Fibrolytic enzyme activity of herbivore microbial ecosystems

By

FABIAN NDE FON

Submitted in partial fulfilment of the academic requirements for the degree of

Master of Science in Agriculture

In the

Discipline of Animal and Poultry Sciences

School of Agricultural Sciences and Agribusiness

Faculty of Science and Agriculture

University of KwaZulu-Natal

Pietermaritzburg

2006

ABSTRACT

The aim of this study was to determine firstly if there exist variations in fibrolysis among herbivore microbial ecosystems and secondly, the effect on fibre hydrolysis of compositing the most active systems with ruminal microbial ecosystem harvested from a Jersey cow. A literature review pointed to the complexity of carbohydrate (fibre) and how the physical and chemical nature of the forage carbohydrate can present barriers that hinder digestion in the rumen, especially its association with hemicelluloses, pectin, lignin and tannins.

Fresh rumen fluid was collected from fistulated herbivores (Jersey cow and sheep) and faecal samples from non-fistulated herbivores (buffalo, horse, impala, camel, elephant, llama, sheep, wildebeest and elephant). Crude protein samples were precipitated with 60% ammonium sulfate. Sample activities were monitored and optimised by incubating with carboxymethyl cellulose (CMC) for 2 h at 39°C. The crude protein samples precipitated from the 11 herbivore microbial ecosystems were active. This was confirmed by an increase in enzyme specific activity with a decrease in total crude protein concentration. *In vitro* pH optimisation showed a broad range of activity for all ecosystems (4.5-8.0) but for the zebra, horse and elephant which peaked at pH 5.

In experiment two (Chapter 4), seasonal variation of the enzymes (exocellulase, endocellulase, cellobiase and xylanase) were monitored through winter and summer. Enzyme specific activity of exocellulase, endocellulase, cellobiase and xylanase were determined by incubation with the specific substrates, crystalline cellulose, CMC, pNPG and xylan, respectively. The amount of reducing sugar released was used to determine the enzyme specific activity. Exocellulase analysis was suitable in winter while summer was preferred for carboxymethyl cellulase and xylanase due to their relative abundance. Cellobiase analysis did not depend on any particular season.

Eleven herbivore microbial ecosystems were characterised according to their fibrolytic enzyme specific activities. Enzyme catalytic activities were calculated from kinetic parameters (Km and Vmax) obtained from Eisenthal and Cornish-Bowded

plots (Chapter 5). Fibrolytic enzyme expression as well as their activities differed among the 11 ecosystems ($P<0.0001$). They were classified into three groups based on fibrolytic enzyme concentrations; group A with high enzyme concentrations (horse, impala, zebra, wildebeest and the elephant), group B with intermediate (cow, llama, camel, buffalo and giraffe) and group C with low enzyme concentrations (sheep). Exocellulase activity was reasonably correlated with endocellulase activity ($r = 0.8978$). Xylanase activity was also correlated with carboxymethyl cellulase actvity ($r = 0.7104$). Enzyme kinetic studies revealed that crude protein samples from the horse, zebra, wildebeest and elephant had the highest enzyme catalytic activities.

Microbial or enzyme composite systems were created from the most active ecosystems (horse, wildebeest and zebra) in an attempt to improve the Jersey cow system. These systems were B (cow and horse), C (cow and wildebeest), D (cow and zebra) and E (cow, horse, zebra and wildebeest). The specific activities and enzyme efficiencies of these new systems were determined and compared with system A (cow). Microbial synergism of these systems was also investigated by measuring the amount of gas produced and true degradability (TD) after 72 h of incubation. The composite systems B and E were the most active fibrolytic enzyme systems while C and D were intermediate when compared to that of A. *In vitro* microbial synergism assays showed that systems B, D, and E had the highest potential of improving milky maize stover (MM) and nutral detergent fibre (NDF) fermentation and degradability in Jersey cows.

It was concluded that: (i) fibrolytic and hemicellulolytic enzyme concentrations vary from one season to another with the changing forages; (ii) microbial fibrolytic activities vary among animals grazing on the same field or different geographical regions; and (iii) lastly microbial synergisms of active ecosystems have the potential of improving fibre hydrolysis. However, there is a need to conduct *in vivo* experimentation to determine the real potential of these *in vitro* observations.

DECLARATION

The experimental work described in this dissertation was carried out in the Discipline of Animal and Poultry Sciences, School of Agricultural Sciences and Agribusiness, Faculty of Science and Agriculture, University of KwaZulu-Natal, Pietermaritzburg Campus, under the supervision of Prof. Nsahlai I.V. and co-supervision of Prof. Dennison C. and Dr. Beukes M.

This is to declare that this thesis has been composed by me and has not been presented in any previous application for a degree. All sources of information are shown in the text and listed in the reference and all assistance by others has been duly acknowledged.

FON F.N
UNIVERSITY OF KWAZULU-NATAL
PIETERMARITZBURG CAMPUS

Signed..... Date 19 MAR 2007

I, Prof. Nsahlai I.V., Chairperson of the Supervisory committee, approved the release of this thesis for examination

Signed ..... Date 19 MAR 2007

Acknowledgement

Many people contributed to the success of this research and it may be unjust to single out individuals. However, I would like to express my sincere and profound gratitude to the following persons and institutions:

My supervisor, Prof. Nsahlai I.V. for his guidance, constructive comments and important sources of information provided throughout this study. His patience and support during rainy days was a source of inspiration.

I am very appreciative of my co-supervisors, Dr. Beukes M. and Prof. Dennison C. for their constructive criticism, advice and encouragement throughout this study.

The Department of Animal and Poultry Sciences for providing research facilities.

The staff at the Ukulinga Research farm for providing experimental animals and their support throughout this period.

The Tala Game Reserve, Umbumbulu, KwaZulu-Natal (SA) for providing most of the experimental animals in this study.

To all my friends, thanks for your help and support especially Mbu D., Ouda J., Achilonu I., Norbert N., Kakundi E. and Joan N.

I would also like to appreciate my late dad Mr. Forcob F. and my Brother Fon F. for making my studies possible in South Africa.

Finally, I would like to thank my treasured wife, Yvonne Jabusun, for her patience and all the moral support she has been offering to me. Thanks for being there for my daughter Shalome and the rest of the family in my absence.

CONFERENCE ABSTRACTS

1. **Fon F.N Nsahlai I.V Beukes M. and Dennison C.** (2006). Activities of microbial cellulolytic and hemicellulolytic enzymes from 11 herbivore microbial ecosystems. *South African Society of Animal Sciences*, 41st Congress. Odeion University of Free State, Bloemfontein, 3 April 2006. pp 110

2. **Fon F.N Nsahlai I.V Beukes M. and Dennison C.** (2006). Activities of microbial cellulolytic and hemicellulolytic enzymes from 11 herbivore microbial ecosystems. *Faculty of Science and Agriculture Postgraduate Research Symposium.* University of KwaZulu Natal Pietermaritzburg Campus, 19 October 2005.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of abbreviations

MM = milky maize stover

CMC = carboxymethyl cellulose

CMCase = carboxymethyl cellulase

TD = true degradability

Km = enzyme kinetic constant

Vmax = Rate of product formation

VFA = volatile fatty acids

CS = concentrate selector

GR = grass and roughage selectors

IM = intermediate selectors

MS = maize stover

Vo = initial velocity

Kcat = enzyme catalytic rate

CoA = acetyl coenzyme A

TCA = tricarboxylic acid cycle

EC = enzyme commission number

*p*NP = *p*-nitrophenol

*p*NP = *p*-nitrophenyl β-D-glucopyranoside

DNS = dinitrosalicylic

DM = dry matter

APD = apparent degradability

MYD = microbial yield

NDF = neutral detergent fibre

Chapter 1

General introduction

1.0 Background

In order for all mechanisms as well as living systems to operate they require energy, which may be food, chemical, kinetic or solar energy. Food energy is the most common form of energy utilised by living organisms with carbohydrates being the most abundant and main source of metabolic energy for plants and animals. Carbohydrates are known to be the major product through which the energy of the sun is harnessed and converted into a form that can be utilised by man and other animals. Therefore they are the primary products of photosynthesis. Photosynthesis is an endothermic reduction condensation of carbon dioxide that harnesses light energy in the presence of the plant pigment chlorophyll to produce carbohydrates and oxygen (Equation 1).

Equation 1.0

$$nCO_2 + nH_2O + \text{energy (light)} \longrightarrow C_nH_2O_n + nO2$$

n = natural numbers

Although carbohydrates are known to be the major source of energy for plants and animals, they also play a vital role as structural components in the plant cell wall (cellulose) as well as in the animal cell membrane (glycolipids). They serve as components of recognition sites on cell surfaces (glycoprotein, e.g. immunoglobulins) and as essential components of DNA and RNA molecules (ribose sugars) (Freier and Karl-Heinz, 1997), which are informative molecules.

Carbohydrates are defined as carbon hydrates with respect to their molecular formula $C_n(H_20)_n$. Peter (1990) further separated them chemically into hydroxyl aldehydes or ketones and their derivatives. Simple carbohydrates are called saccharides or sugars (e.g. glucose, ribose and galactose) while the complex forms include cellulose, starch, glycogen and hemicelluloses. They have also been classified as soluble or non-soluble carbohydrates depending on their interaction with water molecules. Plants are the major source of carbohydrates with cellulose (fibre) being the most plentiful polysaccharide in nature (Schwarz, 2001), contributing 200-400 g/kg of most

abundant plant tissue (Van Soest, 1982). Because of cellulose complexity the rate of consumption varies from one animal to another.

Ruminal microbes are responsible for fibre breakdown in ruminant forestomach. These microbes range from bacteria to protozoa and fungi (Flint, 1994; Fonty and Gouet, 1994; Jouany and Ushida, 1994). They are said to be anaerobic since the rumen environment is known to be oxygen deficient (Kamra, 2005). However, Brooker *et al.* (1994) reported that the rumen environment is not completely anaerobic, as it is inhabited by facultative bacteria such as *Streptococcus caprznus* which help to eliminate traces of oxygen entering the rumen.

The distribution of fibre (cellulose) in plant tissue varies from one plant species to another as well as in different parts of the same species (leaves, stems and fruits) (Ouda *et al.*, 2006). Due to these differences, animals have evolved to select and consume different components of plants and vegetation depending on their mouth parts and fractionations of their fore- and hindgut fermentation chambers. There are a large number of extant species of ruminants found in different ecosystems harbouring a variety of microbes. Hofmann (1989) demonstrated that flora variation among ecosystems as well as seasonal availability of forages influence the nutritional habits of these animals. He classified the extant species according to the physiological evidence obtained from fibre hydrolyses into a flexible system of three overlapping morphological feeding types: concentrate selectors (CS), intermediate selectors (IM) and grass and roughage selectors (GR).

Concentrate selectors (e.g. giraffe) feed on easily digestible forages such as shrubs and fruits while GR graze mainly on grasses (e.g. buffalo and cattle). Intermidiate selectors selectors alternate between CS and GR (goat). According to Hofmann (1989), climatic conditions, habitat pressure, behavioural patterns and ecological opportunities have a great influence on ruminant diversity. Despite the variation of the above factors ruminants have been able to maintain two generic futures such as a complicated morphological master plan of the ruminant digestive system and an incredible flexible adaptation that extends from the level of one animal to another. As one moves from CS to GR some glands, such as the salivary gland regress while the

rumen cavity increases in size. These adaptations are flexible, such that seasonal variations with the availability of forages are very common, e.g. increase gland secretion in browsers such as goats in summer (Hofmann, 1998).

Fibre hydrolysis in ruminants has been found to be a major problem as very little of the total intake is converted into food energy (Varga and Kolver, 1997). Less than 30% of the total fibre consumed by ruminants is converted into chemical energy while the rest is passed out as waste products. This implies that metabolic substrates are far less than that which is lost in faeces. Therefore, this review scans the complexity of fibre, its association with other components of the plant cell wall, factors influencing its hydrolysis and different fibre additives. At the end of the review a possible mechanism will be hypothesized attempting to improve fibre hydrolysis with regards to the available information acquired from the variable substrates and microbial activity from different ecosystems.

1.1 Overall objective

To identify microbial ecosystems with high fibrolytic activities and investigate their fibrolytic potential when merged with that of the jersey cow.

1.2 Specific objectives of this study

The main specific objectives of this study were to:

- Evaluate the seasonal variation of fibrolytic enzymes from seven herbivores microbial ecosystems.
- Evaluate the activities of microbial fibrolytic enzymes from 11 herbivore microbial ecosystems.
- *In vitro* manipulation of Jersey cow rumen ecology with enzymes or microbes from the most active ecosystems.

Chapter 2

Literature review

2.0 Carbohydrates

Carbohydrates, as previously mentioned, are a large group of compounds that can be separated chemically into hydroxyl aldehydes or ketones and their derivatives. However, definitions such as polyhydroxyl aldehydes or ketones are very common. Whether hydroxyl or polyhydroxyl they both contain the aldehydes (R-CHO) or ketone (2RCO) functional group. Carbohydrates are represented by the general formula ($C_nH_{2n}O_n$) although some authors still stick to the empirical formula representation, CH_2O. They are classified as reducing or non-reducing sugars due to their ability to reduce metallic ion (Fehling's solution). For simplicity carbohydrates have been divided into two broad groups known as simple sugars and complex sugars (polysaccharides).

Sugar is the basic building block of carbohydrates, which are sweet, crystalline in nature and soluble in water. Simple sugars are subdivided into monosaccharides and oligosaccharides. Monosaccharides are the simplest form and cannot be broken down into simpler units. They could either be trioses (dihydroxyl acetone), tetroses (erythrulose), pentoses (ribose) or hexoses (glucose and fructose). Glucose is present as a free molecule in biological systems and as a component in disaccharides (cellobiose) as well as in polysaccharides (cellulose, starch and glycogen). Oligosaccharides are made up of two to ten saccharide units, which may be present as a free molecule in biological systems or associated with other molecules (Robert and Robert, 1980). For example, lactose is present as a free molecule in biological systems such as that of *Escherichia coli* (Huber and Hurlburt, 1984). There are many different types of disaccharides with sucrose being the most common (O-β-D-glucopyranosyl (1, 4)-β-D-glucopyranose). Disaccharides can either be obtained from the reaction of two saccharide units or from the hydrolysis of a polysaccharide, e.g. oxidative reduction of two glucose units to sucrose or by hydrolysing fibre molecules with the aid of biological enzymes to yield cellobioses.

Polysaccharides or complex carbohydrates are high molecular weight macromolecules constructed by linking more than ten saccharide units. Acid-catalysed hydrolysis of polysaccharides into saccharide units demonstrated that polysaccharides are made up of thousands of monosaccharides. Although there are many types of complex carbohydrates the most common ones are starch (plants), glycogen (animals) and cellulose (plants and animals), which are all polymers of glucose. The difference among these macromolecules appears to be the type of glycosidic bonds holding the different glucose units together. These bonds could either be alpha or beta bonds.

Starch is the most abundant form of food energy consumed by monogastric animals whereas glycogen is the storage form of this energy in animals. Partial hydrolysis of starch produces the product maltose, low molecular weight dextrans and polysaccharides. Analysis of the by-product maltose shows that the two glucose monomers are joined by an α-glycosidic bond between C-1 and C-4 with α-glycosidic bonds between C-1 and C-6 of the branched polysaccharides. Therefore starch is a complex macromolecule that can be divided into two sub groups called amylose and amylopectin. Amylose is a straight chain polymer of glucose linked together by alpha glycosidic bonds between C-1 and C-4. The intact granules of amylose are insoluble whereas grinding and boiling causes it to become soluble. Amylopectin is of higher molecular weight with approximately a million glucose units. The majority of the existing starch is made up of amylopectin. Alpha glycosidic bonds between C-1 and C-4 hold linear polymers of amylopectin while the branched chains are held by the same bonds but between C-1 and C-6 (Fahey and Berger, 1993). Each branched chain occurs after every 25 glucose units. Amylopectins are insoluble in water due to its tightly bonded structure limiting water molecule penetration. There are many other forms of polysaccharides built from monomers other than glucose e.g. mannose, xylose and arabinose. However some polysaccharides have more than one monomeric unit.

Other carbohydrates include chitin, which is the main component of arthropod shells e.g. crabs, hemicelluloses found in plant cell walls e.g. xylan and gums secreted by some plants for protection with sub units such as D-glucoronic acid, D-galactose and L-arabinose. Complex carbohydrate derivatives include glycoproteins e.g.

immunoglobulins, fibrinogen, glycolipids and nucleotides [ribonucleic acid (RNA) and deoxyribonucleic acids (DNA)]. There are a variety of functions that have been associated with carbohydrate derivatives depending on the specific type e.g. glycoproteins such as immunoglobulins have a protective function. DNA and RNA, which are carbohydrate derivatives, are informative molecules in the system. They carry genetic information passed by an organism to its offspring. Because this review will be focusing on fibre and factors affecting its fermentation, only the main complex carbohydrate will be examined closely.

2.1 Fibre (Cellulose)

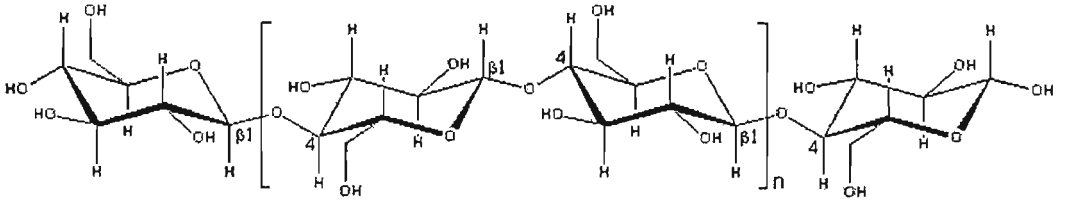

Figure 2.0: The structural unit of cellulose

N = number of disaccharide units in a cellulose molecule [(Martin, 2006) modified]

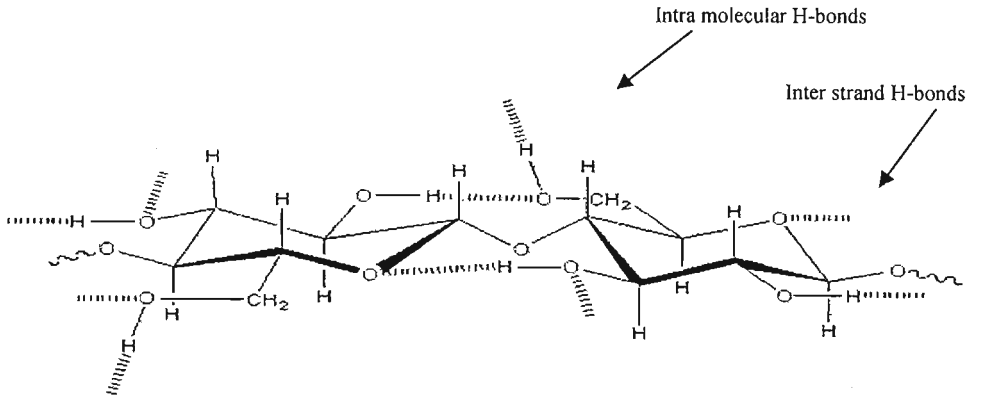

Figure 2.1: Crystalline structure of cellulose (Martin, 2006) modified.

There are many sources of cellulose, with cotton fibre containing cellulose of higher purity while those from wood and grass are of lower purity. Acid hydrolysis of cellulose yields glucose units providing evidence that cellulose comprises glucose monomers. Partial hydrolysis of cellulose produces cellobiose (disaccharide), which is linked by a beta glycosidic bond between C-1 and C-4. This led to the definition of

cellulose as an unbranched polymer of thousands of glucose units (Figure 2.0) linked to each other by beta 1, 4 glycosidic bonds (Ilan *et al.*, 2002). They are generally represented by a molecular formula $(C_6H_{10}O_5)_n$. Although the main source of complex carbohydrates is from plants some bacteria do produce celluloses e.g. *Escherichia coli* (Charles, 2004) as well as algae. Unlike in plants where 50-70% of the cellulose is crystalline, bacteria produce approximately 80% crystalline cellulose (Ross *et al.*, 1991). Irrespective of the different sources of cellulose they are all the same at the molecular level although different in structural orientation (Margaretha *et al.*, 2004). Therefore, there are basically two types of cellulose, namely crystalline and amorphous cellulose (Groleau and Forsberg, 1981).

Crystalline celluloses are characterised by the type of hydrogen bonds that are formed between C-3 hydroxyl group and the pyranose ring within the same molecule (intra molecular hydrogen bonding) and those formed between the C-6 hydroxyl group of one molecule and the oxygen of the glycosidic bond of another molecule (intra-strand) (Figure 2.1). The presence of hydrogen bonds between these fibre molecules is responsible for the tightly packed crystalline structure. Celluloses are insoluble due to the inability of water molecules to penetrate the tightly packed crystalline structures (Ross *et al.*, 1991). Amorphous cellulose is relatively soluble in water because it allows the penetration of water molecules due to fewer frameworks of hydrogen bonds.

2.2 The cell wall

In animals the cell membrane plays a vital role in the protection of cells whereas in plants its protective function is strengthened by the incorporation of a rigid cell wall. The cytoplasmic content of each cell requires some form of protection and this is often rendered by the cell membrane. Plant cells, like any other eukaryotic cells, have a similar structure (e.g. cytoplasmic organelles, nucleus genetic material and a plasma membrane). The name, cell wall, is not universal to plants as bacteria, fungi and algae do have it. Bacterial cell walls are made primarily of peptidoglycans which determine the shape and provide the osmotic pressure balance between the intercellular fluid and its vicinity. Variations in bacterial cell walls have led to the classification of bacteria

into two major groups by Gram (1884), Gram-negative bacteria (dual membrane system e.g. *Escherichia coli*) and Gram-positive bacteria (single membrane system surrounded by a much thicker cell wall). In fungi the main polysaccharide in the cell wall is chitin (the monomeric unit is n-acetylglucosamine). Interestingly, it is also the monomeric unit in the exoskeleton of arthropods (crabs). Primitive plants and higher plants contain cellulose as their main polysaccharide (monomeric unit glucose).

The strength of the plant cell wall is attributed to its multicellularity, paving the way for a rigid form by providing a variation in thickness, chemical composition as well as spatial distribution of cells within the tissue. The name 'cell wall' may sound simple but it is as complex as any other tissue system. It can be subdivided into primary, secondary and tertiary cell wall. These layers will be discussed with an emphasis on the macromolecules that are involved in or affect digestibility in ruminants. Leguminous forages as well as vegetative parts of herbaceous plants and grasses will be accorded prominence. Primary cell walls are formed when expansion of a cell leads to the deposition of components of the cell wall on the plasma membrane. Pectins and hemicelluloses are synthesized in the golgi apparatus before secretion (Hillman *et al.*, 1986) while cellulose is synthesized on the plasma membrane by the enzyme complex, cellulose synthase. Newly synthesized cellulose micro fibrils are deposited at a vertical angle to the direction of cell elongation in a gel-like matrix of interlinked molecules. Macro fibrils comprise micro fibrils, which are made up of about 2000 cellulose chains (fibril). The orientation of fibrils in an upright position is said to play a vital role in cell expansion as well as cell growth.

The distribution of celluloses, hemicelluloses and pectins in the matrix is almost equal. Cells, e.g. parenchyma and meristematic cells, embedded in this matrix still have the ability to grow and expand since the matrix is still flexible and allows for the exchange of material with relative ease. Hemicelluloses are highly branched polysaccharides with saccharide units xylose, mannose and glucoronic acid. They are hydrogen bonded to the surface of cellulose microfibrils forming a network of tough fibre molecules, rendering mechanical strength to the plant cell wall. Pectins are branched polysaccharides with the saccharide units galactose, arabinose and

galactorunic acid.They contain a high number of negative charges allowing it to bind with positively charged ions such as those of calcium ions (Ca^{2+}). The presence of such ions traps water molecules, causing the jelly-like nature of primary cell walls which are often observed in meristematic tissues (young shoots and leaves). At this level of development they are said to be the outermost layer of the cell and are the only layer of protection found in certain cells. Adjacent cells are cemented together by botanic "glues" or pectins in a layer called middle lamellae. Cemented cells communicate through small channels called plasmodesmata. Leaves with only primary cell walls have a relative advantage when it comes to forage availability and digestion, be it mechanical or microbial (thin, flexible and lignin deficient). However as the plant grows older there is a gradual deposition of secondary thickening that becomes more prominent when the cell growth stops. A rigid secondary thickening is formed between the primary cell wall and the plasma membrane. The secondary cell wall components are similar to that of the primary cell wall in terms of macromolecules but for the introduction of lignin and lack of pectin. Therefore it is characterised by the deposition of more cellulose, hemicelluloses with very little pectin. The concentration of hemicelluloses and cellulose increases from 50% to 80% compared to that in the primary cell wall (Ghadaki *et al.*, 1975).

Lignin is an insoluble aromatic polymer made from the polymerisation of phenolic alcohols (Kazuhide *et al.*, 2004) found mainly in mature plant cell walls. Deposition of material into the primary cell wall could either be homogeneous thickening (phloem) or localised thickening (xylem vessel). Further differentiation of the xylem leads to the penetration of lignin from the outside into the secondary thickening. These hydrophobic polymers replace water and encrust all the microfibrils and matrix where the cell walls eventually die. Cell expansion in this layer is practically impossible because it is encrusted with lignin, waterproofing cells, blocking diffusion of water and nutrients into cells. This is the main reason why most lignified cells die on maturity. However, life is still possible for cells that are able to retain enough plasmodesmata as a means of communication between cells. Lignified cells do not only prevent cell expansion but prevent cell division indirectly hence meristematic tissues do not contain lignin. The cells of sclerenchyma, collenchyma and xylem have secondary deposits of lignified cellulose, which provide mechanical strength to the

tissue. Deposition of lignified cellulose is highly ordered when compared to that of the primary cell wall with variable orientations forming a laminated structure that significantly increases cell wall strength.

Although cellulose is the major component of plant cell walls, its distribution differs from one plant to the next (Table 2.0) as well as within different parts of the same plant (stem, fruit and leaves). In the cell walls of a stem, cellulose is closely blended with other cell wall constituents such as hemicellulose and pectin within the meristematic regions (young stems). In mature stems that have undergone secondary growth the percentage of lignin deposited in the matrix is very high. Integration of fibre with other cellular constituents enables it to withstand higher pressure and to support the weight of the plant. However, some stems are modified for storage. These stems have a low percentage of cellulose as well as lignin but are richer in soluble carbohydrates e.g. stems from sugar cane plants have been modified for storage of sugars.

Table 2.0: Cell wall composition of forages and pastures

	Cellulose	Hemicellulose	Lignin
Annual green fodder	26-35	18-25	3-7
Grasses (fresh)	20-32	8-11	4-9
Legumes (fresh)	21-39	20-22	3-6
Lucerne	19-30	10-12	7-11
Temporary grasses and permanent grassland	23-33	14-27	4-9

Typical values during the cutting/grazing phases (g/kg DM) modified from Abreu (1994)

The leaves of plants as well as grasses do not undergo secondary growth and therefore do not increase in width (thickness). Although grasses (leaf blades) do not increase in width they still require strength for support, provided by lignified cellulose, which is abundant around the xylem vessels, middle lamella and vascular bundles. The amount of fibre deposited in these regions is proportional to the weight to be supported so as to keep the leave exposed. Nevertheless some of these leaves have been modified for storage e.g. cabbage and onion leaves, rendering the leaves with a lower level of cellulose. The cell wall constituents of leaves also vary with age. Deinum and Direven (1971) investigated different leaves from the maize plant and found that digestibility declined with age. They concluded that leaves at a later stage of growth were much

less digestible than leaves at initial growth on the same plant species at the same stage of development. Similar effects of leaf succession and age were found in Australia with *Pinicum maximum* (Wilson, 1973a), in Nigeria with *Androgopogon gayanus* (Haggar and Ahmed, 1971) and in Puerto Rico with *Digitaria decumbens* (Van Soest, 1982). A sharp drop in digestibility was observed in leaves grown under high temperatures as opposed to low. Decreased digestibility is due to the fact that higher temperatures cause an increase in cell wall cellulose contents (Deinum, 1976). Generally, the quantity of fibre in leaves is lower than in stems.

