The Rumen Ecosystem : As a Fountain Source of Nobel Enzymes - Review -

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ABSTRACT : The rumen ecosystem is increasingly being recognized as a promising source of superior polysaccharide-degrading enzymes. They contain a wide array of novel enzymes at the levels of specific activities of 1,184, 1,069, 119, 390, 327 and 946 μmol reducing sugar released/min/mg protein for endoglucanase, xylanase, polygalactouronase, amylase, glucanase and arabinase, respectively. These enzymes are mainly located in the surface of rumen microbes. However, glycoside-degrading enzymes (e.g. glucosidase, fucosidase, xylosidase and arabinofuranosidase, etc.) are mainly located in the rumen fluid, when detected enzyme activities according to the ruminal compartments (e.g. enzymes in whole rumen contents, feed-associated enzymes, microbial cell-associated enzymes, and enzymes in the rumen fluid). Ruminal fungi are the primary contributors to high production of novel enzymes; the bacteria and protozoa also have important functions, but less central roles. The enzyme activities of bacteria, protozoa and fungi were detected 32.26, 19.21 and 47.60 mol glucose released/min/mL medium for cellulase; 42.56, 14.96 and 64.93 mmol xylose released/min/mL medium after 48h incubation, respectively. The polysachharide-degrading enzyme activity of ruminal anaerobic fungi (e.g. *Neocallimastix patriciarum* and *Piromyces communis*, etc.) was much higher approximately 3~6 times than that of aerobic fungi (e.g. *Tricoderma reesei*, *T. viridae* and *Aspergillus oryzae*, etc.) used widely in industrial process. Therefore, the rumen ecosystem could be a growing source of novel enzymes having a tremendous potential for industrial applications. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 6 : 988-1001*)

Key Words : Rumen Microbes, Plant Cells, Enzymes, Carbohydrate Utilization, Review

INTRODUCTION

Polysaccharide-degrading enzyme activity in the rumen ecosystem is estimated to be 10 times higher than that of any other known fermentation system in the globes. The rumen ecosystem provides ongoing enrichment and natural selection of microbes adapted to specific conditions. It also represents a virtually untapped resource of novel products (e.g. enzymes, detoxificants and antibiotics, etc.) and provides opportunities to define the processes of nutrient degradation. Thus, ruminal microbes play an important role as a wide source of genetic material for industry.

The global industrial enzyme market estimated in 1994 was worth US$ 400 million per year (Hodgson, 1994). Recently, this market was valued at US$ 1,400 million (Cowan, 1996) and will be increasing 1.5 to 2.5 times annually. Enzymes such as cellulases, xylanases, proteases, lipases, amylases, phosphatases, and pectinases are widely used in the pulp and paper, textile, detergent, food, beverage, and pharmaceutical industries. Industrial enzymes also can be applied in the livestock industry as feed additives. The potential of enzyme supplementation to improve feed utilization by non-ruminant livestock is widely recognized. The cost of enzymes to enhance livestock production and performance can be reduced by selecting for more effective expression and delivery systems. Improvements in this area require the establishment of a larger battery of genes to choose from. Ongoing study on the mechanisms of fiber digestion and finding of more efficacious enzymes for industrial purposes have been made. Recently, technological developments to enable genetic manipulation of ruminal microorganisms have been achieved. All these have contributed to the cloning and characterization of the growing number of genes of ruminal microbes.

This paper focused on ① the distribution of hydrolytic enzymes in the rumen compartments such as enzymes in whole rumen contents, feed associated enzymes, cell associated enzymes and enzymes in the rumen fluid fractions, ② the comparision of enzyme activities between rumen microbial family such as bacteria, protozoa and fungi, and ③ the comparision of few individual pure species of ruminal anaerobes and industrial aerobes such as *Aspergillas oryzae* and *Saccharomyces cerevisiae*. Knowledge of these enzymes and rumen microbes could be very important for biotechnological industries, for producing novel products, and for manipulating rumen ecosystems.

ENZYME ACTIVITY IN THE RUMEN

Cellulolytic enzymes and their activities in the rumen compartment

The plant cell wall is composed primarily of fibrils

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of cellulose which is the most abundant biopolymer on earth. It is a hydrogen-bonded β-1→4-linked *D*-glucan, which accounts for 20~30% of the dry weight of most plant primary cell wall (McNeil et al., 1984). The degree of crystallinity of the cell wall is highly variable. It may be as low as 20% in primary cell walls or as high as 70% in secondary cell walls (Lam et al., 1990). Enzyme systems digesting crystalline cellulose comprise, minimally of endoglucanases (β-1→4-*D*-glucan glucanohydrolase, EC 3.2.1.4), exoglucanase (exocellobiohydrolase; β-1→4-*D*-glucan cellobiohydrolase, EC 3.2.1.91, exoglucohydrolase; β-1→4-*D*-glucan glucohydrolase, EC 3.2.1.74) and β-glucosidase (β-*D*-glucoside glucohydrolase, EC 3.2.1.21). Endoglucanases cleave β-1→4-linkages of cellulose chains at random. The most commonly found exoglucanase is a cellobiohydrolase that removes cellobiose units from the non-reducing ends of cellulose chains. Beta-glucosidase mainly hydrolyses cellobiose produced by exocellobiohydrolases and some β-glucosidases remove glucose residues from short-cellooligosaccharides (Shewale, 1982; Wood, 1991).

