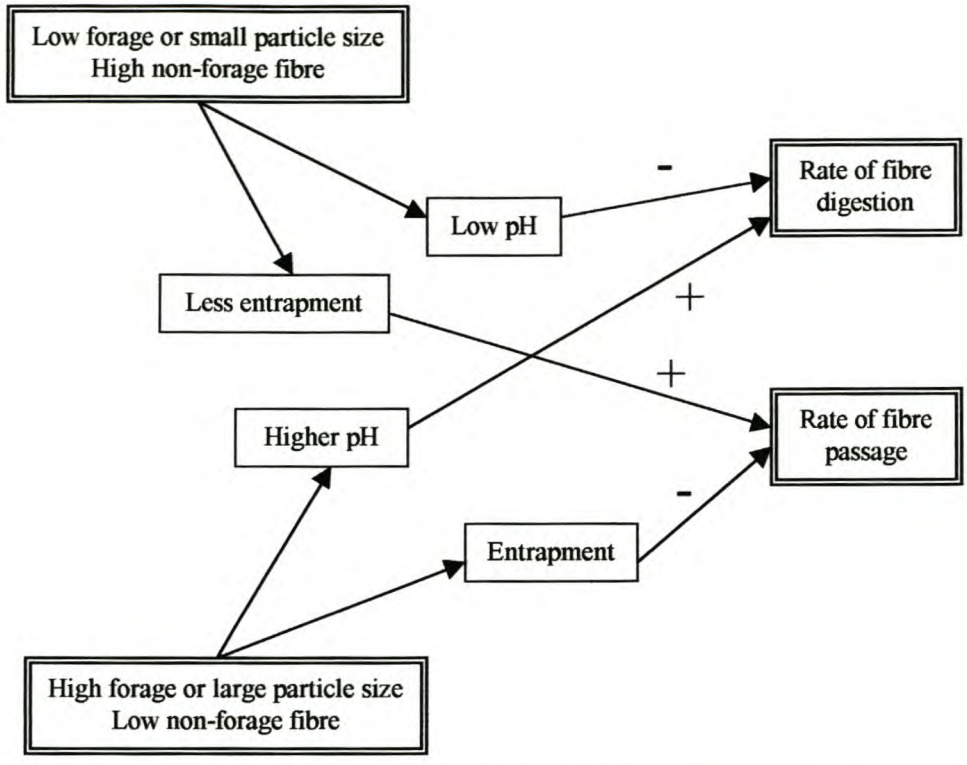


FIGURE 2.5. Potential interactions among forage level and particle size and amount of non-forage fibre on rate of ruminal fibre digestion and passage. The model implies that, when high levels of non-forage fibre are fed instead of forage, the amount of dietary forage is necessarily low; therefore, forage particle size must be adequate to stimulate rumination and entrap small feed particles (adapted from Grant, 1997).

Many feedstuffs contain antinutritional factors that interfere with the digestibility, adsorption, or utilisation of nutrients, and thereby adversely affect animal performance. Antinutritional factors can also be expected to disturb the digestive processes of farm animals, because of similarities in digestive processes, and organisms ingesting them lack the appropriate enzymes in their gastrointestinal tracts to render them ineffective. They work in many ways, from the complexing of important nutrients and mineral ions, to direct enzymatic inhibition (Walsh *et al.*, 1993).

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 a major determinant of energy available. Some fibre cannot be digested no matter how long it remains in the rumen. Lignin interferes with microbial degradation of fibre polysaccharides by acting as a physical barrier and by being cross-linked to polysaccharides by ferulate bridges. Physical and structural barriers may limit fibre digestibility beyond the effect of lignin. Waxes and the cuticle of the epidermis covering plants restrict microbe and enzyme access to forage tissues. Plant anatomy

at the cellular level also influences fibre digestibility, and cell types differ in digestibility in general (Table 2.14 and Fig. 2.6) (Buxton and Redfearn, 1997).

TABLE 2.14. Summary of plant tissues and their relative digestibility (Buxton and Redfearn, 1997).

Tissue	Function	Digestibility	Comments
Mesophyll	Contain chloroplasts	High	Thin wall, no lignin. Loosely arranged in legumes and C_3 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 chloroplasts	Moderate to high	Surrounds vascular tissue in C_4 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, 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.

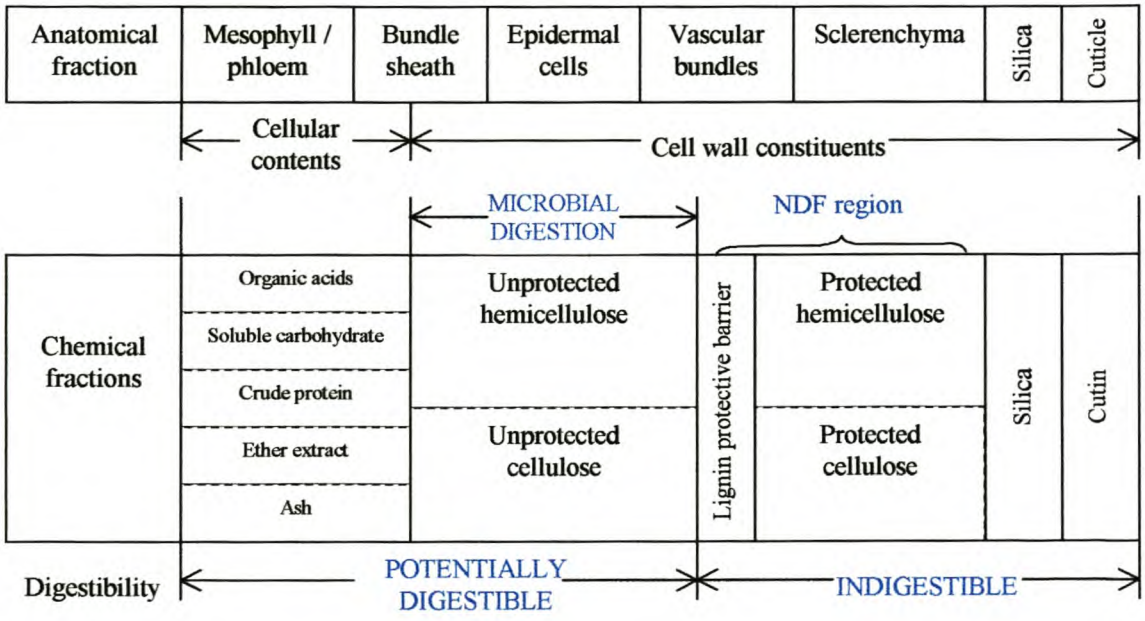


FIGURE 2.6. Conceptual model of the relation between plant anatomy and chemical fractions indicating areas of potential digestibility; NDF ~ neutral detergent fibre (adapted from Minson, 1990)

Intake, dietary interactions, feeding strategies and feed additives influence microbial growth and subsequent fibre digestion. The extent of fibre digestion is the result of competition between the rates of digestion and passage. Rumen available energy normally limits growth of bacteria and any additional organic matter fermented in the rumen. Fibre digestion depends on the various rates of digestion of structural and non-structural carbohydrate. Ferulic and ρ-coumaric acid limit the biodegradability of plant fibre. Fibre digestibility is reduced by 3.3% as a result of reduced residency time in the rumen. The order and frequency of substrate presentation to the rumen may limit fibre digestion (Varga and Kolver, 1997).

Other limitations to fibre digestion in diets include: (1) a high proportion of low pH silages; (2) fermented feeds with a moisture content greater than 50%; an acid detergent fibre less than 19%; (3) a high proportion of feed concentrate; (4) irregular feeding of high levels of feed concentrate; and (5) finely ground feed concentrate. Feed particles are generally retained in the rumen two to three times longer than the fluid, depending on size, density, and susceptibility to digestion. Prolonged residence within the rumen is necessary for slowly growing organisms like ruminal fungi and protozoa, because they are rapidly depleted if unable to attach to feed particles (Varga and Kolver, 1997). According to McAllister *et al.* (1994), most feeds contain a surface layer resistant to microbial attachment and therefore to digestion. Maturity of the microbial consortium and adaptation to a particular type of feed leads to inherent stability, and its participant microorganisms are notoriously difficult to manipulate due to the impenetrable nature of biofilms (McAllister *et al.*, 1994). *In vitro* studies showed that phenolic acid concentrations above 1 mM inhibited growth of many species of ruminal bacteria. It was found that phenolic monomers (5 mM) are toxic to rumen microbes *in vitro* and interfered with the attachment of *Fibrobacter succinogenes* to cellulose. However, the free phenolics concentration in ruminal fluid is generally low (0.15 – 5.15 μM) (Akin *et al.*, 1993).

2.3.3. Enhancement of fibre digestibility by farm animals

Fibre digestibility enhancement is dependent upon advances in a number of related areas, a few of which is summarised in Table 2.15.

TABLE 2.15. Research areas that could lead to the enhancement of fibre digestion (Varga and Kolver, 1997).

Microbial	Animal
Attachment to the substrate	Feeding strategies
Ruminal pH (5.9 – 6.5)	
Increase interaction with fungi	Increase frequency of feeding
Increase substrate accessibility	Evaluate timing of feeding
Identify optimal particle size	Evaluate order of substrate presentation
Increase substrate availability	Evaluate direct use of enzymes
Evaluate microbial growth phase	Increase residence time of substrate
Identify microbial growth factors	Determine optimal carbohydrate availability in the rumen
Increase rate of hydration of substrate	Increase mastication and insalivation
Decrease antiquality factors (tannin, silica)	
Evaluate importance of spirochetes	
Determine serotypes of binding proteins	
Penetration of substrate	For poor and good quality forages
Increase interaction with fungi	Evaluate interaction with non-fibre components
Determine enzyme induction mechanism	Evaluate use of biological and chemical treatments
Determine surface area exposure necessary	Determine interaction with protein
Evaluate plant structure and composition	Determine hydration potential
Evaluate importance of protozoa	Determine cationic exchange capacity
Rate of digestion	Determine optimal particle size
Elucidate enzymatic activity of consortium	
Identify microbial synergistic and/or competitive interactions	
Decrease phenolic concentration of plants	
Decrease generation time of organisms	
Determine importance of catabolic and/or regulatory enzyme expression	
Determine effect of non-fibre components	
Select for bacteria that attack refractory portions of the cell wall	

To improve the dry matter digestibility, changes must occur in both the fibre concentration and the fibre digestibility. According to Buxton and Redfearn (1997), the most logical way of improving forage digestibility would be to reduce fibre concentration.

2.3.4. Microorganisms involved in lignocellulose degradation by farm animals

Rumen bacteria consist of a range of strict anaerobes and facultative anaerobes and include *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Ruminococcus albus*, and *Butyrivibrio fibrisolvens* as the most important species (Table 2.16). They tend to degrade the more readily digestible structures like mesophyll cells. Fungi account for almost 8% of the microbial biomass in the rumen and primarily degrade unlignified cell walls in leaf blades and stems. They are able to penetrate both cuticle and cell wall of lignified tissues. They also can degrade more recalcitrant cell wall materials like the sclerenchyma and vascular tissue. Their fibrolytic activity is enhanced by hydrogen-utilising methanogens, which decrease the repressive effect of hydrogen (Czerkawski, 1986). There are 3 groups of fungi in the rumen of herbivores: (1) facultatively anaerobic and aerobic fungi, described as transient because they continually enter the rumen in feed; (2) two species that parasitise some ciliate protozoa; (3) obligatory anaerobic zoosporic fungi that are saprophytic on plant material (Wubah *et al.*, 1993: Borneman *et al.*, 1990).

Protozoa are responsible for 19 – 28% of the total cellulase activity. They may be limited to digestion of very susceptible tissue like mesophyll cells. There is an increased requirement for non-protein nitrogen in the absence of protozoa (defaunation), due to an increase in the bacterial population. The relationships between protozoa and other microorganisms in the rumen are characterised by competition for nutrients and possibly for space, predation, and synergism. Defaunation of the rumen leads to an increase in amylolytic, pectolytic, and cellulolytic bacteria. It appears that, when the hydrogen concentration is reduced, methanogens adhere to the protozoa to be able to use the hydrogen produced by protozoa via the direct transfer of hydrogen. Protozoal metabolic activity may be negatively affected by the accumulation of hydrogen, whereas its elimination by methanogens probably stimulates protozoal metabolism (Ushida *et al.*, 1991; Varga and Kolver, 1997).

TABLE 2.16. Selection of rumen bacteria, protozoa and fungi and their function (Czerkawski, 1986).

Bacteria	
Fibre digesters	*Bacteroides succinogenes*
	Clostridium lochheadii
	Ruminococcus flavefaciens
	Ruminococcus albus
Starch digesters	*Bacteroides amylophilus*
	Succinomonas amylolytica
	Butyrivibrio fibrisolvens
	Bacteroides ruminocola
	Selenomonas ruminantium
	Streptococcus bovis
Acid utilisers (mainly lactate)	*Veillonella alcalescens*
	Peptostreptococcus elsdenii
	Selenomonas lactolytica
Protozoa	
Sugar utilisers	*Isotricha prostoma*
	Dasytricha ruminantium
Particle ingesters (including bacteria)	*Epidinium ecaudatum*
	Diplodinium deutatum
	Ophryoscoles purkynei
	Entodinium caudatum
Fungi	
Fibre digesters	*Neocallimastrix frontalis*
	Spheromonas communis
	Piromyces communis

2.3.5. Addition of microorganisms to animal feeds to improve digestibility

The survival of adult ruminants and other herbivores fed diets of high fibre content depend on microorganisms. Maximisation of utilisation of forage by the animal depends on maximising the rate and extent of fibre digestion in the rumen, as both voluntary intake and digestibility depend on this (Offer, 1990). The rumen must be considered as an integrated system and therefore it is difficult to rationalise manipulation. Thus, the observed result of any treatment is a combination of several interactive reactions and therefore any change to one component of the system has several uncontrolled effects on other components (Wallace, 1994). The addition of microbial feed additives (direct-fed microbials) is currently one of the present methods for manipulating ruminal fermentation. Others include dietary ionophores, antibiotics, and addition of large amounts of certain natural substrates (Jouany, 1994).

Yeast cultures are microbial feed supplements that contain both viable yeast cells and a dried preparation of the medium in which these cells were grown, with value as a rumen microbial enhancer (Dawson, 1993). The culture benefits the nutrition of the animal and the efficiency of meat and milk production by stimulating the growth of rumen bacteria (Wallace and Newbold, 1993). The ability of yeast to stimulate the viable count in the rumen depends on its respiratory activity. It appears that yeast removes some of the oxygen that occurs in ruminal fluid at various times during the daily feed cycle, thereby preventing toxicity to the ruminal anaerobes (Fig. 2.7) (Wallace, 1994).

Aspergillus oryzae fermentation extract (Amaferm) and *Saccharomyces cerevisiae* are the most common cultures been fed to animals to promote desired responses like increased weight gain, milk production, or total tract digestibility of feed components. These saccharolytic microbes' main metabolic action in aerobic condition entails the use of sugars and oligosaccharides to produce CO_2 and ethanol. They increase the total viable bacteria and cellulolytic bacteria numbers, with no clear effect on protozoa and fungi. This partially explains the improvement in fibre breakdown and increased stability of the fermentation. Apparently, the microbial additives containing both components might have a broader spectrum of efficacy than preparations containing single organisms. However, the effectiveness of *S. cerevisiae* and *A. oryzae* seems to be affected by the diet and nutritional demands of the host (Varel *et al.*, 1993; Jouany, 1994; Wallace, 1994; Wallace and Newbold, 1993; Newbold, 1995).

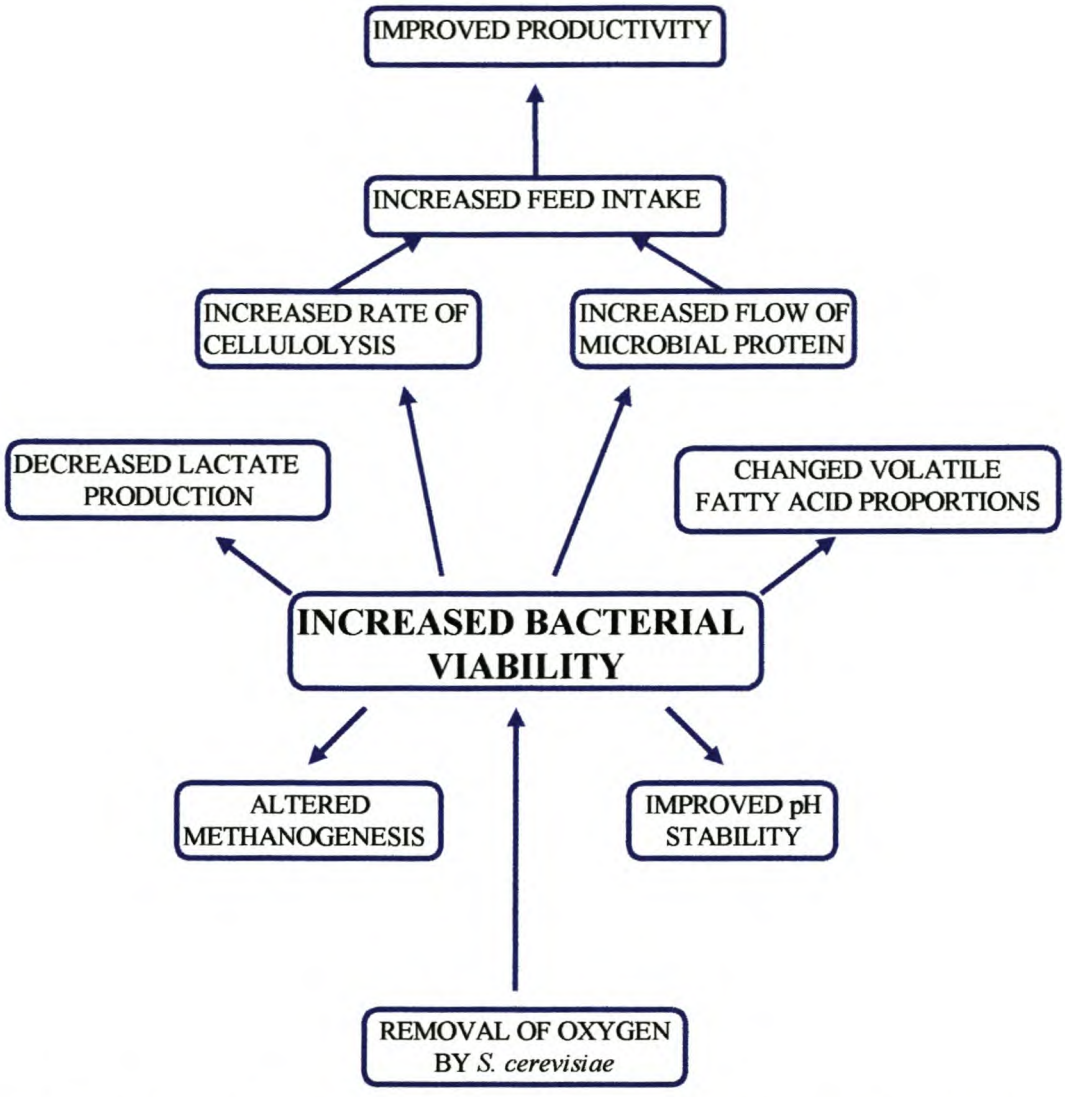


FIGURE 2.7. A scheme describing the mode of action of yeast culture (Wallace, 1994).