In fruit the cell wall is composed of a fibre mesh that provides protection. A very thin cell wall membrane protects fruits such as mangoes and apples. The quantity of fibre deposited within the cell wall matrix is lower than in stems. When fruit ripens the amount of cellulose decreases even futher as some of the cellulose has broken down into oligosaccharides. The fruit becomes softer in nature and can easily be punctured by CS selectors. Generally the strength of the primary cell wall is due to the presence of cellulose macrofibrils embedded in a jelly-like matrix while that of the secondary cell wall is accomplished by lignified celluloses.

2.3 Fibre hydrolyses in ruminants

Ruminants, like any other animal, require some form of energy to survive. This energy is obtained by eating grass, fruit, leaves or stems. Hofman (1989) classified these animals into CS, IM and GR while Van Soest (1988) subdivided the CS group into seed and foliage selectors (suni) and tree and shrub browsers (deer and giraffe). The IM group can be subdivided into forb or browse preference (goats) and grass preference (sheep). The GR group is subdivided into fresh grass grazers (buffalo and cattle), roughage grazers (topi) and dry region grazers (camel). Variation in feeding habits can be ascribed to the availability and nutritional content of forages.The cellulose content of different forages was found to increase as one move from CS through IM to GR; this also implies that their dietary protein content decreases in this order. Ruminal microbes hydrolyse both structural and soluble molecules into volatile fatty acids (butyrate, propionate and acetate), which are then used for energy generation by the host. In order to better understand fibre hydrolyses in ruminants, it

will be examined in two steps, namely, mechanical (physical) breakdown and microbial hydrolyses (fibre fermentation).

2.3.1 Mechanical breakdown of fibre

Mechanical breakdown of fibre in ruminants is similar to the mechanical breakdown of forages in monogastric animals. However, there are some major differences between monogastric animals and ruminants such as regurgitation in ruminants, which is absent in monogastric animals. Mechanical breakdown of fibre is a non-enzymatic process where macro-fibres are broken down into microfibres and cellulose units thereby increasing the surface area for enzymatic hydrolysis. Fibre fermentation was observed to increase with surface area. This was confirmed by *in vitro* experimentation of dry matter digestion rate of alfalfa with variable sizes of feed particles (Robles *et al*, 1980). Not only did a large surface area increase fermentation, it also increased feed intake. Feed intake was higher in animals fed with ground particles than unground particles of the same feed. More time was spent on the unground feed during chewing than on the crushed feed (Weston and Hogan, 1967).

The main site for the physiological breakdown of fibre is the buccal cavity with mastication being the main process. The lips, tongue, lower incisors and the dental pad in front of the hard palate are the prehensile organs of the ruminant. These organs are the important features that influence the animal's nutrition. The oral cavity and the lips are used for holding the food. The size and length of the buccal cavity and lips, respectively, differ from CS through IM to GR. GR have a smaller mouth opening and shorter lips which facilitate cutting and easy manipulation of grass. CS have longer lips and large mouth openings for manipulation of forages such as fruit. Molars and premolars are used for grinding and these are affected by the sideways swing of the mandibles. The mandibles of GR offer a larger surface for masticatory muscle attachment and they have bigger masticatory muscles than CS. In GR, chewing is initially very brief when grazing in the fields but particulate size is later reduced by rumination (chewing of the cud) during resting hours. The group of CS is characterised by a shorter rumination period but with an intense initial chewing since most of the cell content from the dicot material is released. The tongue assists in

holding and movement of forage material in the oral cavity. The shape and size of the tongue varies among the different feeding types. CS generally possesses a plump, piston-like tongue while GR have a pointed slender tongue.

Saliva secreted during mastication by the salivary glands serves: (a) as a rinsing fluid for nutrients released by CS; (b) to dissolve food particles and allow for flexible movement during chewing; (c) as a fermentation buffer in the rumen; and (d) as a transporting fluid. Hofman (1989) showed that the salivary gland varies among the different feeding types. Their sizes regress as one move from CS through IM to GR. Peristaltic movement of chewed material from the oral cavity through the oesophagus to the rumen also plays a minor role in mechanical breakdown. During resting periods ruminants have a special ability to regurgitate the swallowed material for effective chewing. This is a unique feature in ruminants that has been used in their classification. The size of the rumen appears to play a major role in rumen digestion. The larger the size of the rumen the longer the time spent by particulate matters (in GR) hence prolonging the time for microbial activity. This implies that mechanical breakdown will be much better in GR with larger rumen than in CS with smaller rumen. Non-rhythmic contractions of the rumen during mixing also assist in the mechanical process of fibre breakdown.

2.3.2 Microbial fermentation of fibre

Microbial fermentation of fibre is also known as enzymatic hydrolysis since enzymes secreted by microbes carry out the fermentation process. Fermentation of fibre occurs mainly in the rumen or hindgut of herbivores. Although some ruminants still possess a double fermentation chamber (fore- and hindgut) the foregut fermentation chamber is said to be prominent. This review will focus on the foregut fermentation chamber with little attention to the hindgut; hence the rumen will be the main chamber of interest. The rumen harbours a variety of microbes called symbiotic microbes comprising anaerobic bacteria, protozoans and fungi.

These microbes are introduced into the rumen by the host animal during grazing or browsing in the fields. Microbial species vary from one geographical region to

another even within animal species in the same geographical regions (Goncalves and Borba, 1996). The rumen harbours microbes that have evolved through millions of years of selection with the varying nutrional constraints. This is an extraordinary feature that all ruminants possess in order to survive and reproduce while eating high fibre diets. Survival of microbes within this environment depends on their ability to compete with other microbes as well as adapt to the stringent conditions of the rumen. The ability to adapt and survive in the rumen is quite important as an unlimited number of microbes are being introduced each day. The dominant rumen microbes here are saccharolytic since carbohydrates are the main metabolic substrates. The rumen is an anaerobic chamber as most, if not all, microbes that it harbours are anaerobic. However, Yokoyama and Johnson (1998) illustrated that these microbes are not absolutely anaerobic. Consequently, these microbes were classified as either obligate or facultative anaerobes.

Obligate anaerobes are those that grow only in the absence of oxygen while facultative microbes are those that are able to grow in the presence or absence of oxygen. Owens and Goestsch (1998) re-enforced the presence of these microbes by postulating their locations in the rumen environment. Microbes that associate with particulate matter (Figure 2.2) as well as free-floating microbes were classified as obligate anaerobes (Bailey and Jones, 1971). Those that attached firmly to the epithelial wall of the rumen were classified as facultative anaerobes. Studies by Akin (1980) extended the above theory by demonstrating that not all facultative anaerobes adhere to the ruminal wall. Quantitatively, the obligate anaerobes are far more numerous than facultative anaerobes.

Bacteria classification is based on their morphological structure, size and shape (cocci, rods and spirilla). Interestingly, some nutritionists have used substrates hydrolysed by microbes as a primary basis of classification. The objective of this approach was to assist in elucidating the contribution of each bacterium species towards the utilisation of the different components of feed. Preliminary screening of bacteria by this method led to the classification of bacteria into eight different groups with respect to the following substrates, celluloses, hemicelluloses, sugars,

intermediates, proteins, lipids, starch and methane (Yokoyama and Johnson, 1998). The shortcoming of this method of classification was the broad spectrum of certain enzymes, which were capable of hydrolysing and fermenting more than one substrate. Cellulolytic microbes are the most important microbes in the rumen based on the high fibre content of feeds. Bacteria are also said to be the most adaptable species of microbes in the rumen e.g. *Bacterroides succinogenes, Ruminicoccus flavefaciens, Ruminicoccus albus* and *Butyrivibrio fibrisolvens.* The population of these microbes increases with an increase in substrate availability. Other minor cellulolytic microbes like *Clostridium lochheadii* do not vary much even with increased concentration of the substrate.

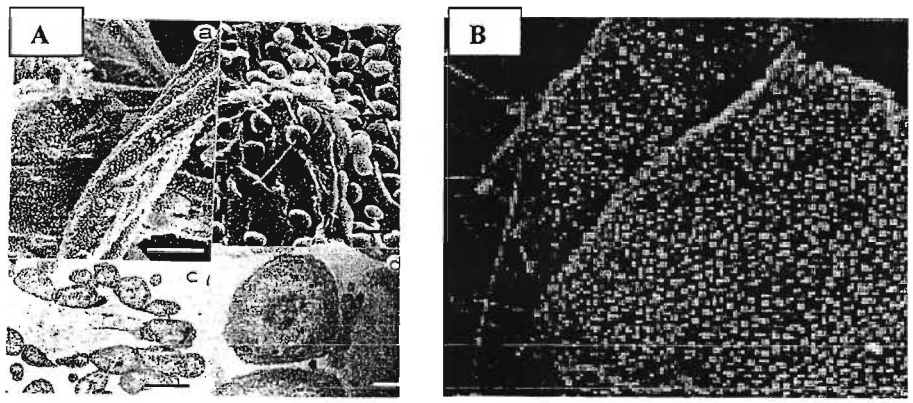

Figure 2.2: Bacteroides succinogenes growing on filter paper cellulose. *(a)* SEM of cells on a cellulose fibre. Bar = 10 um. (b) SEM of cells on a fibre. Bar = 1 um. Groleau and Forsberg, (1981) modified.

Cellulase secreted by cellulolytic bacteria diffuses through the rumen fluid onto the substrate or stays within the vicinity of the bacteria adhering to a substrate. Cellulose molecules are hydrolysed into glucose molecules which are used as metabolic substrates by microbes yielding by-products such as volatile fatty acids (VFA), CO_2, methane and hydrogen. Fibre hydrolysis is not as simple as one might think because the process is catalysed by a complex enzyme system called cellulases. There are three different types of enzymes involved in this system: crystalline cellulase (exocellulase), carboxymethyl cellulase (endocellulase) and glucosidase (cellobiase). The number of enzymes involved in this process clearly indicates that fibre hydrolysis is a stepwise process commencing with the breakdown of crystalline cellulose into cellobiose by exocellulase, amorphous cellulose into cellobiose by endocellulase and cellobiose into glucose by glucosidase. Glucose molecules are then catabolized

through the process of glycolysis yielding VFA, 2ATP, H and CO_2. ATP is used as a primary source of energy by the microbes whereas the by-products are used as metabolic substrates or intermediates by the host.

Hemicellulolytic bacteria secrete hemicellulases that hydrolyse hemicelluloses into monomeric units such as xylose. *Butyrivibrio fibrisolvens* and *Bucroides ruminicola* are top on the list of potent hemicellulolytic bacteria. Besides cellulolytic and hemicellulolytic bacteria, other bacteria that exist in the system ferment only monomeric units such as glucose, xylose and galactose. Symbiotic bacteria living in this ecosystem utilise the by-products of fermentation as metabolic substrates or monomers for synthesizing macromolecules e.g. methane-producing bacteria such as *Methanobrevibacter ruminantium, Methanobacterium formicicum* and *Methanomicrobium mobile* reduce carbon dioxide with hydrogen gas producing methane as an end product. Both carbon dioxide and hydrogen gas are the by-products of fermentation, which when allowed to accumulate can become harmful.

Protozoa are also associated with fibre breakdown as a good number of species have been identified that preferred soluble carbohydrate as a substrate. *Diplodinium diplodinium* and *Diplodinium diploplastron* hydrolyse substrates like cellulose, hemicellulose and starch while *Diplodinium polyplastron* hydrolyse cellulose, glucose, starch and sucrose but not hemicellulose. *Entodinium bursa* utilises starch and hemicellulose but not cellulose as substrates. *Entodinium caudatum* utilises completely soluble molecules such as cellobiose, maltose, glucose and starch.

Anaerobic fungi are found adhering to or within plant materials in cattle and sheep. Their contribution to fibre hydrolysis is minimal although limited degradation of fibre and xylan were observed when incubated with protozoa. Interestingly, protozoa in the rumen may contribute up to 80% of the microbial mass. This high population is yet to be explained as its functional significance is still to be defined. Some bacterial species adhere to protozoa but the rationale behind this adherence is not clear. Logically one might deduce some kind of proximity effect for the bacteria to their substrate since these protozoa are attached to plant materials.

2.3.3 Factors affecting fibre fermentation and utilisation

2.3.3.1 Nature of fibre

There are two types of fibre molecules, namely, crystalline and amorphous cellulose. These two molecules are structurally different due to the type of hydrogen bonding formed within their molecules. The bonding formed within and between these molecules is the primary factor affecting fibre hydrolysis. Intra- and inter-hydrogen bonds formed with crystalline cellulose are more than those formed in amorphous celluloses (Martin, 2006). This implies that crystalline cellulose hydrolysis will be slower than amorphous cellulose hydrolysis upon incubation with microbes.

2.3.3.2 Hemicelluloses

Hemicelluloses are secondary factors affecting fibre hydrolysis. These molecules interact with fibres through the formation of intra- and inter-chain hydrogen bonds. Neidusznki and Marchessault (1971) reported that hemicelluloses like xylan are able to form crystalline structures rendering the matrix less permeable to cellulases. Association of this complex with lignin renders the matrix even more difficult to hydrolyse as the mesh network will be almost impermeable to cellulolytic enzymes. This implies that the association of these molecules with celluloses makes the complex structure even stronger. The degradation of pure fibre when incubated with microbes was higher than that of crude fibre (Kamra, 2005).

2.3.3.3 Pectins

Pectins are polymers of galacturonic acids and are often referred to as "intercellular cement". The term "intercellular cement" was derived from their role in binding two or more cells together. Enzymatic hydrolysis of pectin in a substrate complex was found to separate plant cell walls from each other. Pectins are able to hydrogen bond with celluloses, hemicelluloses and other matrix macromolecules, e.g. proteins. They encrust both celluloses and hemicelluloses, rendering them inaccessible to cellulolytic and hemicellulolytic enzymes, respectively.

2.3.3.4 Lignin

Lignin is formed from the polymerisation of three primary precursors phenylpropanoid precursors, phenylalanine and tyrosine. These primary precursors give rise to p-coumaryl, coniferyl and sinapyl alcohols. Free radicals are formed from these monomers under the influence of phenol oxidase, which undergoes non-enzymatic reactions to form polyphenols. Lignin polymers are chemically stable structures that are mostly attacked by aerobic bacteria, which therefore limit the possibility of extensive breakdown in an anaerobic rumen (Keford, 1958). Lignin found in plants differs mainly in the proportion of free radicals from which they are formed. Lignin in gymnosperm is made up of 80% p-coumaryl, 14% coniferyl and 6% sinapyl alcohols while angiosperms lignin contain 56% p-coumaryl, 4% coniferyl and 40% sinapyl alcohols. In addition to the phenolic monomers grasses and herbage lignin is richer in acids such as p-coumaric, ferulic, diferulic, p-hydroxbenzoic and vanillin. A highly condensed phenyl propanoid matrix of lignin is referred to as "core" lignin while the p-coumaric and ferulic fragments are known as "non-core" lignin (Smith *et al.*, 1971).

Non-core lignin fragments (p-coumaric and ferulic acid) are the most important molecules in terms of the cross linkages formed between lignin and structural carbohydrates due to the presence of hydroxyl and carboxyl groups. Ester bonds are common between "non-core" lignin and hemicelluloses in forages. Therefore, the binding of lignin to hemicelluloses indirectly affects fibre hydrolyses as hemicelluloses are bound to celluloses. However, it does not cancel the fact that bonding between lignin and celluloses as well as other carbohydrates is also possible. Variation in the association of lignin to hemicelluloses was substantiated by analysing carbohydrates extracted by alkali and dimethyl sulfoxide (Ian, 1974). Alkali analysis of carbohydrates from lignin-carbohydrates liberated 70% xylose, 20% arabinose, 5% galactose and 5% glucose. The high percentage of xylose confirms hemicelluloses as the dominant molecules associated with lignin. Analysis of the carbohydrate moiety extracted by dimethly sulfoxide indicated that the percentage of glucose (50%) observed was higher than that of xylose (30%), arbinose (12%) and galactose (5%).

High glucose concentrations imply that lignin associates with other molecules in the cell matrix.

Casler (1987) demonstrated that the concentration of lignin increases with increasing maturity for both grasses and legumes. Generally the concentration of lignin is higher in legumes than in grasses but lignin in legumes is more soluble than lignin in grasses. The solubility of legume lignin is due to higher "core lignin" than "non-core" lignin. Geographical regions from which forages are harvested also play a major role, as lignin content of forages is not stable. Van Soest (1982) demonstrated that lignin content of grasses from the tropics is higher than those harvested from the temperate regions. Consequently, the digestion of forages from the tropics will be more difficult than those from temperate regions. However, the evolution of microbes in the animals that graze in these regions is so magnificent that meeting the nutritional needs of these animals is not problematic. Encrustation is one of the major effects observed on fibre by lignin, which renders fibre inaccessible to cellulolytic enzymes (Huub *et al.*, 1988). The most import mechanism established is the bonding of lignin to other polysaccharides including celluloses forming a polysaccharide complex. The bonds formed within these complexes are watertight and make the diffusion of cellulolytic enzymes extremely difficult. This implies that fermentation will be greatly reduced in the presence of such complexes.

2.3.3.5 Tannins

These are naturally occurring bio-molecules of higher molecular weights synthesized by plants. Notable are the diverse groups of oligomers and polymers of phenols. They are defined as phenolic compounds that precipitate proteins. This definition has some shortcomings as not all tannins bind and precipitate proteins and secondly, not all polyphenols precipitate proteins or form complexes with polysaccharides. However, Horvath (1981) amended the definition by defining tannins as any phenolic compound of high molecular weight containing sufficient hydroxyl groups and other suitable groups to form effectively strong complexes with proteins or other macromolecules under the particular environmental conditions being studied. In the plant kingdom tannins are found both in angiosperms (flowering plants) and gymnosperms (non-flowering plants). In flowering plants dicots are richer in tannin than monocots. An

understanding of the distribution of tannins in plant tissues can assist in the enriching or restructuring of the grazing area of ruminants.

Tannins are prominent in plant parts such as barks, wood, fruit, leaves, and roots. They are mostly located in the vacuoles of surface wax in these tissues, where interference with the plant's metabolism is nil. This implies that it is only after cell lesion or cell death that these macromolecules can interfere with metabolic activities. The outer part of the bud tissue is believed to have a protective function against freezing in winter while the upper epidermis of plant leaves reduces palatability, thus protecting the plant against predators. However, in the tropics tannins are evenly distributed on the leaf surface compared with plants in temperate regions in which tannins are concentrated in the upper epidermis. Stem tissue, secondary phloem and the xylem, which are active growth areas, are richer in tannins. The role tannins play in this area is not well understood but they have been associated with growth regulation in these tissues. Functionally, tannins have been associated with the maintenance of plant dormancy due to their allelopathic and bactericidal properties.

Tannins are divided into two main groups: hydrolysable and condensed (proanthocyanidins) tannins (Fahey and Berger, 1998). They exhibit common characteristics such as being soluble in water (but for some high molecular weight structures), binding to proteins and forming either soluble or insoluble protein-tannin complexes of higher molecular weight and lastly containing oligomeric chains with multiple units having free phenolic groups. Hydrolyzable tannins are macromolecules produced by plants with polyol as a backbone. The polyol central core is generally made up of D-glucose. Carbohydrate hydroxyl groups are partially or totally esterified with phenolic groups of acids such as gallic acid or ellagic acid. Quantitative analysis of tannins in plants shows that hydrolysable tannins are usually present in lower concentrations than condensed tannins. Hydrolysable tannins are subdivided into taragallotannins (gallic and quinic acid) and caffetannins (caffeic and quinic acid). They are hydrolysed by mild acids, mild bases or hot water to yield carbohydrates and phenolic acids. Enzymes called tannases, which are specific for ester bond hydrolysis, also hydrolyse them.

Condensed tannins, on the other hand, are polymers of flavanoid units (flavan-3-ol) linked by carbon bonds not susceptible to cleavage by mild hydrolysis. Condensed tannins are widely distributed in legume pasture species such as *Lotus corniculatus*, *Lotus pedunculatus*, *Onobrychis viciifolia*, in several species of acacia (Degen *et al.*, 1995), in sorghum grain (Kumar and Singh, 1984) and in many other plant species. The complexity of condensed tannins depends on their flavanoid units which vary among constituents and within sites for interflavan bond formation. Anthocyanidin pigment is responsible for the variety of colours observed in leaves, fruits juices and wines. The astringent taste of some leaves, fruit and wines is due to the presence of tannin. Solubility of condensed tannins in aqueous organic solvents depends on its chemical structure and degree of polymerisation. This implies that the higher the degree of polymerisation the less soluble they are. Understanding the interaction of tannins with other macromolecules is vital for designing an approach for negating the harmful effects of these macromolecules or for harnessing tannins' properties for a nutritional benefit. Interaction with other macromolecules such as proteins, carbohydrates and bacterial cell membranes is very common.

The manner in which tannins affect fibre hydrolysis can be classified as direct or indirect. A direct effect is said to occur when the tannin interacts with carbohydrates molecules while an indirect effect is when it interacts with other macromolecules thus slowing down the fermentation process e.g. enzymes and bacterial cell walls (Scalbert, 1991; Fahey and Berger, 1998). Their interaction with carbohydrates is said to be a direct inhibition process because it interferes with microbial breakdown. Tannins interact with the hydroxyl groups of fibres through hydrophobic bond formation. They form complexes with these insoluble materials, reducing the surface area for microbial fermentation hence inhibiting the fermentation process. However, the degree of inhibition depends on the type of microbes available in that ecosystem as well as the evolutional efficiency of the microbes. Some microbes have evolved in their defensive mechanisms so much so that they are able to resist certain amounts of tannins in their diet (Odenyo and Osuji, 1998). They do so by (i) secreting enzymes such as tannases in large quantities to assist in hydrolysing these molecules on fibre surfaces and (or), (ii) secreting higher quantities of nonsense proteins which minimize the amount of functional protein complexing with tannins.

Indigestibility of fibre due to the formation of tannin-carbohydrate complexes is not as potent as the indigestibility of tannin-protein complexes (Hagerman and Butler, 1981). Tannins interact with proteins forming tannin-protein complexes, which can either be classified as soluble or insoluble complexes. Tannin-protein interactions are based on hydrophobic interaction, hydrogen bonding but rarely ionic or covalent bonding. Tannins have a high affinity for proteins hence forming very strong tannin-protein complexes. These proteins can either be dietary, salivary proteins, endogenous enzymes or microbial enzymes. Hydrogen bonding is favoured by the ability of phenolic groups to donate hydrogen to form strong hydrogen bonds with proteins. Protein precipitation is maximal at pH values nearer their isoelectric points. At high pH, tannin-protein complex formation does not or occurs very slowly because both the phenolics and protein molecules are ionised with a net negative charge. Same charges imply that these molecules exert a repulsive force on each other. This emphasizes the need for buffering with large volumes of salivary buffer to higher pH in ruminants fed on tannin-rich diets. Tannin-protein complex formation is said to be the most important anti-nutritional aspect of tannins in ruminants. This is because these complexes are resistant to protease attacks and greatly reduce the availability of cellulolytic enzymes.

Tannin-protein complexes are either soluble or insoluble depending on the availability of each component. Soluble complexes are favoured by high protein concentrations while insoluble complexes are favoured by lower protein concentrations in the reaction medium. Insolubility is due to the exposure of hydrophobic surfaces of many tannin molecules crowding around a single protein molecule hence precipitating it out of solution. Solubility is due to high hydrophilic molecules crowding the surface of the complexes formed. Soluble complexes are a major problem as they are quite difficult to measure in solution. Tannins also react with microbes' cell walls, forming tannin-microbe complexes with a potential of inactivating microbial activity. Rumen microbes such as *Streptococcus bovis*, *Butyvibrio fibrosolvens* and *Fibrobacter succinogens* are inactivated by tannin complexing (McSweeney *et al.*, 1999). Binding induces morphological changes and deprives microbes of certain metal ions.

Sotohy *et al.* (1997) reported that the total number of bacteria in the rumen of goats decreased significantly when the animals were fed with a tannin-rich plant (*Acacia*

nilotica) and the decrease in population was directly proportional to the level of tannin in the diet. Decreasing the microbial population indirectly affects cellulolytic enzymes concentration and hence a decrease in fibre hydrolysis. Therefore, microbial inhibition is said to be an indirect inhibition of fibre hydrolyses. Tannins do not only affect fibre breakdown but also reduce palatability of fibre in ruminants due to their astringent taste. Astringency is due to the precipitation of salivary protein in the mouth. Reducing intake implies decreasing the amount of fibre available for digestion hence indirectly affecting hydrolysis. Complex formation with proteins reduces the amount of nitrogen in the rumen necessary for microbial growth. Nitrogen deficiency implies a decrease in the microbial population and a decrease in celluloytic enzymes, and hence a decrease in fibre breakdown.

2.3.4 The origin and fate of volatile fatty acids in the rumen

Rumen microbial organisms depend on each other with respect to their metabolic activity. Microbes depend on the host to harvest and provide fibres, increase surface area for enzyme activity, regulate rumen pH, as well as maintain the internal temperature (38-39°C) for optimum activity. These microbes in turn break down cellulose and hemicellulose macromolecules into hexoses (glucose) and pentoses (e.g. xylose), respectively. As mentioned earlier, liberated glucose molecules are utilised as a primary substrate for energy production by microbes. Glycolysis is the main process by which these monosaccharides are metabolized to yield high energy molecules called adenosine triphosphate (ATP) and by-products such as pyruvate, and reduced nicotinamide adenine dinucleotide (NADH2); some pathways of pyruvate metabolism are illustrated in Figure 2.2. This process is completely anaerobic since it does not involve oxygen. Microbes utilise ATP liberated from glycolysis for microbial growth (synthesis of cells) and maintenance. Pyruvate is converted into volatile fatty acids (VFA) namely acetate, propionate and butyrate. VFA are the essential metabolic molecules of interest to ruminants. The concentration of VFA produced as endproducts of carbohydrate fermentation vary depending on the bacterial species involved and the type of substrate consumed (Owens and Goestsch, 1998). Different pathways have been described for the conversion of carbohydrates into acetate, propionate and butyrate. Figure 2.3 demonstrates the major pathways that are

involved in the conversion of pyruvate into VFA. Fermentation of hemicelluloses to VFA is very similar to that of celluloses but for the initial conversion stages of hemicellulose. Hemicelluloses are first hydrolysed into pentoses (xyloses) by hemicellulases and then converted into hexoses (fructose phosphates) that can enter the glycolytic pathway. VFA production varies from animal to animal as well as with the type of feed being fed.