Table 1 shows the cellulolytic enzyme activities found in rumen of Hereford cows fed 100% alfalfa hay diets. Endoglucanase activity is the highest and β-glucosidase activity it the lowest among cellulolytic enzymes. Total endoglucanase activity was detected at the range of 362.66~1183.56 IU, whereas exoglucanase activity was detected only at the range of 8.03~38.55 IU. The total and specific enzymatic activities varied considerably ($p<0.01$) with compartments such as WRE (enzymes located in whole rumen contents), RFE (enzymes located in rumen fluids), FAE (ezymes associated with feed particles) and CAE (enzymes associated with microbial cells). Polysaccharide-degrading enzymes such as endoglucanase and exoglucanase may exist in cell surface or cytoplasm, however glycoside-degrading enzymes such as β-glucosidase and β-fucosidase is located mainly in the rumen fluid. It is interesting to note that most of the β-glucosidase activities are located in the rumen fluids. Beta-glucosidase has been known as an intracellular enzymes in most microorganisms (Yazdi et al., 1990). Becuase cellobiose is soluble and potentially transportable to the membrane, it does not require enzymes for its utilization to be extracellular (in the rumen fluids). However, our unpublished observations indicated all the activities of β-glucosidase and β-fucosidase detected in rumen fluids possibly caused by the lysis of rumen microorganism (Wells and Russell, 1996). The review revealed that the ratio of dead (lysed) and live cells in ruminal fluids may be as great as 10:1. Gong and Tsao (1979) reported that β-glucosidase could be released only by autolysis of the producing cells. Relative distributions of endoglucanase in the rumen compartments were 15.3, 28.2 and 56.5%, whereas β-glucosidase were 33.4, 37.0 and 29.6 % for RFE, FAE and CAE, respectively (figure 1 and 2). These results indicated that adhesion of ruminal microorganisms, especially cellulolytic organisms,

Table 1. Specific enzyme activities of cellulase and xylanase in whole rumen contents, rumen fluid, feed associated and cell associated preparations from rumen contents of Hereford bulls fed 100% alfalfa diets

Enzymes	Enzyme activities distributed in			
	WRE[1]	RFE[2]	FAE[3]	CAE[4]
Cellululoytic enzyme activities (IU · mg protein^{-1})[6]				
β-1→4-*D*-endoglucanase	1183.56±20.39^{a}*	206.74±9.03^{c}	378.99±27.93^{b}	761.93±70.04^{a}
β-1→4-*D*-exoglucanase	38.55±13.33^{a}	10.43±0.99	14.55±2.43	10.17±0.62
β-*D*-glucosidase	27.74±0.66^{a}	9.73±0.66^{b}	10.85±0.55^{a}	8.65±0.90^{b}
β-*D*-fucosidase	33.00±0.72^{a}	15.14±0.69^{b}	16.85±0.84^{a}	9.51±0.83^{c}
Hemicelluloytic enzyme activities (IU · mg protein^{-1})[6]				
Xylanse	1068.59±53.48^{b}	300.24±11.34^{d}	682.92±40.17^{c}	1995.50±125.16^{a}
β-Xylosidase	33.69±1.77^{a}	21.45±1.62^{b}	20.40±1.00^{b}	28.62±1.94^{a}
Acetylesterase	210.93±10.01^{a}	245.88±7.74^{a}	160.41±9.03^{b}	95.14±8.59^{c}
α-*L*-Arabiofuranosidase	23.89±0.97^{a}	21.41±0.43^{ab}	19.22±1.01^{b}	13.22±1.12^{c}

(Source; Lee et al., 1998a)

[1] WRE (Enzymes located in whole rumen contens); rumen contents including both of liquid and solid fractions were homogenzied and centrifuged, and the supernatant was assayed.
[2] RFE (Enzymes located in rumen fluids); rumen contents were centrifuged and the supernatant was assayed.
[3] FAE (Enzymes associated with feed particles); feed particles in rumen contents were separated manually, washed with buffer, resuspended in an equal volume of buffer, homogenized, centrifuged, and the supernatant was assayed.
[4] CAE (Enzymes associated with microbial cells); rumen microbial cell fraction was separated by centrifugation, suspended in an equal volume of buffer, sonicated, centrifuged, and the supernatant was assayed.
[5] IU : Enzyme activities are expressed as μmol reducing sugars (polysaccharide-degrading enzymes) or ρ-nitrophenol (pNP: glycoside-degrading enzymes) released by 1ml of crude enzymes in min.
[6] IU · mg protein^{-1}; specific activities (μmol reducing sugars or pNP released mg^{-1} · protein min^{-1}).
* Each value represents Mean±standard error. In the same row mean with different superscript letters are significantly different ($p<0.01$).

to plant cell walls is quite important to degrade cellulosic materials. FAE is very important in rumen forage digestion, in terms of microbial mass as well as enzymatic activity.

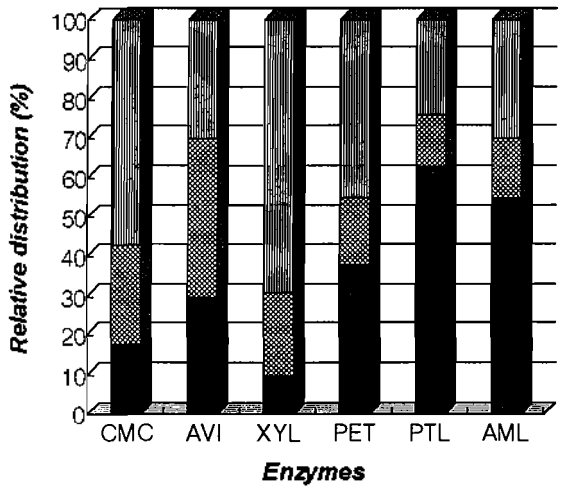

Figure 1. Relative distributions (%) of polysaccharide-degrading enzymes in rumen compartments; RFE (■, rumen fluidal enzymes), FAE(▩, feed associated enzymes) and CAE (▥, cell associated enzymes). CMC, CMCase; AVI, Avicelase; XYL, xylanase; PET, pectinase; PTL, pectate lyase, AML, amylase (Source; Lee et al., 1998a).