Aspergillus spp. produces a wide variety of polisaccharidase enzymes (cellulases, hemicellulases), which could influence plant cell wall degradation (Varel *et al.*, 1993). When the aerobic fungus *A. oryzae* is exposed to an anaerobic environment, it may simply lyse and release enzymes, which enhances the rate of plant cell wall breakdown, but not necessarily the extent of breakdown (Martin and Nisbet, 1992). Supplementation with *A. oryzae* increased ruminal and total tract digestibility of fibre fractions, but ruminal volatile fatty acid and NH_3 production was not affected. Some *A. oryzae* strains produce substances with a wide range of antibacterial activities, but none of the ruminal bacteria was negatively affected (decreased growth rate). Most ruminal bacteria showed no response to *A. oryzae* supplementation; however, some species grew faster when *A. oryzae* was supplemented. Table 2.17 summarises the effect of *A. oryzae* supplementation on growth rate of ruminal bacteria. Some bacteria exhibit higher growth rates in medium containing *A. oryzae* than in medium without it.

Responses between the 2 and 5% (0.2 and 0.5 mg/ml, respectively) concentrations of *A. oryzae* filtrate did not differ (Beharka and Nagaraja, 1998).

TABLE 2.17. Effect of *Aspergillus oryzae* fermentation extract on specific growth rate of ruminal bacteria (adapted from Beharka and Nagaraja, 1998).

Organism	Control Growth rate (/h)	2% AO Filtrate[1] Growth rate (/h)	5% AO Filtrate Growth rate (/h)
Fibrobacter succinogenes S85	0.26[b]	0.35[a]	0.36[a]
Megasphaera elsdenii B159	0.32[b]	0.43[a]	0.42[a]
M. elsdenii T81	0.30[b]	0.40[a]	0.42[a]
M. elsdenii LC1	0.29[d]	0.32[c]	0.39[c]
Ruminococcus albus 7	0.58[b]	0.72[a]	0.69[a]
Selenomonas ruminantium D	0.59[b]	0.71[a]	0.72[a]
S. ruminantium HD1	0.65[b]	0.75[a]	0.75[a]
S. ruminantium HD4	0.62[b]	0.74[a]	0.72[a]
S. ruminantium GA31	0.50[a]	0.54[a]	0.55[a]
S. lactilytica PC18	0.57[b]	0.72[a]	0.74[a]

[a,b] Means within a row with different superscripts differ ($P < 0.01$).
[c,d] Means within a row with different superscripts differ ($P < 0.05$).
[1] A sterile filtrate of 10% *Aspergillus oryzae* (AO) was added to the medium at 2 or 5% (vol/vol), providing a final AO concentration of 2 or 5 mg/ml, respectively.

Microbial feed additives can also be applied in the pre-ruminant animal, thereby manipulating the gut fermentation to reduce diarrhoea and to enhance the development of the active, fibre digesting flora and fauna of the adult ruminant. This also helps to accelerate the transition from liquid to solid feed. Therefore, microbial additives must perform part of the function of probiotics for non-ruminants and should have an additional effect specific to the ruminant. Table 2.18 summarises the general effectiveness of different microbial additives in pre-ruminants (Wallace and Newbold, 1993).

TABLE 2.18. Effects of microbial feed additives in pre-ruminants (Wallace and Newbold, 1993).

Microbial species	Animal	Observed effects
Lactobacillus spp.	Calves	Decreased coliform count
		Reduced scouring
		Improved feed intake / liveweight gain
	Lambs	Lower mortality
		Improved feed intake / liveweight gain
Saccharomyces cerevisiae	Calves	Improved feed intake / liveweight gain
	Lambs	Improved feed intake / liveweight gain
	Calves	Decreased effects of transport stress
Aspergillus oryzae	Calves	Improved feed intake / liveweight gain

2.3.6. Enzymes involved in lignocellulose degradation by farm animals

Enzymes have several distinct advantages relative to chemical and physical processing methods, namely that they:

1) are of natural origin and non-toxic
2) have specific activities
3) work best under mild conditions; i.e. moderate temperatures and broad pH range
4) act rapidly at relatively low concentrations
5) are easily inactivated (Sears and Walsh, 1993).

Rumen fungi produce a wide range of enzymes that can digest the major structural polysaccharides of plant cell walls, hydrolyse a range of glycosidic linkages, and thereby enabling fungi to grow on a number of polysaccharides. Many of the polysaccharide-hydrolysing enzymes are excreted into the extracellular culture medium. It seems that all species studied to date utilise xylan, starch, and hemicelluloses. Cellulases necessary for solubilising both amorphous and highly ordered celluloses present in plant fibre are produced by anaerobic fungi, e.g. avicelase, endoglucanase, and β-glucosidase. Extracellular exoglucanase(s), endoglucanase(s), and cellodextrinase(s) combined makes fungi capable of degrading cellulose into cellobiose. Rumen fungi also produce high levels of hemicellulases; for example, xylanase is the most active of all endo-acting polysaccharide hydrolases. Other enzymes include acetyl xylan esterase, and ρ-coumaroyl and feruloyl esterases (Wubah *et al.*, 1993).

2.3.7. Addition of enzymes to animal feeds to improve digestibility

Supplementation of cattle feed with enzymes containing amylolytic, proteolytic, and cellulolytic activities showed significant improvements in average daily gain and feed conversion ratio. However, exogenous enzymes can only be beneficial when the feed composition and the enzyme preparation are complementary (Beauchemin and Rode, 1996). Maximal effects are obtained when the enzyme additives are applied to the feed in an aqueous form. An increase in the interval between the enzyme application to feed and feeding creates a stable enzyme-substrate complex that increases enzyme effectiveness. Enzyme application to the feed before feeding is more beneficial than direct application into the ruminal environment (Rode and Beauchemin, 1998).

The goals of enzyme supplementation to animal diets would be to: (1) stimulate dry matter intake; (2) maintain rumen pH; (3) increase rumen microbial synthesis of proteins or volatile fatty acids; (4) improve fibre digestion in the rumen; (5) stabilise the rumen environment; (6) improve

animal growth; (7) minimise weight loss; (8) improve animal health; and (9) maintain the immune system and response (Hutjens, 1998).

Feed digestion rate and the absorption of the products of digestion is dependent on the formation of a complex between the digestive enzyme and its substrate, and subsequent release of its product. Fibre digestion is a complex process that is not only affected by the forage, but also by the retention time of forage particles in different segments of the gastrointestinal tract and the amount and activity of enzymes secreted by fibrolytic organisms in the rumen and large intestine (Bedford, 1995). Forage fibre consists of complex carbohydrates that include cellulose, hemicellulose, pectins, and lignin. The complete fermentation of cellulose and hemicellulose are limited by the degree of lignification; therefore they are incompletely fermented to volatile fatty acids, unlike pectin that is completely fermented to volatile fatty acids (Allen and Oba, 1998).

The use of crude enzyme preparations enhances feed digestibility by:

* hydrolysing raw materials (fibre) not usually degraded by natural enzymes;
* hydrolysing raw materials with specific anti-nutritional properties, such as β-glucans, thereby enhancing the nutritive value of poor quality feeds;
* enhancing bio-availability of polysaccharides (starch) and proteins;
* hydrolysing phytate in the intestine; and
* complementing the enzymes in young animals to enhance digestive systems not fully developed at weaning (Teller and Vanbelle, 1990).

Exogenous enzymes may simply supplement those enzymes already present in the digestive tract to a more effective level, or provide hydrolytic capacity that is completely absent (Bedford and Schulze, 1998). According to Howes *et al.* (1998), supplemental fibrolytic enzymes may act by initiating degradation of plant structural polysaccharides prior to ingestion and ruminal digestion, or by complementing the fibrolytic enzymes produced by ruminal microorganisms. *In vitro* studies showed that Fibrozyme, an enzyme preparation from the fermentation extracts of *Aspergillus niger* and *Trichoderma longibrachiatum*, enhanced the digestion of particulate material and carbohydrate metabolism in a 100% grass hay diet by 44% during a 12 hour incubation period. However, it had little consistent long-term effects on digestion over longer periods (Howes *et al.*, 1998). It has not yet been determined whether the major benefit of enzyme application occurs in pre-feeding treatment or after the feed enters the rumen. However, it appears that when enzymes are added directly to grass and silages, they have a definite benefit in improving digestibility of forages. The treatment of feed or forage way in advance of feeding allows the potential for increased rumen enzymatic activity.

In general, it would appear that the means of delivering the enzyme into the rumen and the time allowed between treatment and feeding may be crucial (Lewis *et al.*, 1996). It is important to consider the combined effect of enzyme type, enzyme level, and forage moisture condition when forage is treated with enzymes. When fibrolytic enzymes are added to dry grass immediately before feeding, *in vivo* data indicate improved intake, digestibility, particulate passage, and ruminal degradability (Feng *et al.*, 1996).

CHAPTER 3

METHODS

3. METHODS

3.1. MICROORGANISMS AND ENZYME PREPARATIONS

Aureobasidium pullulans NRRL Y2311-1, *Aspergillus aculeatus* DSM 2344, *Trichoderma reesei* Rut C30, and *Thermomyces lanuginosus* ATCC 34626 served as microbial sources for enzyme production. Fibrozyme, an enzyme supplement for ruminants contains fermentation extracts from *Aspergillus niger* and *Trichoderma longibrachiatum* (Alltech, USA), was used as the one commercial enzyme preparation. Celluclast 1.5 L (Novo Nordisk, Denmark), a liquid cellulase preparation made via submerged fermentation of a selected strain of *Trichoderma reesei*, served as the other commercial enzyme preparation. All enzyme preparations were held at 4 °C until use in experiments.

3.2. MEDIA AND CULTIVATION CONDITIONS

All the organisms were routinely maintained by cultivation at 30 or 50 °C on YMX slants containing (g/l): xylose, 10; yeast extract, 3; malt extract, 3; peptone, 5; agar, 15 (Christov *et al.*, 1997).

Growth media for *A. pullulans* and *A. aculeatus* consisted of (g/l): Birchwood xylan (Roth), 20; Yeast nitrogen base (YNB), 6.7; Asparagine, 2.0; KH_2PO_4, 5.0 (O'Neill *et al.*, 1996). Growth media for *T. reesei* consisted of (g/l): Cellulose (Solka floc), 10.0; Bacto-peptone, 0.735; $(NH_4)_2SO_4$, 6.66; Urea, 1.5; $CaCl_2$, 1.5; $MgSO_4$, 1.5; Citric acid, 5.84; K_2HPO_4, 7.71; Trace elements, 10 ml. Trace elements consisted of (mg/l): $FeSO_4 \cdot 7H_2O$, 5.0; $MnSO_4 \cdot H_2O$, 1.56; $ZnSO_4 \cdot 7H_2O$, 1.40; $CoCl_2$, 2.0 (Biely *et al.*, 1988; Mandels and Weber, 1968). Growth media for *T. lanuginosus* consisted of (g/l): Birchwood xylan (Roth), 20; Yeast extract, 14.3; $(NH_4)_2SO_4$, 2.1; $MgSO_4$, 3.0; $CaCl_2 \cdot 2H_2O$, 0.3; KH_2PO_4, 10.0 (Bennett *et al.*, 1998). All the above organisms were also grown on media containing (g/l): wheat straw, 20; Yeast nitrogen base (YNB), 1.7; and $(NH_4)_2SO_4$, 5.0. All organisms were cultivated in liquid media at 30 or 50 °C, with agitation at 150 rpm for 5 days.

3.3. ENZYME RECOVERY

The enzymes were recovered from the spent medium by centrifugation (10 000 x g for 20 minutes at 4 °C). The supernatant was subjected to ultrafiltration (10 000 MW cut-off; Millipore, Bedford, MA, USA) with a Minitan apparatus and concentrated up to 30-fold by using a high-pressure ultrafiltrate-membrane apparatus (10 000 MW cut-off; Amicon, Beverly, MA, USA). Finally 0.02 % (w/v) of sodium azide was added to prevent microbial growth.

The commercial enzyme product, Fibrozyme (Alltech, USA) was dissolved in distilled water at a final concentration of 0.2 mg/ml dry weight, thoroughly mixed, and the enzymes extracted via vortexing in the presence of 0.5 mm glass beads (20 minutes). The mixture was centrifuged at 10 000 rpm for 10 minutes, and the supernatant harvested and stored at 4 °C until further use.

3.4. ANALYSIS OF ENZYME PROPERTIES

3.4.1. Enzyme assays

3.4.1.1. Protein assay

The protein concentration was determined using the Coomassie Brilliant Blue dye-binding method as described (Bio-Rad; Bradford, 1976).

3.4.1.2. Xylanase assay

β-Xylanase activity was determined by following the release of reducing sugars from a 1.0 % birchwood xylan (Roth) solution at 50 °C for 5 minutes (Bailey *et al.*, 1992). The reaction was terminated by addition of dinitrosalicylic acid (DNS) reagent and subsequently boiled for 5 minutes. The reducing sugar concentration was determined spectrophotometrically at 540 nm. One unit (IU) of activity was defined as the amount of enzyme that released 1 µmol of xylose as reducing sugar equivalents per minute.

3.4.1.3. Total cellulase assay

Total cellulase activity was determined by measuring the release of reducing sugars from Whatman no. 1 filter paper at 50 °C for 60 minutes (Wood and Bhat, 1988). The reaction was stopped by addition of DNS reagent and subsequent boiling for 5 minutes. The release of reducing sugars from

filter paper was measured spectrophotometrically at 540 nm. One unit (IU) of activity was defined as the amount of enzyme that released 1 µmol of glucose as reducing sugar equivalents per minute.

3.4.1.4. Cellobiase assay

Cellobiase (β-glucosidase) activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucoside at 50 °C for 30 minutes (Wood and Bhat, 1988). The reaction was terminated via addition of 0.4 M glycine-NaOH (pH 10.8) buffer. The liberated *p*-nitrophenol was measured at 430 nm. One unit (IU) of activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol as reducing sugar equivalents per minute.

3.4.1.5. CMC assay for endo-glucanase

Endoglucanase activity was determined by measuring the release of reducing sugars from carboxymethyl cellulose (CMC) at 50 °C for 30 minutes (Wood and Bhat, 1988). The reaction was terminated with DNS reagent and subsequent boiling for 5 minutes. The release of reducing sugars from CMC was determined spectrophotometrically at 540 nm. One unit (IU) of activity was defined as the amount of enzyme that released 1 µmol of glucose as reducing sugar equivalents per minute.

3.4.1.6. Acetyl esterase assay

Acetyl esterase activity was determined by measuring the release of *p*-nitrophenol from 2 mM *p*-nitrophenyl acetate at room temperature over a 10 minute period (Biely *et al.*, 1996a). The absorbance of *p*-nitrophenol was measured spectrophotometrically at 410 nm. One unit (IU) of acetyl esterase activity hydrolyses 1 µmol of the substrate in 1 minute.

3.4.1.7. β-Xylosidase assay

β-Xylosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-xylopyranoside (5 mM in 0.1 M sodium acetate buffer, pH 5.0) substrate at 37°C for 10 minutes. The reaction was stopped by addition of saturated sodium borate (Borax) solution. Released *p*-nitrophenol was quantified spectrophotometrically at 410 nm. One unit (IU) of activity was defined as the amount of enzyme that released one µmol of *p*-nitrophenol per minute.

3.4.1.8. α-L-Arabinofuranosidase assay

α-L-Arabinofuranosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-arabinofuranoside (5 mM in 0.1 M sodium acetate buffer, pH 5.0) substrate at 37°C for 10 minutes. The reaction was stopped by addition of saturated sodium borate (Borax)

solution. Released *p*-nitrophenol was quantified spectrophotometrically at 410 nm. One unit (IU) of activity was defined as the amount of enzyme that released one µmol of *p*-nitrophenol per minute.

3.4.1.9. Pectinase assay

Pectinase activity was determined on plates as described by Hagerman *et al.* (1985). The pectin medium contained (w/v): 0.1 % pectin, 0.1 % maltose, 0.1 % KNO_3 and 0.5 % agarose in buffer (0.05 M Na_2HPO_4; 0.01 M citric acid, pH 6.3). Enzyme (10 µl) was spotted onto the plate and incubated at 30 °C for 4 hours (if necessary plates were stored at 4 °C for up to 15 hours before staining for activity). Afterwards plates were stained with freshly prepared 0.02 % (w/v) Ruthenium red in distilled water and then refrigerated for ca. 2 hours. The staining solution was discarded and plates destained for 15 minutes in distilled water.