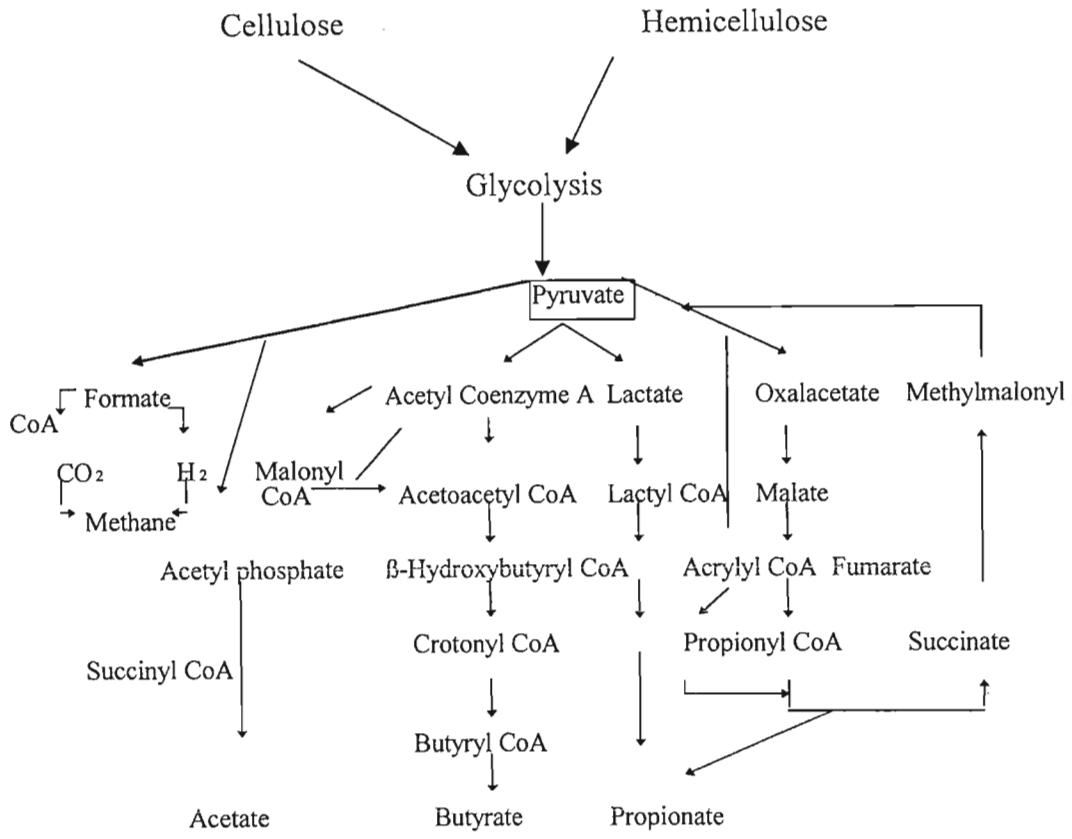

Figure 2.3: The production and conversion of pyruvate to volatile fatty acids in the rumen. McDonald et al. (1995) modified.

Understanding the fate of VFA is as important as probing their origin. VFA are essential metabolic substrates in ruminant nutrition since approximately 60% of the energy of feed comes from them. They are absorbed mainly from the rumen by simple diffusion across the rumen wall into the epithelial tissue and then into the blood stream. Acetate and propionate diffuses through the rumen epithelium without any modification while it appears a reasonable amount of butyrate is modified to beta-hydroxybutyric acid (ketone bodies) before diffusion (Leng and West, 1969). This

accounts for most of the ketone bodies in the blood stream of animals during feeding. Some of the VFA (e.g. acetate and butyrate) are metabolised in the rumen epithelium to carbon dioxide.

The fate of VFA when absorbed is not the same because they are used as metabolic substrates in different pathways. All three VFAs are involved in the Tricarboxylic Acid Cycle (TCA) (Figure 2.4). TCA is a major energy generation pathway in ruminants and can only take place in the presence of oxygen (aerobic process). VFA are modified with coenzyme A (CoA) before introduction at different stages of the TCA cycle.

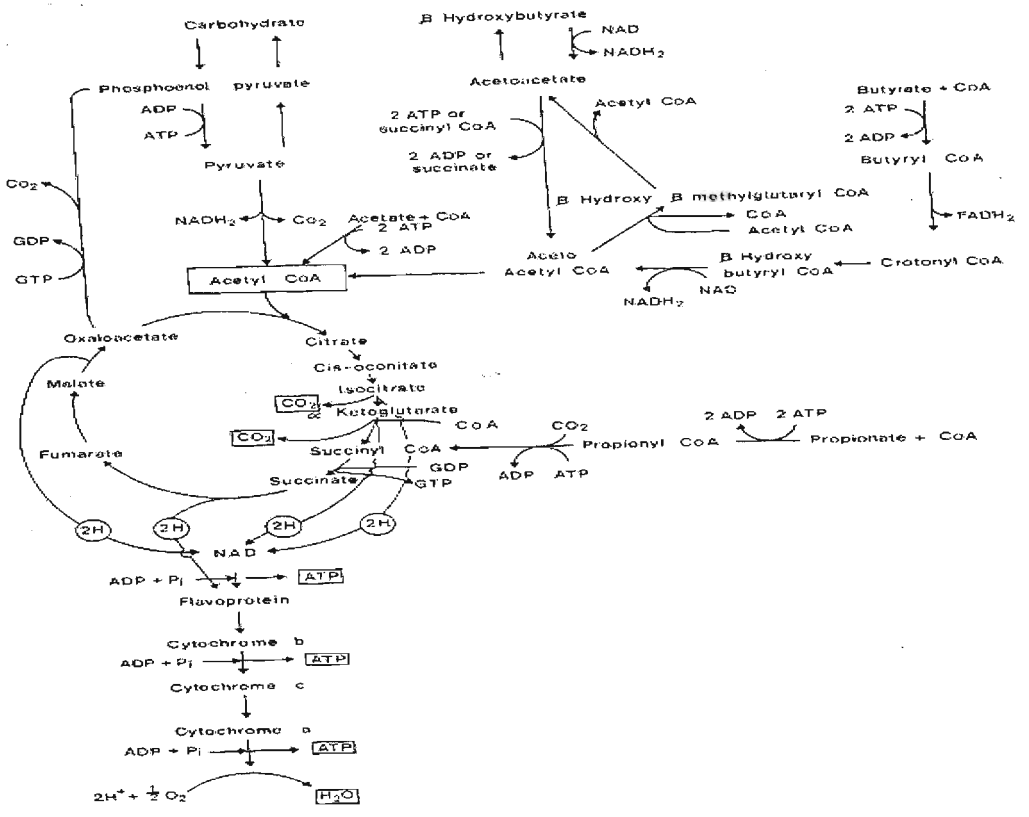

Figure 2.4: A schematic diagram illustrating the fate of VFA.

In the cytoplasm of the liver cells, free acetate is activated to acetyl CoA in the presence of 2 ATP molecules and coenzyme A synthetase. Acetyl CoA is an intermediate in the TCA cycle which when completely oxidized yields 12 ATP molecules. Butyrate is converted to butyryl CoA in the presence of butyryl CoA

synthetase and 2 ATPS. The conversion of butyryl CoA to aceto acetyl CoA involves two stages with the release of reduced flavin adenine dinucleotide ($FADH_2$) and $NADH_2$. Aceto acetyl CoA is then hydrolysed into acetyl CoA, an intermediate in the TCA cycle. This undergoes the same oxidative process as any other acetyl CoA molecule. The theoretical value of the numbers of moles of ATP released from the oxidation of butyrate is higher than that of acetate. Propionyl CoA is an intermediate product formed from the reaction between propionate and CoA in the presence of 2 ATP. Propionly CoA is then converted to succinyl CoA which is an intermediate in the TCA by the introduction of carbon dioxide. The oxidation of succinyl CoA yields a theoretical value of 20 moles of ATP which is lower than that produced from butyrate but higher than those from acetate.

Propionate is also a major substrate in the gluconeogenic pathway. Gluconeogenesis is the generation of glucose from non carbohydrate sources e.g. lactate, glycerol, amino acids and intermediates of the TCA cycle. Glucose generated from propionate is the main source of glucose in ruminants. Energy required at the tissue level of ruminants is provided mainly by the gluconeogenic pathway as very little or no bypass of glucose is observed except in cases where ruminants are fed with high concentrate feeds. Small quantities of propionate are converted to lactate in the rumen epithelium during absorption while the rest enters the portal vein blood to the liver. In the liver these molecules are converted to lactate which enter the gluconeogenic pathway for glucose synthesis. Alternatively, acetyl CoA is used for the synthesis of fatty acids, which are the building units of lipids. However the conversion of glucose molecules to the building units of lipids (acetyl CoA) is limited by the presence of ATP citrate lyase. Butyrate is converted into three major ketones, namely, β-hydroxyl butyrate, acetone and acetoacetate. About 80% of the total ketone produced is β-hydroxyl butyrate with some of it being used in fatty acid synthesis in the adipose and mammary gland tissues. To conclude, VFA are produced from pyruvate which is a by-product of glycolysis. They are the main metabolic energy substrates in ruminants resulting from microbial fermentation with propionate being the most important in gluconeogenesis. This process is very active immediately after feeding in ruminants. VFA are not only utilised by ruminants for energy generation but also by microbes

with limited supply of glucose. However, energy generation for microbial growth from VFA is lower than that generated from glucose molecules.

2.4 Cellulases

Cellulases are enzymes that hydrolyse fibre into oligosaccharides and monosaccharides (Hoshino *et al.*, 1994). The term cellulase may sound simple but it is a complex enzyme system. Studies by Howell and Stuck (1975) revealed that cellulases are complex enzymes made up of different subunits with specific functions. Reese *et al.* (1950) incubated cellulose with cellulases and demonstrated that cellobiose and glucose were the main by-products. They also noticed that the concentration of cellobiose decreased with time while that of glucose increased. King and Versal (1969) also showed that cellulases obtained from *Trichoderma viride* operate in a similar manner. They postulated that hydrolysis of cellulose occurs in two basic steps: firstly, hydrolysis of beta-1, 4 glycosidic bond by beta-1, 4 exocellulase producing cellobiose as the end product and secondly, the hydrolysis of glycosidic linkage of cellobiose by beta-1, 4 cellobiase yielding glucose units (Equation 2.0).

Equation 2.0

$$\text{Cellulose} \xrightarrow{\beta\text{-1, 4 exocellulase}} \text{n cellobiose} \xrightarrow{\beta\text{-1, 4 cellobiase}} \text{2n glucose}$$

n = natural numbers

The complexity of cellulose existing in both crystalline and amorphous forms has led to the secretion of different cellulolytic enzymes by microbes with respect to the type present in their environment. This implies that the activity of these enzymes will vary among species. Even within species the cellulolytic activity as well as molecular weight also varies as their genetic material is not identical. Freelove *et al.* (2001) demonstrated that there was another component of these enzymes that was responsible for the recognition of substrate (carbohydrate binding sites (CBH)). This protein component that recognises and binds to cellulose surfaces is said to be inert but enhances enzyme activity due to the proximity effect. Figure 2.5 demonstrates the

hydrolysis of amorphous and crystalline cellulose by a complexed and non-complexed enzyme system. Endocellulases cut randomly within the amorphous molecule while exocellulases chip off cellobiose molecules from the non-reducing end of crystalline molecules (Teeri, 1997).

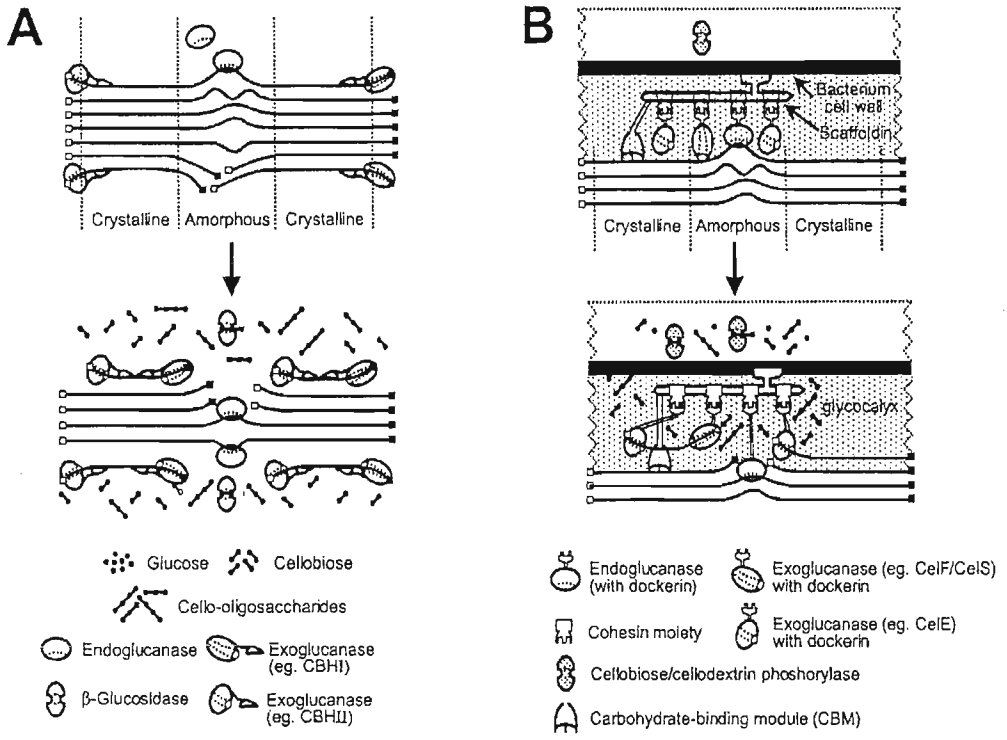

Figure 2.5: Schematic representation of the hydrolysis of amorphous and microcrystalline cellulose by a non-complexed (A) and a complexed (B) cellulase system. Solid squares represent reducing ends and open squares represent non-reducing ends (Lee et al., 2002) modified.

2.5.0 Cellulose additives

More than 70% of forage content consumed by ruminants when grazing on the fields is made up of fibre (Buxton *et al,* 1995a). Fibre breakdown has been a major problem in these animals as less than 40% of it is hydrolysed for metabolic purposes while the rest is passed out as faeces. Agriculturists have found it very difficult to sustain the cost of their forages as much of it is wasted as faeces. The high cost and wastage of fibre intake has instigated many researchers to carry out different studies on how to improve digestibility of fibre. Cellulose additives are substances that are administered to ruminants to enhance fibre breakdown. The different substances include enzymes, antibiotics, probiotics and buffers.

2.5.1 Enzymes

Cellulases secreted by microbes in the rumen are responsible for fibre breakdown. Theoretically it is possible that an increase in enzyme concentration will increase the rate of enzyme activity. Biotechnological advancement has made it possible for large quantities of enzymes to be synthesized at moderate cost. Microbes from different ecosystems have been isolated and chemically engineered to express cellulases (Cavicchioli and Watson, 1991). Supplementing roughage feeds with variable quantities of exogenous enzymes showed minimal increment in fibre hydrolysis (Colombatto *et al.,* 2003). This small increase irrespective of the large amount of enzyme applied was due to the dynamic nature of the rumen physiology. Secondly, these enzymes are protein in nature and are rapidly hydrolysed by the potent microbes in the rumen. The success of applying this theoretical knowledge has been very difficult to demonstrate practically.

2.5.2 Buffers

Buffers are solutions that resist minimal changes in pH. Like any other microbes, cellulolytic microbes have an active pH range (6.2-8). Any alteration of this pH to a very high or lower value will have a negative impact on their activity. Fluctuations in ruminal pH are common especially when forages with high content of soluble carbohydrates are consumed e.g. an increase in lactatic acid concentration due to the fermentation of glucose by *Lactobacillus ruminis* decreases the pH leading to a serious clinical condition known as lactic acidosis. Decrease in pH by lactic acid implies inactivation of enzymatic activity. In order to regulate the fluctuating pH levels in the rumen, a variety of buffers have been introduced in ruminant feed and investigated e.g. magnesium oxide, sodium carbonate and calcium carbonate. These buffers regulate the concentration of hydrogen ions as well as the dilution rate of fluid in the rumen. A decrease in hydrogen ion concentration by these buffers increases the efficiency of microbial activity (Edwards and Poole, 1983). Increased dilutions were found to be relatively advantageous as soluble carbohydrates and proteins were easily flushed from the reticulo-rumen into the abomasum. The shorter the retention time of soluble carbohydrates the smaller the quantity of lactic acid hence a decrease in pH fluctuation.

2.5.3 Antibiotics

Antibiotics are chemical substances that halt the growth of microbes e.g. pathogenic microbes. These chemical compounds are very effective when applied in small quantities. Antibiotics are produced by micro-organisms for protection against other microbes e.g. fungi.The development of advanced biotechnology has made it possible for laboratory synthesis. The rumen is inhabited by a variety of microbes but not all of these microbes are important to the host. The following negative effects have been associated with non-symbiotic microbes in the rumen: production of toxins, competition with the host for nutrients, decreased absorptive capacity of the ruminal wall and synthesis of unwanted macromolecules and by-products e.g. lactic acids and methane. *Megasphaera elsdenii* has been found to compete with cellulolytic microbes and host for glucose. The presence of *Lactobacillus ruminis* and *Streptococcus bovis* increases the concentration of lactic acid, thereby promoting lactic acidosis. Antibiotics administered through injection, food or water was associated with a relative decrease in such bacteria and an increase in cellulolytic bacteria activity (Chen and Wolin, 1979).

2.5.4 Probiotics

Probiotics are antagonistic to antibiotics. They are chemical compounds or microbes (fungi and yeast) that enhance the growth of certain bacteria (Fuller, 1989). As mentioned in the above paragraph, the rumen habours a variety of microbes, some of which are very important to the host and others that are of less importance. The elimination of the non-essential bacterial population is also important as this action favours the growth of the population of essential bacteria. Some of the essential microbes in the rumen include *Ruminococcus flavifaciens* (Gram-positive bacterium) and a Gram-negative *Fibrobacter succinogenes*. *Ruminococcus flavifaciens* has the ability of secreting both hemicellulases and cellulases in the ruminal growth medium. The presence of these two enzymes plays a vital role in fibre breakdown as one digests the hemicellulose mesh network cross-linking cellulose exposing the cellulose for cellulases to act on. *Fibrobacter succinogenes* ferments glucose with by-products such as acetate, succinate and energy (ATP). Apart from cellulolytic microbes, other microbes in the rumen utilise these by-products to generate energy.

Different cellulolytic microbes hydrolyse fibre with a net positive change being energy production but the by-product depends on the species. The different by-products include methane, fatty acids, lactic acid, and succinate, hydrogen (NADH) and carbon dioxide. *Veillonella parvula* is a Gram-negative bacterium that utilises lactic acid as growth substrate from which it produces end-products such as acetate and propionate. *Veillonella parvula* has a double function because it eliminates lactic acid from the rumen preventing lactic acidosis but at the same time producing propionate which is a gluconeogenic substrate. Therefore, it enhances the growth of both the host and the bacteria. The introduction of chemical compounds that enhance the growth of such bacteria is essential in enhancing growth. *Acetaculum ruminis* and *Ruminococcus schinikii* utilise carbon dioxide as a source of carbon and hydrogen from NADH to supply reducing equivalence.

2.6.0 Microbial variations in herbivores

Herbivores cannot digest fibre but depend on microbial fermentation in the rumen and hindgut. These microbes are subjected to a variety of forages and environmental constraints that affects degradability. The efficiency of herbivores to utilise such a wide variety of substrates of feeds is due to the highly diversified rumen microbial ecosystems consisting of bacteria, protozoa, fungi and even bacteriophages (Krause *et al.,* 1999). Microbial species, dominant microbial population and the type of forage involved may play a vital role in influencing microbial activity and efficiency.

Krause *et al.* (1999) demonstrated that cellulose fermentation varies among the following microbial species: *Butyrivibris fibriosolvens, Ruminococcus albus, Ruminococcus flavefaciens* and *Fibrobacter succinogens*. When a group of 22 *Butyrivibris fibriosolens* strains were assessed for their ability to degrade spear grass few could digest more than 10 % of the DM (McSweeney *et al.,* 1998). A similar study by Krause *et al.* (1998) also demonstrated that microbial activity varies greatly among strains of *Ruminococcus albus* and *Ruminococcus flavefaciens.* They called the most active strains superior fibre degraders. *Butyrivibris fibriosolens* strains also

showed a potential to digest xylan hence exposing cellulose for fermentation (Hesepell *et al.*, 1997).

Microbial species are also said to vary among ruminant hosts of the same or different species coming from the same or different geographical regions. This was demonstrated by Leng (1990) when *Ruminococcus albus* from the same host species demonstrated variable activity. They also showed that the activities of isolated strains from the same host as well as different hosts from different geographical regions vary even more.

Forage type is said to play a major role in microbial activity as they influence microbial species, population as well as their evolution (Varga and Kolver, 1997). Microbes sense environmental signals and adapt with the nutrient available (Stock *et al.,* 1989; Stock, 1999). Therefore, microbial ecosystems subjected to roughage will switch on the fibrolytic enzyme system. Two bacterial membrane proteins (histidine kinase and cytoplasmic regulator protein) were activated and hydrolytic enzyme expression was switched on when subjected to soluble polysaccharides (Stock, 1999). Although little is known about how bacteria sense the presence, and alter gene expression in response to cellulose, four major protein phosphatases are said to be involved in this process (Shi *et al.*, 1998).

Digestibility depends on the rate and extent of colonisation of fibre and the biomass of adherent organisms (Cheng *et al.,* 1990). Therefore, fibre digestibility increases with an increase in the microbial population of superior degraders. Although microbial population and activity are said to play a vital role, Maglione *et al.* (1997) demonstrated that cellulose digestion is not only limited by microbial population or the activity of cellulolytic microbes but also by the amount of cellulose available for attack. This emphasises the need for active symbiotic microbes to degrade encrusting substances (e.g. lignin and phenolics), and expose more cellulose (Burk, 1991).

Screening many ecosystems, both domestic and wild, might lead to the revelation of more superior fibre degraders which when merged can lead to a more superior ecosystem. Secondly, the exposure of more active non-cellulolytic microbes with the

potential to degrade anti-nutritional factors will be advantageous to cellulolytic microbes in symbiotic systems.

2.6.1 A hypothesised model for the improvement of fibre breakdown

The amount of fibre wasted as by-products from animals or industries has been unavoidable. Numerous experiments have been carried out on the improvement of fibre hydrolysis as a major source of energy in ruminants but with little success. Earlier attempts included the chopping and grinding of fibre material to increase the surface area for enzymatic hydrolysis (Bourquin *et al.* 1990). Increasing the surface area showed a very small increase in fibre utilisation as there were other inhibiting factors such as hemicellulose and lignin encrusting the cellulose. Getting rid of these macromolecules for the exposure of cellulose will be a major task for the improvement of fibre digestibility. Isolating and purifying the different components of cellulases responsible for fibre breakdown has been the main topic of the century. Different research groups have engaged in purifying and investigating the activities of exocellulases and endocellulases from different strains of bacteria while hoping to find strains with higher activity. Others tend to research bacteria strains with the capacity to synthesize cellulase, hemicellulase and ligninase. However, Wanapat (2001) looked at fibre hydrolysis in a different direction where he transferred digesta from swamp buffalos into cattle. He established that buffalo digesta were sustained in cattle for up to 14 days. This is an indication that microbes with superior fibrolytic activity can be transferred from endowed ruminants into ruminants deficient of such bacteria. With respect to the information available on cellulases and the different research carried out on the improvement of fibre hydrolysis, it is correct to hypothesize that if microbes from a variety of rumen ecosystems with higher activities are merged to form a new ecosystem (composite system) fibre breakdown may be improved (Figure 2.6). The rate of fibre hydrolysis might be higher in the new system than in the control systems.

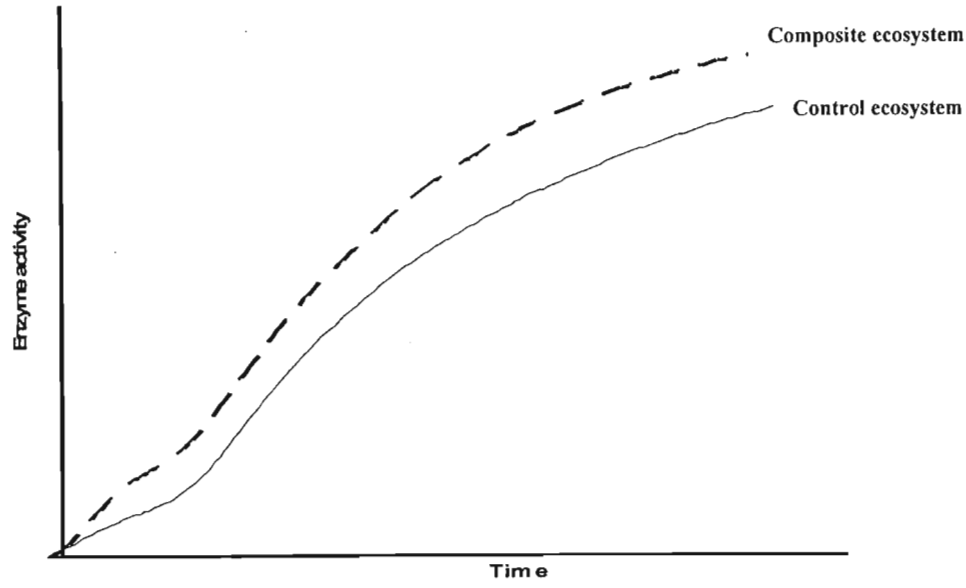

Figure 2.6: A hypothetical graph predicting the activity of a composite ecosystem

2.7 Conclusion

Ruminants derive a considerable proportion of their dietary needs from the digestion of cell wall polysaccharides of plants. These polysaccharides comprise crystalline cellulose in a matrix of hemicelluloses, which may associate physically and chemically with lignin. Its nutritional functions are very limited as herbivores depend on microbes for fermentation. The digestibility of fibre is greatly influenced by the amount and distribution of hemicelluloses, pectin and lignin. Ruminants are the only animals that seem to handle fibre breakdown successfully due to the fact that they harbour microbes that synthesize cellulolytic enzymes. However hydrolysis by these enzymes is a major problem as the fibre content lost as faeces is still very high. There have been a number of positive attempts with regards to fibre hydrolysis but there is still a lot that needs to be uncovered. The merging of two ecosystems might pave the way for improvement of fibre breakdown rather than using purified enzymes. This may be more efficient and cheaper because if cellulolytic microbes are able to compete and survive in the new ecosystem, enzyme secretion will be a continuous process and will proliferate.

Chapter 3

Isolation of fibrolytic enzymes from 11 herbivore ecosystems and optimisation of enzyme assay conditions

3.0 Abstract

Herbivore microbial populations attack, degrade and ferment structural carbohydrates in forage cell walls, producing VFA and microbial proteins for the host. Hydrolysis is carried out by the secretion of a complex cellulase enzyme system involving three major enzymes: (a) exocellulase (crystalline cellulase) (b) endocellulase (carboxymethyl cellulase) and (c) cellobiase (glucosidase). Hemicellulases (xylanases) also play a vital role in cellulose exposure for fermentation. The objective of this study was to isolate proteins that hydrolyse or assist fibrolysis in any way from 11 herbivore microbial ecosystems and optimising their working conditions. Rumen fluid was collected from fistulated herbivores and faeces from non-fistulated herbivores *in situ*. Microbes in both the solid and liquid phases of the sampled solutions (0.1 mM PMSF) were lysed by sonication to liberate periplasmic cellulases and hemicellulases. Proteins were precipitated from 10 ml solutions using 60% ammonium sulfate and dialysed. Protein samples were assayed for endocellulase specific activity, optimal pH and crude protein concentration upon incubation with cellulose, carboxymethyl cellulose (CMC), xylan and cellobiose. Fibrolytic enzymes were successfully isolated as demonstrated by their high carboxymethyl cellulase (endocellulase) specific activities. A broad *in vitro* pH range (4.5-8.0) of activity was observed for all the experimental microbial ecosystems. Despite the broad pH range, some peaks of endocellulase activity (CMCase) were observed within the pH range 4.5–5.0 (elephant), at pH 5 (for horse and zebra), pH 5.0–6.0 (for sheep) and pH 5.0–7.0 for the wildebeest. While CMCase activity in the rest of the animals (cow, llama, giraffe and camel) responded minimally within pH 4.5–8.0. Optimal crude protein concentrations for enzyme assays were 320μg/ml for cellulases and carboxymethyl cellulases while 80μg/ml was suitable for both xylanases and cellobiase analysis.