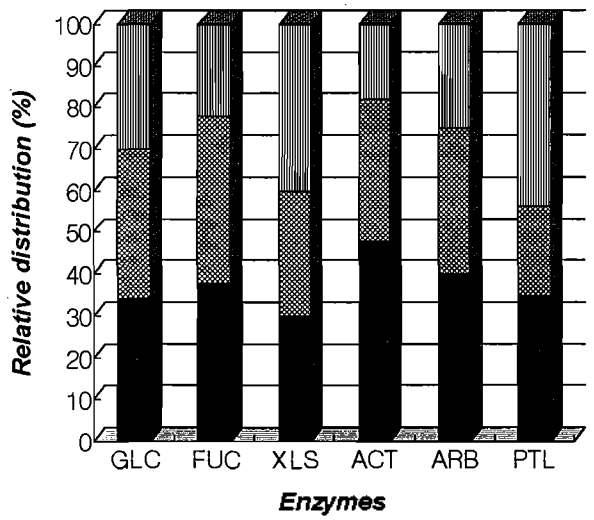

Figure 2. Relative distributions (%) of glucoside-degrading enzymes in rumen compartments; RFE (■, rumen fluidal enzymes), FAE (▩ , feed associated enzymes) and CAE (▥ , cell associated enzymes). GLC, glucosidase; FUC, fucosidase; XLS, xylosidase; ACT, acetylesterase; ARB, arabinofuranosidase; PTL, pactate lyase (Source; Lee et al., 1998a).

However, we observed that FAE was the lowest ($p<0.01$) in enzymatic distributions in the rumen. Thus, to manipulate ruminal forage fermentation, it is essential to know how to efflux enzymes associated with feed particles and/or how to release enzymes associated to microbial cell wall facing rumen fluid. To increase the FAE or RFE for manipulation of ruminal forage digestion, it would be necessary to use nonionic surfactant such as Tween 80 and Montanox 20, and/or acrylic acid polymers such as Junlon PW110. In the comparison between FAE and RFE, the specific endoglucanase activity of FAE constituted a larger enzymatic proportion (65%) than that of RFE (35%). These results were supported by the report of Martin and Michalet-Doreau (1995) that the amount of 15*N*-marked microbial biomass in the different series of ruminal contents showed that solid- adherent microorganisms (SAM) constituted a large proportion (74% in mean) of the total rumen microbial mass. This enzymatical result showed the same trend with the microbial population, as were in the results of Craig et al. (1987a, b), Forsberg and Lam (1977), and Legacy-Carmier and Bauchart (1989) who showed that the SAM constituted a large proportion of the total rumen microbial mass at the range of 70~80%, and the other 20~30% were composed of LAM (liquid associated microorganisms).

Hemicellulolytic enzymes and their activities in the rumen compartment

Hemicellulose is the second most abundant polysaccharide in nature and is a heteropolymer composed primarily of β-1→4-linked β-*D*-xylose backbone with various amounts of arabinose, glucose, arabinofuranose, uronic acid, methylated glucuronic acid, and other sugars as side groups, depending on the plant source (Whistler and Richards, 1970). The complete degradation of xylan requires the synergistic action of at least two types of enzymes, endo-β-1→4-xylanase that cleaves β-1→4-glycosidic bonds to produce xylooligosaccharides and β-*D*-xylosidase that cleaves small xylooligosaccharides to produce xylose. Enzymes degrading hemicellulose are produced by various microorganisms including bacteria, protozoa and fungi in the rumen. Hemicellulose composes a substantial proportion of herbage. It has been known that for some time the rumen has hemicellulolytic activity. Hemicellulolytic enzymes have been described in a number of ruminal bacteria (Clarke et al., 1969; Pettipher and Latham, 1979a, b). Ruminal protozoa have also shown to possess hemicellulolytic activity (Prins, 1977). For a long time these organisms were considered to be the major degraders of hemicellulose in the rumen. However, it is now considered that ruminal fungi produce novel hemicellulolytic enzymes which give high yields and activities of the enzymes.

Hemicellulolytic enzyme activities found in rumen compartments is shown in table 1. The activities of all enzymes that can complete hydrolysis of xylan into monosaccharides have been detected at high levels. This is the same trend as cellulolytic enzymes activities. Xylanase is located mainly in the cell while other hemicellulose degrading enzyme such as acetyl-esterase and arabinofuranosidase are located in the rumen fluid. Relative distributions of xylanase in the rumen compartments were 10.1, 22.9 and 69.0% for RFE, FAE and CAE, respectively (figure 1). Most of xylanase activity was detected in microbial cells, but a lower activity was detected in rumen fluid. The specific xylanase activity of FAE was two times higher than that of RFE. This is in agreement with the results of Martin and Michalet-Doreau (1995) as discussed above. Even though the nature and composition of diets, host animal, freguency of and sampling can affect enzyme activities in the rumen, we believe that the specific enzyme activities of CAE fraction constituted a large proportion (more than 65%) of the total rumen polysaccharide-degrading enzyme activities, based on our unpublished observation. The actual contribution of this greater activity to overall feed digestion is unknown, but it may be important in the process of ruminant adaptation to severe feed restriction.

Pectinolytic and other enzymes and their activities in the rumen compartment

Plant cell walls are composed of the structural polysaccharides such as cellulose, hemicellulose and pectin. Pectin polymers, chains of predominantly β-1 →4-*D*-galacturonic acid and methoxylated derivates are major consistituents of the middle lamellae and primary cell walls of higher plants (Collmer et al., 1988). The most important pectinolytic microorganisms in the rumen are bacteria and protozoa (Wojciechowicz and Ziolecki, 1984; Paster and Canale-Parola, 1985). A few strains of fungi isolated from rumen also have pectinolytic activities (Kopecny and Hodrova, 1995). In the rumen, several species of bacteria, protozoa and fungi are responsible for the degradation and fermentation of pectin (Szymanski, 1981; Orpin, 1983/84; Kopecny and Hodrova, 1995). These microbes produce exo-polygalacturonase, endo- and exo- pectate lyase and pectin esterase (Wojciechowicz and Ziolecki, 1984; Paster and Canale-Parola, 1985).