3.4.1.10. Laccase assay

Laccase activity was determined on plates as described by Buswell *et al.* (1995). The laccase medium contained (w/v): 0.15 % 2,2'-azino-*bis*-ethylbenthiazoline (ABTS) and 0.5 % agarose in 0.1 M sodium acetate buffer (pH 5.0). Enzyme (10 µl) was spotted onto the plates and incubated at 30 °C for ca. 24 hours.

3.4.2. Gel electrophoresis

SDS-PAGE (denaturing) was done according to the method of Laemmli (1970) with 12 % polyacrylamide gels. Gels were stained with 0.25 % Coomassie Brilliant Blue R250 (Sigma) in 45 % methanol / ethanol and 10 % glacial acetic acid. Excess dye was removed with a solution containing 25 % methanol / ethanol and 10 % glacial acetic acid. The various bands were identified by relating their position on the gel with a standard marker preparation (Full range rainbow marker, RPN 800) from Amersham. These consisted of the following molecular weights (kDa): 250, 160, 105, 75, 50, 35, 30, 25, 15, 10 (all recombinant proteins).

3.4.3. Iso-electrofocusing (IEF)

IEF was done with pre-cast gels in the pH range 3.5 to 10.0. The anode solution consisted of 25 mM L-aspartic acid, 25 mM L-glutamic acid and the cathode solution was 1 M NaOH. Gels were run with the Flat Bed apparatus (FBE 3000) connected to the Computer controlled power supply (Bio-Rad Model 3000XI). The gels were pre-run at 600 V for 20 minutes and thereafter under standard mode conditions of 1000 V, 200 mA & 50 W for ca. 1 hour. Gels were placed in

fixing solution for 0.5 – 1.0 hour, followed by washing in destaining solution (see SDS-PAGE) for 5 minutes. Subsequent staining (in staining solution (see SDS-PAGE), pre-heated to 60 °C) and destaining was followed by a final preservation step in preservation solution (10 % glycerol in destain solution). The pI of the various bands was established using standard pI marker proteins (IEF mix 3.5 - 9.3, product no. I-3018, Sigma).

3.4.4. Zymogram / activity staining

This was performed according to the method of Biely *et al.* (1985). The substrate gel contained either Remazol Brilliant Blue (RBB)-dyed xylan (Sigma) or Ostazin Brilliant Red (OBR)-hydroxyethyl cellulose (Sigma), depending on enzyme being tested. Protein gels (non-denatured) were overlaid with the different dye-containing gels to detect the activity of either xylanases or cellulases.

3.5. *IN VITRO* DIGESTIBILITY ASSAYS WITH RUMEN FLUID

3.5.1. Enzyme sample preparation prior to *in vitro* digestion

Fibrozyme powder was dissolved at the various concentrations in 1.0 ml distilled water prior to addition to the 0.5 g feed sample. The crude enzyme extracts were diluted in 1.0 ml distilled water, relative to the xylanase concentration of each enzyme sample, prior to addition to the 0.5 g feed sample. Celluclast was added in concentrated form (1.0 ml) to the feed samples.

3.5.2. Experimental procedure

In vitro digestibility assays were performed according to the methods of Tilley and Terry (1963) and Engels (1966). The rumen fluid was obtained from cannulated Dohne Merino wethers, which were routinely fed a roughage diet consisting of mainly oats hay (60 to 70 %) and lucerne hay (30 to 40 %). The animals abstained from feed and water for ca. 12 hours prior to removing rumen fluid.

The following feed samples were evaluated: wheat straw, lucerne hay, oats hay, oats silage (all the previous obtained locally), and NaOH-treated wheat straw (obtained from Meadow Feeds, Paarl, South Africa). Before the *in vitro* digestibility assay, the feed samples were dried at 60 °C for ca. 8 hours and hammer-milled to pass through a 1 mm sieve.

Fresh rumen fluid was strained through four layers of cheesecloth and then kept in a pre-warmed thermos flask. Before use, the fluid was diluted five-fold with a synthetic saliva/buffer solution consisting of: $NaHCO_3$, 15.2 g; Na_2HPO_4, 5.84 g; Urea, 1.74 g; KCl, 0.91 g; NaCl, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.19 g; $CaCl_2 \cdot 2H_2O$, 0.064 g, and distilled water (up to a final volume of 1.6 l). The solution was degassed by bubbling CO_2 for ca. 80 minutes or until the pH reached 6.9.

The diluted rumen fluid (50 ml) was added to feed samples containing the added enzyme (in 100 ml tubes), degassed with CO_2, sealed with rubber corks with a Bunsen gas release valve, hand mixed and incubated at 39 °C in a shaking water bath (ca. 30 oscillations/minute) for 48 hours. At the completion of incubation, the pH was reduced to 1.2 by addition of 2N HCl to stop microbial activity. A pepsin solution (pepsin A, BDH Chemicals Ltd, Poole, England) was added to each tube to simulate the complete digestion period in the ruminant. Distilled water (at 40 °C) was added to the tubes to the 80 ml mark, the corks replaced and the tubes were further incubated at 39 °C for 48 hours.

Afterwards, the tube contents were filtered through no. 2 sintered glass crucibles under vacuum and dried overnight at 100 °C, to determine the dry mass (DM). Finally, the feeds insoluble fibre content was determined via the neutral detergent fibre (NDF) analysis method of Van Soest *et al.* (1991). Samples were brought to boiling in a neutral detergent solvent containing (per litre): lauryl sulphate, 30 g; EDTA, 18.61 g; sodium borate (Borax), 6.81 g; Na_2HPO_4, 4.56 g; ethylene glycol, 10 ml. In addition, 2 ml decahydrophthalene and 0.5 g anhydrous sodium sulphite were added to the samples prior to the 60 minutes boiling period. Samples were washed with water, followed by rinsing with acetone and then dried at 100 °C overnight. After determining the samples' dry weight, they are incinerated at 500 °C for 5 hours, the ash weight determined and brought into account of the final calculations.

3.5.3. Calculations

Abbreviations:

DM ~ dry mass

MM ~ sample mass

OM ~ organic matter

ADF ~ acid detergent fibre

NDF ~ neutral detergent fibre

(1) % DM = DM / MM x 100

(2) % Ash = Ash / DM x 100

(3) gOM = gDM – gAsh

(4) Fraction DM

(4.1) % DM digestibility = $\frac{[(MM \times \%DM) / 100] - gDM}{[(MM \times \%DM) / 100]} \times 100$

(4.2) % ADF = $\frac{\text{Weight after boiling}}{\text{Weight before boiling}} \times 100$

(4.3) gADFout = Weight before boiling x (% ADF/ 100)

(4.4) % ADF digestibility = $\frac{MM \times \text{fraction ADF} - gADFout}{MM \times \text{fraction ADF}} \times 100$

(4.5) % $NDF_{as\ is}$ = $\frac{(\text{Weight after boiling} - \text{Weight after incineration})}{\text{Weight before boiling}} \times 100$

(4.6) % $NDF_{dry\ basis}$ = $\frac{\%\ NDF_{as\ is}}{\%\ DM} \times 100$

(4.7) g NDF_{out} = Weight before boiling x fraction $NDF_{as\ is}$

(4.8) NDF digestibility = $\frac{MM \times \text{average } NDF_{dry\ basis} \text{ fraction} - g\ NDFout}{MM \times NDFdry\ basis\ fraction} \times 100$

(4.9) % OM digestibility = $\frac{MM \times (100 - \text{Ash fraction}) - g\ OM}{MM \times (100 - \text{Ash fraction})} \times 100$

3.6. ENZYME HYDROLYSIS IN THE ABSENCE OF RUMEN FLUID

Enzyme hydrolysis experiments with Celluclast 1.5 L (Novo Nordisk) was done via the method of Colombatto *et al.* (1999). Feed samples that were evaluated included: sugarcane bagasse, wheat straw, NaOH-treated wheat straw, lucerne hay, oats hay, and oats silage. These feed samples were incubated at 50 °C for 48 hours, in the presence of 0.05 M sodium acetate (pH 4.5) and 0.02 % (w/v) sodium azide. Samples were withdrawn after 0, 24 and 48 hours of incubation. Withdrawn samples were boiled for 5 minutes, cooled and centrifuged (12 000 rpm for 15 minutes), and the supernatant harvested and stored at 4 °C until further use. Samples were evaluated for release of reducing sugars via a modified protocol. The samples were added to 0.1 M sodium acetate buffer, pH 5.0. Dinitrosalicylic (DNS) reagent was added to indicate a possible release of reducing sugars, with subsequent boiling and cooling afterwards. The release of reducing sugars was determined spectrophotometrically at 540 nm.

CHAPTER 4

RESULTS

4. RESULTS

4.1. ENZYME ASSAYS AND PROPERTIES OF CRUDE ENZYME EXTRACTS

4.1.1. Background

The four different microorganisms employed during this study were chosen mainly for their excellent enzyme producing abilities. *Trichoderma reesei* Rut C30 is a cellulase overproducing mutant, with very high endoglucanase activity, relative to the carbon source. It also produces considerable amounts of other enzymes, such as endo-β-1,4-xylanase and acetyl esterase (Schülein, 1988; Sunna *et al.*, 1997). *Thermomyces lanuginosus* is a xylanase overproducer, with little or no other enzyme activity (Singh *et al.*, 2000). *Aureobasidium pullulans*, the black yeast, is known for producing a vast array of enzymes, with moderate levels of mainly endo-β-1,4-xylanase and acetyl esterase (Myburgh *et al.*, 1991). Species of *Aspergillus* also produces moderate to high enzyme levels, depending on the carbon source. Important enzymes being produced by this fungus include the various cellulases, xylanase, and xylosidase (Bailey and Poutanen, 1989).

4.1.2. Enzyme assays

Concentrated crude extracellular enzyme extracts of fungi, grown on Birchwood xylan or Solka-floc (cellulose), are shown in Table 4.1.

TABLE 4.1. Enzyme activities (U/mg protein) of different organisms grown on 2 % Birchwood xylan (Roth) or 1 % Solka-floc cellulose (*T. reesei* only) as carbon sources for ca. 5 days

Assay [Enzyme]	*T. reesei* 10x	*A. pullulans* 80x	*A. aculeatus* 60x	*T. lanuginosus* 40x
Xylanase	2660.0±20.0	5878.0±42.0	4085.0±238.0	23750±250.0
Xylosidase	19.2±1.0	5.3±0.2	36.0±5.0	0.3±0.01
Acetyl esterase	111.2±15.0	74.0±3.0	139±8.0	19.1±0.7
Arabinofuranosidase	2.75±0.23	1.0±0.1	4.81±0.65	0.1±0.01
Cellulase (Total)	4.77±0.54	0.2±0.0	1.5±0.2	0.1±0.0
Endoglucanase	66.0±19	1.2±0.0	53.3±10.0	0.3±0.0
Cellobiase	3.0±0.2	1.0±0.1	5.0±0.3	0.3±0.0
[a]Pectinase	+	++	+	++
[a]Laccase	—	—	—	++
[Protein]	9.0 mg/ml	7.6 mg/ml	13.0 mg/ml	10.0 mg/ml

Values are indicated as the average of two determinations ± range.
[a]Both pectinase and laccase assays were plate assays.
Key: + indicate a zone diameter of 5.0 - 8.0 mm; ++ indicate a zone diameter of 12.0 - 18.0 mm; — indicate no result.

All the organisms' crude enzymes tested show moderate to high enzyme levels, except for *T. lanuginosus*, which produced mainly xylanase (Singh *et al.*, 2000). The other organisms show high xylanase and acetyl esterase activities, with high endoglucanase activity for both *T. reesei* and *A. aculeatus* (Table 4.1). Therefore, the array of enzymes from the crude extracts of *T. reesei* and *A. aculeatus* might be most suitable for enhancing fibre digestibility in feed. Also, no laccase activity was detected for *T. reesei*, *A. pullulans* and *A. aculeatus*.

The four microorganisms were also grown on wheat straw as carbon source to produce crude enzyme extracts that might contain a wider range of enzyme activities appropriate for *in vitro* digestion. The assumption was made that if the organisms were grown on the same substrate as the enzymes were required to digest, more enhanced digestibility might occur. The protein concentrations of the four organisms, after growth on wheat straw as carbon source and subsequent concentration (ca. 30-fold), are given in Table 4.2.

TABLE 4.2. Protein concentration (mg/ml) of the culture fluid from different organisms grown on wheat straw (2 %) as carbon source and concentrated ca. 30-fold

Organism	[Protein] (mg/ml)
T. reesei	1.5±0.20
A. pullulans	0.5±0.03
A. aculeatus	0.72±0.14
T. lanuginosus	0.23±0.00

Values are indicated as the average of two determinations ± range.

Wheat straw was shown to be an unsuitable substrate for the growth of all the organisms tested (Table 4.2), as their growth resulted in approximately 10-fold lower amounts of protein, compared to growth on other substrates (Table 4.1). Therefore, the enzyme activities of the crude extracts from microorganisms grown on wheat straw as carbon source were not evaluated further, but only SDS-PAGE analysis was performed to compare the banding pattern of these enzymes with that of enzymes from the same fungi grown on either Birchwood xylan or Solka-floc cellulose as carbon sources (see section 4.1.3).

4.1.3. SDS-PAGE analysis

SDS-PAGE analysis was performed for all the crude extracellular enzyme extracts obtained during fungal growth on the various carbon sources (Fig 4.1).

Fig. 4.1A reveals a diverse electrophoretic banding pattern for all the organisms cultivated on Birchwood xylan or Solka-floc. *T. reesei* showed major bands at ca. 140, 70, 50, 35, 33 and 24 kDa. *A. aculeatus* showed the most diverse banding pattern of all the organisms tested; with major bands at ca. 55, 33, 31, 28, 25 and 20 kDa. However, the function of the individual protein bands could not be determined. *T. lanuginosus* had only a few major bands at ca. 50, 26 and 20 kDa; with the very prominent xylanase band at ca. 26 kDa as shown previously (Singh *et al.*, 2000). *A. pullulans* showed 3 major bands at ca. 250, 43 and 26 kDa; the latter being the xylanase band as confirmed by the studies of Li *et al.* (1993).

When the various fungi were cultivated on wheat straw, fewer bands were revealed by SDS-PAGE analysis (Fig. 4.1B). *T. reesei* showed prominent bands at ca. 60 and 50 kDa. *A. aculeatus* gave bands at ca. 75, 70, 50 and 33 kDa, with *T. lanuginosus* and *A. pullulans* showing prominent bands at ca. 34 and ca. 25 kDa, respectively. Therefore, it is apparent that substrates such as Birchwood xylan and Solka-floc induce a wider range of enzymes than wheat straw. All subsequent enzyme preparations used in this study were derived from organisms grown on either Birchwood xylan or Solka-floc cellulose.

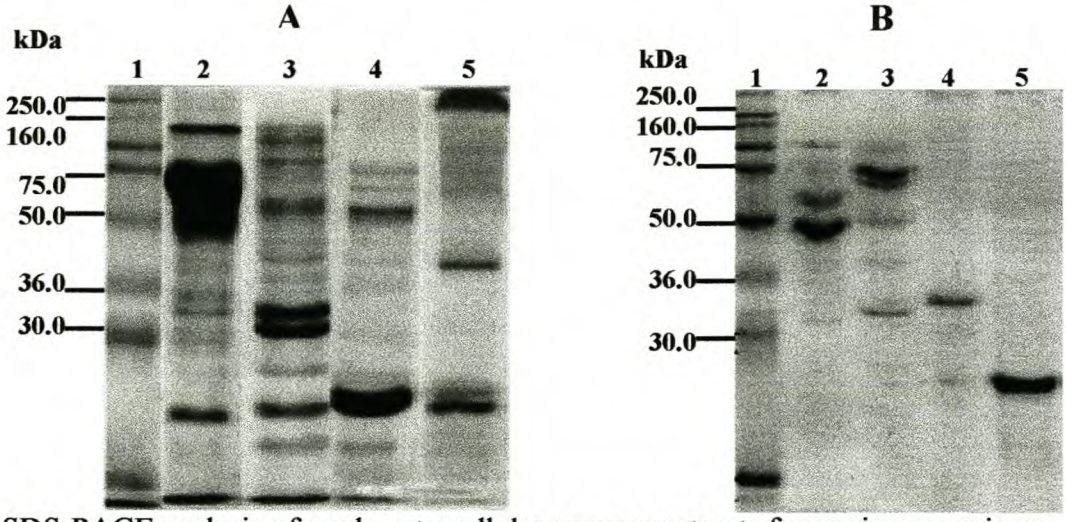


FIGURE 4.1. SDS-PAGE analysis of crude extracellular enzyme extracts from microorganisms

Samples: 1, Molecular weight (MW) Marker; 2, *T. reesei*; 3, *A. aculeatus*; 4, *T. lanuginosus*; 5, *A. pullulans*.
A. Organisms grown on either 2 % Birchwood xylan (Roth) or 1 % Solka-floc (*T. reesei* only) as carbon sources
B. Organisms grown on 2 % wheat straw as carbon source

4.1.4. Iso-electrofocusing (IEF) and activity staining

Iso-electrofocusing analysis was conducted on the crude extracellular enzyme extracts of the four fungi, grown on either Birchwood xylan or Solka-floc (cellulose) as carbon sources. IEF analysis (Fig. 4.2) indicated that most proteins have an optimal activity in the acidic region (3.6 – 5.3) for all organisms tested. Only a few proteins function optimally in the basic region (9.1 – 8.3) for most organisms, except *T. lanuginosus*. *T. reesei* shows strong bands at ca. 5.3, 4.6, 4.3, 4.1 and 4.0 pI; with weak bands at ca. 8.9, 5.9 and 3.8 pI. *A. aculeatus* reveals a broader optimal enzyme range, with prominent bands at ca. pI 9.0, 8.4, 5.5 and 3.7. *T. lanuginosus* have only two bands in the acidic region; one more prominent band at ca. pI 3.9 and a less prominent band at ca. 3.7. The former band is the xylanase band as confirmed by Singh *et al.* (2000). *A. pullulans* have one prominent band at ca. pI 8.4, and weak bands at ca. 9.2, 4.3 and 4.0. The band sizes of 4.0, 8.4, and 9.2 almost fully corresponds to three of the four major xylanase bands of 4.0, 7.9, and 9.4, as described by Li *et al.* (1993). In the studies of Li *et al.* (1993), *A. pullulans* was grown on 1.0 % oat spelts xylan, whereas during this study it was grown on birchwood xylan.