3.1 Introduction

Cellulolytic microbes possess three types of enzymes which enable partial or complete solubilisation of plant cell walls. These enzymes are exocellulases (EC 3.2.1.91), endocellulases (EC 3.2.1.4) and cellobiases (EC 3.2.1.21). These complex enzyme systems function synergistically to hydrolyse plant cell walls. Exocellulases remove cellobiose units from the non-reducing ends of the cellulose chain, endocellulases act in a random fashion on the region of low crystalinity on the cellulose chain and β-glucosidase produces glucose units from cellobiose (Lee *et al.*, 2002). Although glucosidase is not strictly necessary for cell wall solubilisation, its presence enhances cellulose hydrolysis because these three enzymes function synergistically and inhibition of endocellulase and exocellulase by cellobiose is avoided.

Solubilisation of plant cell walls does not solely depend on cellulase complex enzymes because of the complexity of its association with other macromolecules (Lee *et al.*, 2002). Hemicellulose, lignin, pectin and tannins are examples of macromolecules that are often found in close association with cellulose. Therefore for solubilisation to be successful the enzymes hemicellulase, ligninase, tannase and pectinase are required to solubilise these macromolecules and expose cellulose chains for proper hydrolysis. It is possible that the population structure of microbes in a particular ecosystem is greatly influenced by forage availability.

Apart from celluloses, hemicelluloses are the second most abundant plant fraction available in nature with xylan being the most abundant of all the hemicelluloses. Therefore in roughage feed cellulases and hemicellulases are expected to be the most prominent enzymes. However, not all the microbes (bacteria, fungi and protozoa) that are found in both the fore- and hindguts have fibrolytic or hemicellulolytic properties but can assist in the fermentation process either directly or indirectly. Fermentation yields volatile fatty acids which can reduce the pH of the rumen fluid to less than 6, thereby halting the activity of most rumen microbes which function optimally at pH 6.2 and above (Russel and Dombrowski, 1980). The presence of non-fibrolytic

microbes that utilise acetic acid as a source of energy reduces its concentration hence maintaining the optimal pH of the rumen fluid.

Recent studies have shown that adding exogenous enzymes to herbivore diet increases milk production due to an increase in feed digestion (Lewis *et al.,* 1999, Rode *et al.,* 1999, Schingoethe *et al.*, 1999, Kung *et al.*, 2000; Yang *et al.*, 2000). In a similar study on fibre rich forages, Feng *et al.* (1996), Krause *et al.* (1998), Rode *et al.* (1999), Yang *et al.* (1999), Beauchemin *et al.* (2000) and Kung *et al.* (2000) observed only small increments in fibre digestion. *In vitro* increases in DM digestibility have also been reported by Nakashima *et al.* (1988), Feng *et al.* (1996) and Yang *et al.* (1999).

It is possible that microbial enzymes from herbivorous species consuming different food items in the pasture can vary in plant cell wall hydrolysis. This study intends to extract and evaluate the activities of fibrolytic enzymes from herbivore microbial ecosystems. Several methods have been applied to isolate proteins from the rumen fluid or faecal samples including ammonium sulfate precipitation, three phase partitioning precipitation, gel filtration and acetone precipitation. The ammonium sulfate precipitation method was preferred in this study for protein precipitation because of its simplicity, availability and cost effectiveness (Henry *et al.*, 1974). The precipitated crude protein samples were investigated for fibrolytic activity as well as used to determine the working conditions for optimising enzyme assays (pH and crude protein concentrations).

3.2 Materials and Methods

3.2.1 Materials

Cellulase from *Aspergillus niger*, Cellobiase (Novozyme®), and xylanase from *Thermomyces ianuginosus* (Novozyme®) purchased from Sigma (USA) were the commercial enzymes utilized in this study (positive controls). The substrates were carboxylmethyl cellulose sodium salt (CMC) from FLUKA Bichemica (Germany), crystalline cellulose (powder) from ALDRICH® (Germany), p-nitrophenyl β-D-glucopyranoside (pNP-G), and xylan from beech wood (high grade) from SIGMA (USA). Phenylmethylsulfonyl fluoride (PMSF), D-(+)-xylose, D-(+)-glucose and

phenol were chemicals from Sigma (USA). Polyethylene glycol 20 000 (PEG 20 000) from MERK Laboratory suppliers, South Africa, Micro BCA™ protein assay kit from PIERCE (USA) and 3,5-Dinitrosalicylic from FLUKA (Switzerland). All the other common chemicals such as glacial acetic acid, sodium azide (NaN_3), ammonium sulfate, ethylene diamine tetra acetic acid (EDTA), sodium carbonate, and sodium hydroxide were bought locally from Capital Suppliers, South Africa. Dialysis tubing cellulose membrane (10 000 molecular weight cut-off) was from Sigma-Aldrich. All spectral scans were carried out with Virian Scan 50® Bio UV-Visible spectrophotometer from Varian Australia Pty (Ltd), Australia.

3.2.2 Experimental Animals

Animals were chosen with no preference to sex or species. *Bos taurus* (fistulated Jersey cows), *Equus caballus* (horse) and *Ovis aries* (fistulated sheep) were from Ukilinga Research farm, University of KwaZulu Natal, Pietermaritzburg. *Llama glama* (llama), *Loxodonta africana* (elephant) and *Camelus ferus bactrianus* (camel) were provided by Brian Boswell Circus, Pietermaritzburg. *Equus quagga boehmi* (zebra), *Connochaetes taurinus albojubatus* (wildebeest), *Aepyceros melampus* (impala), *Syncerus caffer* (buffalo) and *Giraffa camelopardalis reticulata* (giraffe) were provided by the Tala Game Reserve, Umbumbulu, KwaZulu-Natal (SA)

3.2.3 Animal nutrition

This study was conducted in winter (April –August, 2005). Camel, elephant and llama were kept in an enclosed area and fed with hay on a regular basis. Game animals such as the impala, wildebeest, zebra and buffalo were grazing on a dry land in an open field where *Pennisetum clandestinum* (Kikuyu grass), hay and other fibres were dominant. The giraffe browsed on a variety of tree leaves. At the Ukulinga research farm, the horse and fistulated cow were fed entirely on hay but sheep received some concentrate in their daily ration.

3.2.4 Sample collection

A slightly modified procedure previously described by Smith *et al.* (1974) was employed when collecting rumen samples from fistulated cows and sheep. Rumen digesta (200 ml) was collected through a fistula, strained through four layers of cheese

cloth, and treated immediately with 150 µl of phyenylmethylsulfonyl fluoride (0.1 mM PMSF) to inhibit proteases from lysing enzymes of interest (Owolabi *et al.*, 1988; Vercoe *et al.*, 2003). On the other hand, faeces were collected *in situ* from the herbivores (horse, giraffe, buffalo, impala, wildebeest, zebra, elephant, llama and camel) and transported in an airtight thermo flask maintained at 38°C.

3.2.5 Protein isolation

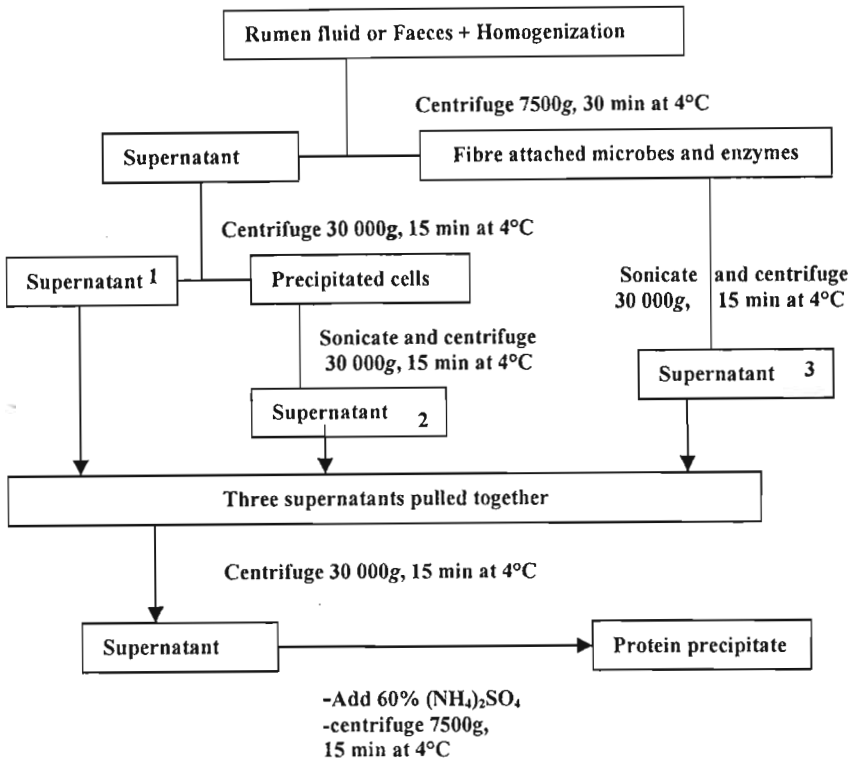

Figure 3.0: A flow chart for the precipitation of cellulase and hemicellulase from rumen fluid and faeces

Protein isolation was done using a slightly modified procedure described by Henry *et al.* (1974). Both rumen fluid (100 ml) and faeces were used for protein isolation. In the case of faeces, 20 g was dissolved in 80 ml of a homogenization buffer (50 mM sodium-acetate buffer, 0.02% (m/v) NaN_3, 0.1 mM PMSF and 0.1 mM EDTA at pH 5.0) before topping to 100 ml. Sample solutions in sealed centrifuge tubes were placed on a shaker for 30 min to facilitate bacteria detachment from fibres before centrifuging (6500x*g*, 30min at 4°C) to sediment particulate matter. The supernatant was centrifuged (30 000x*g*, 15 min at 4°C) to sediment bacteria cells. The sediment particles and bacteria cells were dissolved in 10 ml and 5 ml of homogenisation

buffer, respectively, sonicated (to lyse bacteria cells) and centrifuged (30 000x*g*, 15 min at 4°C). The supernatants of the three different steps were pooled (Figure 3.0) and centrifuged (30 000x*g*, 15 min at 4°C) to sediment any unlysed cells. Ammonium sulfate (60% (m/v) $(NH_4)_2SO_4$) was dissolved in the sample solution to facilitate protein precipitation before centrifuging (7000x*g*, 15 min at 4°C). The precipitate was dissolved in 10 ml of storage buffer (20 mM sodium acetate, 0.02 % (m/v) NaN_3, and 0.1 mM EDTA at pH 5.0) before dialyzing.

3.2.6 Protein dialysis

Each sample solution was pipetted into a dialysis membrane and immersed in a 2*l* storage buffer solution overnight (12 h). Dialyzed sample solutions were concentrated using polyethylene glycol 20 000.

3.2.7 Determination of protein concentrations

Micro BCA™ Protein assay kit, previously used by Richardson (2000) was utilized in this study for protein determination. A standard protein curve was derived as described by the kit manual with few modifications. A standard curve for a micro assay was prepared with bovine serum albumin (BSA) concentrations 0–40 µg/500ul of reaction buffer (20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA at pH 5.0). The MCA™ detecting reagent (500 µl) was pipetted into the sample solution and incubated for 1 h at 39°C. For the unknown protein sample solutions, 1µl was diluted into 499 µl of the reaction buffer before adding MCA™ detecting reagent (500µl) and incubating for 1 h at 39°C. Each assay was performed in triplicates and absorbancies were measured at 562 nm. Unknown protein concentrations were determined from the standard curve.

3.2.8 Determination of the activeness of isolated crude protein samples

The method described by Seyis and Aksoz (2005) for the optimisation of xylanase activity from Trichoderma 1073 D3 was modified slightly and used in this assay. Enzyme stability within a pH range of 4.0 – 8.0 was determined by pre-incubating the crude enzyme extracts in 500 µl sodium-acetate buffer (400 ug/500 µl of 20 mM sodium-acetate buffer) at different pH values for 30 min before adding 500 µl of the substrate (1% (m/v) CMC) and incubating for 2h at 39°C. The substrate

concentrations as well as the protein concentration were constant throughout the experiment. Enzyme reactions were stopped by heating at 100°C. Incubated samples were centrifuged at maximum speed on a desktop centrifuge for 5 min and 400 µl of the sample solution was used for reducing sugar analysis.

3.2.9 pH optimization

The method described by Seyis and Aksoz (2005) for the optimisation of xylanase activity from Trichoderma 1073 D3 was modified slightly and used in this assay. Enzyme stability within a pH range of 4.0 – 8.0 was determined by pre-incubating the crude enzyme extracts in 500-µl sodium-acetate buffer (400 ug/500 µl 20 mM sodium-acetate buffer) at different pH values for 30 min before adding 500 µl of carboxymethyl cellulose (1% (m/v) CMC in 20 mM sodium acetate, 0.02% (m/v) NaN_3 and 0.1 mM EDTA) and incubating for 2 h at 39°C. The substrate concentrations as well as the protein concentration were constant throughout the experiment. Enzyme reactions were stopped by heating at 100°C. Incubated samples were centrifuged at maximum speed on a desktop centrifuge for 5 min and 400 µl of the sample solution was used for reducing sugar analysis.

3.2.10 Quantification of reducing sugars

Several methods have been used for the quantification of reducing sugars in sample solution including: Somogyi-Nelson method (Nelson, 1944), Phenol-sulfuric method, Dinitrosalicylic (DNS) method (Miller *et al.*, 1960), Peterson (1979) and glucose oxidase peroxidase assay (Zuping *et al.*, 2000). DNS technique was chosen because of its sensitivity, simplicity and availability. A standard curve of micrograms of reducing sugars (glucose or xylose) against absorbancies at 540 nm was plotted as described by Wood and Bhat (1988). DNS reagent, 600 µl (0.001 M sodium metabisulfate, 0.708 M potassium sodium tatrate, 0.25 M sodium hydroxide, and 0.021 M phenol) was pipetted into 400 µl of the sample solution and boiled for 5 min. The reaction mixture was then cooled under running water and the absorbance measured at 540 nm. The absorbance values were translated into reducing sugars using the standard curve.

3.2.11 Enzyme assays

3.2.11.1 Crystalline cellulase activity (exocellulase)

Crystalline cellulase operates by removing glucose or cellubiose molecules from the non-reducing ends of crystalline cellulose (Reese *et al.* 1950; Teeri, 1997). Crystalline cellulase activity was assayed following a slightly modified version of the method described by Gerrit *et al.* (1984). Crystalline cellulase activity was measured by pipetting 0.5 ml of 1% (m/v) crystalline cellulose in the reaction buffer into 0.5 ml of crude protein solution obtained from different herbivores and incubating for 48 h at 39°C (standard assay condition). The enzyme reaction was stopped by boiling at 100°C, following which the reaction mixture was centrifuged (6000x*g*, 5 min at 4°C) and 400 µl sample analysed for reducing sugars using DNS method. Each ecosystem was represented by three samples each of which was analyzed in triplicates. Specific enzyme activity was measured in µg of glucose/ min/µg protein.

3.2.11.2 Carboxymethyl cellulase activity

A modified version of the method described by Gerrit *et al.* (1984) was used to investigate the activity of CMCase. The reaction mixture contained 0.5 ml of 0.5% (m/v) CMC in the reaction buffer at pH 5.5 and 0.5 ml of crude enzyme. The enzyme reaction was stopped by boiling at 100°C after incubating at 39°C for 2 h. Samples were centrifuged at 6000x*g* at RT for 5 min, then 400 µl of the reaction mixture was analyzed for reducing sugars using DNS method.

3.2.11.3 Cellobiase activity

Cellobiases are not strictly cellulases but they are nevertheless very important components of cellulase synergistic systems because they complete the hydrolysis of cellobiose, released by cellulase, to glucose molecules. Cellobiase activity was measured in a mixture of 0.5 ml of 0.1% (m/v) *p*NP-G in the reaction buffer and 0.5 ml of crude protein solution. The mixture was incubated in a water bath for 1 h at 39°C. Each enzyme reaction was stopped with 100 µl of sodium carbonate solution (200 mM $NaHCO_3$) and the reaction mixture centrifuged (6000x*g* for 5 min), following which *p*-nitrophenol (pNP) liberated was analysed by reading the

absorbance at 407 nm. The molar absorption coefficient (ε) of *p*NP at 407 nm was taken to be 18300 M^{-1} cm^{-1} from similar calculations done by Frutos *et al.* (2002). The number of moles of *p*NP released was assumed to be directly proportional to the amount of glucose.

3.2.11.4 Xylanase activity

Xylanase hydrolyses xylan into xylose and oligosaccharides (Chivero *et al.*, 2001). A modified procedure described by Khanna (1993) and Seyis and Aksoz (2005) was used to assay xylanase activity. The assay was carried out by pipetting 0.6 ml of 0.1% (m/v) xylan solution in the reaction buffer (pH 5.0) into 0.4 ml of crude protein solution and incubating at 39°C for 1 h. The reaction was stopped with 100 µl of 200 mM $NaHCO_3$, centrifuged (at 6000xg for 5 min) and analysed for reducing sugars using the DNS method. Each ecosystem was represented by three samples each of which was analysed in triplicates. Specific activities of the above enzymes were defined as µg of xylose/ mg crude protein / min.

3.3 Results

3.3.1 Crude protein isolate (fibrolytic enzymes)

The colour of the protein precipitates consisted of variable shades of brown and green. The horse, wildebeest, zebra, buffalo and impala were characterized by a light reddish brown precipitate while those from the elephant, giraffe, llama and camel were characterised by a whitish-brown precipitate. Precipitates from the cow and sheep were characterized by a greenish colour. The crude protein solutions were active to the extent that their enzyme specific activities on carboxymethyl cellulose increased as protein concentration decreased following purification (Table 3.0). The activity recovered (purification fold) was highest in the horse and, zebras, intermediate in the wildebeest, impala and buffalo, and lowest in the camel, sheep and giraffe. It was interesting to note that the camel with the highest percentage yield (84.1%) had the lowest recovery (1.06 fold).

Table 3.0: Purication of crude protein extracts from herbivores using ammonium sulphate

Sample source	Sample fraction	Total Protein (mg)	Total Activity (nmol glucose/min)	Specific Activity (nmol glucose/mg/min)	Purificatin (fold)	Yield (%)
Cow	-Supernatant	310	2927.8	9.4	1	100
	-$(NH_4)_2SO_4$ precipitate	123	1981.7	16.1	1.706	39.7
Sheep	-Supernatant	313.1	2783.1	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	137.75	1377.5	10	1.125	43.9
Horse	-Supernatant	588	5553.3	9.4	1	100
	-$(NH_4)_2SO_4$ precipitate	232.4	8263.1	35.6	3.765	39.6
Camel	-Supernatant	396	3300	8.3	1	100
	-$(NH_4)_2SO_4$ precipitate	333	2960	8.9	1.067	84.1
Elephant	-Supernatant	462	5390	11.7	1	100
	-$(NH_4)_2SO_4$ precipitate	145.8	4536	31.1	2.667	31.6
Zebra	-Supernatant	876	7786.7	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	159.6	5231.3	32.8	3.688	18.2
Llama	-Supernatant	518	4316.7	8.3	1	100
	-$(NH_4)_2SO_4$ precipitate	201	2010	10	1.2	38.8
Wildebeest	-Supernatant	938	8337.8	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	182.6	3854.9	21.1	2.375	19.5
Giraffe	-Supernatant	336	2800	8.3	1	100
	-$(NH_4)_2SO_4$ precipitate	84	746.7	8.9	1.067	25
Impala	-Supernatant	451	3758.3	8.3	1	100
	-$(NH_4)_2SO_4$ precipitate	135	2025	15	1.8	29.9
Buffalo	-Supernatant	486	4320	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	170.2	2742.1	16.1	1.813	35.0

Substrate = Carboxymethyl cellulose

3.3.2 pH optimization

The trial for optimizing pH examined the specific CMCase activity of the various crude protein extracts (Figure 3.1). CMCase had a broad pH range of activity for all the experimental microbial ecosystems. Despite the broad pH range, some peaks of CMCase activity were, however, observed within the pH range 4.5–5.0 for the elephant, at pH 5 for the horse and the zebra, within pH 5.0–6.0 for the sheep, and within pH 5.0–7.0 for the wildebeest. CMCase activity for the rest of the animals (cow, llama, giraffe and camel) responded minimally within pH 4.0–8.0 range.

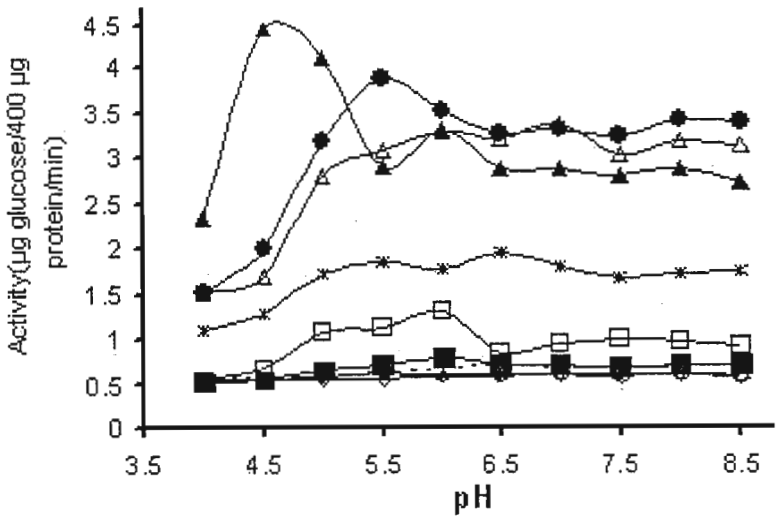

Figure 3.1: The effect of pH on carboxymethyl cellulase activity. The effect of pH on CMCase activity was measured by pipetting 500 µl of the substrate (1%CMC) into 500 µl of enzyme solution (400 µg/500 µl) and incubated for 2 h at 39°C. The reaction mixture was stopped by100 µl of 200 mM sodium carbonate and centrifuge at maximum speed on the desktop centrifuge for 5 min. 400 µl of the sample solution was analyzed for reducing sugars using DNS method. Product formation was monitored for sample from the reaction mixture of cow (■),sheep (□), horse (●), camel (○), elephant (▲), zebra (Δ), llama (◊), wildebeest (⋆), giraffe (+), impala (--) and buffalo (▪).

3.3.3 Optimization of crude protein concentrations for enzyme assay

Generally, product formation increased with increasing crude protein concentration from the sampled herbivore microbial ecosystems. Glucose liberated from crystalline cellulose (Figure 3.2 A), carboxymethyl cellulose (Figure 3.2 B) and *p*NP-G (Figure 3.2 C) following incubation with crude extracts, increased non-linearly. Xylose liberated from xylan by xylanase from crude protein extracts also increased non-linearly (Figure 3.2 D).

Figure 3.2 demonstrated the differences in enzymes activities among the sampled herbivore microbial ecosystems. In Figure 3.2 A, the highest increase was obtained in the zebra, horse and wildebeest while the elephant and impala showed moderate increases. High levels of product formation were also observed in horse, impala and wildebeest when CMCase concentrations were varied (Figure 3.2 C). A similar situation was also observed when cellobiase was incubated with *p*NP-G (Figure 3.2 B).

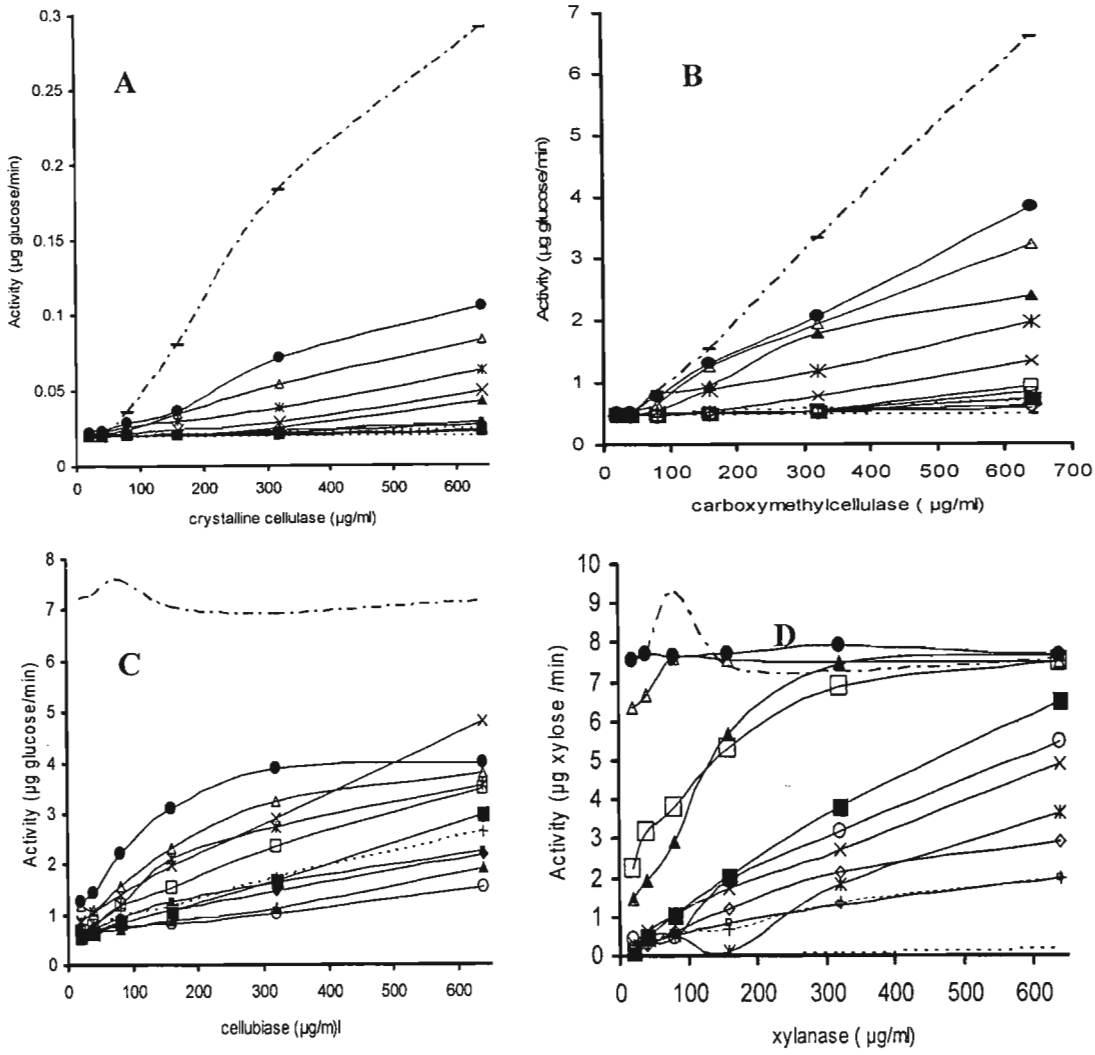

Figure 3.2: The effect of enzyme concentrations on product formation. *The reaction mixture containing crystalline cellulose (3.1 A), carboxymethylcellulose* (3.1 B), *p*-nitrophenyl ß-1,4-glucopyranoside (3.1 C) or xylan (3.1 D) were incubated with precipitated protein samples under standard assay conditions as described in the method section. The amount of reducing sugar liberated was determined by DNS reagent (Miller, 1960). Product formation was monitored from the reaction mixture of cow (■),sheep (□), horse (●), camel (○), elephant (▲), zebra (Δ), llama (◊), wildebeest (•),giraffe (+), impala (×) and buffalo (▫). The positive controls (-·-) were commercial cellulase from *Aspergillus niger* (3.1 A and 3.1 B) cellubiase (3.1 C) and xylanase from *Thermomyces ianuginosus* (3.1 D) while the negative control (...) was BSA for all experiments

Product formation from the reaction mixtures of the horse, zebra, impala, wildebeest and sheep were dominant while the rest were crowded together at low product formation. The result was not very different when hemicellulases were incubated with xylan (Figure 3.2 D). The product formed in the sheep was as high as those observed

from the horse and zebra, while the cow, camel and giraffe demonstrated relatively high activities

3.4. Discussion

Collecting fresh samples with limited exposure to oxygen maximised the presence of anaerobic cellulolytic and hemicellulolytic microbes as well as their enzymes. Limited exposure to oxygen prevents cell death, which was vital for the release of periplasmic proteins during sonication (Rachel *et al.,* 2000). Ammonium sulfate precipitation reduced the amount of unwanted proteins in the samples. This was confirmed by the increase in specific activity of crude protein samples with a decrease in protein concentration (Groleau and Forsburg, 1983). The quantities of crude protein loss from the wildebeest (243.6%), zebra (231.1%), horse (114.7%), impala (101%) and buffalo (101%) were higher than that observed in camel (20.3%), sheep (56.5 %) and cow (60%). The high values observed in horse and zebra were accounted for by the loss of both enzymes and unwanted proteins because their supernatants were relatively active while low activity was observed in buffalo, camel, cow or sheep. The reddish-brown protein precipitate observed in horse, wildebeest, impala and zebra was due to high cellobiase content while the greenish colour in cow and sheep precipitates was due to the plant pigment chlorophyll.