Pectate lyase activity was the highest and polygalacturonase activity was the lowest among pectinolytic enzymes we tested (unpublished observations). Total pectin lyase activity was detected at the range of 49~223 IU, whereas galacturonase activity was detected only at the range of 71~204 IU. Their specific activities were in the range of 57~128 and 63~117 mol galacturonic acid · h^{-1} · mg $protein^{-1}$ for pectin lyase (pectinase) and galacturonase, respectively (table 2). These are the most important pectinolytic enzymes, and their total and specific activities located mainly in the RFE. Specific pectinase activity is located mainly in the microbial cells and has a similar distribution to cellulolytic and hemicellulolytic enzyme activities. However, the specific pectinase activity of FAE was lower than that of RFE, which is different trend than cellulolytic and hemicellulolytic enzyme activities. On the other hand, pectate lyase activities were higher in the rumen fluid fraction (more than 50%) than in the other fractions

Table 2. Specific enzyme activities of pectinolytic and other enzyme in whole rumen contents, rumen fluid, feed associated and cell associated preparations from rumen contents of Hereford bulls fed 100% alfalfa diets

Enzymes	Enzyme activities distributed in			
	WRE[1]	RFE[2]	FAE[3]	CAE[4]
Specific activity of pectinolytic enzymes (IU · mg $protein^{-1}$)[5]				
Polygalacturonase	119.44±7.01[a]*	117.22±13.58[a]	63.11±2.77[b]	106.41±7.49[a]
Pectate lyase	267.71±7.60[a]	245.68±1.41[a]	152.60±11.73[b]	312.49±29.04[a]
Pectin lyase	116.83±15.28[ab]	127.86±16.19[a]	67.59±4.53[b]	56.72±18.01[b]
Hemicelluloytic enzyme activities (IU · mg $protein^{-1}$)[6]				
Protease[6]	76.72±4.70[a]	48.34±1.85[b]	28.31±0.84[c]	51.44±5.72[b]
Amylase	390.18±25.68[a]	250.86±14.82[b]	88.22±5.80[c]	146.90±11.45[c]
β-1→3-D-glucanase	164.05±5.71[a]	56.90±1.74[c]	113.17±7.14[b]	162.15±6.78[a]
β-1→3,1→4-D-glucanase	326.84±19.13[a]	184.31±0.24[b]	183.98±11.01[b]	319.31±26.97[a]
Arabinase	946.36±57.41[a]	901.41±14.55[a]	648.47±48.79[b]	961.03±67.11[a]
Pachimanase	20.73±7.11	nd[7]	nd	nd

(Source; Lee et al., 1998a)

[1] WRE, [2] RFE, [3] FAE and [4] CAE : see table 1 for details.
[5] IU · mg $protein^{-1}$; specific activities (μmol reducing sugars or pNP released mg^{-1} · protein min^{-1}).
[6] Protease activity was expressed as μg azocasein hydrolyzed hr^{-1} · ml of crude enzyme $solution^{-1}$).
[7] nd : No activity detected.
* Each value represents Mean±standard error. In the same row mean with different superscript letters are significantly different ($p<0.01$).

and were quite similar to FAE and CAE fraction, but different from cellulolytic and hemicellulolytic enzyme activities.

The enzyme activities confirmed to exist in the rumen are diverse and include plant cell wall polymer-degrading enzymes (e.g. cellulolytic, hemicellulolytic and pectinolytic enzymes), amylase, proteases, phytases and specific plant toxin-degrading enzymes (e.g. tannases). The variety of enzymes present in the rumen arises not only from the diversity of the rumen microbial community but also from the multiplicity of fibrolytic enzymes produced by individual micro-organisms (Doerner and White, 1990; Malburg and Forsberg, 1993; Flint et al., 1994; Ali et al., 1995). Efficient digestion of complex substrates in the rumen requires the coordinated activities of many enzymes. Many of the bacterial, protozoal and fungal inhabitants of the rumen exhibit proteolytic and amylolytic activities (Coleman, 1986; Wallace and Cotta, 1988; Michel et al., 1993; Attwood and Reilly, 1995). Proteolytic and amylolytic activities in the rumen were detected at high levels. The ranges of total activities detected were 362.66~1183.56 IU and 362.66~1183.56 IU for protease and α-amylase, respectively, and specific activities of such enzymes were at the range of 8.03~38.55 IU and 8.03~38.55 IU. The relative distributions of specific enzymatic activities varied considerably with compartments such as WRE, RFE, FAE and CAE ($p<0.01$). The values of protease were calculated as 37.7, 22.1 and 40.2%, whereas those of α-amylase were 51.6, 18.2 and 30.2 % for RFE, FAE and CAE, respectively (figure 2). These results indicates that protease is located mainly in rumen fluid and microbial cell, whereas α-amylase was located mainly in rumen fluid, and has different trend compared with cellulolytic, hemicellulolytic and pecti-nolytic enzymes. No endo-1→3-β-glucanase (pachimanase) activity was detected in feed particles (FAE) and microbial cells (CAE) while the higher values were found in rumen fluids (RFE).