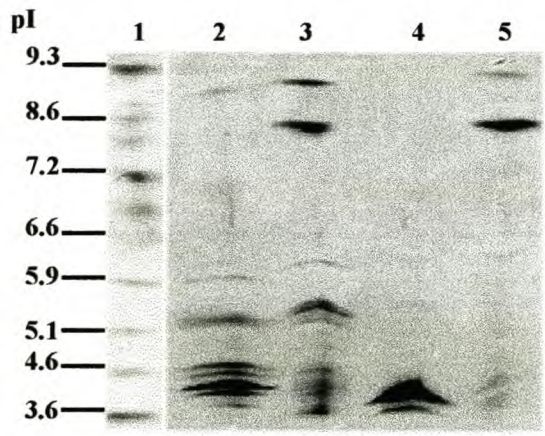


FIGURE 4.2. IEF analysis of crude extracellular enzyme preparations from microorganisms cultivated on either Birchwood xylan or Solka-floc for ca. 5 days

Samples: 1,Molecular weight (MW) Marker; 2, *T. reesei*; 3, *A. aculeatus*; 4, *T. lanuginosus*; 5, *A. pullulans*.

Activity staining of the IEF gel revealed that a number of bands contained protein with either cellulase or xylanase activity (data not shown). *T. reesei* and *A. aculeatus* were found to have a larger number of bands containing either cellulase or xylanase activity than either *A. pullulans* or *T. lanuginosus*; suggesting the presence of a wider range of isozymes in the former two organisms. *T. lanuginosus* possessed a single band with xylanase activity and no bands with cellulase activity.

4.2. ENZYME ASSAYS AND PROPERTIES OF COMMERCIAL ENZYME PREPARATIONS

4.2.1. Fibrozyme analysis

4.2.1.1. Background

Fibrozyme is an enzyme supplement for ruminants (Alltech, USA), containing fermentation extracts from *Aspergillus niger* and *Trichoderma longibrachiatum*. Due to its ability to degrade xylan in the hemicellulose fraction, it was designed to aid in the breakdown of the fibrous matrix in plant structural carbohydrate complexes. Thereby, many bound nutrients are exposed to the digestive activities in the rumen. Fibrozyme also contains measurable amounts of cellulase and protease activity, which enhances enzymatic activity in the rumen. Many of its component proteins are produced in a naturally glycosylated form, thereby protecting the enzymes in the rumen (Dawson and Tricarico, 1999).

Previous studies showed an enhanced degradability of grass hay in animals receiving Fibrozyme (Tricarico *et al.*, 1998). The digestive activities were often more pronounced in animals receiving a high level of grain supplement, rather than a hay based diet. However, enhanced application of Fibrozyme and other exogenous enzymes will also require investigation of other determining parameters, such as: feed evaluation, enzyme addition, and time of incubation (Dawson and Tricarico, 1999).

The purpose of the biochemical analysis of Fibrozyme was to obtain more details of the range of enzymes present in the preparation and the activities. Fibozyme is a protected enzyme supplement, therefore certain techniques were applied to release its enzymes for further analysis. The Fibrozyme powder was dissolved in distilled water and the release of the enzymes was attempted by: 1) exposure in a ultrasonic waterbath at room temperature and subsequent centrifugation for 30 seconds; 2) exposure on a bead mill (cell homogenizer) in the presence of glass beads for 20 minutes, followed by centrifugation; 3) only centrifugation and harvesting of the supernatant, and 4) incubation at 39 °C (rumen temperature) for 20 minutes, followed by centrifugation. Exposure in the presence of glass beads was the more successful extraction method (showing the highest protein concentration) and therefore used further during this study (Table 4.3). Subsequent SDS-PAGE analysis also revealed that the sample subjected to glass beads treatment yielded the most intense bands and the larger number of bands (data not shown). Fibrozyme was therefore subjected to glass bead extraction prior to further biochemical analysis in subsequent experiments.

TABLE 4.3. Comparison of Fibrozyme protein concentrations obtained by various extraction methods.

Technique	[Protein] (mg/ml)
[1]Ultrasonication	0.106±0.05
[2]Glass beads	0.397±0.06
[3]Centrifugation	0.100±0.05
[4]Incubation	0.09±0.01

[1]Ultrasonication was performed at room temperature for 30 seconds.
[2]Homogenisation in the presence of glass beads on a bead mill for 20 minutes.
[3]Fibrozyme powder was dissolved in water and centrifuged.
[4]Incubation at 39 °C (rumen temperature) for 20 minutes.
All samples were centrifuged (10 000 rpm for 15 minutes) after their respective treatments and the supernatants stored at 4 °C until further use.

4.2.1.2. Enzyme assays

Compared to the preparations described above, analysis of Fibrozyme showed that the preparation contained relatively low enzyme activities (Table 4.4). The reason for these low activities is unknown, but could be related to the formulation of Fibrozyme or the instability of enzymes.

TABLE 4.4. Enzyme activities (U/mg protein) of Fibrozyme extracted by homogenisation with glass beads.

Assay	Activity (U/mg)
Xylanase	3.98±0.33
Xylosidase	0.04±0.01
Acetyl esterase	0.24±0.03
Arabinofuranosidase	0.01±0.00
Cellulase (Total)	0.01±0.00
Endoglucanase	0.1±0.01
Cellobiase	0.02±0.00
[a]Pectinase	+
[a]Laccase	++
[Protein]	0.397 mg/ml

Values are indicated as the average of two determinations ± range.
[a]Both pectinase and laccase assays were plate assays. KEY: + indicate a zone diameter of 5.0 - 8.0 mm; ++ indicate a zone diameter of 12.0 - 18.0 mm; — indicate no result.

4.2.1.3. SDS-PAGE analysis

SDS-PAGE analysis was conducted on Fibrozyme to determine the molecular weight of the proteins present (Fig. 4.3).

A range of proteins is revealed, differing in size from ca. 70 to ca. 30 kDa, with prominent bands at 70, 50, 36 and 29 kDa. Although a large number of proteins are present, which suggests that Fibrozyme is composed of a number of different enzymes, the identities and enzyme activities of these main protein bands are still unknown.

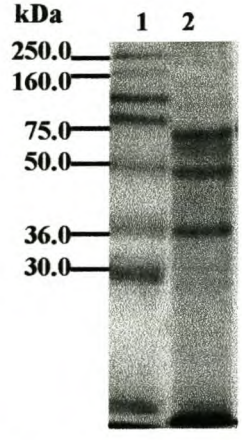


FIGURE 4.3. SDS-PAGE analysis of Fibrozyme extracted by homogenisation with glass beads.
Samples: 1, Molecular weight (MW) Marker; 2, Fibrozyme.

4.2.1.4. Iso-electrofocusing (IEF) and activity staining

Fig. 4.5 reveals four weak bands at pI 8.6, 7.2, 4.3 and 4.1, suggesting the presence of proteins that function over a broad pH range. Subsequent activity staining indicated a xylanase at 5.0, and a cellulase at 4.3 and 4.1 (data not shown). However, the xylanase band was not visible by IEF analysis.

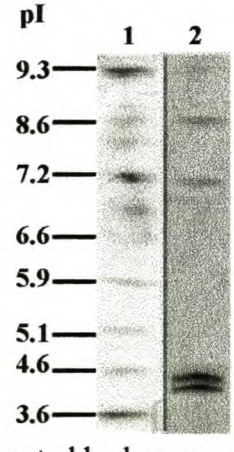


FIGURE 4.5. IEF analysis of Fibrozyme extracted by homogenisation with glass beads
Samples: 1, Marker; 2, Fibrozyme.

4.2.1.5. Further analysis

The Fibrozyme powder smelt faintly of active dried yeast and motivated a search for the presence of any viable cells. Therefore the enzyme product was streaked out on a yeast peptone dextrose (YPD) slant and incubated at 30 °C overnight. Growth of a single yeast type was observed, and after microscopic analysis, it was identified as an Ascomycete (probably *Saccharomyces* sp.).

The Fibrozyme powder was also dissolved in sterile distilled water and incubated at both room temperature and 37 °C overnight. Both mixtures showed fermentation, but better fermentation was observed at 37 °C. This might indicate that Fibrozyme contained adequate nutrients to sustain the yeast, and also that the yeast could function in the rumen, where it possibly takes up excess oxygen and produces volatile fatty acids (Wallace and Newbold, 1993; Wallace, 1994). A slight change in pH, from 5.6 to 5.0, was also observed.

In conclusion it is apparent that Fibrozyme consists of a range of enzymes involved in fibre digestion, with mainly xylanase activity and little cellulolytic activity. It also contains yeast, but the enzymes do not seem to be associated with the yeast, as yeast separated from the enzyme mixture showed no enzyme activity on cellulose and xylanase activity plates.

4.2.2. Celluclast analysis

4.2.2.1. Background

Due to the low enzyme activities of the Fibrozyme sample, a second commercial enzyme product was chosen for evaluation during this study. Celluclast 1.5 L (Novo Nordisk), a cellulase preparation produced by submerged fermentation of a selected strain of *Trichoderma reesei* was selected because of the promising enzyme levels obtained with *T. reesei* Rut C30 during a study by Schülein (1988).

Celluclast has optimal activity at about 50 to 60 °C and a pH of 4.5 to 6. It catalyses the breakdown of cellulose into glucose, cellobiose and other higher glucose polymers. It also has a pronounced viscosity-reducing effect on soluble cellulosic substrates (Celluclast information sheet).

4.2.2.2. Enzyme assays

The various enzyme activities tested (Table 4.5) showed relatively high values, especially the different cellulases and xylanase, which play the major roles in lignocellulosic breakdown. *T. reesei* is an established source of cellulolytic enzymes which produces two cellobiohydrolases (CBH-I and CBH-II) and four endoglucanases (EG-I, II, III, and IV), with CBH-I as the most abundant enzyme (Medve *et al.*, 1998; Schülein, 1988).

TABLE 4.5. Enzyme activities (U/mg protein) of Celluclast 1.5 L.

Assay	Activity (U/mg)
Xylanase	23016.8±1739.4
Xylosidase	1814.9±50.3
Arabinofuranosidase	1103.5±36.7
Cellulase (Total)	338.5±41.0
Endoglucanase	197.1±59.0
Cellobiase	227.9±12.0
[Protein]	26.0 mg/ml

Values are indicated as the average of two determinations ± range.

4.2.2.3. SDS-PAGE analysis

SDS-PAGE analysis (Fig. 4.4) showed prominent bands at ca. 65, 55, and possibly 45 kDa, with few other bands present. This confirmed the findings of Schülein (1988), who observed purified enzymes from Celluclast at 65 (cellobiohydrolase, CBH), 52 (endoglucanase I, EG-I), and 48 kDa (endoglucanase II, EG-II). In addition, Schülein (1988) determined their respective pI to be 3.8 to 4.0 (CBH), 4.0 to 5.0 (EG-I), and 7.0 (EG-II) (Schülein, 1988). Therefore, this commercial product was considered to have good potential to increase the digestibility of various feeds and was used in some digestion experiments. *T. reesei* has several isoforms of endoxylanase II (XYL II), with a molecular mass of 20 kDa as determined by SDS-PAGE analysis (Lappalainen *et al.*, 2000). This might explain the high xylanase yield detected in Celluclast.

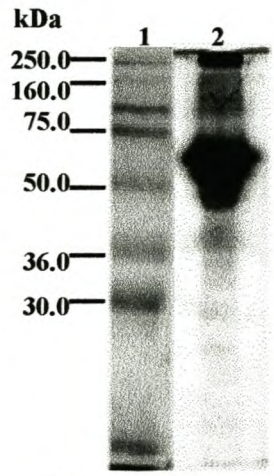


FIGURE 4.4. SDS-PAGE analysis of Celluclast

Samples: 1, Molecular weight (MW) Marker; 2, Celluclast.

4.3. DIGESTION OF FEED SAMPLES WITH RUMEN FLUID WITHOUT ADDED ENZYMES

The relative *in vitro* dry matter (DM) and neutral detergent fibre (NDF) digestibility values of both wheat straw and lucerne hay was determined over time, without the addition of enzymes. Figs. 4.6 and 4.7 presents these values for wheat straw and lucerne hay without addition of enzymes.

Wheat straw normally has DM and NDF digestibility values in the order of 40 to 45 %, whereas lucerne hay normally has a DM digestibility value ranging between 50 and 60 % and a NDF digestibility value of 50 %. In rare cases, the NDF digestibility value might be higher than the DM digestibility value (National Research Council, 2001). Considerable variation was found in the *in vitro* digestibility of wheat straw, whereas the variability was considerably less with lucerne hay. Furthermore, the NDF digestibility value was greater than the DM digestibility value of wheat straw, which is unusual. The reasons for the variability in digestibility are uncertain. The diet of the ruminant, as well as the environment where the ruminant is kept, can influence the composition of the rumen fluid. For example, the rumen fluid was obtained from sheep kept either at the Welgevallen Experimental farm of the University of Stellenbosch or at the Elsenburg Experimental farm, and this might contribute to the variability of the control data. The sheep were kept on an oat hay (60 to 70 %) and lucerne hay (30 to 40 %) diet and had *ad libitum* access to feed and water, which might have led to variability in rumen fluid among different animals. In addition, this might have stimulated competition between animals within the same pen, resulting in each animal receiving different levels of feed. Therefore, the small variations in the experimental procedure might have influenced the digestion ability of the rumen fluid (Jones and Theodorou, 2000).

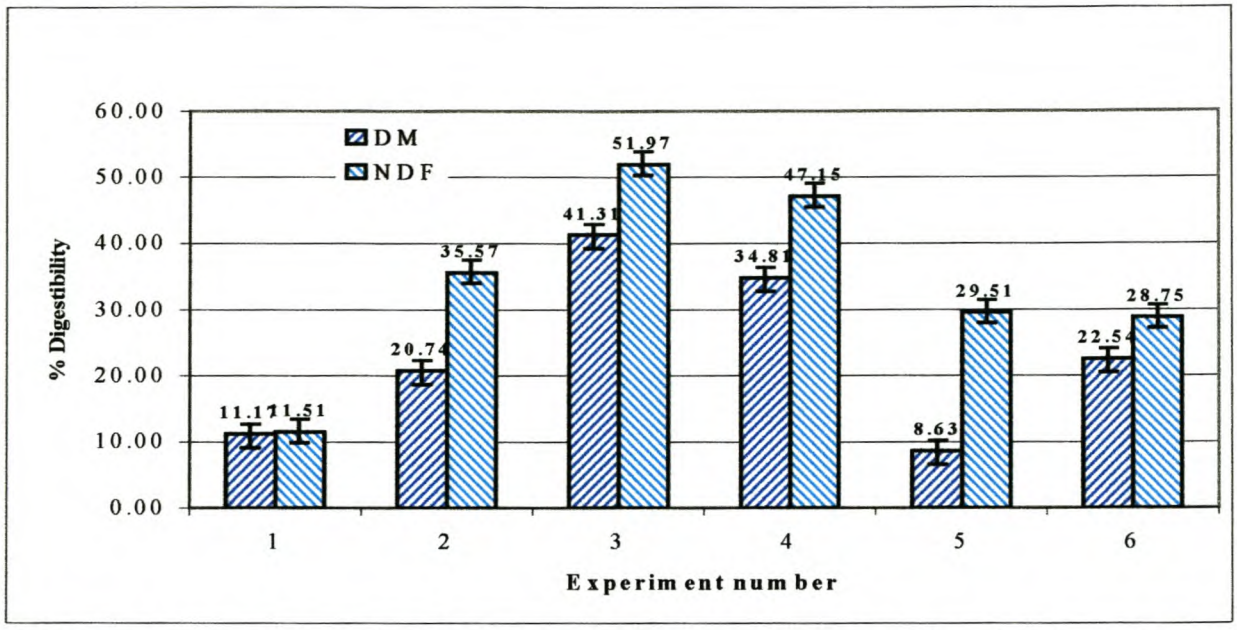


FIGURE 4.6. Mean changes in dry matter (DM) and neutral detergent fibre (NDF) of wheat straw in the absence of enzymes, as determined in various experiments. Error bars indicate the standard deviation of triplicate values.

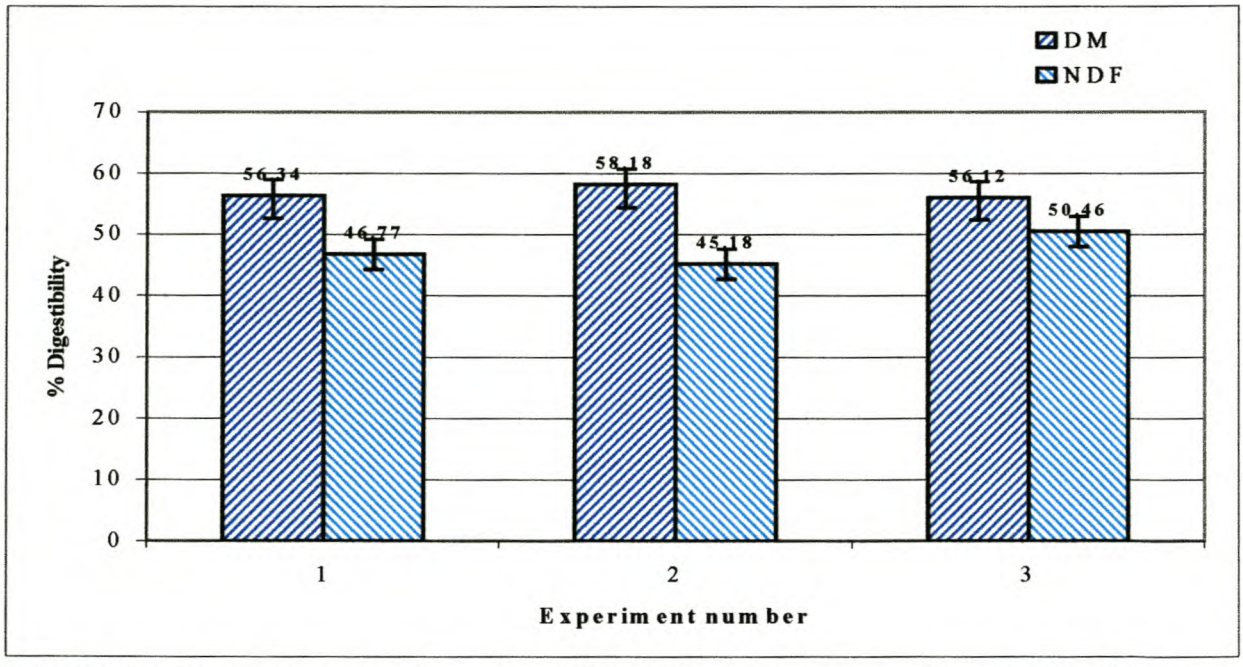


FIGURE 4.7. Mean changes in dry matter (DM) and neutral detergent fibre (NDF) of lucerne hay in the absence of enzymes, as determined in various experiments. Error bars indicate the standard deviation of triplicate values.