Specific activity and enzyme stability were both affected by pH and temperature. The rate of enzyme reactions might be compromised if these factors were neglected. pH optimisation was vital as enzymes were isolated from 11 different ecosystems which might vary greatly. The pH profile showed that activity occurs with a wide range as opposed to the narrow *in vivo* pH range (5.6-8) described by many authors (Russel and Dombrowski, 1980). This disparity can be ascribed to the presence of enzymes only in *in vitro* systems unlike in *in vivo* where very low or high pH might compromise microbial growth and survival. However the peaks observed in the horse (pH 5.5) and elephant (pH 5) demonstrated that these ecosystems were harbouring microbes that were active in slightly acidic conditions. Variations can also be attributed to the genetic differences among strains of microbes in various ecosystems in the expression of these enzymes.

Other factors also affecting activity include enzyme concentration and substrate concentration. Suitable substrate concentrations were obtained from literature (Henry *et al.* (1974) while the optimisation of enzyme sample concentration was needed because optimal enzyme activity varies among microbial ecosystems. The results arising from incubating different crude protein concentrations with the different substrates (CMC, xylan, crystalline cellulose and cellobiose) established that 340 µg was the ideal concentration to apply in enzyme assays. It was also noted that as the crude protein concentration increases the rate also increases linearly but linearity was lost at high crude protein concentrations. This was expected because at a high crude protein concentration, substrate concentration would become rate limiting. This was similar to the results obtained by Groleau and Forsburg (1983) in studies on cellobiase and CMCase.

In an attempt to optimise extract concentrations for enzyme assays with the different substrates, it was noted that the rate at which protein samples from the different ecosystems hydrolyse specific substrates was not the same. Crude protein samples from the horse, zebra and wildebeest were the most active when incubated with all four substrates but for the elephant which was also very active when incubated with CMC. Interestingly, the hindgut fermenters contained the most active protein samples. Although many studies have stated that there are no major differences among the microbes in both fore- and hindgut fermenters (Smith and Mackie, 2004), the results obtained from this study clearly indicate that there might be some factors influencing activity in these chambers. If time spent by digesta is a major factor (Schingoethe, 1993) then it is imperative to postulate that microbes in the hindgut will be more efficient in order to extract sufficient nutrients before it is being eliminated as faeces. However not all the foregut fermenters exhibited low activities as the wildebeest; the impala and cow showed intermediate activities with all four substrates. Variation in these activities might have been influenced by differences in ecological niches or composition of microbial species. For herbivores within the same geographical region, the differences in enzyme activity could be attributed to differential genetical evolution of microbes which might have resulted in variable fibrolytic efficiencies.

3.5 Conclusion

Crude proteins (containing cellulases) precipitated from all 11 herbivore ecosystems were active as demonstrated by their increase in specific activity with decreasing protein concentrations. A wider pH range was observed for these enzymes at 39°C. An enzyme concentration (of 340 μg/ml) for optimal activity was established as suitable for cellulose and carboxymethyl cellulose. Crude protein samples from the zebra, horse and wildebeest were the most active when incubated with all four substrates while those from the elephant, impala, cow and giraffe were intermediate. Future studies to confirm high activity among these ecosystems would include determining the affinity of enzyme to substrate, maximum velocity as well as their catalytic efficiencies.

Chapter 4

Seasonal variation of fibrolytic enzymes in seven herbivores

4.0 Abstract

Cellulose, the major component of plant cell walls, is the most abundant polysaccharide in nature and a virtually inexhaustible source of renewable bioenergy. In nature, cellulose is hydrolysed to glucose by microorganisms, mainly fungi and bacteria that produce a set of extracellular enzymes. Hemicelluloses are soluble plant cell wall polysaccharides that are hydrolysed by hemicellulases. In this study rumen fluid was collected in summer and winter from fistulated herbivores (sheep and cow) and faeces from non-fistulated herbivores (horse, zebra, impala and buffalo) *in situ*. Microbes in both the solid and liquid phases of the sampled solutions were lysed by sonication to liberate periplasmic cellulases and hemicellulases. Crude proteins were precipitated by 60% ammonium sulfate and dialysed. Protein samples were assayed for exocellulase, endocellulase, cellobiase and xylanase by incubating with crystalline cellulose, carboxymethylcellulose, p-nitrophenyl ß-1, 4-D-gulcopyranoside and xylan at optimum pH (4.5, 5.5, 6 and 6.5) for 48, 2, 2 and 1 h, respectively. The specific activities of each enzyme from the 11 ecosystems were determined. By relating specific activity to enzyme concentration, it was found that exocellulase concentrations in all seven herbivores varied from winter to summer ($P < 0.0001$). Although there was a general increase in exocellulase concentrations, the increase in sheep (269.96%) and horse (233.96%) was outstanding. This increase was attributed to an increase in fibre content of forages in winter, while the concentration of endocellulases and xylanase decreased from summer to winter due to a decrease in soluble carbohydrates and hemicelluloses. These results suggest that *in vitro* analysis of rumen fluid or faecal innoculum for exocellulases will be best in winter while sampling for endocellulases and xylanases will be advantageous in summer.

4.1 Introduction

In natural pastures (veld) herbivores browse and graze on forages that are richer in fibre while enclosed livestock depend on fibrous feeds (hay, kikuyu, lucerne and alfalfa) and supplementation. Abreu (1993) reported that less than half of the fibrous feeds eaten by animals in the European countries are wasted as faeces (although it may be higher in certain countries). This waste is due to the inability of herbivore microflora to efficiently hydrolise fibre. The unsatisfactory response of microbes towards fibre hydrolysis has led to speculation on how fermentation can be improved to enhance production and animal welfare. In order to better understand fibre hydrolysis different approaches have been pursued beginning with several levels of aggregation, pure cultures of fibrolytic microorganisms (Varel *et al.*,1991), mixed cultures of fibrolytic microorganisms (Colombatto *et al.*, 2003), components of cellulase enzymes (Beldman *et al.,* 1985) and unfractionated enzyme systems (Lynd *et al.*, 2002). An active and diverse microflora (bacteria, fungi and protozoa) is responsible for the breakdown in both fore- and hindgut of herbivores. Although bacteria (Flint, 1994), fungi (Fonty and Gouet, 1994) and protozoans (Jouany and Ushida, 1994) have been shown to exhibit cellulolytic activities not all species living in the fore- and hindgut are fibrolytic (Chen and Weimer, 2001).

Microbes function by expressing three major enzyme systems: exocellulases, endocellulases and cellobiases which act synergistically on the substrate to release soluble sugars (Gilbert and Hazlewood, 1993). These soluble sugars are consumed by microbes liberating end-products such as volatile fatty acids that are essential metabolites to the host. Cellulase enzyme systems are often more complex in the rumen than always stated due to the nature of cellulose substrates. Cellulose is made up of a heterogeneous intertwined polysaccharide chains with varying degrees of crystalinity, hemicelluloses and pectins embedded in lignin. Therefore, an efficient ruminal system will require hemicellulase, pectinase and ligninase to expose the embedded fibre. The supplementation of ruminant dietswith exogenous fibrolyticenzymes of has been shown to improve animal performance and milk production (Beauchemin *et al.*, 2003). Pure or mixed cultures of microbes utilised to monitor fibre breakdown still involve the expression of enzymes which implies that cellulolytic enzymes are vital entities for fibrolysis. It is, therefore, possible that

enzyme expression is affected when the cellulolytic microbial population is maintained by supplementing ruminant feed with concentrates (Tafaj *et al.*, 2001), protein and nitrogen (Yang, 2002) and phosphorus.

Russel and Hespell (1981) classified microbes according to their substrate specificity into two broad categories, namely primary (complex cell wall fermenters) and secondary (products formed by primary fermenters) fermenters. Bergen *et al.* (1982) refined the studies by showing that the growth rate of bacteria is partially a function of the availability of substrates at any given time interval while Cassia *et al.* (1999) showed that the growth rate of endoglucanases was higher in cellobiose fed cultures but its production was greater in cultures grown on amorphous cellulose. Although the source of these microbes, in many cases, is similar among enzyme products, the types and activity of enzymes produced can vary widely depending on the strain selected, the growth substrate and the culture condition used. The composition of pastures and forages can vary significantly. Knowing the distribution of these polysaccharides would assist in choosing forages with high energy content and limited anti-nutritional factors so as to improve animal performance.

Most studies on enzyme systems focus on isolated specific microbial strains (Matulova *et al.*, 2005; Weimer and Weston, 1985) while a few studies collect rumen fluid and sample for enzyme activity (Smith *et al.*, 1973; Weimer *et al.*, 1990). The rate and extent of fibre digestion in the rumen are largely dependent on the population size of cellulolytic bacteria that indirectly affect enzyme concentration. Rumen fluid obtained *in vivo* as opposed to *in vitro* cultures can give a better reflection of the actual fibrolytic process. Studies specifying appropriate seasons for enzyme sampling are limited (Koike *et al.*, 2000). In this study, rumen fluid and faeces will be collected from seven herbivores in winter and summer and their activities will be monitored. The specific activity of these crude proteins will be determined and related to enzyme concentrations to deduce the relative abundance of each enzyme in the cellulase enzyme system, with the aim of determining the appropriate season to obtain samples of microbial ecosystems for the fibrolytic enzyme assays.

4.2 Materials and Methods

4.2.1 Materials

The chemicals, commercial enzymes and equipments utilized in this experiment were the same as those described in section 3.3.1.

4.2.2 Experimental animals

Animals were chosen with no preference to sex or species. *Bos taurus* (fistulated Jersey cows), *Equus caballus* (horse) and *Ovis aries* (fistulated sheep) were from the Ukulinga Research farm, University of KwaZulu Natal, Pietermaritzburg. *Equus quagga boehmi* (zebra), *Connochaetes taurinus albojubatus* (wildebeest), *Aepyceros melampus* (impala) and *Syncerus caffer* (buffalo) were also made available by the Tala Game Reserve, Umbumbulu, KwaZulu-Natal (SA).

4.2.3 Protein isolation

Protein samples were collected from herbivores as described in chapter 3 (section 3.2.4). Crude protein samples were precipitated using ammonium sulfate, dialyzed overnight and concentration determined (section 3.2.7).

4.2.4 Enzyme assays

Exocellulase, endocellulase, xylanase and cellobiase specific activities were determined by incubating with crystalline cellulose, carboxymethyl cellulose, xylan and *p*NP-G respectively, as described in chapter 3 (section 3.2.11). Reducing sugars liberated from the different enzyme assays were measured as previously described by Miller (1959). Samples were analysed to determine the effects of season and microbial ecosystem using the GLM procedure in SAS (2001).

4.3 Results

4.3.1 Protein precipitation

The final step of the isolation procedure was protein precipitation using 60% (m/v) ammonium sulfate. The colour of the precipitates did vary from one animal to another. Crude protein extracts from the horse, wildebeest, zebra, buffalo and impala were characterized by a light reddish brown precipitate while those from the cow, and

sheep were characterised by a greenish colour. The crude protein solutions were active as demonstrated by their specific activities on carboxymethyl cellulose. Generally, the isolation process was successful as there was an increase in enzyme specific activity with a decrease in protein concentration following the purification process (Table 4.0). The recovered activity (purification fold) was highest in the horses and zebras while that of the wildebeest was relatively high. The impala, buffalo and cow had low recovery values.

Table 4.0: Purification of crude protein extracts from 11 hervore microbial ecosystems using ammonium sulphate.

Sample source	Sample fraction	Total Protein (mg)	Total Activity (nmol glucose/min)	Specific Activity (nmol glucose/mg/mi)	Purificatin (fold)	Yield (%)
Cow	-Supernatant	310	2927.8	9.4	1	100
	-$(NH_4)_2SO_4$ precipitate	123	1981.7	16.1	1.706	39.7
sheep	-Supernatant	313.1	2783.1	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	137.75	1377.5	10	1.125	43.9
Horse	-Supernatant	588	5553.3	9.4	1	100
	-$(NH_4)_2SO_4$ precipitate	232.4	8263.1	35.6	3.765	39.6
Zebra	-Supernatant	876	7786.7	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	159.6	5231.3	32.8	3.688	18.2
Wildebeest	-Supernatant	938	8337.8	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	182.6	3854.9	21.1	2.375	19.5
Impala	-Supernatant	451	3758.3	8.3	1	100
	-$(NH_4)_2SO_4$ precipitate	135	2025	15	1.8	29.9
Buffalo	-Supernatant	486	4320	8.9	1	100
	-$(NH_4)_2SO_4$ precipitate	170.2	2742.1	16.1	1.813	35.0

Substrate = Carboxymethyl cellulose

4.3.2 pH optimization

Carboxymethyl cellulase (CMCase) was the specific enzyme that was targeted in this assay. The results obtained from incubating 400 µg protein/0.5 ml with 1% CMC demonstrated that, CMCase had a broad pH range of activity for all the experimental animals. Although the pH range of activity was broad some peaks were recorded at different intervals within the incubation process. In the horse the pH increases gradually from 4.0 to 4.5 and peaks at 5.5. A similar observation was found in the zebra with the only difference being the optimal activity at pH 6. In the sheep, peaks of optimum activity were observed between pH 5.0–6.0 while a range of 5.0–7.0 was

chosen as the active range for wildebeest. The cow responded minimally to pH changes within pH 4.0–8.0.

4.3.3 Enzyme specific activity

4.3.3.1 Exocellulase specific activity

Exocellulase specific activity differed between seasons and among herbivore microbial ecosystems (Table 4.1). With the exception of the sheep ecosystem for which the exocellulase activity was similar between seasons, the activity of this enzyme increased in all other ecosystems by 1.12 to 3.33 folds. The impala, buffalo, cow and wildebeest had the lowest improvements in exocellulase concentrations while the horse and zebra had the highest.

Table 4.1: Seasonal variation of exocellulase, endocellulase, cellobiase and xylanase specific activities of crude protein extracts from seven herbivore microbial ecosystems

	Endocellulase specific activity (µg glucose/mg /min)		Exocellulase specific activity (µg glucose/mg /min)	
Substrate	Carboxylmethyl cellulose		Crystalline cellulose	
	Summer	Winter	Summer	Winter
cow	13.304	5.871	0.199	0.306
sheep	6.044	3.586	0.254	0.252
Horse	19.451	12.945	0.27	0.899
Zebra	25.395	11.948	0.269	0.732
Wildebeest	3.924	7.436	0.259	0.525
Buffalo	8.209	5.667	0.26	0.292
Impala	3.43	5.238	0.274	0.381
Standard error	0.569		0.052	
P<	0.0001	0.0001	0.0001	0.0001
	Xylanase specific activity (µg glucose/mg /min)		Cellobiase specific activity (µg glucose/mg /min)	
Substrate	Xylan		cellobiose	
	Summer	Winter	summer	winter
cow	100.077	88.532	0.54	0.286
sheep	67.415	83.981	0.834	0.248
Horse	160.665	106.573	1.446	0.63
Zebra	363.516	100.517	2.725	0.754
Wildebeest	20.929	23.363	0.244	0.563
Buffalo	13.561	22.588	0.067	0.156
Impala	41.481	29.04	0.311	0.243
Standard error	0.809		0.011	
P<	0.0001	0.0001	0.0001	0.0001

4.3.3.2 Endocellulase specific activity

Endocellulase specific activity differed ($P<0.0001$) among sampled herbivore microbial ecosystems and between seasons (Table 4.1), with the overall effect that the endocellulase activity was lower in winter than in summer for all but impala and wildebeest microbial ecosystems. Among species, endocellulase specific activities in summer was higher in the zebra and horse, intermediate in the cow and lowest in buffalo, sheep, impala and wildebeest.

4.3.3.3 Xylanase specific activity

The xylanase specific activity differed ($P<0.0001$) among sampled herbivore microbial ecosystems and between seasons. The zebra recorded the highest specific activity, followed by the horse with about half the specific activity in the zebra (Table 4.1). The cow and sheep systems had intermediate activities, while impala, buffalo and wildebeest recorded the lowest xylanase activity. The xylanase specific activity increased noticeably in summer for zebra and horse ecosystems, and slightly for impala and cow.

4.3.3.4 Cellobiase specific activity

A significant variation in cellobiase specific activity was observed among animal species between seasons ($P<0.0001$). Apart from the high variation of cellubiase specific activity observed in the zebra and horse, the rest of the animals responded minimally to seasonal changes (Table 4.1). Among herbivore species, the zebra, horse and sheep recorded high enzyme specific activities while the rest of the animals were relatively similar in specific activities.

4.3.4. Correlation of enzyme specific activities among experimental animals

Apart from the seasonal variation observed in herbivores, variations among herbivore cellulolytic and hemicellulolytic enzymes were considerable (Table 4.2). The mean exocellulase concentration of the horse was significantly different ($P < 0.05$) from that of the zebra which was in turn was greater than that of the wildebeest and impala (Table 4.2). There was no significant difference in exocellulase concentrations observed among the buffalo, sheep and cow. Endocellulase, xylanase and cellobiase activities of the zebra were significantly different from the rest of the herbivores.

However the horse showed the second most distinctive enzyme concentrations while the rest of the animals were slightly different from each other.

Table 4.2: Comparison of the *in vitro* hydrolyis of crystalline cellulose, carboxymethyl cellulose, cellobiose and xylan by exocellulase, endocellulase, xylanase and cellobiase from 7 herbivores microbial ecosystems

Specific activity	Cow	Sheep	Horse	Zebra	Wildebeest	Impala	Buffalo	SED
Exocellulase (µg glucose/mg /min)	0.253^{e}	0.253^{e}	0.585^{a}	0.501^{b}	0.392^{c}	0.327^{d}	0.276^{e}	0.0188
Endocellulase (µg glucose/mg /min)	12.921^{c}	4.8152^{f}	19.198^{b}	81.672^{a}	5.679^{e}	4.334^{f}	6.938^{d}	0.4312
Xylanase (µg xylose/mg /min)	94.503^{c}	75.698^{d}	133.619^{b}	232.016^{a}	22.146^{f}	35.261^{e}	18.074^{f}	4.5663
Cellobiase (µg glucose/mg /min)	0.419^{d}	0.541^{c}	1.038^{b}	1.739^{a}	0.4033^{d}	0.277^{e}	0.112^{f}	0.0766

^{a,b,c,d,e,f} Mean within a row with unlike superscript letters were significantly different ($P<0.05$)

4.4 Discussion

The crude protein extract and pH activity profile observed in this experiment were the same as those observed and discussed in chapter 3 (section 3.4).

For comparative studies, specific activity per unit protein was the parameter exploited to estimate the seasonal variation of celluloytic enzyme concentrations (Bruce *et al.*, 1984). Variation in specific activities observed from crude protein samples within herbivore ecosystems in summer and winter could be attributed to forage availability, chemical composition of forages, composition of microbial population, microbial requirements, voluntary intake of forage by herbivores and the efficiency of the different fermentation chambers (Kamra, 2005).

The distribution of forages in winter and summer is very diverse. Summers are characterised by young and (or) green pastures while the winters are characterized by mature and dry plants. The high concentrations of cellulases targeting crystalline cellulose (horse and zebra) in winter were associated with the abundance of dry and fibre rich forages while their concentrations in summer decrease due to the low content of crude fibre in young and vegetative plants. High concentrations of carboxymethyl cellulase and xylanase in summer were due to the richness of soluble

carbohydrates (amorphous cellulose and hemicelluloses) in young and vegetative plants while in winter their concentration decreased as the quantity of soluble carbohydrates decreased.

Forages grazed by herbivores can be grouped into grasses, legumes and shrubs (grazers, intermediates and concentrates). The chemical composition of these forages varies among the groups as well as in summer and winter. Generally the cellulose content of grasses is higher than those of shrubs and legumes. Legumes and shrubs are in turn richer than grasses in both protein and soluble carbohydrates. At the beginning of summer when young plants (vegetative stages) are dominant the level of proteins and soluble carbohydrates is higher in legumes and shrubs than that observed in grasses which are in turn richer in cellulose than legumes and shrubs. As the plant matures towards winter, the level of soluble carbohydrates and proteins decreases with an increase in cell wall components such as cellulose, fibre and lignin in all three groups. Lignification is greater in shrubs and grasses than in legumes. Due to seasonal changes in the chemical composition of forages (high fibre) between seasons, it is possible that crystalline cellulase concentrations would increase in winter since crystalline cellulose is predominant in forages; correspondingly, carboxymethyl cellulase and xylanase would be high in summer because of the high concentration of these constituents in forages. Also, the low nutritive quality of some forages during the growth period might be due to environmental stresses such as high temperatures (van Soest, 1988) and infertile soils (Roberts, 1987).

Voluntary intake is also another factor that influences enzyme concentration indirectly. When an animal selects forages higher in fibre contents whether in winter or summer, the concentration of fibre degrading enzymes is bound to increase relatively. This implies that the concentration of fibre-degrading enzymes will be much lower in an animal grazing on very young shoots in winter or entirely on green leaves in the veld, than in those that are fed with hay (cows, horses, and sheep). The concentration of cellulases targeting crystalline cellulose observed in sheep in winter was not different from that observed in summer. This could be accounted for by the supplementation of hay with concentrates which might have diverted the overall microbial fermentation to affect mainly soluble carbohydrates, as such decreasing cellulolytic enzyme expression. This result was similar to that obtained by Hiltner and

Dehority (1983) and Bergen *et al.* (1982) where endoglucanase concentrations increased when carboxymethyl cellulose was supplemented with concentrates. Supplementation of feed possibly increased the rate of expression but not the relative abundance as compared to the expression on pure CMCase. Although supplementing hay with concentrates does enhance microbial growth, exocellulase expression and activity are still relatively low. High intake of fibre rich forages in winter and/or prior intestinal digestion (by horse, impala and wildebeest) allows cellulose to be the most available microbial substrates in winter which is associated with an increase in fibrolytic enzymes concentration.

Microbial population and microbial nutritional requirements are also two important factors that affect fibrolytic enzyme concentrations indirectly. While the carbon compounds serve as the main energy source, nitrogen is a major constituent of proteins (el-Shazly *et al.*, 1961); phosphorus is required for the synthesis of phospholipids, nucleic acids and the energy carrier, ATP. Sulphur is required for the synthesis of the amino acids, methionine and cysteine, and vitamins, B12 (Scott and Dehority, 1965) and potassium is required for the functioning of enzymes involved in protein synthesis. A deficiency will slow down cell division hence decreasing microbial population, mass and enzyme expression. It is possible that in winter, fibre-rich ingesta that leaves the intestines and enters the caeco-colon of the zebra or wildebeest is enriched and supplemented with endogenous losses which can boost microbial growth and hence increase enzyme activity. It is not certain why a herbivore such as buffalo, which also grazes on fibrous forages, would be deficient in fibrolytic enzymes relative to the wildebeest. Forage selection might partly explain this observation. For instance, it was typical for the buffalo to graze mainly on very dry substrates while the zebra, wildebeest and impala focused on mature sun-cured kikuyu areas of the veld, which likely could have been relatively richer in digestible fiber as well as proteins. The most adapted rumen fibrolytic microbes will increase the microbial population to dominate the ecosystem and by so doing will increase cellulolytic enzyme concentrations, hence an increase in fibre digestion. The influence of substrates on the microbial population was similar to the results obtained by Waldo (1972), van Soest (1973) and Weimer *et al.* (1990), which did increase with substrate availability.

4.5 Conclusion

In this study herbivores were sampled both in winter and summer to analyse the relative abundance of cellulolytic enzymes (exocellulase, endocellulase, cellobiase and xylanase) associated with the breakdown of fibrous forages. Available feed appeared to play a role in the relative abundance of these enzymes both in winter and summer. Exocellulases were higher in winter than summer due to the abundance of fibre-rich and lignin-cemented polysaccharides while endocellulase and xylanase concentrations were higher in summer than in winter due to the abundance of soluble polysaccharides and hemicelluloses. Minimal changes in cellobiase concentrations were observed in both seasons.

This study indicated that experiments involving exocellulase analysis from wild herbivores will be advantageous in winter while summer will be preferable for endocellulase and xylanase analysis. There is no seasonal preference for the analysis of cellobiases due to insignificant changes in cellobiase activity between seasons. Although enzyme concentrations did increase from one season to another the degree of activity of enzymes from these microbial populations varied, so it is of interest to determine the different enzymes involved. A further investigation to establish differences among enzymatic activities within and among herbivore species grazing on the same piece of land or in different geographical regions will be very important.

Chapter 5

Activities of microbial fibrolytic enzymes from eleven herbivore microbial ecosystems

5.0 Abstract

The objective of this study was to classify 11 herbivore microbial ecosystems in terms of their richness in cellulases and hemicellulases and their activities (antelope, camel, cow, buffalo, elephant, goat, horse, llama, sheep, wildebeest and zebra). Rumen fluid was collected from fistulated herbivores and faeces from non-fistulated herbivores *in situ*. Microbes in both the solid and liquid phases of the sampled solutions were lysed by sonication to liberate periplasmic cellulases and hemicellulases. Proteins were precipitated using 60% ammonium sulfate and dialysed. Protein samples were assayed for exocellulase, endocellulase, cellobiase and xylanase by incubating with crystalline cellulose, carboxymethyl cellulose, p-nitrophenyl ß-1, 4-D-glucopyranoside and xylan at optimum pH (4.5, 5.5,6 and 6.5) for 48 h, 2 h, 2h and 1h, respectively. Enzyme specific activities of each cellulase from the 11 ecosystems were determined. By relating specific activity to enzyme concentration, the ecosystems were classified into three groups. Group A with high enzyme concentrations comprised the zebra, horse, wildebeest, impala and elephant. Group B with intermediate concentrations comprised the cow, llama, camel, buffalo and giraffe. Group C, comprising only one member (the sheep), recorded the lowest enzyme concentration. Exocellulase concentrations had strong, moderate and weak relationships with endocellulases (r = 0.90), cellobiase (r = 0.67) and xylanases (r=0.54). However, systems that were rich in xylanases also had high concentrations of endocellulases (r = 0.7104). A kinetic study revealed that crude protein extracts from the microbial ecosystems of wildebeest, horse, zebra and elephant were the most active in all four enzymes analysed with high catalytic activity (Kcat/Km). So, it is possible that exogenous cellulolytic and hemicellulolytic enzymes from wild herbivores can supplement the farm animals' fibrolytic enzyme system and perhaps improve the fermentation of fibre.