ENZYME ACTIVITY OF RUMINAL BACTERIA, PROTOZOA AND FUNGI

Many species of bacteria, protozoa and fungi are involved in plant cell wall digestion in the rumen. This is due to the difficulty in separation of each microbial group in the rumen and measurement of fungal biomass, and the complex nature of the rumen ecosystem. This involves many microorganisms, which of each microbial group contributes to the degradation and fermentation of plant cell wall. In spite of this complicated interrelationship between microorganisms throughout the whole rumen ecosystem, bacteria play the major role because of their numerical predominance and metabolic diversity (Cheng et al., 1991) while protozoa can digest from 25 to 30% of total fiber. The extent of the involvement of fungi, however, has not yet been estimated. On the other hand, the interaction affects the range from synergism to antagonism depending on the kind of microbial groups, species and the type of substrate used. The interrelationship among bacteria, protozoa and fungi in the rumen also have not yet been estimated. *In vitro* examinations to estimate the roles that bacteria, protozoa and fungi play in plant cell wall digestion in the rumen microbial ecosystem have been attempted. Nevertheless many methodological problems remain in preparing in vitro microbial suspensions and simulating natural environment. To solve these problems, many artificial rumen ecosystems have been tested. To clearly assess the overall effect of individual rumen microbial fractions on rumen digestion and ruminant production, more systematic approaches will be required on the rumen microbial ecosystem. Fundamental knowledges provided from further research would be important to achieve the ultimate aim of the biological manipulation of forage fiber digestion (Flint and Thomson, 1991; Ørskov, 1991).

Lee et al. (1998b) reported that the relative roles of bacteria, protozoa and fungi in plant cell wall digestion inhibited the growth and activity of specific microbial groups (table 3 and 4). The time courses of the degradation rates of Orchard grass cell wall by fractionated microbial groups or its possible combination are presented in table 3. The various monocultural treatments used to evaluate the potential roles and relative contribution of bacterial, protozoal and fungal fractions of the rumen fluids in the degradation of Orchard grass cell wall resulted in three distinct groups of data corresponding to positive and F systems > B system > P and negative system. The greatest overall degradation rate of cell wall occurred in the positive and F systems (50.82 and 52.18% in 96 h incubation for positive and F systems, respectively) indicating that fungal activity was potentially sufficient to account for all of the observed degradation. Bacterial fraction (B system) alone resulted in significantly ($p<0.05$) less degradation at the late stage of incubation time (46% in 96 h incubation). Protozoal fraction (P system) alone could not progressively degrade the cell wall material. In addition, degradation (about 4~8%) occurring in the absence of microbial activity was not attributed to the loss of soluble components. This might be due to the experimental error from dry matter measuring step, because the cell wall used in the experiment did not contain soluble components.

Coculture systems (B+P, B+F and P+F system) to assess the interaction of component microbial groups showed a decrease in cellulolysis, when compared with the monoculture systems. When one microbial fraction

Table 3. Degradition rate (%) of cell wall extracted from Orchard grass by various microbial fractions separated from bovine rumen fluids through physical and chemical treatments

Treatements*	Incubation times (h)					
	12	24	36	48	72	96
Positive system	23.51^{a}	28.27^{a}	35.17^{a}	40.36^{a}	45.50^{b}	51.03^{ab}
B (Bacterial) system	18.54^{b}	24.33^{c}	31.85^{abc}	35.89^{bc}	42.20^{cd}	41.21^{d}
P (Protozoal) system	12.42^{c}	18.16^{ef}	19.33^{d}	13.18^{d}	20.16	20.48
F (Fungal) system	9.90^{d}	15.85^{f}	30.11^{bc}	38.48^{ab}	49.35^{a}	52.58
B+P system	19.31^{b}	25.59^{ab}	32.06^{ab}	34.26^{c}	39.53^{d}	40.31^{d}
B+F system	19.38^{b}	21.71^{cd}	32.62^{ab}	41.10^{a}	43.57^{bc}	48.24^{bc}
P+F system	18.00^{b}	19.71^{de}	27.84^{c}	36.13^{bc}	42.09^{cd}	44.39^{cd}
Negative system	3.50^{e}	4.46^{g}	5.85^{e}	6.57^{e}	4.85^{f}	5.41^{f}
SEM^{g}	1.23	1.42	1.86	2.49	2.87	3.11

(Source: Lee et al., 1998b)

^{a,b,c,d,e,f} Means in the same column with different superscript letters are significantly different ($p<0.05$).

^{g} SEM : standard erro of pool means (n=24).

* For details of procedures of microbial fractonation from the rumen contents, see the text.

Table 4. Celluase and xylanase activity in the culture supernatants grown various microbial fractions separated from bovine rumen fluids through physical and chemical treatments with the Orchard grass cell wall as a substrate

Treatments*	Incubation times (h)					
	12	24	36	48	72	96
Cellulase activity (μ mol glucose · min^{-1} · ml^{-1})						
Positive system	19.00^{a}	22.82^{bc}	30.22^{b}	34.63^{bc}	44.55^{ab}	47.04^{a}
B (Bacterial) system	13.04^{b}	20.08^{cd}	26.24^{c}	32.26^{c}	36.96^{c}	36.89^{c}
P (Protozoal) system	14.56^{b}	28.44^{a}	28.14^{bc}	19.21^{d}	19.52^{d}	19.55^{d}
F (Fungal) system	8.44^{c}	13.63	25.30^{c}	47.60^{a}	48.92^{a}	47.07^{a}
B+P system	17.30^{a}	21.83^{bc}	33.82^{a}	32.52^{c}	36.08^{c}	34.93^{c}
B+F system	17.52^{a}	18.40^{d}	26.38^{c}	36.98^{b}	38.04^{c}	43.60^{ab}
P+F system	18.39^{a}	23.74^{b}	29.66^{b}	31.92^{c}	39.73^{bc}	38.99^{bc}
Negative system	1.89^{d}	3.11^{f}	4.51^{d}	3.44^{e}	3.67^{e}	3.72^{e}
SEM^{f}	1.13	1.47	1.69	2.48	2.80	2.86
Xylanase activity (mmol xylose min^{-1} · ml^{-1})						
Positive system	15.84^{a}	18.89^{a}	34.25^{a}	44.74^{b}	54.41^{b}	51.21^{a}
B (Bacterial) system	12.45^{b}	17.01^{ab}	26.70^{b}	42.56^{b}	40.21^{c}	41.77^{b}
P (Protozoal) system	8.49^{d}	13.15^{c}	16.22^{c}	14.96^{c}	21.29^{d}	19.84^{c}
F (Fungal) system	12.33^{b}	15.92^{abc}	34.29^{a}	64.93^{a}	64.21^{a}	54.28^{a}
B+P system	10.55^{c}	16.44^{ab}	27.67^{b}	40.82^{b}	39.86^{c}	37.48^{b}
B+F system	12.46^{b}	16.26^{ab}	31.18^{a}	37.56^{b}	51.23^{b}	47.84^{a}
P+F system	9.92^{cd}	14.14^{bc}	26.11^{b}	39.77^{c}	41.15^{c}	49.83^{a}
Negative system	2.10^{e}	2.56^{d}	5.29^{d}	5.25^{d}	3.91^{e}	4.44^{d}
SEM^{f}	0.77	0.98	1.88	3.63	3.59	3.72