4.4. DIGESTION OF FEEDS WITH COMMERCIAL AND LABORATORY ENZYME PREPARATIONS

4.4.1. Fibrozyme as feed supplement

The digestibility of wheat straw and lucerne hay, by the addition of Fibrozyme, at different concentrations, was evaluated to determine the optimal enzyme levels for both wheat straw (Fig. 4.8) and lucerne hay (Fig. 4.9).

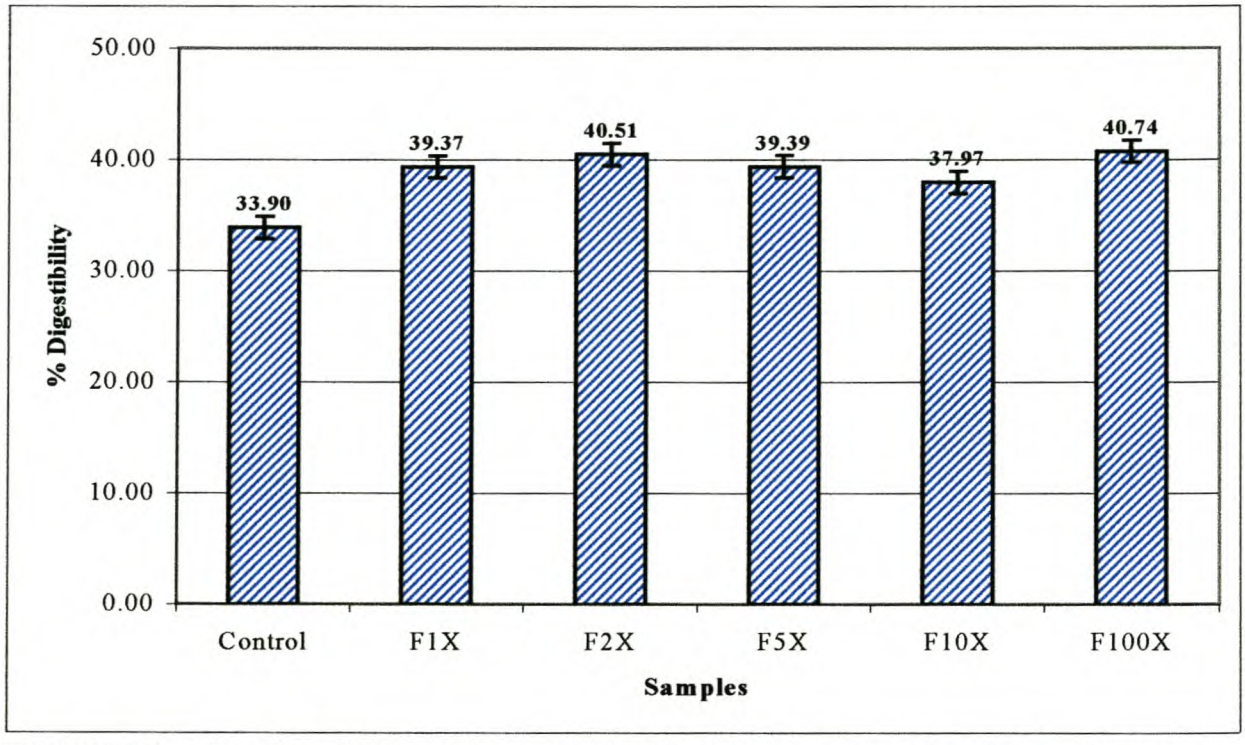


FIGURE 4.8. Mean digestibility of dry matter (DM) of wheat straw with addition of Fibrozyme to rumen fluid. Error bars indicate the standard deviation of triplicate values. F ~ Fibrozyme; 1x (0.03U xylanase), 2x (0.06U xylanase), 5x (0.15U xylanase), 10x (0.30U xylanase), 100x (3.0U xalanase) ~ enzyme level added per gram feed.

The addition of the recommended level of Fibrozyme (1-fold) to wheat straw resulted in a ca. 16 % improvement in digestibility, relative to the control. Addition of higher levels of Fibrozyme also resulted in similar levels of digestibility improvement (Table 4.6). Beauchemin *et al.* (1995) also reported an optimum level of fibrolytic enzyme addition, above which no further increase in digestion improvements could be found. However, the DM digestibility value of wheat straw's control is ca. 6 % below its normal value, and therefore this result is inconsistent.

The addition of Fibrozyme to lucerne hay resulted in only a slight or no increase in digestibility (Fig. 4.9; Table 4.6). This result suggests that the lucerne hay might not be suitable as a

feed where enzyme addition could lead to increased digestibility. It appears that an enzyme supplement's ability to enhance feed digestibility is dependent on the diet's nature (Howes *et al.*, 1998).

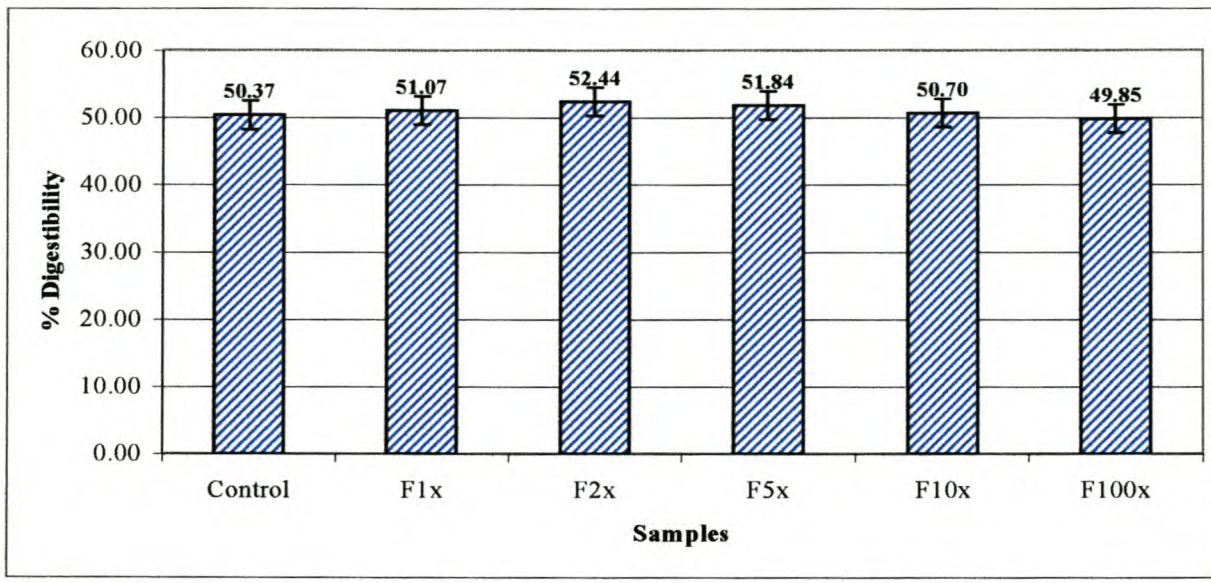


FIGURE 4.9. Mean digestibility of dry matter (DM) of lucerne hay with addition of Fibrozyme to rumen fluid. Error bars indicate the standard deviation of triplicate values. F ~ Fibrozyme; 1x (0.03U xylanase), 2x (0.06U xylanase), 5x (0.15U xylanase), 10x (0.30U xylanase), 100x (3.0U xalanase) ~ enzyme level added per gram feed.

TABLE 4.6. Effect of Fibrozyme addition to wheat straw and lucerne hay on change in *in vitro* digestibility relative to the control[a].

Fibrozyme level (U xylanase)	% Increase (-decrease)	
	Wheat straw	Lucerne hay
1x (0.03U)	16.0	1.4
2x (0.06U)	19.5	4.1
5x (0.15U)	16.2	2.9
10x (0.30U)	12.0	0.7
100x (3.0U)	20.2	-1.0

[a]The digestibility of the control sample of wheat straw and lucerne hay was 33.9 % and 50.4 %, respectively.

Digestion assays (dry matter and neutral detergent fibre) were also performed with Fibrozyme to assess the effect of an incubation of the enzyme with the feed prior to *in vitro* evaluation with rumen fluid. Fig. 4.10 revealed that Fibrozyme failed to enhance the digestibility of wheat straw, even after a pre-incubation period of 2 hours between enzyme and feed. However, the control's DM and NDF values are not in the normal range expected for wheat straw. Therefore, this result might

also reflect problems with the rumen fluid used in the experiments. Fig. 4.11 also shows no significant effect on enhancing the digestibility of lucerne hay, even after a pre-incubation period of 2 hours.

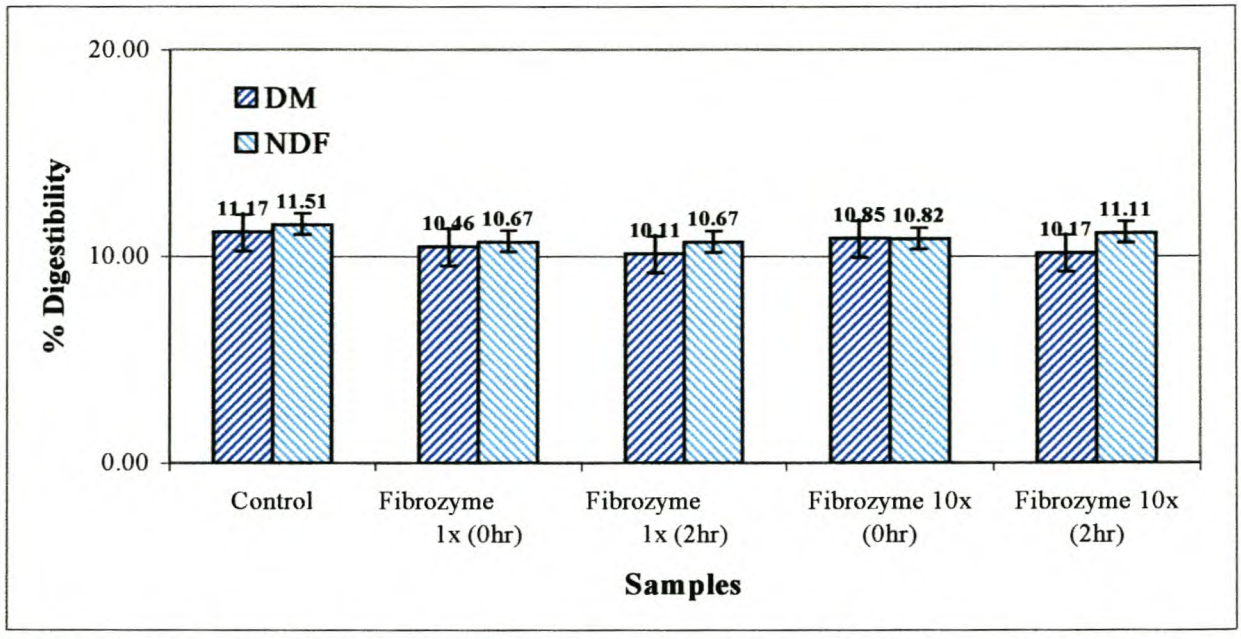


FIGURE 4.10. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of wheat straw with addition of Fibrozyme to rumen fluid. Error bars indicate the standard deviation of triplicate values. F ~ Fibrozyme; 1x (0.03U xylanase), 10x (0.30U xylanase) ~ enzyme level added per gram feed ; 0hr, 2hr ~ time of adding enzyme prior to feeding.

The results obtained in Figs. 4.10 and 4.11 do not correspond with the results shown in Figs. 4.8 and 4.9 and, where the experiments were conducted in a similar way except for pre-incubation with Fibrozyme. This variation might be due to the changes related to the rumen fluid source. According to Jones and Theodorou (2000), the main disadvantage of using rumen fluid in feed digestibility studies is the variability in the potency of the microbial inoculum from week to week. In addition, significant variation in the inoculum's activity occurs between different donor animals and even for the same animal on different days. This might also be dependent on water consumption at varying intervals before collecting rumen fluid (Jones and Theodorou, 2000).

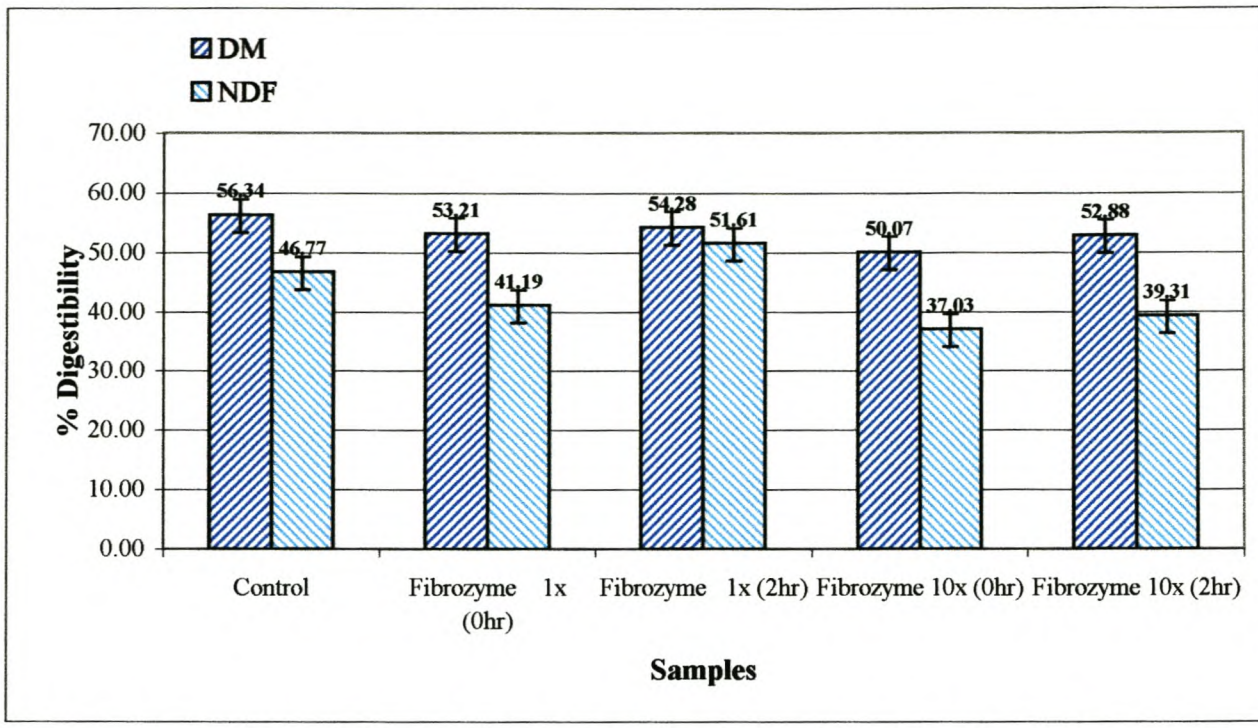


FIGURE 4.11. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of lucerne with addition of Fibrozyme to rumen fluid. Standard error bars indicate the standard deviation of triplicate values. F ~ Fibrozyme; 1x (0.03U xylanase), 10x (0.30U xylanase) ~ enzyme level added per gram feed ; 0hr, 2hr ~ time of adding enzyme prior to feeding.

4.4.2. Crude enzyme extracts as feed supplements

The *in vitro* digestibility of lucerne hay and wheat straw was evaluated by addition of crude enzyme extracts obtained from fungi (see 4.1.3 for details of enzyme preparations). Results are shown in Figs. 4.12 to 4.15 and Table 4.7 below.

Figure 4.12 shows a definite increase in DM and NDF digestibility of wheat straw for all the enzyme preparations tested. However, the samples, including the control (no enzyme supplement), had a 10 to 15 % lower digestibility value than normally expected since wheat straw normally has a DM and NDF value of ca. 45 % and 55 %, respectively. Nevertheless, *T. reesei* increased DM and NDF digestibility by ca. 45 and 25 % respectively. *A. pullulans* showed increases of ca. 59 and 35 %, and *T. lanuginosus* ca. 62 and 35 %.