5.1 Introduction

Herbivores (ruminants) are not capable of hydrolysing plant cell wall polysaccharides by utilising endogenous enzymes, thereby paving the way for a symbiotic relationship between ruminants and microbes (Lee *et al.*, 2002). Anaerobic bacteria, protozoans and fungi make up the microbial population (Hungate, 1966). Microbial distribution within the rumen is not homogenous, with bacteria contributing the largest population, followed by protozoa (Bryant, 1973) and then fungi (Bauchop, 1979). A sustainable microbial population is highly dependent on the availability of forages (Bryant and Burkey, 1953) and forage type (Stewart *et al.*, 1979). Herbivores occupy different environments and forage on a variety of different plant species, with an expected consequence that their microbial ecosystems are exposed to varied and perhaps different challenges inhibiting the extraction of nutrient from ingested substrates. There are also possibilities of intra- and inter-specific competition for the utilisation of substrates which could compel micro-organisms to evolve strategies/mechanisms that safeguard their dominance in the exploitation of substrate(s) and ensure their survival within their ecosystem. With fibrolytic organisms, their competitiveness may be enhanced by superior attachment processes, microbial ability to etch into a hemicellulolytic hindrance by removing chunks of xylan to expose cellulose substrate, utilisation of hemicellulose, cellulose or multiple substrates, the production of metabolites that are either inhibitory to rival organisms or facilitatory to the micro-organisms with which they interact synergistically (Varga and Kolver, 1997). Some of these possible strategies may be a translation or expression of fundamental changes within the micro-organisms. Since the microbial generation interval is very short, microbial adaptation to variable fibrous substrates might have evolved an adaptation of the microbial gene expressions towards the production of more active fibrolytic enzymes.

Herbivores are characterised by their fermentation chambers which can either be before (foregut fermenters) or after (hindgut fermenters) the true stomach. Fermentation varies with the relative size of these organs as well as the length of time spent by the digesta (Schingoethe, 1993). Microbes that inhabit these chambers secrete enzymes according to their needs, which implies that the system will be flooded with an array of enzymes from different microbes. However, not all microbes

in this system secrete cellulases or hemicellulases, but may be adapted in the utilisation of end products, hence reducing the extent of negative feedback inhibition (Bruce *et al.,* 1984). Accumulation of other end products such as H, methane and lactic acids may lead to anti-nutritional conditions such as bloating and lactic acidosis. Microbial sampling in sheep demonstrated that starch and sugar favour the growth of protozoans which can reach densities of 4 000 000/ml but would decreases to 100 000/ml on fibrous diets (Preston and Leng, 1987). Thus understanding the physiology of microbes and maintaining a population of optimum activity is vital. Different approaches for improving fibre breakdown have been proposed and tested, including components of cellulase enzyme systems (Gerrit *et al.,* 1984, Owolabi *et al.,* 1988; Xiao and Brent, 2000), unfractionated cellulase systems (Ali and Tirta, 2001), pure cultures of cellulolytic micro-organisms (Seyis and Aksoz, 2005), and mixed cultures of cellulolytic micro-organisms. Supplementing diets with fibre-degrading enzymes improved milk production in dairy cows and feed efficiency in growing cattle (Kung *et al.*, 1988, Pioneer Hi-bred International, 1988, Beauchemin *et al.*, 1995; Beauchemin *et al.*, 2003). The utilisation of these enzymes as feed additives is yet to be elucidated as only a minimal increase in fibre hydrolysis has been reported (Beauchemin *et al.,* 2003). Besides, enzymes are very expensive and difficult to manage since they are susceptible to lyses by ruminal proteases.

This study is an inventory of microbial fibrolytic enzyme activities in selected herbivore microbial ecosystems. It will quantify the microbial cellulolytic and hemicellulolytic enzymes from unfractionated ecosystems (protein extracts from 11 herbivores) based on their specific activity on crystalline cellulose, carboxylmethy cellulose (CMC), cellubiose and xylan. Secondly, the kinetic parameters viz: affinity of an enzyme to its substrate (Km), the rate at which products are being formed from a substrate (Vmax) and enzyme catalytic efficiency or specificity constant (Kcat/Km) will be determined.

5.2 Material and Methods

5.2.1 Material

Chemicals, commercial enzymes and equipments utilized in this experiment were the same as those described in section 3.2.1 apart from the Hyper3 Software Programme that was utilized for data analysis (Easterby, 2003).

5.2.2 Experimental animals

The eleven animals were chosen as previously described in section 3.2.2

5.2.3 Animal nutrition

This study was conducted during the winter months (April–August) 2005. Camel, elephant and llama were kept in an enclosed area and fed hay regularly. Game animals such as the impala, wildebeest, zebra and buffalo were grazing on a dry land in an open field where *Pennisetum clandestinum* (kikuyu grass), hay and other fibres were dominant. The buffalo migrated often to the valley where they chewed hay and stems while the impala selected green pastures. The giraffe browsed on a variety of tree leaves. At the Ukulinga research farm, horses and fistulated cows were fed entirely on hay but sheep received supplementary concentrate.

5.2.4 Sample collection

Rumen fluid and faecal samples were collected by slightly modifying the method of Smith *et al.* (1974) as previously described in section 3.2.4.

5.2.5 Protein extraction and determination

Crude protein extracts were isolated following the procedure described in section 3.2.5. Each sample solution was pipetted into a dialysis membrane and immersed in a 2*l* storage buffer solution overnight (12 h), before being concentrated using polyethylene glycol 20 000. The Micro BCA^{TM} Protein assay kit (Richardson, 2000) was used for protein determination (section 3.2.7).

5.2.6 Quantification of reducing sugars

Reducing sugars were determined as previously described by Miller (1950) with a few modifications (section 3.2.10).

5.2.7 Enzyme assays

Exocellulase was assayed following the method described by Gerrit *et al.* (1984) (section 3.2.11.1). The activity of endocellulase was determined following the method described by Gerrit *et al.* (1984) (section 3.2.11.2). Cellobiase activity was measured in a mixture of 0.5 ml of 0.1% (m/v) *p*NP-G in the reaction buffer and 0.5 ml of crude protein solution, based on the method described by Frutos *et al.* (2002)(section 3.3.11.3). Xylanase was essayed by the method described by Khanna (1993) and Seyis and Aksoz (2005) (section 3.3.11.4). Each ecosystem was represented by three samples, each of which was analyzed in triplicates. Specific activity was defined as µg of glucose/ mg crude protein /min.

5.2.8 Determination of enzyme kinetic parameters (Km and Vmax)

Enzyme assays were the same as those described for enzyme specific activities (section 2.11) but for the fact that reducing sugar was measured at intervals to determine the kinetic parameters, Km and Vmax. CMC, *p*NP-G, and xylan concentrations were varied from 0.25 – 2 mg/ml while samples were collected at 5 min intervals and analyzed for glucose and *p*NP. Crystalline cellulose concentration was varied between 0.5 – 4 mg/ml and the sample mixture incubated at 39°C for 12 h during which sample were collected at 2 h intervals and analyzed for glucose. The initial velocity (V_0) at each substrate concentration was determined by calculating the slope of a progressive curve of time versus reducing sugar per hour. Km and Vmax were calculated based on procedure described by Eisenthal and Cornish-Bowded (1974) using the HYPER software programme (Easterby, 2003). Enzyme catalytic rate or rate of product formation (Kcat) was equal to Vmax at constant enzyme concentration. Kcat is also called turnover number which is defined as the maximal number of product formed per active site per unit time. The ratio Kcat/Km (specificity constant) measures the catalytic efficiency of an enzyme-substrate pair (Che-Chang *et al.*, 2005; Sophie *et al.*, 1999).

5.3 Results

The protein extracts and colour variation was same as that described in section 3.3.1

5.3.1 Enzyme specific activity

Cellulases targeting crystalline cellulose specific activity differed ($P<0.0001$) among the sampled herbivore microbial ecosystems. The horse and zebra recorded the highest crystalline cellulase activity while the wildebeest and impala recorded intermediate activities (Table 5.0). The cow, sheep, giraffe, llama, buffalo, elephant and camel exhibited low crystalline cellulase activities.

Table 5.0 Monitoring the specific activities of exocellulase, endocellulase, cellobiase and xylanase from crude protein extracts of 11 herbivore microbial ecosystems

Enzyme source	[protein] (mg/ml)	Total Protein (mg)	Exocellulase specific activity (µg glucose/mg/min)	Endocellulase specific activity (µg glucose/mg/min)	Cellulbiase specific activity (µg glucose/mg/min)	Xylanase specific activity (µg xylose/mg/min)
cow	8.2	87	1.83	2.93	0.36	38.05
sheep	7.25	116.2	1.49	1.77	0.31	35.96
Horse	8.3	134.98	5.39	6.47	0.79	46.38
Camel	7.94	104	1.58	1.63	0.16	10.13
Elephant	8	134.4	1.96	5.49	0.19	34.48
Zebra	8.4	101.64	4.39	5.97	0.94	43.59
Llama	6.6	108.16	1.65	1.69	0.29	7.58
Wildebeest	8.32	89.6	3.15	3.72	0.70	8.00
Giraffe	5.6	129.75	1.65	1.80	0.49	7.73
impala	7.5	81.4	2.28	2.62	0.30	10.62
Buffalo	7.4	14	1.74	2.83	0.19	7.64
Standard error			0.14	0.15	0.012	0.38
P <			0.0001	0.0001	0.0001	0.0001

The horse, zebra and elephant had the highest ($P<0.0001$) CMCase specific activity while the cow, wildebeest, impala and buffalo had intermediate carboxymethyl cellulase specific activities (Table 5.0). The lowest CMCase specific activities were exhibited in sheep, camel, llama and giraffe.

Cellobiase specific activities differed ($P<0.0001$) among the sampled herbivore microbial ecosystems. Wildebeest, zebra and horse recorded the highest cellobiase

specific activities while giraffe, impala, sheep and cow demonstrated intermediate activities (Table 5.0). The buffalo, camel, elephant and llama exhibited the lowest cellobiase specific activities.

The sampled herbivore microbial ecosystems differed in xylanase specific activities (P<0.0001). The horse and zebra recorded the highest xylanase specific activities whereas the sheep, cow and elephant were observed with intermediate activities (Table 5.0). Impala, giraffe, wildebeest, buffalo, llama and elephant had the lowest xylanase specific activities.

A correlative analysis showed that crystalline cellulase activity was strongly correlated to CMCase activity (r = 0.90; P<0.0001), had moderate correlation with cellobiase (r = 0.67; P<0.0001) and a low correlation with xylanase activity (r = 0.54, P<0.0006). Whereas xylanase activity was moderately correlated with CMCase activity (r = 0.71, P<0.0001). Analysis of cellobiase activity showed similar correlations with all the other enzymes, crystalline cellulase (r = 0.67, P<0.0006), CMCase (r = 0.67, P<0.0001) and xylanase (r = 0.56, P<0.0012).

Table 5.1: Kinetic constants of crude carboxylmethyl cellulase and cellobiase on their specific substrates carboxylmethyl cellulose and p-nitrophenyl β-D-glucopyranoside

	Carboxymethyl cellulase			Cellobiase		
Herbivore	Vmax (µg glucose/mg protein/min)	Km (µg/ml)	Efficiency (/µg/ml/ugS)	Vmax (µg glucose/mg protein/min)	Km (µg /ml)	Efficiency (/µg/ml/µgS)
Zebra	15.81	0.986	16.034	1.75	1.219	1.436
Horse	16.42	1.39	11.813	1.18	0.174	6.782
Wildebeest	1.61	0.02	80.500	0.15	0.305	0.492
Impala	10.38	0.908	11.432	0.33	0.24	1.375
Elephant	27.62	3.061	9.023	5.45	0.205	26.585
Sheep	3.49	2.829	1.234	0.95	0.131	7.252
Camel	1.35	0.182	7.418	0.01	0.686	0.015
Cow	62.11	1.528	40.648	0.71	0.168	4.226
Buffalo	0.25	0.046	5.435	0.29	0.533	0.544
Giraffe	1.26	0.364	3.462	0.1	0.266	0.376
Llama	ND	ND	ND	0.02	1.08	0.019

Vmax = µg glucose/min, Km = µg substrate, ND = not determined. Kcat = turn over number, S=substrate

5.3.2 Enzyme activity (enzyme rates)

The kinetic parameters, km, Vmax and Kcat/Km, of CMCase and cellobiase for the microbial herbivore ecosystems are shown on Table 5.1. From the results obtained, the wildebeest and cow had the highest catalytic efficiencies. The zebra, camel, impala and horse on the other hand had moderately active ecosystems with intermediate values of Kcat/Km. The elephant, sheep, buffalo, giraffe and llama recorded the lowest Kcat/Km. With respect to cellobiase activity, the sheep had the most active microbial ecosystems, with the highest Kcat/Km value. The horse, sheep and cow had intermediate Kcat/km values while the buffalo, wildebeest, zebra, camel, giraffe and llama had the lowest cellobiase efficiency.

The kinetic parameters, km, Vmax and Kcat/Km, for crystalline cellulase and xylanase enzymes are shown on Table 5.2. The horse and wildebeest had the highest Kcat/Km values for crystalline cellulase, followed by the giraffe and zebra with intermediate values. The least efficient systems with the lowest Kcat/Km values for crystalline cellulase were the cow, elephant and impala.

Table 5.2: Kinetic constants of crude crystalline cellulase and xylanase on their specific substrates crystalline cellulose, and xylan respectively

	Crystalline cellulase			Xylanase		
Herbivore	Vmax (µg glucose/mg protein/min)	Km (µg/ml)	Efficiency (/µg/ml/ugS)	Vmax (µg glucose/mg protein/min)	Km (µg/ml)	Efficiency (/µg/ml/µgS)
Zebra	40.79	0.387	105.401	21.57	0.744	28.992
Horse	26.29	0.031	848.065	206.27	1.329	155.207
Wildebeest	25.24	0.048	525.833	3.81	0.012	317.500
Impala	1.58	0.162	9.753	1.24	0.745	1.664
Elephant	4.25	1.423	2.987	279.19	1.143	244.261
Sheep	ND	ND	ND	79.52	1.591	49.981
Camel	ND	ND	ND	11.73	1.292	9.079
Cow	12.56	1.278	9.828	191.34	2.823	67.779
Buffalo	ND	ND	ND	2.13	0.578	3.685
Giraffe	7.63	0.058	131.552	3.1	3.324	0.933
Llama	ND	ND	ND	3.85	2.089	1.843

Vmax = µg glucose/min, Km µg substrate, ND = not determined. Kcat = turn over number, S = substrate

The highest Kcat/Km ratios for xylanase were observed in the wildebeest, horse and elephant. Intermediate values of Kcat/Km for xylanase were observed in the cow,

sheep, camel and zebra with moderate activity, while the llama, giraffe, buffalo and impala, which were the least active microbial ecosystems, had the lowest Kcat/Km values for xylanase.

5.4 Discussion

For comparative studies, specific activity per unit protein was the parameter exploited which was similar to that utilised by Bruce *et al.* (1984). The differences in crude protein specific activities among the ecosystems were expected due to the diversity of microbes within or among herbivore species. Apart from microbial diversity, the size of the microbial population (indexed by enzyme concentrations) as well as enzyme activity could also characterise these ecosystems since samples were harvested from different geographical regions and from animals feeding in different ecological niches. Wanapat (1989), Wanapat *et al.* (1994), Kenedy and Hogan (1994) and Wanapat (2001) in their studies on cattle and buffaloes reported that buffaloes utilise feed 2-3% units better than cattle kept under the same conditions as a consequence of increased cellulolytic bacteria and decreased protozoans in buffalo.

Protein solutions from the horse and zebra had the highest specific activities when incubated with cellubiose that was more soluble than crystalline cellulose. This was in agreement with others (Groleau and Forsberg, 1981) who reported that cell-free culture fluids and cell extracts from *B. succinogenes* had low hydrolytic activity on crystalline cellulose but high endocellulase (CMCase) and cellobiase activities. The horse, zebra and wildebeest were 264.7%, 111.4% and 194.6% richer, respectively, in crystalline cellulases when compared to the sheep with the lowest enzyme concentrations. Crude protein extracts from impala, cow and elephant demonstrated moderate crystalline cellulases which were 53.0, 31.5 and 22.8% more active, respectively, relative to the sheep.

The horse and zebra were again richer in endocellulase concentrations when compared to domesticated ruminants. This was associated with microbial diversity, substrate availability and the ability of most exocellulase to hydrolyse CMC. The elephant that was observed with moderate concentration of crystalline cellulases had a high concentration of endocellulases. Buffalo, impala and cow showed low enzyme

concentrations while very low concentrations were observed in llama and giraffe. Based on *p*NP-G hydrolysis the zebra, horse, wildebeest and giraffe had the highest concentration of cellobiases. Cellobiase specific activities of the cow, impala, llama and sheep were relatively high, which implies that cellobiase expression was also relatively high in all systems.

The microbial ecosystem of the giraffe achieved the highest specific hemicellulolytic activity due to microbial adaptation being influenced by substrate availability (Hofmann, 1989). With respect to the activity of hemicellulases, the cow and sheep were comparable to the horse and zebra with the highest specific activities, which demonstrate high levels of enzyme concentrations.

Apart from the horse, game animals (zebra, wildebeest, impala and giraffe) were noted for their high expression of fibrolytic enzymes. This suggested that microbes from different geographical regions had different enzyme expression and competence. Hindgut fermenters (zebra and horse), except the elephant had, a higher exocellulase activity relative to ruminants. This was associated with the efficiency of microbes in the hindgut rather than their geographical locations because ruminants grazing on the same field had lower activities. Besides digesta entering the caecum has very little degradable substrate relative to rumen contents (Mould *et al.*, 2005). In addition, digesta passage rate is high; hence fibrolytic organisms living in this organ, by necessity of survival, should be more efficient in fibre breakdown. It is also possible that the absence of protozoan preying on bacteria in the caecum would lead to an increase in bacteria population which are the most active cellulolytic microbe (Hobson, 1971).

Among the foregut fermenters the impala and wildebeest had the highest exocellulase activity when compared to the farm animals. This can be associated with the evolutional efficiency of microbes of higher competence in different geographical regions that differ in substrate availability (Latham *et al.*, 1971). Secondly, individual animals harbour different microbes that have evolved with different survival mechanisms for maximising nutrient extraction from plant cell walls with limited soluble polysaccharides. Therefore, microbes in animals of the same species

occupying the same ecological niche might differ in fibrolytic competence depending on competition and the type of substrates selected by the animal.

Increased exocellulase specific activity was positively associated with endocellulase activity, with the exception of the elephant microbial ecosystem, which had low exocellulase activity but higher endocellulase activity. This correlation could be due to the fact that some exocellulases have endocellulase activity but the reverse is not true for most CMCases (Hurst *et al.,* 1978). However, cellobiase activity was not strongly related to CMCase activity. Cellobioses are said to be readily hydrolysed by most microbes (Shi and Weimer, 1997). Therefore, hydrolysis of cellobiose is not much influenced by the presence or absence of CMCase.

Hindgut fermenters (zebra, horse and elephant) had high xylanase. This was primarily ascribed to the diversity of microbial species harbouring the different ecosystems since they came from three geographical regions. These animals (zebra and horse) were also noted for their ability to select fresh grasses or leaves (which are rich in hemicelluloses); this might influence the type of microbes and microbial population. Thirdly, some hindgut fermenters have been associated with the ability to manage plant anti-nutritional factors (tannins or antibacterial secondary compounds e.g. sagebrush essential oil) better than ruminants (Nagy *et al.*, 1964). In hindgut fermenters salivary proteins will detoxify the effect of tannins released after mastication by forming tannin-protein complexes before it reaches the caecum, hence reducing the amount of tannin-enzyme or tannin-microbe complex formation in the caecum. While in the rumen tannin-protein complexing will be competitive against microbial proteins and salivary proteins hence reducing the amount of microbial enzymes available for hydrolysis (Robbins *et al.*, 1995).

Based on the current results, the sampled herbivores can be classified into three main groups, viz: (i) group A, with high enzyme concentration ,composed of a horse, zebra, wildebeest, impala and elephant.; (ii) group B, with average enzyme concentration, comprising cow, buffalo, llama, camel and giraffe and (iii) group C, with low enzyme concentration, comprising the sheep alone .

Kinetic studies revealed that crude proteins from the wildebeest, horse, zebra and elephant had relatively high activity for all the four enzymes analysed. These results demonstrated that not all ecosystems with high enzyme concentrations had higher activity. The horse and zebra with the highest exocellulase concentrations were observed with low Km and high Vmax. This implies that they harbour microbes that have evolved with higher fibrolytic activities. The wildebeest with low exocellulase concentrations had a lower Km when compared to that of the zebra with high enzyme concentrations, which implies that enzyme expression in the zebra was higher than that of the wildebeest but the enzymes were less active when compared to the wildebeest. The high enzyme activity in the wildebeest can be attributed to the efficiency of the microbial species as well as to the substrate available to the wildebeest in winter, which was basically standing hay. The elephant was observed with the highest xylanase activity. Because the elephant browses on forages with compounds (tannins) detrimental to microbial growth and enzyme catalysis, its system might have developed better survival mechanisms such as an increase in microbial population (increasing activity) and a certain degree of tannin tolerance (Kamra, 2005). The giraffe naturally browses on tannin-rich leaves and has a lower xylanase specific activity. This is probably due to the fact that the microbial species that it harbours in the caecum have ill-evolved mechanisms to digest fibre.

5.6 Conclusion

In a search for ecosystems with high fibrolytic enzyme expression and activity, 11 herbivore microbial ecosystems were classified into three groups based on their activities on cellulose, CMC, xylan and cellobiose. Group A with high enzyme concentrations comprised horse, zebra, wildebeest, impala and elephant. Group B with intermediate concentration comprised cow, giraffe, camel, buffalo and llama while Group C comprising only one member (sheep) recorded the lowest enzyme concentration. Enzyme kinetics confirmed that most of the ecosystems with high enzyme expression had high enzyme activity (zebra, wildebeest and horse).

Chapter 6

In vitro manipulation of Jersey cow rumen ecology with enzymes or microbes from the wildebeest, horse and zebra

6.1 Abstract

Ruminal microbes have the ability to convert low quality feeds into high quality protein and to utilise feeds (roughage) from land not suitable to grow crops for human consumption. Only 10–35% of energy intake is captured as net energy while 20–70% of cellulose may not be digested by herbivores. This study was designed to attempt an increase in the amount of energy harvested by the cow system (A) without necessarily increasing intake. Primarily four *in vitro* composite enzyme systems B (cow and horse), C (cow and wildebeest), D (cow and zebra) and E (cow, horse, wildebeest and zebra) were created. Exocellulase, endocellulase, xylanase and cellobiase specific activities of these systems upon incubation with cellulose, carboxymethyl cellulose, xylan and cellobiose respectively were determined by measuring the amount of reducing sugars liberated. The kinetic parameters Km, Vmax and Kcat were also determined. Thirdly, *in vitro* microbial fermentation and degradability of milky maize stover (MM) and NDF with inoculums from the four systems over 72 h at 39°C were determined by measuring the amount of gas released and true degradability (TD). Systems E (Kcat = 17.08) and B (Kcat = 8.54) were the most active enzyme systems upon incubation with cellulose with specific activity 9.57 and 4.47 μg glucose/mg /min respectively. Upon incubation with CMC, system B (Kcat = 15.37) was more active than E (Kcat = 9.48) with a higher enzyme specific activity. Xylan hydrolysis was highest in E and D while B and C were intermediate. Gas released during MM fermentation was highest in system C (141ml) and intermediate in systems B, D and E (134.6, 132.8, and 134.2 ml respectively). NDF fermentation was higher in B and E with gas volumes of 127.82 and 118.62 ml respectively. TD of MS showed that system D was the most active while the rest were relatively higher than that of A. Systems D and E had the highest TD values of NDF. These results demonstrated that *in vitro* transfer of active enzymes or microbes from other herbivores to the Jersey cow was possible while maintaining their fibrolytic potential.

6.2 Introduction

Milk yield and animal performance are limited in animals consuming low quality forages because of their low voluntary intake and digestibility (Hungate, 1984). Low digestibility is partly a consequence of the forage property, host animal adaptation and partly associated with the extent of microbial activity in the rumen and the caecum. Microbial fermentation yields VFAs and the energy derived from fermentation is used for microbial growth. Both the VFAs and microbial proteins synthesised are the main energy and protein source for herbivores. Several factors have been associated with a decrease in fibre breakdown including: forage quality, intake and passage rate, forage composition, type of forage, microbial species and efficiency of microbes and microbial population (Leng, 1990).

Forage type, microbial species and evolution have been the major focus of most studies. Although forage type is still a major problem, the advancement of plant biotechnology has allowed the modification of plants through the process of genetic engineering, producing forages of higher nutritive values. However, in the tropics where these technologies are limited, herbivores depend largely on low quality roughages and agricultural crop residues which are lower in both fermentable carbohydrate and protein content. With the high variation in forage quality and quantity, fermentation depends largely on the efficiency of microbes in the rumen and hindgut of herbivores. Different studies have shown that microbes from the same or different herbivore species grazing on the same or different fields vary in their ability to ferment fibre (Goncalves and Borba, 1996). This was observed in our previous studies (chapter 5), where cellulose hydrolysis by horse enzymes was higher than in cow and sheep grazing on the same field. Among the game animals the zebra, wildebeest and impala had a higher specific fibrolytic activity than that observed in the giraffe, elephant, camel and llama. It is, however, yet to be determined if microbes from these ecosystems can co-exist in the same medium and if such co-existence can confer positive synergism on fibrolysis.

In this study enzyme rate and catalytic efficiency of the cow system (A) will be compared to the newly created *in vitro* enzyme systems B (cow and horse), C (cow and zebra), D (cow and wildebeest) and E (cow, wildebeest, horse and zebra).

Secondly, an *in vitro* digestibility of maize stover (MS) and NDF will be used to compare the Jersey cow microbial system (A) against microbial systems corresponding to the enzyme systems B, C, D and E, defined above.

6.3 Materials and methods

6.3.1 Materials

The materials for the enzyme assays were the same as those used in chapter 3. For the *in vitro* microbial assay the substrates were maize stover harvested at milk stage (MS) of maturity, neutral detergent fibre (NDF) extracted from maize stover using the method described by van Soest *et al.* (1991). Maize stover was harvested from the Ukulinga Research farm, University of KwaZulu Natal, Pietermaritzburg.

6.3.2 Sources of microbial systems

Sources of microbial systems are described under section 3.3.2, but only ecosystems with high cellulolytic activity (arising from the horse, zebra and wildebeest) and the cow's system were evaluated in this study.

6.3.3 Animal nutrition

Animal nutrition was the same as described in section 3.3.3.

6.3.4 Sample collection

6.3.4.1 Crude protein samples

Enzyme samples were collected by slightly modifying the method of Smith *et al.* (1974) as previously described in section 3.3.4.

6.3.4.2 Microbial inoculums

The method was similar to that described in section 3.3.4 but for the fact that the protease inhibitor (PMSF) was not applied. Carbon dioxide was generated in an airtight thermo flask while in the field by reacting calcium carbonate with sulphuric acid.

6.3.5 Protein isolation

The method described by Henry *et al.* (1974) was applied for protein isolation as described in section 3.3.5. Crude protein precipitates were dialysed as previously described (section 3.3.6) and protein concentrations determined (section 3.3.7). The method described by Seyis and Aksoz (2005) for the optimization of xylanase activity from Trichoderma 1073 D3 was used for pH optimization (section 3.3.8).

6.3.6 Enzyme and microbial systems

Microbial and enzyme systems resulted from mixing either crude enzyme extracts or inoculums as described in Table 6.1.

Table 6.1: Mixtures of enzyme systems and microbial inoculums

Systems	Enzyme composition (ratio)	Microbial sample proportions (ml)
Cow (A)	1	33
Cow + horse (B)	0.5 : 0.5	16.5 : 16.5
Cow + wildebeest (C)	0.5 : 0.5	16.5 : 16.5
Cow + Zebra (D)	0.5 : 0.5	16.5 : 16.5
Cow + horse + wildebeest + zebra (E)	0.25 : 0.25 : 0.25 : 0.25	8.25 : 8.25 : 8.25 : 8.25

6.3.7 Enzyme assays

The activities of exocellulase, endocellulase, cellobiase and xylanase in these enzyme mixtures were investigated following the methods described under section 3.3.10. The kinetic parameters (Km and Vmax) of mixtures of these enzyme systems were determined as described under section 3.3.10.