(Source: Lee et al., 1998b)

^{a,b,c,d,e} Means in the same column with different superscript letters are significantly different ($p<0.05$).

^{f} SEM : standard erro of pool means (n=24).

* For details of procedures of microbial fractonation from the rumen contents, see the text.

was associated with another microbial fraction, two types of results were obtained. Protozoal fraction reduced the degradation rate of cell wall by both bacterial and fungal fraction. In the coculture of bacterial fraction with fungal fraction, a synergistic interaction was detected.

To measure endoglucanase, carboxymethyl cellulose (CMC) was used for the assay substrate. The endoglucanase activity of culture supernatant for positive and F system was higher than that of other monoculture systems, with the same trend of the cell wall degradation (table 4). The results also showed

that the amount of endoglucanase released from the bacterial fraction was not much larger than the amount from the fungal fraction. The protozoal enzyme activity was lowest except negative system. There was little or no activity (usually less than 5 μmol · ml^{-1} · min^{-1}) in the negative system. Also, endoglucanase activity was higher for cultures of B+F system than for the other coculture (B+P and P+F system).

Xylanase production for F system was more rapid and higher than that of the B system (table 4). The xylanase activity in the culture of F system after 48 h incubation was 1.3 times higher than that of B system. There was little xylanase activity in the P system, detected usually less than 20 mmol · ml^{-1} · min^{-1}. Coculture of bacterial fraction with fungal fraction (B+F system) also increased the xylanase activity over the cultures of the bacterial or fungal fraction alone. Thus, increased cellulase and xylanase activities would reflect the increase in CW digestion by microbial fractions. Although the interactions occurring between rumen microbes (bacteria, protozoa and fungi) have been reviewed by Wolin and Miller (1988), and interactions involved in fiber degradation are also reviewed by Dehority (1993), the relative contribution of bacteria, protozoa and fungi to CW degradation is still poorly understood. The first study was conducted to assess the relative contribution to the overall process of cell wall digestion by microbial fractions in rumen fluids and to assess the interactions of component microbial groups.

In the monoculture, the cellulolysis of Orchard grass cell wall by bacterial fraction was high at the early stages of incubation. However, cellolysis by fungal fraction was high at the late stages. Protozoal fraction alone did not degrade the cell wall material.

These results indicates that rumen bacteria quickly die. Lyse on prolonged incubation and anaerobic rumen fungi showed a marked lag in their *in vitro* degradation of cell wall materials, whereas rumen protozoa could not survive under the optimized conditions studied. The relative contribution of microbial fractions to the overall process of cell wall digestion was higher in an order of fungal fraction > bacterial fraction > protozoal fraction. Although the rumen bacteria are believed to be responsible for most of the feed digestion in the rumen, because of their numerical predominance and metabolic diversity (Cheng et al., 1991), the results obtained in our study suggested that the contribution of fungal fraction to cell wall degradation in the rumen may greatly exceed that of the bacteria. In the gut fermentation of mammals, the ability of the anaerobic fungi to penetrate deeply into plant tissues not normally accessible to bacteria (Bauchop, 1981) suggests that fungi have a special role in fiber digestion. The ability of the fungal fraction to utilize cell wall component of plant material has been demonstrated, and fungal activity could potentially be sufficient to account for all the observed degradation. Onodera et al. (1988a) confirmed that mixed rumen protozoa participate in cellulose digestion in the rumen ecosystem, with direct attack at the enzyme level by their own β-1→4-glucanase. Coleman (1989), Newbold et al. (1989) and Williams and Withers (1991) also confirmed as much as 62% of the cellulolytic activity associated with plant material in the rumen may be protozoa. However, the protozoal fraction alone did not degrade the cell wall material to a great extent in this study nor in a following study which is concerned protozoa alone did not degrade cell wall under the conditions studied (Coleman, 1986; Craig et al., 1987b; Onodera et al., 1988b; Coleman, 1992). Protozoa are able to digest bacterial and fungal material, and apart from the fungus itself, nutrients from the culture medium, microbial fermentation products, and protozoa themselves may also have been utilized. Small feed particles are readily ingested by protozoa (Coleman, 1992). Our results may indicate that the rumen protozoal fraction was not able to take up insoluble large feed particles prepared by extractions of cell walls and by grinding through 1 mm screen. We failed to assess the direct quantitative contribution of protozoal fraction on cell wall degradation. The manner of food intake and digestion is quiet different between bacteria, protozoa and fungi, and this difference should be taken into account to assess the relative contribution of nutrient digestion. Mixed ruminal bacteria absorb nutrients onto the cell wall, and hydrolysis occurs at this site (Wallace, 1985). In this process, solubility and the primary sequence of amino acids are the most important determinants of hydrolysis of nutrients. On the other hand, hydrolysis of nutrients by rumen protozoal fraction can occur intracellularly, and factors affecting engulfment are more important. However, in case of rumen fungi which have motile stages in their life cycle, attachment to the feed particle and germination are the most important factors to digest feed. Thus, chemical and physical characteristics of nutrients are the most important factors in the relative contribution of bacteria, protozoa and fungi to cell wall degradation. In our experiment, the relative contribution of bacterial and protozoal fraction to cell wall digestion may be underestimated, because cell wall substrates used have no soluble components and are composed of relative particle to large for protozoa to engulf.