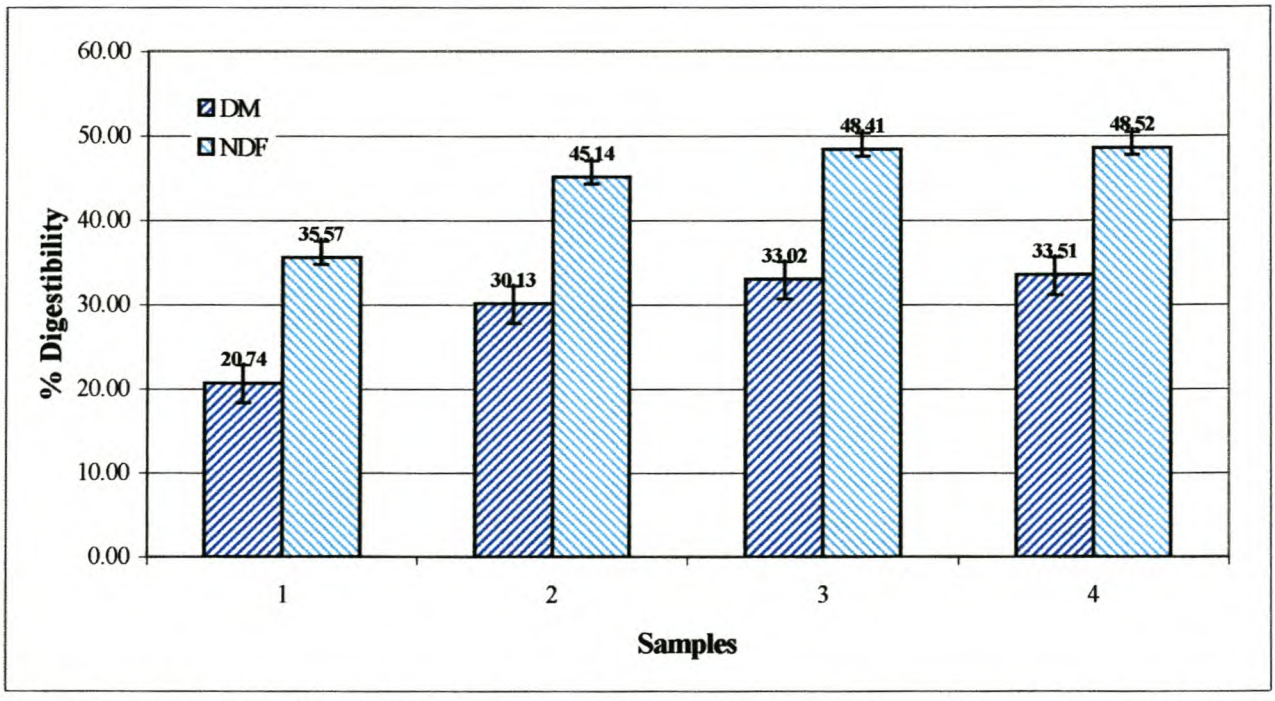


FIGURE 4.12. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of wheat straw with addition of crude enzyme levels of 2U xylanase per gram feed to rumen fluid. Error bars indicate the standard deviation of triplicate values. 1, Control (without enzyme); 2, *T. reesei*; 3, *A. pullulans*; 4, *T. lanuginosus*.

Figure 4.13 indicates no increase in both the DM and NDF digestibility of wheat straw due to addition of enzyme preparations, thereby implying that the enzymes had no effect on increasing wheat straw's digestibility. Considering the result obtained in Fig. 4.12, this current result (Fig. 4.13) is probably more reliable because the control's DM and NDF values are in the normal range expected for wheat straw.

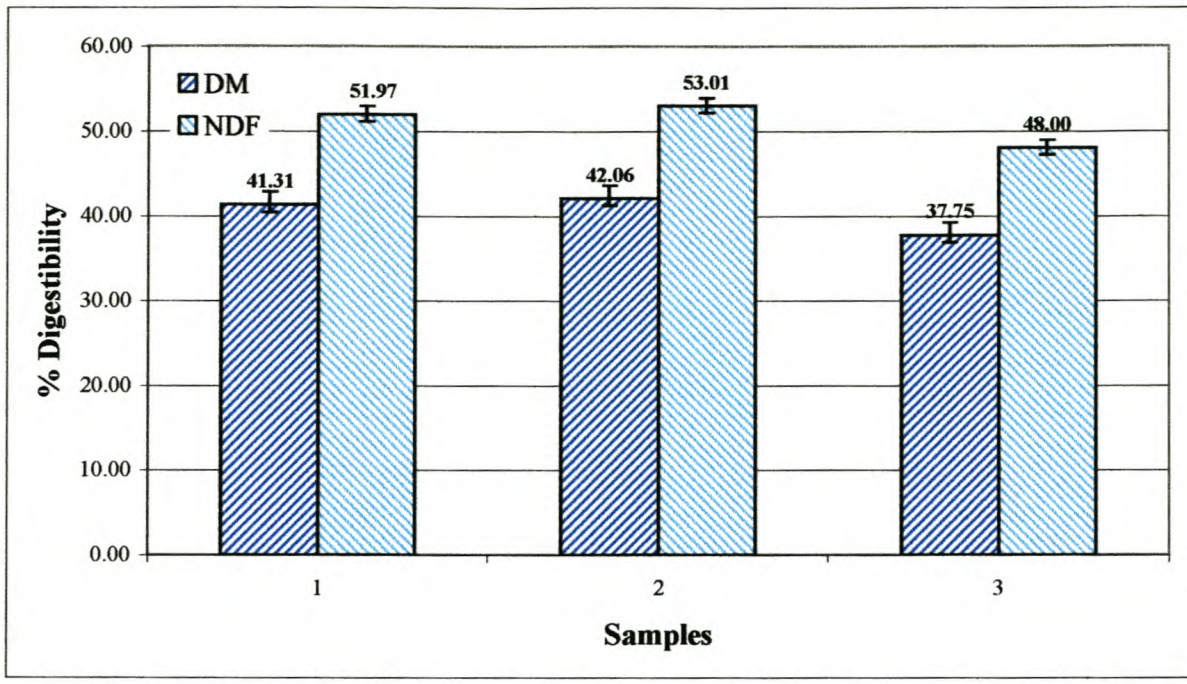


FIGURE 4.13. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of wheat straw with addition of crude enzyme levels of 2 or 4U xylanase per gram feed to rumen fluid. Error bars indicate the standard deviation of triplicate values. 1, Control (without enzyme); 2, *A. aculeatus* (2U); 3, *A.pullulans* (4U).

Fig. 4.14 indicates no increase in either the DM and NDF digestibility of lucerne hay with the various enzyme applications, while Fig. 4.15 shows only marginal increases in both the DM and NDF digestibility. Although these results (Figs. 4.14 and 4.15) were obtained at different weeks, it shows a reasonable correlation between data, and therefore indicates that *in vitro* digestibility assays with rumen fluid can yield a constant result at different time intervals. However, this observation was only achieved with lucerne hay, and almost never with the poorly digestible wheat straw.

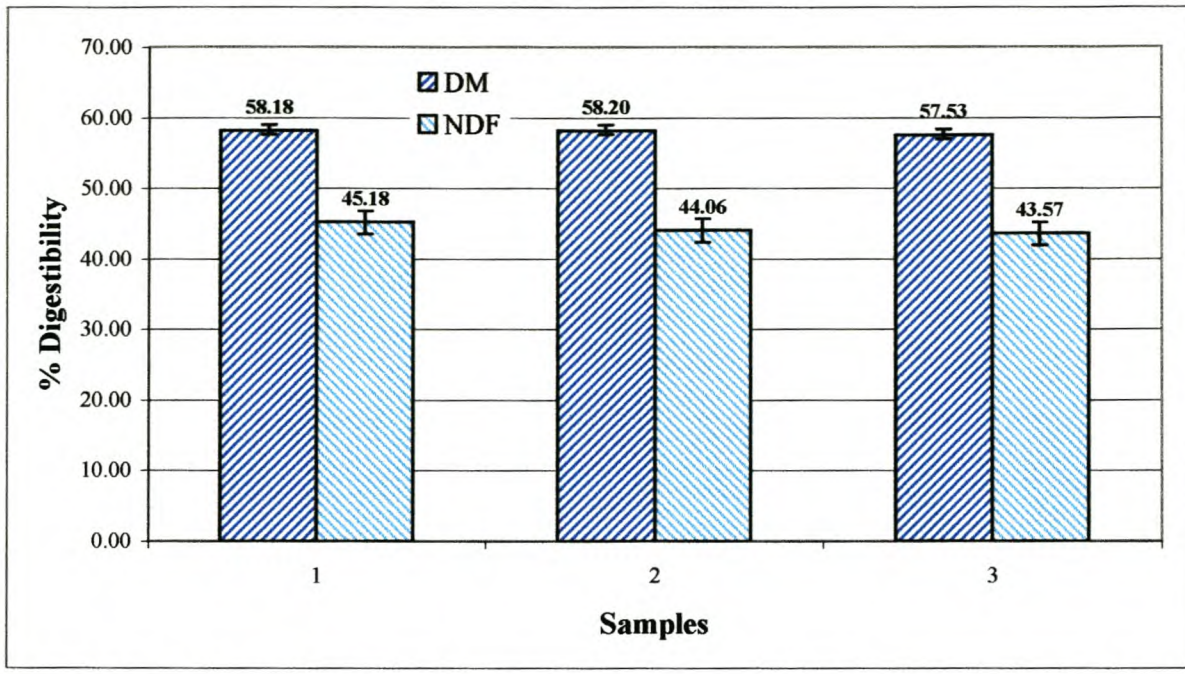


FIGURE 4.14. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of lucerne hay with addition of crude enzyme levels of 2U xylanase per gram feed to rumen fluid. Error bars indicate the standard deviation of triplicate values. 1, Control (without enzyme); 2, *A. pullulans* (2U); 3, *T. reesei* (2U).

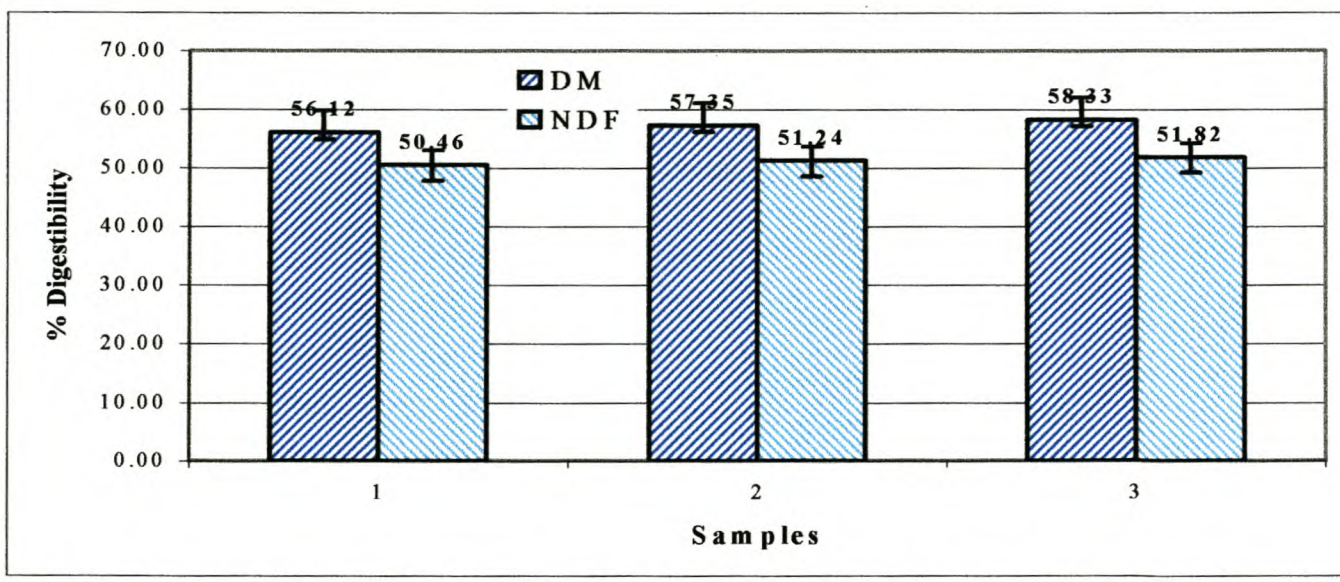


FIGURE 4.15. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of lucerne hay with addition of crude enzyme levels of 2 or 4U xylanase per gram feed to rumen fluid. Error bars indicate the standard deviation of triplicate values. 1, Control (without enzyme); 2, *A. aculeatus* (2U); 3, *A. pullulans* (4U).

Table 4.7 summarises the effect of enzyme addition from various fungi on the *in vitro* digestibility as presented in Figs. 4.12 to 4.15. It is apparent that the largest increase in digestibility by enzyme addition occurred with wheat straw, while a much lower or no increase was found with lucerne hay. However, the inconsistencies in the data made it impossible to draw firm conclusions as to whether enzymes improve digestibility, or whether the enzyme extract from one fungus was better than another. Therefore there is a need for a means of evaluation that would yield consistent and reliable data.

TABLE 4.7. Effect of the addition of crude enzyme preparations from various fungi on change in *in vitro* digestibility.

Organism (xylanase activity)	**% Increase (-decrease)**	
	Wheat straw DM / NDF	**Lucerne hay DM / NDF**
T. reesei (2U)	45.3 / 26.9	-1.1 / -3.6
A. pullulans (2U)	59.2 / 36.1	0.03 / -2.5
A. pullulans (4U)	-8.6 / 7.6	3.9 / 2.7
T. lanuginosus (2U)	61.6 / 36.4	N.D.
A. aculeatus (2U)	1.8 / 2.0	2.2 / 1.5

N.D., not determined.

4.4.3. Celluclast as feed supplement

Because of the difficulties with the *in vitro* digestibility experiments using wheat straw and lucerne hay, as well as the inconsistent contribution of the enzyme preparations to the improvement in digestibility, it was decided to evaluate digestion of other feed samples using Celluclast, an efficient fibrolytic enzyme preparation containing a range of enzyme activities (Table 4.5). The *in vitro* digestibility of four different feed samples, oat hay, lucerne hay, oat silage and NaOH-treated wheat straw, was evaluated for a possible increase in their digestibility, by addition of the commercial enzyme product Celluclast 1.5 L (Novo Nordisk). The obtained results are given in Figs. 4.16 to 4.19 below.

Oat hay gave a ca. 5 % increase in DM and NDF digestibility relative to the control, whereas lucerne hay showed no positive effect after enzyme addition (Fig 4.16). The failure of the higher enzyme activities in Celluclast to improve digestibility was unexpected. This might be due to the rumen fluid containing all necessary enzymes for digestion and therefore enzyme addition was unable to further improve digestibility. Incubation of the Celluclast with the feed for 24 hour prior to the *in vitro* digestibility assay (Fig. 4.17) also failed to improve the digestibility in both DM and NDF for oat hay, whereas lucerne hay showed a ca. 4 % increase in both DM and NDF digestibility. It was also noticeable that the percent digestibility of both feeds showed a ca. 10 % decrease relative to the values obtained and shown in Fig. 4.16. Both results were inconsistent in Fig. 4.17, especially that of oat hay, which shows higher NDF values (ca. 15 % higher than the DM digestibility value). The *in vitro* assay and the rumen fluid might be responsible for the fluctuation in results. Therefore, the results obtained were not conclusive in showing a possible improvement in the digestibilities of these two feeds by Celluclast addition or by incubation of the enzymes with the feed prior to the digestibility assays.

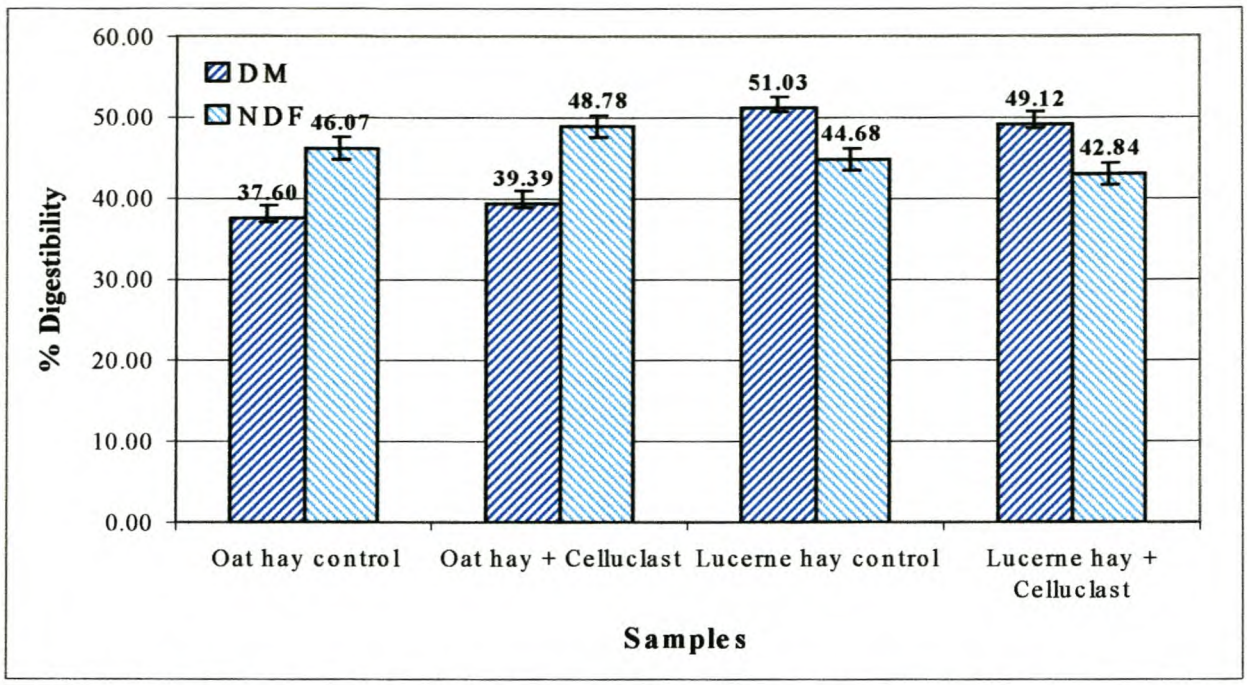


FIGURE 4.16. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of oat hay and lucerne hay after Celluclast addition to rumen fluid. The enzyme was added at enzyme levels of ca. 443U xylanase and 4U cellulase per gram feed (52 mg protein per gram feed). Error bars indicate the standard deviation of triplicate values.