6.3.8 Gas production

The automated gas production method exploited in this study was that described by Blummel and Becker (1997) and Pell and Schofield (1993) using the Computerized Pressure transducer system. MS (milled to pass through a 1-mm sieve) or NDF was weighed (co. 1.0 ± 0.002g) and transferred into a 250 ml Duran bottles containing 67

ml of incubation buffer (buffer C)[1]. The substrate-buffer mixture was placed in the incubator to equilibrate to 39°C (for 1 h) before inoculation. Rumen fluid was squeezed through a four layered muslin cloth into a pre-warmed (39°C) vacuum flask flushed with carbon dioxide and 33 ml of the filtrate was pipetted into the sample bottles under a stream of carbon dioxide. In the case of faeces inoculum, 100 g of faecal samples were dissolved in 100 ml of distilled water (flushed with CO_2, 39°C) and squeezed through a four layered muslin cloth (pre-warmed and flushed with CO_2) before pipetting the required volumes into the substrate solution. The bottle lids were tightened and pressure sensors fitted. A settlement time of 0.5 h was allowed before starting pressure logging for 72 h incubation. The negative control was the same mixture as above with no substrate but the positive control was lucerne and grass hay (1:1) incubated with rumen fluid. Substrate incubation with each ecosystem was done five times. The terminal pressure data was converted to gas volumes (mL) using a predetermined calibration equation (Equation 3).

6.3.9 Degradability

This experiment was designed to determine apparent (APD) and true degradability (TD) of MS and NDF. The method utilized in this experiment was that described by Tilley and Terry (1963) or Blummel *et al.* (1997) with some modifications. Rumen fluid from the cow and faecal samples from horse, zebra or wildebeest were collected and prepared for inoculation as described above. Substrate incubation with each ecosystem was done five times. After 72 h of incubation the terminal pH was measured and samples centrifuged at 18,000x*g*. The supernatant was discarded and the pellet residue dried in a fanned oven at 100°C for 48 h until constant weight was attained. The difference in weight between the initial DM and residue was used to calculate APD. The residue was refluxed with neutral detergent solution (NDS) to determine the NDF. The weight of NDF was subtracted from that of the incubated material and the difference was used to calculate TD (van Soest *et al.,* 1991). The difference between the calculated values of TD and APD was taken to represent microbial matter (MY), as explained by Blümmel *et al.* (1997).

[1] Buffer C was made by titrating 2*l* of warmed solution A (solution A, 19.60, 7.40, 1.14, 0.94 and 0.26 g of NaHCO3, Na2HPO4, KCl and MgCl.6H2O, respectively in 2 L distilled water) with 2 ml of Solution B (5.3 g CaCl2.2H2O in 100 mL distilled water) just before use while continuously stirring to form a complete buffer solution. 5.8 g of ammonium sulfate was dissolved in buffer C as a nitrogen supplier (Tilley and Terry, 1963).

6.4 Results

Table 6.2: Kinetic constants of crude exocellulase, endocellulase, xylanase and cellobiase on their specific substrates (crystalline cellulose, carboxymethyl cellulose, xylan and p-nitrophenyl β-D-glucopyranoside)

		Type of enzyme system					SED
		Cow (A)	Cow+H (B)	Cow+Z (C)	Cow+WB (D)	Cow+H+Z+WB (E)	
Endocellulase							
	Vmax (µg glucose/mg protein/min)	10.57	16.67	10.66	10.76	32.08	1.532
	Km (µg/ml)	0.18	0.4	0.83	0.96	2.07	0.545
	Efficiency (/µg/ml/ugS)	5.38	8.54	5.75	5.86	17.08	1.279
	Specific activity (µg glucose/mg /min)	2.78	4.47	3.29	3.41	9.57	0.052
Exocellulase							
	Vmax (µg glucose/mg protein/min)	2.66	3.89	12.09	9.96	22.91	2.599
	Km (µg/ml)	2.26	0.25	3.29	1.85	2.56	0.085
	Efficiency (/µg/ml/ugS)	1.16	15.37	3.68	5.46	9.46	0.410
	Specific activity (µg glucose/mg /min)	19.98	17.97	17.65	16.07	54.7	0.001
Xylanases							
	Vmax (µg glucose/mg protein/min)	82.54	141.91	109.89	38.23	33.09	7.803
	Km (µg/ml)	0.85	1.09	0.38	3.16	0.12	0.125
	Efficiency (/µg/ml/ugS)	98.52	130.75	289.06	12.11	276.28	10.776
	Specific activity (µg glucose/mg /min)	100.38	182.38	175.07	61.66	240.04	3.192
Cellobiases							
	Vmax (µg glucose/mg protein/min)	0.44	0.36	0.54	0.24	1.37	0.033
	Km (µg/ml)	0.28	0.04	0.35	0.25	0.13	0.068
	Efficiency (/µg/ml/ugS)	1.58	9.94	1.55	0.98	10.22	0.813
	Specific activity (µg glucose/mg /min)	0.56	0.76	0.78	0.49	2.53	0.028

H=horse, WB=wildebeest, Z=zebra

6.4.1 Specific activity of mixtures of enzyme systems

The highest endocellulase specific activity was observed in system E while systems B, C and D were intermediate (Table 6.2). System B was the most active endocellulase mixture with a high catalytic efficiency while system E was intermediate. Relative to system A, minimal increments in endocellulase Kcat were observed in systems C and D.

Based on the catalytic efficiencies of exocellulases, system E was more active than system A (Table 6.2). Intermediate catalytic efficiency was observed in B while C and D showed the least increments.

Xylanase specific activities were generally high in all but system D enzyme system. The highest specific activity was observed in system E (Table 6.2). The systems, B and C recorded intermediate activities while system D was inferior to system A. Systems E and B had the most active xylanases with high Kcat values while system B was intermediate.

Cellobiase specific activity was similar among the systems A, B, C and D (Table 6.2). A high cellobiase specific activity was observed in system E. System E and B recorded the highest Kcat for cellobiase while systems C and D were inferior to system A.

6.4.2 Effect of mixtures of microbial ecosystems

Table 6.3: In vitro digestibility, microbial yield and gas production of maize stover incubated in inoculums from four microbial ecosystems

	Type of microbial system					
	Cow (A)	Cow+H (B)	Cow+WB (C)	Cow+Z (D)	Cow+H+Z+WB (E)	SED
DM digestibility (MS)						
Apparent (g/kg)	350	477	375	610	446	52.6
True(g/kg)	624	694.0	661	765	695	38.4
Microbial yeild (g/kg)	274	216	286	155	248	53.5
Gas volume (ml/g)	112	134	141	132	134	9.9
NDF digestibility						
Apparent (g/kg)	132	345	260	355	316	39.1
True (g/kg)	600	658	658	709	700	32.1
Microbial yeild (g/kg)	467	313	398	354	383	12.3
Gas volume (ml/g)	71	127	95	82	118	58.1

6.4.2.1 Dry matter digestibility

There was a significant difference ($P<0.0001$) in gas production among the systems A, B, C, D and E when incubated with MS (Table 6.3). System C recorded the highest volume of gas after 72 h when compared to system A. The systems B, D and E showed similar values, which were relatively higher than that for system A.

The results of both the apparent and true degradability of MS and microbial yields of the five systems are shown on Table 6.3. APD and TD of MS differed ($P<0.001$) among the five systems. The highest APD value for MS was observed in system D while systems B and E were intermediate when compared to system A. System D was noted for its high TD value of MS while the systems B, C and E were intermediate when compared to system A. Microbial yield (MYD) differed among the five systems when it was incubated with MS. MYD of the different systems were classified as high (system C) or relatively low (B, D and E) when compared to that of system A. MYD was greater in system A than in systems B, D and E.

6.4.2.2 NDF digestibility

The volume of gas produced after 72 h of incubation with NDF differed ($P<0.0001$) among the five systems. The cumulative gas volume after incubation is shown on Table 6.3. The smallest increment of gas volume when compared to that produced by system A was observed in system C and D while that of B demonstrated the highest increase. An intermediate gas volume was observed in system E.

APD and TD of NDF differed ($P<0.001$) among the five systems. Systems B and D demonstrated high APD values (Table 6.3), while the APD on NDF for E was intermediate when compared to that of system A. A much lower APD value was observed in system C when compared to system A. The highest TD values were recorded in systems D and E while a relatively small increment when compared to system A was observed in systems B and D. MYD upon incubation with NDF was much higher in system A than in any other system (Table 6.3). Intermediate MYD were observed in systems C and D while systems B and C had the lowest MYD.

6.5 Discussion

Jung and Allen (1986) reported that feeding accounts for up to 60% of the cost of livestock production yet forages are abundant, cheap and poorly utilised. It is thus necessary to investigate various means of harnessing maximal energy from the abundant roughages as a means of minimising the cost of production. Three herbivore ecosystems with high specific fibrolytic and catalytic activities were investigated if

any would exhibit a positive synergistic fibrolytic activity with the cow's system. Enzyme specific activities and enzyme efficiencies demonstrated that all four systems (B, C, D and E) were more active than the cow's system alone (system A). The superior enzyme activities in combined systems might be due to the introduction of active enzymes from the corresponding ecosystems, which according to Beauchemin *et al.* (1995) could contribute to a net positive synergistic effect.

Upon incubation with exocellulases, system E was found to be the most active system. This activity was associated with a rich variety of active enzymes coming from all four systems with a net positive synergistic effect. This agrees with a three-fold increase of cellulase activity on insoluble cellulose reported by Koichiro *et al.* (2002). Systems C and D with similar exocellulase activity had slightly higher activities than system A but lesser activities than system E. The decreased activity observed in systems C and D relative to system E might be indicative of an increased variety of active enzymes in E.

The similarity between systems C and D could be explained by the fact that the wildebeest and zebra grazed on the same pasture and could have cross inoculated each other while feeding. Interestingly, system B arising from the horse and cow grazing on the same piece of land showed a higher exocellulase activity compared to system A. It is possible that the horse might be harbouring microbes that are more evolved in fibrolytic activity than the cow. Statistical analysis of Km and Vmax showed that only system E was different to system A, while the rest of the systems were similar. However, analysis of exocellelulase enzyme efficiencies demonstrated that the rate at which it catalyses cellulose hydrolysis was higher in E and B but remained the same among the systems A, C and D even though they appeared to be biologically different.

The specific activities of endocellulases were higher in system A than in systems B, C and D but lower than in system E. The high activity in system A was due to high endocellulase concentration influenced by the availability of substrates that are rich in soluble carbohydrates on the grazing field (Muetze *et al.*, 2003). Conversely, kinetic studies on the catalytic activities of endocellulases demonstrated that systems C, B, D and E were more active than system A even though they showed lower specific activities. This implies that system A was richer in enzyme concentration but that

these enzymes had a low catalytic rate. Systems A and C exhibited the same endocellulase activity when statistically analysed even though they were biologically different by 6.87%.

Xylanase activity was higher in system E than in systems B and C, both of which were inferior to system A. High activity in system E could indicate a positive synergistic effect of a richer variety of active xylanases from the four systems and microbial proteins that might aid in hydrolysis. System D demonstrated an activity that was lower than that of A.

It is worthwhile mentioning that the specific activities of cellobiases were similar among systems A, B, C and D but that system E was relatively more active. This can be explained by the fact that cellobioses are very soluble and can easily be hydrolysed by most microbes. However, kinetic studies revealed that although cellobioses are easily hydrolysed, systems E and B had the highest ability.

Even though the five systems were statistically similar in gas production after 72 h of incubation with MS, these differences appear to be biologically important and warrant some discussion. All composite systems (B, C, D and E) generally produced more gas than the control system A. These gas volumes were slightly lower than had been observed when MS was incubated with rumen fluid by Ouda *et al.* (2006), perhaps as a result of variation in animal species, sample collection, inoculum preparation, date and time of collection or sampling season (Huntington *et al.*, 1998). System C produced more gas than systems B, D and E as well as the control system A. The high fermentation could be due to more active microbial species in the amalgamated inoculums from the wildebeest. System E was expected to produce more gas than any other system because it is richer in microbial species than any other systems but this was not the case. The amalgamation of a wide variety of microbial species might have led to a very competitive system in which some species secrete inhibitory proteins (antibiotics) or proteases that prevented the growth of other microbes (de Rossi *et al.*, 1994). It could well be that the dominant species might have been very active but fewer relative to system B.

The volume of gas produced from the incubation of the five systems with NDF was generally lower than that observed in MS fermentation. This appears to be different from the results obtained by Mertens *et al.* (1998), showing that celluloses produce more gases than soluble carbohydrates. MS contained 80% fibre and 20% solubles (Ouda *et al.*, 2006), the solubles might have been used as a primary source of energy to initiate microbial growth, which could have increased fermentation (Manal and EL-Shazly, 1969). System B had the highest gas volume upon fermentation. Although the cow and the horse were grazing on the same field their microbial species might have been different (Goncalves and Borba, 1996). The merging of such systems led to an overall positive synergistic activity. This also suggests that the *in vitro* system B accommodates active microbial species from the horse. Increased variety of microbial species in system E might have led to a more competitive system in which microbes with high ability to ferment NDF survived. One would expect that an increase in MYD would be inversely proportional to fermentation products, which is true as system A with the highest MYD produced lesser gas than system B.

Generally, degradability of both MS and NDF differed among the systems. All the composite systems (B, C, D and E) had a higher degradability when compared to that of the cow (system A). APD and TD of MS were highest in system D. This could be attributed to a positive synergistic effect of active microbial species from different ecosystems (cow and zebra). Similar experiments performed by Wanapat (1998) on cow and buffaloes demonstrated that digesta transferred from buffalo to cow survive for up to 14 days and improved degradability. The merging of inoculums from different ecosystems might have introduced microbial proteins that were essential for degradation but absent in system A. Although system B comprised fewer systems than system E, both systems have similar APD and TD of MM. This is in agreement with the volume of gas that both systems produced during fermentation. It is possible that competition among the microbial species in system E might have led to lysing and inhibition of some important fibrolytic microbes.

High APD and TD values of NDF in both systems D and E were probably due to an overall positive synergistic effect of active microbes from different ecosystems. It was also noted that system E was the most active ecosystem in degrading MS and NDF, while systems B and E were most active in fermenting MS and NDF, respectively.

Therefore system E appears to be the superior system based on the fermentation of both MS and NDF.

6.6 Conclusion

The results obtained from the *in vitro* investigation of enzyme composite systems demonstrated that synergism had an overall positive effect on cellulose, carboxymethyl cellulose and xylan hydrolysis. Systems B and E were the most active enzyme systems while systems C and D were intermediate when compared to the control system (A). Secondly, *in vitro* gas production from the fermentation of MS and NDF showed that microbial synergism was positive among the four systems (B, C, D and E). Again system B and E were classified as the most active systems with respect to MS fermentation while D and E were the most active with respect to NDF fermentation. Although the results were reasonably positive, further research is still required to confirm the survival of microbes from different inoculums *in vivo* since biological systems may respond differently to foreign material.

Chapter 7

7.0 Introduction

As mentioned earlier, feeding accounts for more than 60% of the cost of livestock production yet forages are abundant. Kossila (1988) reported that more than 340 million tonnes of fibrous crop residues are produced in Africa per year with the majority coming from cereals. Most of the pastures are from the veld while the number of cultivated pasture species are very limited hence the dependence of livestock production on uncultivated pastures (low quality). Although seasonal shortages and low nutritive values of available feed resources have been blamed as the main constraints of livestock production in the tropics (Osuji and Odenyo, 1997), seasonal shortages were considered a more significant factor than the nutritive value of forages. This is because if only 10% of the total amount of energy wasted as faeces was extracted, energy shortage as a major constraint will disappear.

Carbohydrates are the main products into which the energy of the sun is harnessed through the process of photosynthesis. Structural carbohydrates (celluloses and hemicelluloses) constitute the most abundant source of energy for both hindgut and foregut fermenters (Lynd *et al.*, 2002). Despite the abundance of this substrate it is under utilised as the majority is wasted as feaces (Varga and Kolver, 1997). Factors responsible for the underutilization of fibre are the nature of fibre (crystalline), complex formation with lignin, hemicellulose, saponins and tannins and the inefficiency of ruminal microbes to digest this recalcitrant substrate (Lynd *et al.*, 2002). Feed additives such as buffers, antibiotics, probiotics, exogenous microbes and enzymes were found to contribute minimally to fibre hydrolysis (Lynd *et al.*, 2002). A lot of information is available on the use of exogenous enzymes as an additive; however, fibrolytic enzymes from a single or few microbial species have been the main focus. Secondly studies on enzyme or microbial composite systems assessing cellulose hydrolysis or fermentation were scanty. Therefore this study was designed to: (i) isolate and determine cellulolytic enzyme concentration suitable for enzyme assays from 11 herbivores; (ii) investigate seasonal variation in fibrolytic enzymes (exocellulases, endocellulases, xylanase and cellobiases) in herbivores; (iii)

investigate the activity and efficiency of microbial enzymes from eleven microbial ecosystems; and (iv) determine *in vitro* the effect of compositing microbial ecosystems from the jersey cow with that of the horse, wildebeest and/or zebra.

7.1 Isolation and determination of suitable microbial fibrolytic enzyme concentrations necessary for enzyme assays

Crude proteins (containing cellulases) precipitated from all 11 herbivore ecosystems were active as demonstrated by an increase in specific activity with decreasing protein concentrations. This implies that the purification method was successful and can possibly replace other ambiguous and expensive methods for the intial extraction of cellulases from rumen fluid. Although the active pH varies from one animal to the other, the pH profile observed in this study could possible serve as a guide to other researchers involved in *in vitro* assays of cellulose targeting enzymes. Secodly, the establishment of a suitable crude protein extract quatity for the different enzyme assays may be vital information to on-farm researchers on cellulose targeting enzymes.

7.2 Seasonal variation of fibrolytic enzymes in herbivores

Herbivores were sampled both in winter and summer to analyse the relative abundance of cellulolytic enzymes (exocellulase, endocellulase, cellobiase and xylanase) associated with the breakdown of fibrous forages. Available feed appeared to play a role in the relative abundance of these enzymes both in winter and summer. Exocellulases were higher in winter than summer due to the abundance of fibre-rich and lignin-cemented polysaccharides while endocellulase and xylanase concentrations were higher in summer than in winter due to the abundance of soluble polysaccharides and hemicelluloses. Minimal changes in cellobiase concentrations were observed in both seasons.

These results indicate that experiments involving exocellulase analysis from wild herbivores will be advantageous in winter while summer will be preferable for endocellulase and xylanase analysis. There is no seasonal preference for the analysis of cellobiases due to insignificant changes in cellobiase activity between seasons.

Although enzyme concentrations did increase from one season to another the degree of activity of enzymes from these microbial populations varied, so it is of interest to determine the different enzymes involved. A further investigation to establish differences among enzymatic activities within and among herbivore species grazing on the same piece of land or in different geographical regions will be very essential.

7.3 Activities and efficiencies of fibrolytic enzymes from microbial ecosystems

In a search for ecosystems with high fibrolytic enzyme expression and activity, the 11 herbivore microbial ecosystems were classified into three groups based on their activities on cellulose, CMC, xylan and cellobiose. Group A, with high enzyme concentrations comprised horse, zebra, wildebeest, impala and elephant. Group B, with intermediate concentration comprised cow, giraffe, camel, buffalo and llama while Group C, comprised of sheep alone recorded the lowest enzyme concentration.

Enzyme kinetics demonstrated that most of the ecosystems with high enzyme expression had a higher enzyme activity. The ecosystems (horse, wildebeest and zebra) richer in cellulolytic enzymes were also the most active systems as shown by their high catalytic efficiencies. The giraffe with lower cellulolytic specific activity had a higher rate of fibre hydrolysis. This implies that the low specific activity initially demonstrated was due to low enzyme concentration rather than the activeness of fibrolytic enzymes.

The results obtained when investigating the rate of CMC hydrolysis, showed a higher activity for the cow and wildebeest but intermidiate activities for the horse, zebra and elephant. This implies that the cow contained the most active endocellulase system but their expression by microbes was lower. These results demonstrated that microbes show two mechanisms of increasing fibre hydrolysis: (i) increasing enzyme expression when enzymes are less active; and/or (ii) maintaining low enzyme concentrations when enzymes are more active. The elephant with an intermediate xylanase specific activity was noted for a remarkable high catalytic efficiency.

7.4 Composite enzyme and microbial ecosystems

In vitro studies on composite enzyme systems demonstrated an overall positive effect on the hydrolysis of cellulose, carboxymethyl cellulose and xylan. Systems B (cow and horse) and E (cow, horse, wildebeest and zebra) were the most active enzyme systems while systems C (cow and wildebeest) and D (cow and zebra) were intermediate when compared to that of the control system A (cow). Secondly *in vitro* gas production from the fermentation of MS and NDF showed that, microbial synergism was positive among the four composite systems (B, C, D and E). Again system B and E were classified as the most active system with respect to MS fermentation while D and E were the most active with respect to NDF fermentation. Although the results were reasonably positive, further research is still required to confirm the survival of microbes from different inoculums *in vivo* since biological systems will respond differently to foreign material.

Conclusion

Cellulases and hemicellulases were successfully isolated from samples collected in all 11 herbivores. Samples collected in winter were richer in exocellulases while those collected in summer were richer in endocellulases and xylanases. The horse, wildebeest and zebra, with high cellulolytic enzyme concentrations, were also the most active ecosystems with high catalytic efficiencies. The enzyme composite systems E (cow, horse, wildebeest and zebra) and B (cow and horse) were the most active cellulolytic systems when compared to that of the Jersey cow alone. *In vitro* analysis of MS and NDF degradability or fermentation demonstrated that systems E and D (cow and wildebeest) were the most superior microbial composite systems.

References

Abreu M. (1994). Fibrous crops and residues for animal production in Europe. Micro-organisms in ruminant nutrition. Prins R. and Stewart C. (Eds.) Nottingham University Press. pp 1-13

Akin E. (1980). Attack on lignified grass cell walls by a facultatively anaerobic bacterium. *Applied and Environmental Microbiology*. **40**: 809-820

Ali M. and Tirta K. (2001). Correlation between microbial enzyme activities in the rumen fliud of sheep under different treatments. *Reproduction Nutrition Development*. **41**: 513-529

Bailey H. and Jones R. (1971). Hydrolysis of ryegrass structural carbohydrates with carbohydrases in relation to rumen digestion. *New Zealand Journal of Agricultural Research.* **14**: 847-860

Bailey R. Mornro J. Pickmere S. and Chesson A. (1976). Herbage hemicellulose and its digestion by Ruminants. *Carbohydrate Research in Plants and Animals*. **12**: 1-15

Bauchop T. (1979). Rumen anaerobic fungi of cattle and sheep. *Applied Environmental Microbiology*. **38**: 148-158

Beauchemin A. Colombatto D. Morgavi P. and Yang Z. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization. *Ruminants Journal of Animal Science*. **81**: E37-E47

Beauchemin K. Rode M. and Sewalt H. (1995). Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. *Canadian Journal of Animal Science*. **75**: 641–644

Beauchemin K. Rode M. Maekawa M. Morgavi D. and Kampen R. (2000). Evaluation of a non-starch polysaccharidase feed enzyme in dairy cow diets. *Journal of Dairy Science.* **83**: 543–553

Beldman G. Searle-Van Leeuwen M. Rombouts F. and Voragen G. (1985).The cellulase of Trichoderma viride. Purification, characterization and comparison of all detectable endoglucanases, exoglucanases and beta-glucosidases. *European Journal of Biochemistry.* **146**: 301-308

Bergen W. Bates D. Johnson E. Waller C. and Black R. (1982). Ruminal microbial protein synthesis. Protein 99 in protein requirements for cattle:Proceedings of an international Symposium. Owens F. (Ed.).Oklahoma State University.

Blummel M. and Becker K. (1997). The degradability characteristics of fifty-four roughages and roughage neutral detergent fibers as described by *in vitro* gas production and their relationship to voluntary feed intake. *British Journal of Nutrition.* **77**: 757-768

Blümmel M. Steingga H. and Becker K. (1997). The relationship between *in vitro* gas production, *in vitro* microbial biomass yield and 15N incorporation and its implications for prediction of voluntary feed intake of roughages. *British Journal of Nutrition.* **77**: 911-921

Bourquin L. Garleb K. Merchen N. and Fahey G. (1990). Effect of intake and forage level on site and extent of digestion of plant cell wall monomeric components by sheep. *Journal of Animal Science.* **68**: 345-352

Brooker D. O'Donovan L. Skene I. Clarke K. Blackall L. and Muslera P. (1994). *Streptococcus caprznus sp.*, a tannin-resistant ruminal bacterium from feral goats. *Letters in Applied Microbzology* . 18: 313-18

Bruce H. Paul C. and Rex E. (1984). Resolution of multienzyme cellulase complex of *Trichoderma reesei* QM9414. *Journal of Applied Biochemistry.* **6**: 156-183

Bryant M. (1973) Nutritional requirement of the predominant rumen cellulolytic bacteria. *Feed Processing.* **32**: 1809-1813

Bryant M. and Burkey A. (1953). Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *Journal Dairy Science.* **36**: 205-217

Burk A. (1991). Effects of microbial synergism on fibre digestion in the rumen. *Proreedings of the Nutrition Societry.* **50**: 149-159

Buxton D. Mertens D. and Moore K. (1995a). Forage quality for ruminants: plant and animal considerations. *The Professional Animal Scientist.* **11**: 121-131

Casler M. (1987). *In vitro* digestibility of dry matter and cell wall constituents of smooth bromegrass forage. *Crop Science.* **27**:931–934

Cássia R. Clarita S. and Heloiza B. (1999). Growth and endoglucanase activity of Acetivibrio cellulolyticus grown in three different cellulosic substrates. *Revista Microbiologia.* **30**: 310-314

Cavicchioli R. and Watson K. (1991). Molecular Cloning, Expression, and Characterization of Endoglucanase Genes from Fibrobacter succinogenes ARI. *Applied and Environmental Microbiology.* **57**: 359-365

Charles C. (2004). Bacteria cellulose makes e-paper, bandages. United Press International.www.washtimes.com/upi-breaking/20040402-105340-9744r.htm.

Che-Chang H. Zhangyong H. Masaru W. Dirk F. and Chi-Huey W. (2005). Directed evolution of D-sialic acid aldolase to L-3-deoxy- manno-2-octulosonic acid (L-KDO) aldolase. *Proceedings of the National Academy of Sciences of the United States of America.* **102**: 9122–9126

Chen J. and Weimer J. (2001). Competition among three predominant ruminal cellulolytic bacteria in the absence or presence of non-cellulolytic bacteria. *Microbiology.* **14**: 21–30

Chen M. and Wolin J. (1979). Effect of Monensin and Lasalocid-Sodium on the Growth of Methanogenic and Rumen Saccharolytic Bacteria. *Applied and Environmental Microbiology.* **38** : 72-77

Cheng K. Forsberg C. Minato H. and Costerton J. (1990). Microbial ecology and physiology of feed degradation within the rumen. Paper presented at the International Symposium on Ruminant Physiology. Sendai, Japan.