In general, the coculture systems showed a decrease in cellulolysis compared with the monoculture systems. When microbial fractions were divided with one another, two types of results were obtained in our experiment. The protozoal fraction inhibited the digestion of cellulose in the plant cell wall by both

Table 5. Comparison of carbohydrate utilization patterns of ruminal anaerobic microorganisms

Species	Utilization of saccharides[1]		
	Poly-	Di/Tri-	Mono-
BACTERIA			
Primary celluloytics			
Fibrobacter succinogenes	Cel	Cl	G
Ruminococcus flavefaciens	Cel, Xyl, Pec	Cl	-
Ruminococcus albus	Cel, Xyl	Cl	G, X*, A*
Secondary cellulolytics			
Butyrivibrio fibrisolvens	Cel, Xyl, Dex, Pec	Ml*, Lc*, Cl*	G, Ga, M, F, X*
Clostridium longisporum	Cel	Cl, Ml, Lc, Sc	G, Ga, F
Clostridium locheadii	Cel, Dex	Ml, Sc	G
Noncellulolytics			
Prevotella ruminicola	Pec, Sta*, Dex*	Lc, Cl, Ml*	G, Ga, F, X*, A*, R*
Ruminobacter amylophilus	Sta	Ml	-
Selenomonas ruminantium	Sta, Dex	Cl, Ml, Lc, Sc*	G, Ga, F, X, A
Streptococcus bovis	Sta	Ml, Lc, Cl, Sc	G, Ga, M, F
Succinomonas amylolytica	Sta, Dex	Ml	G
Succinivibrio dextrinosolvens	Dex, Pec	Ml, Sc*	G, Ga, M, X, A*, F*
PROTOZOA			
Holochrichs			
Isotricha intestinalis	Sta, Pec	Sc	G
Prostoma	Sta, Pec	Sc	G
Dasytricha ruminantium	Sta	Cl, Ml	G
Entodinomorphs			
Entodinium bursa	Sta, Xyl		
Caudatum	Sta	Cl, Ml, Sc	G
Simplex	Sta	-	-
Diplodinium diplodinium	Cel, Xyl, Sta	-	-
Polyplastron	Cel, Sta	Sc	G
Ostracodinium	Cel, Xyl, Sta	-	-
Eremoplastron	Cel, Xyl, Sta	-	-
Epidinium ecaudatum	Cel, Xyl, Sta	Sc, Ml	-
Ophryoscolex caudatus	Cel, Xyl, Sta	-	-
FUNGI			
Monocentrics			
Neocallimastix frontalis	Cel, Xyl, Sta, Pec*	Cl, Gn, Ml, Lc, Ra, Sc	F, G, X
patriciarum	Cel, Xyl, Sta, Pec*	Cl, Gn, Ml, Ra, Sc	G, X
Piromyces communis	Cel, Xyl, Sta*, Pec*	Cl, Gn, Ml, Lc, Ra, Sc*	F, G, X
Caecomyces communis	Cel, Xyl, Pec*	Cl, Gn, Ml, Lc	F, G*, X
Polycentrics			
Anaeromyces mucronatus	Xyl, Sta	Cl, Gn, Ml, Lc, Sc	F, G, X
Orpinomyces joyonii	Cel, Xyl, Sta	Cl, Gn, Ml, Lc*, Sc*	F, G, X

* Positive for some strains.

[1] Abbreviations : (Polysaccharides : Cel=cellulose, Xyl=xylan, Pec=pectin, Sta=Starch, Dex=dextrin).
(Di/trisaccharides : Cl=cellobiose, Gn=Gentiobiose, Lc=lactose, Ml=maltose, Ra=raffinose, Sc=sucrose).
(Monosaccharides : A=arabinose, F=fructose, G=glucose, G=galactose, M=mannose, R=rhamnose, X=xylose).

bacterial and fungal fractions, but in coculture of bacterial fraction with fungal fraction, a synergistic interaction was detected. Although protozoal fraction alone did not degrade the cell wall material to a great extent in our experiment, the cocultures of protozoal fraction with bacterial fraction (B+P system) or fungal fraction (P+F system) significantly affected the degradation rate of Orchard grass cell wall, compared to bacterial or fungal fraction monoculture. In general, the early stages (1~2 d incubation) of incubation, did not make differences in degradation rate, but as the incubation time increased the differences between monoculture and coculture became more pronounced. When the fungal fraction was incubated with protozoal fraction, a steady decline in the degradation rate was observed, accounting for an 18.5% reduction at the