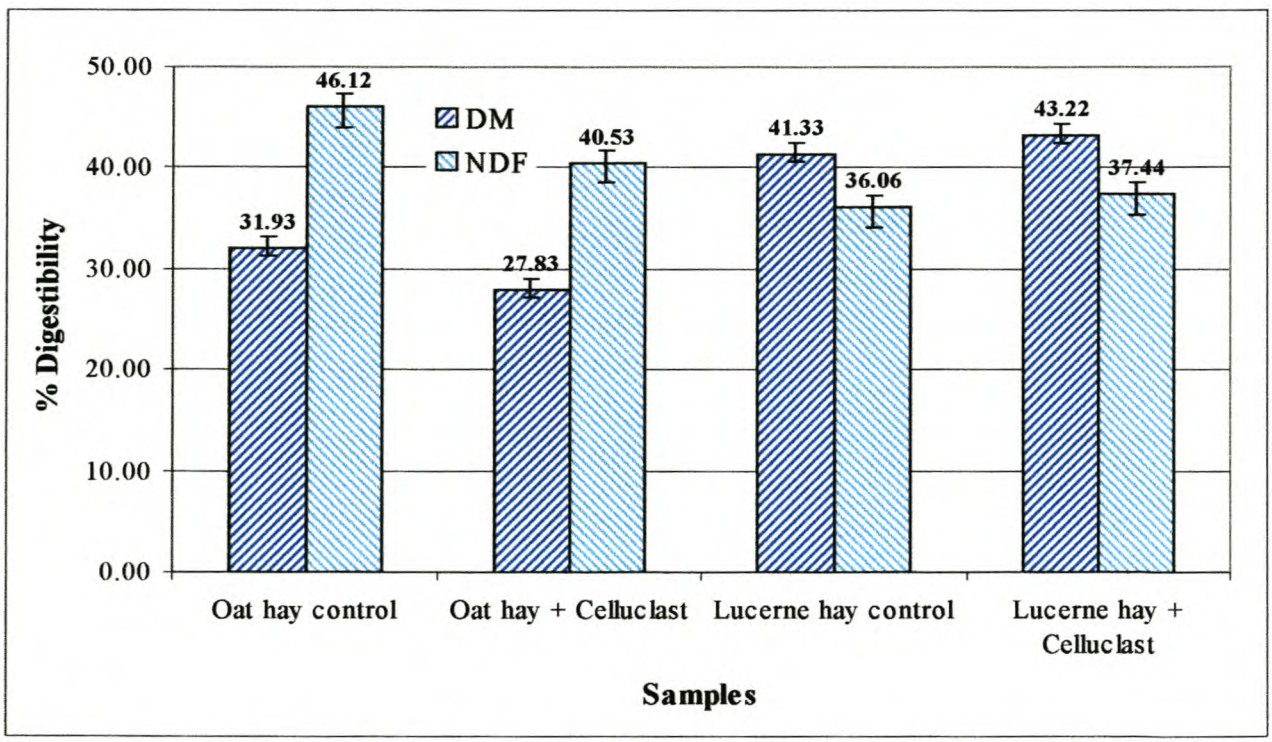


FIGURE 4.17. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of oat hay and lucerne hay after Celluclast addition, 24 hours prior to the *in vitro* assay. The enzyme was added at levels of ca. 443U xylanase and 4U cellulase per gram feed (52 mg protein per gram feed). Error bars indicate the standard deviation of triplicate values.

Figs. 4.18 and 4.19 give the results of *in vitro* digestibility assays performed with oat silage and NaOH-treated wheat straw under the same experimental conditions as for oat hay and lucerne hay given above. In Fig. 4.18 oat silage shows a ca. 8 and 13 %, and NaOH-treated wheat straw a ca. 6 and 10 % increase in DM and NDF digestibility, respectively. However, both their NDF values are higher than the DM values, which indicate possible inconsistencies within the *in vitro* assay and the rumen fluid. Again, the effect of enzyme addition to the feed samples 24 hours prior to the *in vitro* digestibility assay was evaluated (Fig. 4.19). Oat silage showed DM and NDF digestibility increases of ca. 43 and 70 %, respectively, with no enhanced effect for NaOH-treated wheat straw (ca. 10 % decrease in digestibility). However, the percentage digestibility increases are ca. 10 to 20 % lower for oat silage in Fig. 4.19 relative to that shown in Fig. 4.18. Also, all the NDF values are higher than the DM values, indicating that both the results shown in Figs. 4.18 and 4.19 are inconclusive and therefore are not an indication of the real effect that Celluclast have on the feed samples under study.

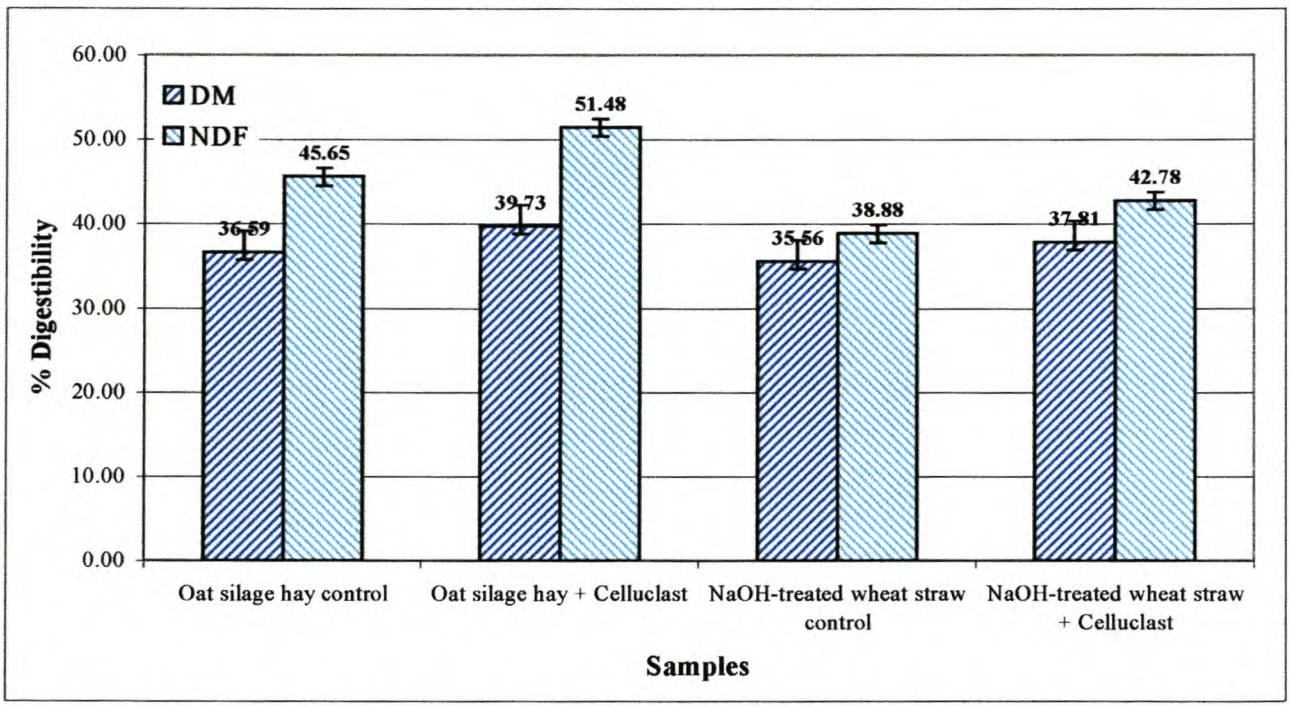


FIGURE 4.18. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of oat silage and NaOH-treated wheat straw after Celluclast addition to rumen fluid. The enzyme was added at enzyme levels of ca. 443U xylanase and 4U cellulase per gram feed (52 mg protein per gram feed). Error bars indicate the standard deviation of triplicate values.

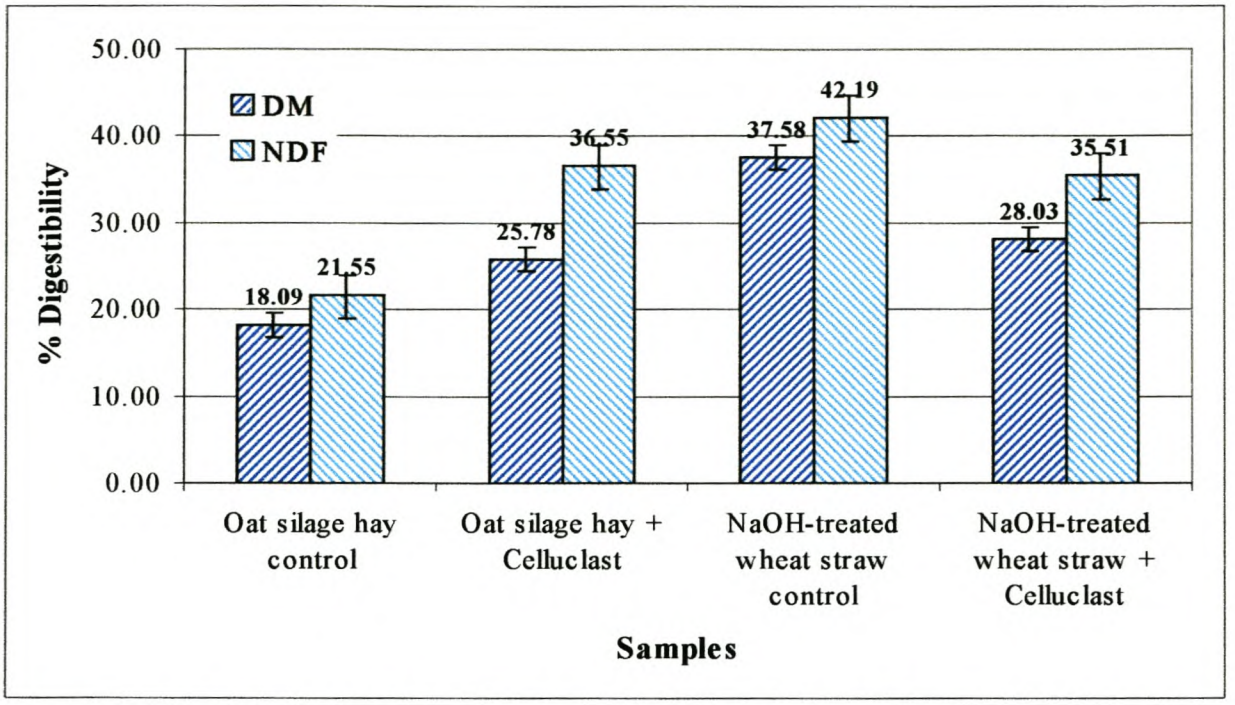


FIGURE 4.19. Mean digestibility of dry matter (DM) and neutral detergent fibre (NDF) of oat silage and NaOH-treated wheat straw after Celluclast addition, 24 hours prior to the *in vitro* assay. The enzyme was added at enzyme levels of ca. 443U xylanase and 4U cellulase per gram feed (52 mg protein per gram feed). Error bars indicate the standard deviation of triplicate values.

4.5. ENZYME HYDROLYSIS WITH CELLUCLAST

As a result of the inability to show conclusively that enzymes could improve the digestibility of various feeds, an attempt was made to establish whether an enzyme preparation such as Celluclast is able to hydrolyse feed samples in the absence of rumen fluid. Five feed samples were evaluated and sugarcane bagasse was included as a poor digestible feed. The results are shown in Figs. 4.20 to 4.24 and the data is summarised in Tables 4.8 and 4.9.

Sugarcane bagasse hydrolysis reached saturation at a lower enzyme level than the other feed samples, but lower amounts of reducing sugars was released. All the feed samples tested show optimal values with the 520 mg protein treatments. Oat hay, lucerne hay, and oat silage show the highest release of reducing sugars, with the two low quality feeds, sugarcane bagasse and wheat straw performing the poorest. The protein levels used in the *in vitro* experiments with Celluclast (52 mg per gram feed) showed significant hydrolysis and release of reducing sugars. This shows that Celluclast would be able to contribute to the digestibility of feed. The failure to observe significant *in vitro* digestibility suggest that the enzymes might be inactive in the assay. This requires further investigation.

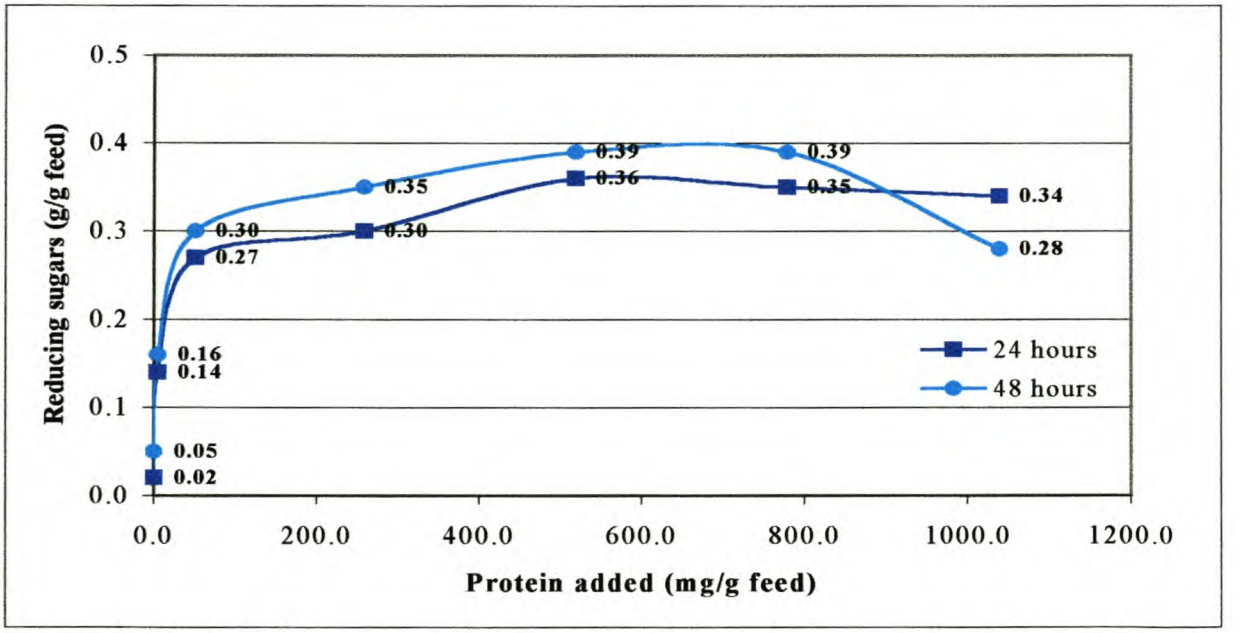


FIGURE 4.20. Release of reducing sugars from sugarcane bagasse (g/g feed) via Celluclast addition after 24 and 48 hours incubation at 50 °C. Values are plotted as the mean of duplicate determinations. Controls (no enzyme added) showed minimal release of reducing sugars (data not shown).

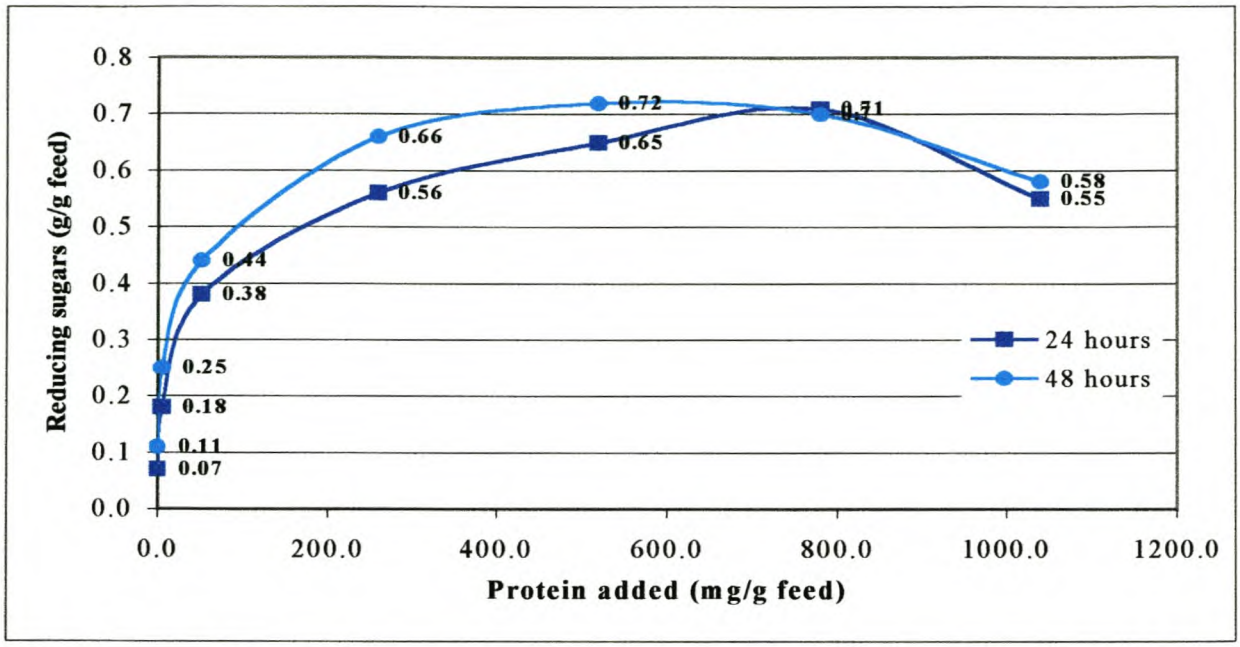


FIGURE 4.21. Release of reducing sugars from lucerne hay (g/g feed) via Celluclast addition after 24 and 48 hours incubation at 50 °C. Values are plotted as the mean of duplicate determinations. Controls (no enzyme added) showed minimal release of reducing sugars (data not shown).