Chivero T. Mutukumira A. and Zvauya R. (2001) Partial purification and characterisation of a xylanase enzyme produced by a microorganism isolated from selected indigenous fruits from Zimbabwe. *Food Chemistry.* **72**: 179-185

Colombatto D. Mould L. Bhat K. Morgavi P. Beauchemin A. and Owen E. (2003). Influence of fibrolytic enzymes on the hydrolysis and fermentation of pure cellulose and xylan by mixed ruminal microorganisms in vitro. *Journal of Animal Sciences.* **81**: 1040-1050

Degen A. Makkar H. Becker K. and Borowy N. (1995). *Acacia saligna* as a fodder tree for desert livestock. *Journal of the Science and Food Agriculture.* **68**: 65–71

de Rossi E. Brigidi P. Welker N. Riccardi G. and Matteuzzi D. (1994). New shuttle vector for cloning in *Bacillus stearothermophilus. Research Microbiology.* **145**: 579–583

Deinum B. (1976). Effect of age leaf number and temperature on cell and digestibility of maize. *Carbohydrate Research in Plants and Animals.* **12**: 29-41

Deinum B. and Dirven P. (1971). The influence of age on the chemical composition and in vitro digestibility of maize *(Zea mays L.)* and Tall fescue *(festuca arundinacea)*. *Neitherlands Journal of Agricultural Science.* **20**: 125-130

Easterby J. (2003). HYPER software programme. (http://www.liv.ac.uk/%7Ejse/software.html).

Edwards S. and Poole D. (1983). Effects of sodium bicarbonate in the diet of dairy cows. *Animal Production.* **37**: 183- 195

Eisenthal R. and Cornish-Browden A. (1974). The direct linear plot. A new graphical procedure for estimating enzyme kinetics parameters. *Biochemistry Journal.* **139**: 715-720

el-Shazly K. Dehority A. and Johnson R. (1961). Effect of starch on the digestion of cellulose in vitro and *in vivo* by rumen microorganisms. *Journal of Animal Science.* **20**: 268-273

Fahey G. and Berger L. (1998). Carbohydrate Nutrition of Ruminants. Church C. (ed) The Ruminant Animal Digestive Physiology and Nutrition. Waveland Press, Inc. US America. pp 269-297

Feng P. Hunt C. Pritchard G. and Julien W. (1996). Effect of enzyme preparations on in situ and in vitro degradation and in vivo digestive characteristics of mature cool-season grass forage in beef steers. *Journal of Animal Science* **74**: 1349–1357

Flint J. (1994). Degredation of plant cell wall polysaccharides by rumen bacteria. Prins R. and Stewart C. (Eds.) Nottingham University Press. pp 49-61

Fonty G. and Gouet H. (1994). Plant cell wall degredation by anaerobic fungi. Prins R. and Stewart C. (Eds.) Nottingham University Press. pp79-91

Freelove J. Bolam N. White P. Hazlewood P. and Gilbert J. (2001). A Novel Carbohydrate-binding Protein Is a Component of the Plant Cell Wall-degrading Complex of *Piromyces equi. The Journal of Biological Chemistry* **276**: 43010-43017

Freier M. and Karl-Heinz A. (1997). The ups and downs of nucleic acid duplex stability: structure–stability studies on chemically-modified DNA:RNA duplexes. *Nucleic Acids Research.* **25**: 4429–4443

Frutos C. Sonsoles P. Chiquinquirá C. and Eduardo C. (2002). Reverse micelles in organic solvents: a medium for the biotechnological use of extreme halophilic enzymes at low salt concentration. *Archaea* **1:** 105–111

Fuller R. (1989). Probiotics in man and animals. *Journal of Applied Bacteriology.* **66**: 365-375

Gerrit B. Marjo F. Frank M. and Fons G. (1984). The cellulase of Trichoderma Viride. Purification characterization and comparison of all detectable endoglucanases, exoglucanases and β-glucosidases. *European Journal of Biochemistry.* **146:** 301-308

Ghadaki M. Van Soest P. Mcdowell R. and Malekpour B. (1975). Chemical composition and in vitro digestibility of some range forage species of Iran. Evaluation and mapping of tropical African rangelands Seminar. Bamako-Mali

Gilbert H. and Hazlewood G. (1993). Bacterial cellulases and xylanases. *Journal General Microbiology.* **139**: 187–194

Goncalves L. and Borba A. (1996). Study of gas production capacity by three sources of inocula. *Journal of Agricultural Science.* **127**: 511 - 515

Gram C. (1884). Ueber die isolirte Farbung der Schizomyceten in Schitt-und Trockenpreparaten. *Fortschritte der Medicin.* 2: 185-189

Groleau D. and Forsberg W. (1981). Cellulytic activity of the rumen bacterium Bacteroides succinogenes. *Journal of Microbiology.* **27**: 517-530

Groleau D. and Forsburb N. (1983). Partial characterization of the extracellular carboxymethylcellulase activity produced by the rumen bacterium *Bacteroides succinogenes. Canadian Journal of Microbiology.* **29**, 504-517

Hagerman A. and Butler G. (1981). The specificity of proanthocyanidin-protein interactions. *The Journal of Biological Chemistry.* **256**: 44-94

Haggar R. and Ahmed M. (1971). Seasonal production of Andropogon gayanus. III. Changes in crude protein content and in vitro dry-matter digestibility of leaf and stem portions. *Journal of Agricultural Science Cambridge.* **77**: 47-58

Henry B. Nick V. Desh P. and Gordon A. (1974). Purification and characterisation of two cellulases from Auxin-treated Pea Epicoltyls. *Journal of Biological Chemistry*. **10**: 1012-1018

Hespell R. Akin D. Dehority B. (1997). Bacteria, fungi, and protozoa of the rumen. In: Mackie RI, White BA, Isaacson RE (Eds) Gastrointestinal Microbiology vol. 2. Chapman and Hall, New York, pp 59-141

Hillman L. Peters G. Fisher A. and Pomare E. (1986). Effects of the fibre components pectin, cellulose, and lignin on bile salt metabolism and biliary lipid composition in man. *Gut*.**27**: 29-36

Hiltner P. and Dehority B. (1983). Effect of soluble carbohydrates on digestion of cellulose by pure cultures of rumen bacteria. *Applied Environmental Microbiology*.**46**: 642-8

Hobson P. (1971). Rumen micro-organisms. Prog. Indust. Microbiology. 9, 42-77

Hofmann R (1998). Anatomy of the gastro-intestinal tract. Church C. (ed) The Ruminant Animal Digestive Physiology and Nutrition. Waveland Press, Inc. USA. pp 269

Hofmann R. (1989). Evolutionary steps of ecophysical adaptation and diversification of ruminants: A comparative view of their digestive system. *Oecologia*. **78**: 443-457

Horvath P. (1981). The nutritional and ecological significance of Acer-tannins and related polyphenols. Masters of Science Thesis, Cornell University, Ithaca, **New York.**

Hoshino E. Kubota Y. Okazaki M. Nisizawa K. and Kanda T. (1994). Hydrolysis of cotton cellulose by Exo- and endo-type cellulases from Irpex lacteus: differential scanning calorimetric study. *Journal of Biochemistry*. **115**: 837-842

Howell J. and Stuck J. (1975). Kinetics of Solka Floc, cellulose hydrolysis by *Trichoderma viride* Cellulase. *Biotechnology Bioengeering*. **17**: 873-892

Huber R and Hurlburt K. (1984). *Escherichia coli* growth on lactose requires cycling of beta-galactosidase products into the medium. *Canadian Journal of Microbiology*. **30**: 411-415

Hungate R. (1966). The rumen and its microbes. Academic Press, New York.

Hungate R. (1984). Microbes of nutritional importance in the alimentary tract. *The Proceedings of the Nutrition Society*. **43**: 1-11

Huntington J. Rymer C. and Givens D. (1998). The effect of host diet on the gas production profile of hay and high-temperature dried grass. *Animal Science*. **67**: 59-64

Hurst L. Sullivan A. and Shepherd G. (1978). Substrate specificity and mode of action of a cellulase from *Aspergillus niger. Biochemistry journal*. **169**: 389-395

Huub J. Verhagen M. Kivaisi K. and de Windt E. (1988). Effects of lignin on the anaerobic degradation of (ligno) cellulosic wastes by rumen microorganisms. *Journal of Applied Microbiology and Biotechnology*. **29**: 408-412

Ian M. (1974). Structural Investigations on the Lignin-Carbohydrate Complexes of Lolium perenne. *Biochemistry Journal*. **139**: 197-204

Ilan L. Ziv S. and Oded S. (2002). Modification of polysaccharides and plant cell wall by endo-1,4-glucanase and cellulose-binding domains *Biomolecular Engineering*. **19**: 17-30

Jouany P. and Ushida K. (1994). Plant cell wall degredation by rumen protozoa. Prins R. and Stewart C. (Eds.) Nottingham University Press. pp 69

Jung H. and Vogel P. (1986). Influence of lignin on digestibility of forage cell wall material. *Journal of Animal Science*. **62**: 1703-1723

Kamra D. (2005). Rumen Microbial Ecosystem. *Current Science*. **89**: 124-135

Kazuhide O. Xin M. Mitsuo U. Seiichi T. and Tadafumi A. (2004). Efficient conversion of lignin into single chemical species by solvothermal reaction in water–p-cresol solvent. *Journal of Physics: Condensed Matter*. **16**: 1325-1330

Keford N. (1958). Lignification of plants in relation to ruminant nutrition. *Journal of Australian Institute of Agricultural Science*. **24**: 297-305

Kenedy P. and Hogan P. (1994). Digestion and metabolism in buffaloes and cattle:are there consistent diffences. In: Proc. The 1st Asian buffalo Association Congress. M. Wanapat and K. Sommart (Eds.), Khon Kaen University, Khon Kaen, Thailand.

Khanna S. (1993). Regulation, purification and properties of xylanase from *cellulomanas fimi. Enzyme and Microbial Technology.* **15**: 990-995

King K. and Vessal M. (1969). Enzymes of the Cellulase Complex, Advances in Chemistry, Series No. 95, R. Gould, Amer. Chem. Soc., Washington.

Koichiro M. Akihiko K. and Roy H. (2002). Synergistic Effects on Crystalline Cellulose Degradation between Cellulosomal Cellulases from *Clostridium cellulovorans. Journal of Bacteriology.* **184**: 5088–5095

Koike S. Shingu Y. Inaba H. Kawai M. Kobayashi Y. Hata H. Tanaka K. and Okubo M. (2000) Fecal bacteria in Hokkaido native horses as characterized by microscopic enumeration and competitive polymerase chain reaction assays. *Journal of Equine Science.* **11**: 45-50

Kossila V. (1988). The availability of crop residues in developing countries in relation to livestock populations. Reed J. Capper B. and Neate P. (eds), *Plant breeding and the nutritive value of crop residues.* Proceedings of a workshop held at the International Livestock Centre for Africa, Addis Ababa, Ethiopia.

Krause D. Bunch R. Smith J. McSweeney C. (1998). Diversity of *Ruminococcus* strains: a survey of genetic polymorphisms and plant digesting ability. *Journal of Appllied Bacteriology.* **86**: 487-490

Krause M. Beauchemin K. Rode L. Farr B. and Nørgaard P. (1998). Fibrolytic enzyme treatment of barley grain and source of forage in high-grain diets fed to growing cattle. *Journal of Animal Sciences.* **96**: 1010–1015

Krause O. McSweeney S. Forster J. (1999). Molecular ecological methods to study fibrolytic ruminal bacteria: Phylogeny, competition, and persistence. Proceedings of the 8th International Symposium on Microbial Ecology Bell C. Brylinsky M. Johnson-Green P (ed) Atlantic Canada Society for Microbial Ecology, Halifax, Canada.

Kumar R. and Singh M. (1984). Tannins: their adverse role in ruminant nutrition. *Journal of Agriculture and Food Chemistry.* **32**: 447-453

Kung L. Treacher R. Nauman G. Smagala A. Endres K. and Cohen M. (2000). The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. *Journal of Dairy Science*. **83**: 115–122

Latham M. Sharpe M. and Sutton J. (1971). The microflora of the rumen of cows fed hay and high cereal rations and its relationship to the rumen fermentation. *Journal of applied bacteriology.* **34**: 559-564

Lee R. Paul J. Willem H. and Isak S. (2002). Microbial cellulose utilization: Fundamentals and Biotechnology. *Microbiology and Molecular Biology Reviews*. **66:** 506-577

Leng A. (1990). Factors affecting the utilization of 'poor-quality' forages by ruminants particularly under tropical conditions. *Nutrition Research Reviews.* **3**: 277-303

Leng R. and West C. (1969). Contribution of acetate, butyrate, palmitate, stearate and oleate to ketone body synthesis in sheep. *Research in Veterinary Science.* **10**: 57-65

Lewis G. Sanchez W. Hunt C. Guy M. Pritchard G. Swanson B. and Treacher R. (1999). Effect of direct-fed fibrolytic enzymes on the lactational performance of dairy cows. *Journal of Dairy Science.* **82**: 611–617

Lynd R. Weimer J. van Zyl H. and Pretorius S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology Molecular Biology Reviews*. **66**: 506–577

Maglione G. Russell J. and Wilson D. (1997). Kinetics of cellulose digestion by *Fibrobacter succinogenes* S85. *Applied and Environmental Microbiology*. **63**: 665-669

Manal Z. and El-Shazly K. (1969). Some Factors Affecting Fermentation Capacity andNet Growth of Rumen Microorganisms. *Applied Microbiology*. **18**: 313-317

Margaretha Å. Barbara H. and Lennart S. (2004) Characterization of the crystalline structure of cellulose using static and dynamic FT-IR spectroscopy. *Carbohydrate Research.* **339**: 569-578

Martin C. (2006). Cellulose. http://www.lsbu.ac.uk/water/hycel.html

Matulova M. Nouaille R. Capek P. Pe´an M. Forano E. and Delort M. (2005). Degradation of Wheat Straw by *Fibrobacter succinogenes* S85: a Liquidand Solid-State Nuclear Magnetic Resonance Study. *Applied and Environmental Microbiology*. **71**: 1247–1253

McDonald P. Edwards R. Greenhalgh J. and Morgan C. (1995). Animal Nutrition. Fifth edition. Longman Scientific and Technical, Harlow, UK

McSweeney C. Dulieu A. Bunch R. (1998). *Butyrivibrio sp* and other xylanolytic microorganisms from the rumen have cinnamoyl esterase activity. *Anaerobe*. **4**: 57-65

McSweeney C. Palmer B. Krause D. and Brooker J. (1999). Rumen microbial ecology and physiology in sheep and goats fed a tannin containing diet. In Tannins in Livestock and Human Nutrition. Proc. International Workshop, Adelaide, Australia, pp 140

Mertens D. Weimer P. and Waghorn G. (1998). Inocula differences affect *in vitro* gas production kinetics. Deaville E. Owen E. Adesogen A. Rymer C. Huntington J. and Lawrence T. (Eds) *In vitro* techniques for measuring nutrient supply to ruminants. BSAS Occasional Publication No. 22, BSAS Edinburgh. pp 95

Miller G. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*. **31**: 426–428

Miller G. Blum R. Glennon W. and Burton A. (1960). Measurement of CMC-ase avtivity. *Analytical Biochemistry*. **2**: 127-132

Mould F. Kliem K. Morgan R. and Mauricio R. (2005). In vitro microbial inoculum: A review of its function and properties. *Animal Feed Science and Technology*. **123**: 31-50

Muetze S. Hoffman E and Becke K. (2003). Supplementation of barley straw with Sesbania pachycarpa leaves in vitro: effects on fermentation variables and rumen microbial population structure quantified by ribosomal RNA-targeted probes. *British Journal of Nutrition*. **89**: 445-453

Nagy J. Steinhoff H. and Ward G. (1964). Effects of essential oils of sagebrush on deer rumen microbial function. *The Journal of Wildlife Management*. **28**: 785-795

Nakashima Y. Orskov E. Hotten P. Ambo K. and Takase Y. (1988). Rumen degradation of straw. 6. Effect of polysaccharidase enzymes on degradation characteristics of rice straw. *Animal Production.* **47**: 421–427

Neidusznki I. and Marchessault R. (1971). Structure of B-D-(1-4)Xylan hydrate. *Nature.* **232**: 46-60

Nelson N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry.* **153**: 375-380 New York.

Odenyo A. and Osuji P. (1998). Tannin-tolerant ruminal bacteria from East African ruminants. *Canadian Journal of Microbiology.* **44**: 905–909

Osuji P. and Odenyo A. (1997). The role of legume forages as supplements to low quality roughages. *Animal Feed Science and Technology.* **69** : 27-38

Ouda J. Nsahlai I. and Mahundu P. (2006) *In vitro* degradability and gas production parameters of sericea lespedeza (*Lespedeza cuneata*) mixed with varying types and levels of roughages. *South African Journal of Animal Science.* **32**: 111-121

Owens F. and Goestsch A. (1998). Ruminal fermentation. Church C. (Ed) The Ruminant Animal Digestive Physiology and Nutrition. Waveland Press, Inc. US America. pp 145

Owolabi B. Beguin P. Kilburn G. Miller C. and Antony J. (1988). Expression in Escherichia coli of the cellulomonas fimi structural Gene for Endoglucanase B. *Appllied Enviromental Microbiology.* **54**: 518-523

Pell A. and Schofield P. (1993). Computerised monitoring of gas production to measure forage digestion. *Journal of Diary Science.* **76**: 1063-1073

Peter A. (1990). Carbohydrates of Physiological significance. Daryl K. Robert K. and Vector W. (Eds.), Herper's Biochemistry, Prentice Hall International Inc., London, United Kingdom. pp 124

Peterson G. (1979). Review of the folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Analytical Biochemistry.* **100**: 201-220

Pioneer Hi-bred International. (1988). Summary of the overall effect of Probios brand microbial products on the performance and health of incoming feedlot cattle. Pioneer Hi-bred International. Johnston, IA.

Preston T and Leng R. (1987). Digestive Physiology of ruminants. Matching Ruminant Production systems with available Resources in the Tropics and Sub-Tropics. pp 21-27

Rachel V. Valerie R. John F. Michel F. Jeremy C. and Roy M. (2000). Enzyme activity and dynamics: Xylanase activity in the absence of fast and harmonic dynamics. *Biochemistry Journal.* **346**: 355-358

Reese E. and Levinson H. (1950). The Biological Degradation of Soluble Cellulose Derivatives and Its Relationship to the Mechanism of Cellulose Hydrolysis, *The Journal of Applied Bacteriology.* **59**: 485-1950

Richardson R. (2000). Characterization of the histon H1-binding protein, NASP, as a cell cycle-regulated somatic protein. *Journal of Biological Chemistry.* **275**: 30378-86

Robbins C. Spalinger D. and Van Hoven W. (1995). Adaptation of ruminants to grass and browse diets: Are anatomically based browser-grazer interpretations valid? *Oecologia.* **103**: 789-799

Robert E. and Robert F. (1980). Carbohydrates. Cell and Molecular Biology Saunders. College Philadelphia, US America. pp78

Roberts B. (1987). The availability of herbage. The nutrition of herbivores. pp Hacker J B and Ternouth J H (Eds), 47-64. Academic Press, Sydney, Australia.

Robles A. Belyea R. Martz F. and Weiss M. (1980). Effects of particle size upon digestible cell wall and rate of *in vitro* digestion of alfalfa and orchard grass forages. *Journal of Animal Science.* **51**: 783-90

Rode L. Yang W. and Beauchemin K. (1999). Fibrolytic enzyme supplements for dairy cows in early lactation. *Journal of Dairy Science.* **82**: 2121–2126

Ross P. Mayer R. and Benziman M. (1991). Cellulose Biosynthesis and Function in Bacteria . *Microbiological Reviews.* **55**: 35-58

Russel J. and Dombrowski D. (1980). Effect of pH on the efficiency of growth by pure culture of rumen bacteria in continuous culture *Applied and Environmental Microbiology.* **39**: 604-610

Russel J. and Hespell R. (1981). Microbial rumen fermentation. *Journal of Dairy Science.* **64**: 11-53

Ryu D. and Mandels M. (1980). Cellulase: Biosynthesis and Application. *Enzyme Microbiology Technology.* **2:** 91-102

Scalbert A. (1991). Anti-microbial properties of tannins. *Phytochemical Analysis.* **12**: 3875-3883

Schingoethe J. (1993). Nutrient needs of ruminants versus monogastric species. Church C. (ed) The Ruminant Animal Digestive Physiology and Nutrition. Waveland Press,Inc. USA. pp 448

Schingoethe J. Stegeman G. and Treacher R. (1999). Response of lactating dairy cows to a cellulase and xylanase enzyme mixture applied to forages at the time of feeding. *Journal of Dairy Science.* **82**: 996–1003

Schwarz H. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology Biotechnology.* **56**: 634–649

Scott H. and Dehority B. (1965). Vitamin Requirements of Several Cellulolytic Rumen Bacteria1. *Journal of Bacteriology.* **89**:1169-1179

Seyis I. and Aksoz N. (2005). Investigation of factors affecting xylanase activity from Trichoderma harzianum 1073 D3. *Brazilian Archives of Biology and Technology.* **48**: 187-193

Shi L. Potts M. and Kennelly P. (1998). The serine, threonine, and/or tyrosine-specific protein kinases and protein phosphatases of prokaryotic organisms: a family portrait. *FEMS Microbiology Reviews.* **22**: 229–253

Shi Y. and Weimer P. (1997). Competition for cellobiose among three predominant ruminal cellulolytic bacteria under substrate-excess and substrate-limited conditions. *Applied and Environmental Microbiology.* **63**: 743-8

Smith L. Goering K. Waldo D. and Gorden C. (1971). *In vitro* digestion rate of forage cell wall components. *Journal of Dairy Science.* **54**: 71-78

Smith H. and Mackie I. (2004). Effect of Condensed Tannins on Bacterial Diversity and Metabolic Activity in the Rat Gastrointestinal Tract. *Applied and Environmental Microbiology.***70**: 1104–1115

Smith R. Moussa N. and Hawkins G. (1974). Utilization of the nucleic acids of Escherichia coli and rumen bacteria by sheep. *British Journal of Nutrition.* **32:** 529-537

Smith W. Yu I. and Hungate R. (1973). Factors affecting cellulolysis by Ruminococcus albus. *Journal Bacteriology*. **114**: 729-733

Sommart K. Parker D. Rowlinson P. and Wanapat M. (2000). Fermentation characteristics and microbial protein synthesis in an *in vitro* system using cassava, rice straw and dried ruzi grass assubstrates. *Asian-Australian Journal of Animal Science*. **13**: 1084-1093

Sotohy S. Sayed A. and Ahmed M. (1997). Effect of tannin rich plant (*Acacia nilotica*) on some nutritional and bacteriological parameters in goats. *Deutsch.-Tierarztl. Wochenschr.* **104**: 432–435

Sophie R. Michèle B. Maria A. Luiz J. Francis G. and Thierry M. (1999). New, Sensitive Fluorogenic Substrates for Human Cathepsin G Based on the Sequence of Serpin-reactive Site Loops. *Journal of Biological Chemistry*. **274**: 13810-13817

Stewart C. Dinsdale D. Cheng K. and Paniagua C. (1979). The digestion of straw in the rumen. In straw decay and its effect on disposal and utilization. New York. pp 123-130

Stock J. (1999). Sensitivity, coopertivity and gain in chemotaxis signal transduction. *Trends in Microbiology*. **7**: 1–4

Stock J. Ninfa A. and Stock A. (1989). Protein phosphorylation and regulation of adaptive response in bacteria. *Microbiological Reviews*. **53**: 450–490

Tafaj M. Steingass H. and Drochner W. (2001). Influence of hay particle size at different concentrate and feeding levels on digestive processes and feed intake in ruminants. 2. Passage, digestibility and feed intake. *Journal of Dairy Science*. **54**: 243-59

Teeri T. (1997). Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends in Biotechnology*. **15**: 160–167

Van Soest J. (1988). Effect of environment and quality of fibre on the nutritive value of crop residues. *Plant breeding and the nutritive value of crop residues.* Reed J. Capper B. and Neate P. (Eds), Proceedings of a workshop held at ILCA, Addis Ababa, Ethiopia,

Van Soest P. (1973). The uniformity and nutritive availability of cellulose. *Federation Proceedings*. **32**: 1804–1808

Van Soest P. (1987). Nutritional Ecology of the ruminant: Ruminant metabolism, nutritional stratagies, the cellulolytic fermentation and chemistry of forages and plant fibres. O & B Books.

Van Soest P. Robertson J. and Lewis B. (1991). Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*. **74**: 3583-3597

Varel H. Jung G. and. Knunholz R.(1991). Degradation of cellulose and forage fiber fractions by ruminal cellulolytic bacteria alone and in co-culture with phenolic monomer-degrading bacteria. *Journal of Animal Sciences*. **69**: 4993-5000

Varga A. and Kolver S. (1997). Microbial and Animal Limitations to Fiber Digestion and Utilization. Conference: New Developments in Forage Science Contributing to Enhanced Fiber Utilization by Ruminants. American Society for Nutritional Sciences. pp 819s-823s

Vercoe P. Kocherginskaya S. and White B. (2003). Differential protein phosphorylation–dephosphorylation in response to carbon source in Ruminococcus flavefaciens FD-1. *Journal of Applied Microbiology*. **94**: 974–980

Waldo D. Smith W. and Cox E. (1972). Model for cellulose disappearance from the rumen. *Journal of Dairy Science*. **55:** 125–129

Wanapat M. Sommart K. Wachirapakorn C. Uriyapongson S. and Wattanachant C. (1994). Recent advances in swamp buffalo nutrition and feeding. In:Proc.The 1st Asian Buffalo Association Congress. Wanapat M. and Sommart K (Eds),Khon Kaen. University, Khon Kaen, pp 17

Wanapat W. (2001). Swamp buffalo rumen ecology and its manipulation. Proceedings of Buffalo Workshop. www.mekarn.org/procbuf/wanapat.htm

Weimer P. and Weston W. (1985). Relationship between the fine structure of native cellulose and cellulose degradability by the cellulase complexes of *Trichoderma reesei* and *Clostridium thermocellum. Biotechnology and Bioengineering*. **27:** 1540-1547

Weimer P. Lopez-Guisa M. and French D. (1990). Effect of cellulose fine structure on the kinetics of its digestion by mixed ruminal microflora in vitro. *Applied Environmental Microbiology*. **56**: 2421–2429

Weston R. nnd Hogan J. (1967). The digestion of chopped and ground roughages by sheep i. The movement of digesta through the stomach. *Australian Journal of Agricultural Research.* **18**: 789-801

Wilson J. (1973a). The influence of aerial environment, nitrogen supply and ontogenetical changes on the chemical composition and digestibility of Panicum maximun Jacq. var. trichoglume Eyles. *Australain Journal of Agricultural Research.* **24**: 543

Wood T. and Bhat M. (1988). Methods for measuring cellulase activities. *Methods of Enzymology.* **160**: 87-112

Xiao Q. Brent S. Yanke J. and Cheng J. (2000). Isolation and analysis of two cellulase cDNAs from *Orpinomyces johonii. Gene.* **245**: 119-126

Yang C. (2002). Response of forage fibre degradation by ruminal microorganisms to branched-chain volatile fatty acids, amino acids, and dipeptides. *Journal of dairy science.* **85**: 1183-90

Yang W. and Beauchemin K. (2006). Effects of physically effective fiber on chewing activity and ruminal pH of dairy cows fed diets based on barley silage. *Journal of Dairy Science.* **89**: 217-28

Yang W. Beauchemin K. and Rode L. (1999). Effects of enzyme feed additives on extent of digestion and milk production of lactating dairy cows. *Journal of Dairy Science.* **82**: 391–403

Yang W. Beauchemin K. and Rode L. (2000). A comparison of methods of adding fibrolytic enzymes to lactating cow diets. *Journal of Dairy Science.* **83**: 2512–2520

Yokoyama M. and Johnson A. (1998). Microbiology of the rumen and intestine. Church C. (Ed) The Ruminant Animal Digestive Physiology and Nutrition. Waveland Press, Inc. US America. pp 125

Zuping T. Xiaogu D. Richard F. and Gerald J. (2000). Effects of pH on Glucose Measurements With Handheld Glucose Meters and a Portable Glucose Analyzer for Point-of-Care Testing. *Archives of Pathology and Laboratory Medicine.* **124**: 577–582