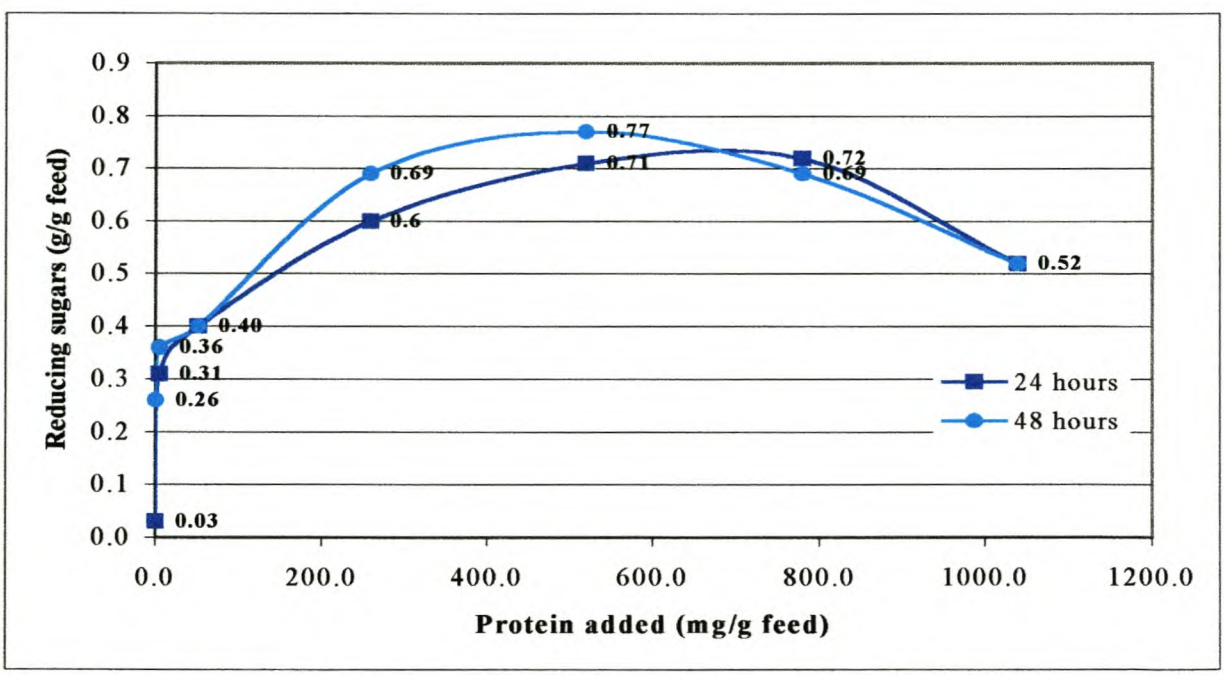


FIGURE 4.22. Release of reducing sugars from oat hay (g/g feed) via Celluclast addition after 24 and 48 hours incubation at 50 °C. Values are plotted as the mean of duplicate determinations. Controls (no enzyme added) showed minimal release of reducing sugars (data not shown).

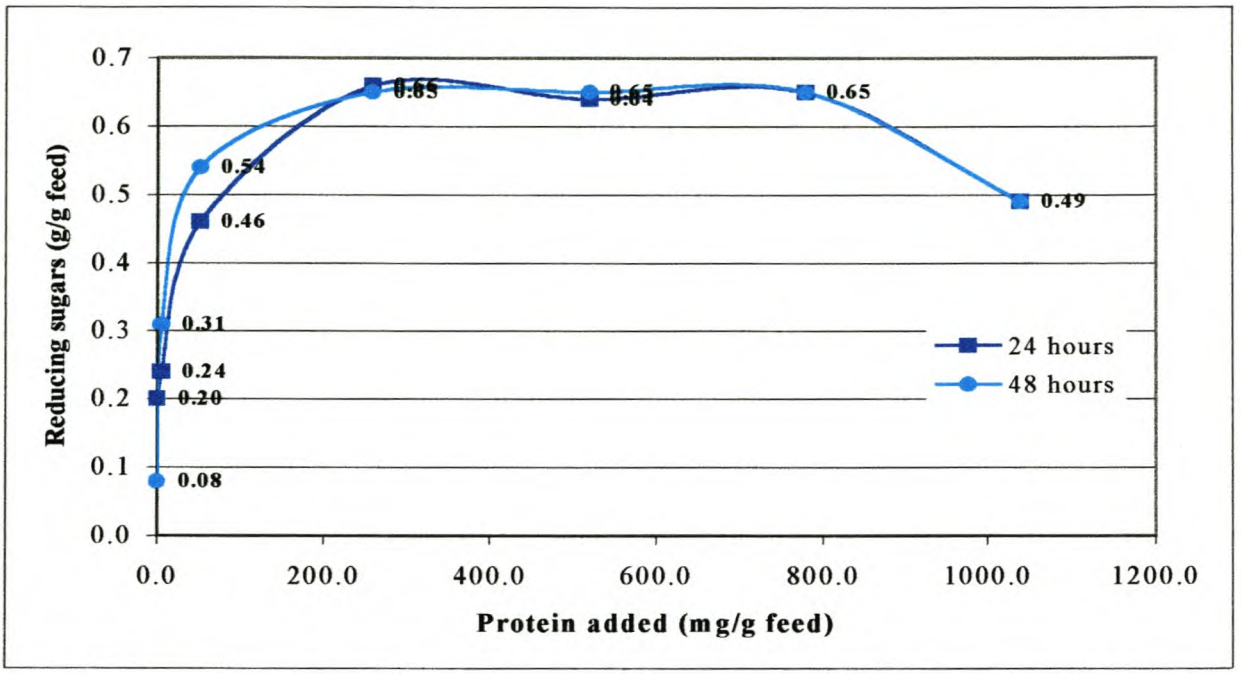


FIGURE 4.23. Release of reducing sugars from oat silage (g/g feed) via Celluclast addition after 24 and 48 hours incubation at 50 °C. Values are plotted as the mean of duplicate determinations. Controls (no enzyme added) showed minimal release of reducing sugars (data not shown).

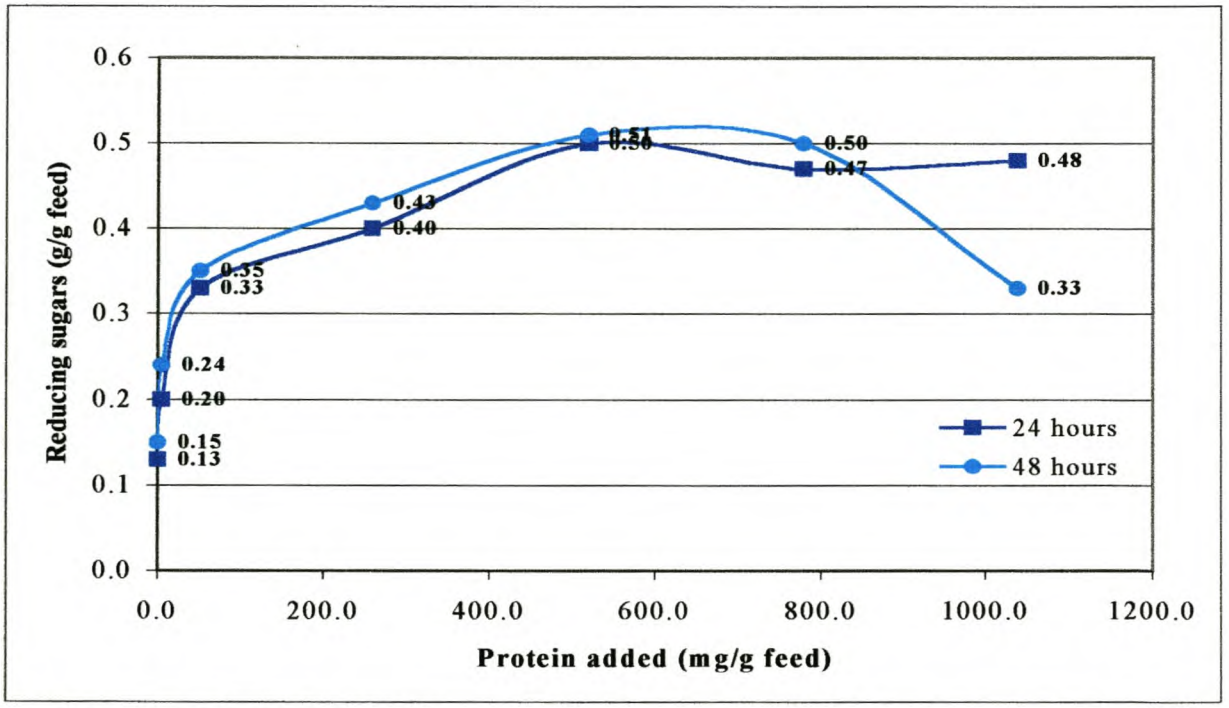


FIGURE 4.24. Release of reducing sugars from NaOH-treated wheat straw (g/g feed) via Celluclast addition after 24 and 48 hours incubation at 50 °C. Values are plotted as the mean of duplicate determinations. Controls (no enzyme added) showed minimal release of reducing sugars (data not shown).

TABLE 4.5. Release of reducing sugars from ruminant feeds (g/g feed) via Celluclast addition after 24hr incubation at 50 °C.

Feed sample	Enzyme added (mg protein per gram feed)							
	0.00	**0.52**	**5.2**	**52**	**260**	**520**	**780**	**1040**
Sugarcane bagasse	0.00±0.00	0.02±0.01	0.14±0.00	0.27±0.02	0.30±0.01	0.36±0.03	0.35±0.08	0.34±0.20
Lucerne hay	0.01±0.00	0.07±0.02	0.18±0.01	0.38±0.02	0.56±0.09	0.65±0.02	0.71±0.22	0.55±0.03
Oat hay	0.08±0.05	0.03±0.00	0.31±0.00	0.40±0.05	0.60±0.00	0.71±0.05	0.72±0.20	0.52±0.09
Oat silage	0.00±0.00	0.20±0.01	0.24±0.02	0.46±0.01	0.66±0.03	0.64±0.08	0.65±0.02	0.49±0.08
NaOH-wheat straw	0.00±0.00	0.13±0.02	0.20±0.01	0.33±0.01	0.40±0.04	0.50±0.005	0.47±0.07	0.48±0.20

Values are indicated as the mean of duplicate determinations ± range of variation.
Control values are shown as 0 mg protein added per gram feed.

TABLE 4.6. Release of reducing sugars from ruminant feeds (g/g feed) via Celluclast addition after 48hr incubation at 50 °C.

Feed sample	Enzyme added (mg protein per gram feed)							
	0.00	**0.52**	**5.2**	**52**	**260**	**520**	**780**	**1040**
Sugarcane bagasse	0.00±0.00	0.05±0.01	0.16±0.01	0.30±0.03	0.35±0.04	0.39±0.14	0.39±0.03	0.28±0.01
Lucerne hay	0.08±0.02	0.11±0.01	0.25±0.03	0.44±0.03	0.66±0.03	0.72±0.01	0.70±0.01	0.58±0.01
Oat hay	0.19±0.05	0.26±0.00	0.36±0.00	0.40±0.04	0.69±0.03	0.77±0.02	0.69±0.09	0.52±0.10
Oat silage	0.02±0.00	0.08±0.01	0.31±0.05	0.54±0.00	0.65±0.02	0.65±0.05	0.65±0.20	0.49±0.20
NaOH-treated straw	0.00±0.00	0.15±0.00	0.24±0.02	0.35±0.01	0.43±0.01	0.51±0.05	0.50±0.06	0.33±0.01

Values are indicated as the mean of duplicate determinations ± range of variation.
Control values are shown as 0 mg protein added per gram feed.

CHAPTER 5

DISCUSSION

5. DISCUSSION

The results obtained during this study failed to consistently show that enzyme supplements improve the digestibility of all the feed samples evaluated. However, all the enzyme preparations showed diverse enzyme activities with mainly xylanase and cellulase activities. The crude enzyme preparations and Celluclast had high xylanase and cellulase levels. However, the commercial fibrolytic enzyme Fibrozyme had low activity for all the enzymes tested.

Fibrozyme addition to both wheat straw and lucerne hay did not improve their respective *in vitro* digestibilities. Possibly the enzyme activity is not optimal for the two feeds' composition, or the pre-incubation period of 2 hours is too short, relative to a 12 hour incubation period that gave a ca. 44 % increase in the *in vitro* dry matter disappearance of a 100 % grass hay diet in the studies of Howes *et al.* (1998). In the studies of Tricarico *et al.* (1998), Fibrozyme showed a ca. 44 % increase in the *in vitro* dry matter disappearance of a grass hay diet, but no effect on a 50 % concentrate diet. In addition, Fibrozyme supplementation to a basal diet increased the ruminal NDF digestion of Holstein steers by ca. 23 % and by ca. 4.5 % in the DM intake of crossbred steer calves (Howes *et al.*, 1998). In conclusion, it is apparent that while some reports show an improvement in feed digestibility by addition of Fibrozyme, in our hands this improvement was not consistent. These differences could be due to feed composition such as high lignin content, or presence of pectins that make the feed recalcitrant to enzymatic hydrolysis (Buxton and Redfearn, 1997).

Crude enzyme supplementation to both wheat straw and lucerne hay gave inconsistent results. Possible reasons for no improvement in the *in vitro* digestibility might be: 1) the enzyme levels are too low; 2) the enzymes might not be resistant to microbial degradation in the rumen, and 3) the *in vitro* assay protocol used is too unreliable. Hristov *et al.* (1998), indicated that CMC-ase and xylanase activities could be resistant to microbial degradation in the rumen, but is dependent on the enzyme source, and also the specific enzyme activities contained within the crude enzyme preparation (Hristov *et al.*, 1998). Morgavi *et al.* (2000), also found that the β-1,4-endoglucanase activity of *Aspergillus niger* remained stable for ca. 6 hours after incubation in rumen fluid. The β-1,4-endoglucanases *Trichoderma viride* and *Irpex lacteus* had half-lives of ca. 2 hours and ca. 4 hours, respectively. The xylanase activity of both *A. niger* and *T. viride* remained stable for more than 6 hours, while *I. lacteus* lost 60 % of its activity after ca. 2 hours of incubation in rumen fluid. The β-glucosidase and β-xylosidase activities of all three organisms were unstable in rumen fluid, particularly those of *T. viride* (Morgavi *et al.*, 2000). Therefore, crude enzyme preparations apparently remain stable long enough for digestion by the enzymes to occur. However, it is of utmost importance that the enzymes be applied to the feed prior to incubation, to establish a significant enzyme-feed complex (Rode and Beauchemin, 1998).

Enzyme activity is normally measured at optimal pH, which generally differs from that of rumen fluid. In principle, this means that exogenous enzymes are likely to contribute less fibrolytic activity than estimated, after been incubated in the rumen (Beauchemin and Rode, 1997). In addition, the change in pH (when differing from the optimal pH of an enzyme) can also mean that enzyme supplements first have to adapt to the rumen environment. The same applies for a change in temperature. These adaptations can therefore be time-consuming and enzyme supplements can be completely degraded before adapting to its new environment. Therefore, by increasing the interval between applying the enzyme to the feed and feeding creates a stable enzyme-feed complex that increases the enzyme's effectiveness (Beauchemin and Rode, 1997).

Celluclast supplementation gave marginal increases in the *in vitro* feed digestibility of oats hay, oats silage, and NaOH-treated wheat straw. However, it was supplemented at ca. 200-fold higher concentrations relative to the crude enzyme preparations. Celluclast have optimal activity at about 50 to 60 °C and a pH of 4.5 to 6.0 (Celluclast information sheet). *In vitro* digestibility assays were performed at 39 °C and pH 6.5, which meant that Celluclast's activity was not optimal. Optimal enzyme activity conditions therefore needs to be taken into account when deciding on using enzymes as feed supplements, relative to the conditions within the rumen environment. This might explain why supplemented Celluclast did not enhance the *in vitro* digestibility significantly, because it had to deal with a poor substrate in its new environment, as well as functioned below its optimal activity conditions.

Enzyme hydrolysis with Celluclast was optimal at the same enzyme concentrations for all the feed samples tested. Oats hay, lucerne hay, and oats silage showed the highest release of reducing sugars, whereas the 2 low quality feeds, sugarcane bagasse and wheat straw performed the poorest. Colombatto *et al.* (1999) found that a commercial enzyme preparation, containing mainly xylanase and cellulase activities, enhanced the hydrolysis of maize silage and a total mixed ration. The optimum pH appeared to be 4.5 for hydrolysis of all the feeds used (Colombatto *et al.*, 1999). In the current study higher hydrolytic increases was obtained for all the feed samples evaluated, relative to the results of Colombatto *et al.* (1999). This indicates that Celluclast was a better enzyme product than the one used in their studies. Also, this result was more positive than the *in vitro* digestibility analysis during this study, because Celluclast supplementation improved all the feed samples' nutritive values. This might be due to the fact that enzyme hydrolysis was performed at 50 °C and pH 4.5, which is in the enzyme's optimal activity range (50 °C, pH 4.5 to 6.0). In addition, the enzyme hydrolysis experiments were done in a buffer solution, whereas the *in vitro* digestibility analysis took place in a rumen solution that varied over time. Therefore, enzyme hydrolysis experiments should probably exceed the *in vitro* digestibility analysis to evaluate an enzyme's effect on feed samples at its optimal conditions.

The *in vitro* digestibility assays in this study showed too much variation to yield reliable data and this suggest that alternative assay methods should be considered. This should include the *in vivo*, *in sacco*, and *in situ* methods of analysis, as well as gas production (CO_2, CH_4 and traces of H_2) measurement (Stern *et al.*, 1997). Another alternative would be the DAISY^{II} system, which contains four 4 litre digestion vessels that slowly rotate in a digestion chamber maintained at 39.5 °C (Holden, 1999). However, these methods have the disadvantage of being expensive and more time consuming.

In future a more detailed study of feed digestibility should be investigated by determining which feeds are hydrolysable, their chemical composition, i.e. how accessible the feeds are, and also evaluation of feed mixtures. The enzyme supplements also need to be evaluated for optimum temperature and pH, as well as the compilation of enzyme cocktails.

CHAPTER 6

REFERENCES

6. REFERENCES

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