end of the incubation period. Rumen protozoal fraction also adversely affected the cellulolysis of the rumen bacterial fractions, and the total amount of cell wall degraded was smaller to the bacterial-protozoal fraction coincubations. These results differ from previous work; Yoder et al. (1966) reported that the addition of washed rumen protozoa to a washed suspension of rumen bacteria substantially increased cellulose digestion and acid production. Onodera et al. (1988b) also observed that the addition of protozoa to bacteria increased cellulose digestion. Orpin (1983, 1984) reported that anaerobic fungi and rumen protozoa may be complementary rather than competitive in nature system. These negative effects observed in B+P and P+F systems seem to be mainly a consequence of the predatory activity of the protozoa. The culture condition used in current experiment was quite limited to grow protozoal fraction probably due to the substrate which was composed of large particle without soluble fraction. Therefore, predatory activity on bacterial and fungal fractions by protozoal fraction might be accelerated. The negative relationship could be partially accounted for by predatory activity against fungal zoospores and bacteria (Hungate, 1966). Another possible explanation was that fungal sporangium could be degraded by protozoal chitinolytic enzymes (Morgavi et al., 1994), although these were not observed in the present study. The results of our experiments also indicated that control of the population size by rumen protozoal fractions may offer an opportunity for altering rumen fermentation and productivity of ruminant animals. Anaerobic fungal numbers have been shown to increase in defaunated animals. Romulo et al. (1986 and 1989) showed 2- to 4-fold increases in zoospores and zoosporangia of anaerobic fungi in defaunated sheep. Soetant et al. (1985) and Ushida et al. (1989) found increased fungal populations in defaunated animals, as well as increased digestion of the high fiber diet fed to these animals. In contrast, Newbold and Hillman (1990) observed only small increases in fungal zoospores in defaunated ruminants.

The rumen is a highly complex ecosystem, which contains many different microbial species and has a great potential for inter-microbial associations. In interactions of B+P system, we have observed a synergistic interaction by detecting higher enzyme activities in B+P system than in fungal monoculture, as same trend shown in the results of degradation rate of Orchard grass cell walls. Relationships are known to exist between microorganisms in the rumen, and many researchers have shown that anaerobic fungi interact with hydrogen-utilizing bacteria (Bernalier et al., 1992; Roger et al., 1993). In the presence of hydrogen-utilizing bacteria such as methanogens, anaerobic fungi are more effective in degrading cellulose. However, recent study on the interactions between anaerobic fungi and rumen cellulolytic bacteria showed the inhibition of fungal action to hydrolyse cellulose (Bernalier et al., 1992, 1993). The inhibition of fungal activity is caused by an extracellular protein released by the cellulolytic bacteria (Bernalier et al., 1993). It is well known that the enzymatic activities of fungi, combined with the particular penetrating growth of the rhizoidal system, lead to weakening and particle size reduction of plant cell walls (Akin et al., 1983; Bauchop and Mountfort, 1981; Orpin, 1983, 1984). This could be due to the synergism between late stage fungus and protozoa *in vitro*.

ENZYME ACTIVITY OF INDIVIDUAL PURE SPECIES BETWEEN RUMINAL ANAEROBES AND COMMERCIAL AEROBES

The major structural polysaccharides of the plant cell wall can only become available to the ruminant animal through the activities of rumen microbes that possess the necessary enzyme systems for the degradation and utilization of such carbohydrates. The ability to digest cellulose has been described in a large number of bacteria, protozoal and fungal species isolated from the rumen (table 6). However, it has been generally accepted that cellulolysis in the rumen is primarily due to the activities of the ruminal cellulolytic bacteria, in particular three predominant species: *Fibrobacter* (*Bacteroides*) *succinogenes, Ruminococuus flavefaciens* and *Ruminococuus albus*. These three species have common characteristics that set them apart from other ruminal bacteria (including secondary cellulolytic species, such as *Butyrivibrio fibrisolvens, Clostridium longisporium* and *Clostridium locheadii*) and from cellulolytic bacteria from habitats other than the intestine. One of more obvious characteristics of the predominant cellulolytic bacteria is their nutritional specialization. As shown in table 6, most ruminal bacteria that ferment carbohydrates are capable of using numerous mono-, di-/tri- and polysaccharides as growth substrates, and even those species with limited capability for digesting cellulose can utilize at least a few of these sugars.

In contrast, *F. succinogenes* and *ruminococci* (predominant cellulolytics) are nearly restricted to cellulose, xylan and its hydrolytic products as growth substrates (Hungate, 1966). The consequence of this nutritional specialization is that the primary means by which these species gain selective advantage in the rumen is by optimizing only two catabolic activities: cellulose hydrolysis (depolymerization) and efficient utilization of the hydrolytic products (cellodextrins). However, these tasks are challenging by the nature of the substrate (an insoluble, well-ordered biopolymer

Table 6. Comparison of rate constants (h^{-1}) for digestion of crystalline cellulose by various ruminal anaerobic and nonruminal aerobic microorganisms

Microbes	Sustrate[1]	Rate constant	Basis	Reference
Bacteria				
Clostridium thermocellum 27405	AV	0.16	Weight loss, chemostat	Lynd et al. (1986)
Ruminococcus albus 8	AV	0.05	Weight loss, chemostat	Pavlostathis et al. (1986)
Ruminococcus flavefaciens FD-1	SC	0.08	Weight loss, chemostat	Shi & Weimer (1992)
Fibrobacter succinogenes S85	SC	0.07	Weight loss, chemostat	Weimer (1992)
Cellulomonas uda ATCC 21399	AV	0.027	Heat production, batch	Dermoun & Belaich (1985)
Cellulomonas flavigena JC3	AV	0.006	Weight loss, batch	Chesson (1988)
Fungi				
Neocallimastix frontalis RE1	FP	0.040	Weight loss, batch	Lee et al. (1997a)
Orpinomyces joyonii SG4	FP	0.037	Weight loss, batch	Lee et al. (1997b)
White-rot fungi (5 species)	CT	<0.004	Weight loss, soil block	Highley (1988)
Brown-rot fungi (8 species)	CT	<0.004	Weight loss, soil block	Highley (1988)

AV=Avicel microcrystalline cellulose PH 101, SC=Sigmacell 20 microcrystalline cellulose, FP=Filter paper cellulose Whatman No. 1, and CT=cotton cellulose.

Table 7. Comparison of cellulase activities (U/ml · h^{-1}) of the supernatant of medium containing rice straw as a caron source incubated with various microorgranisms

Microbial strains	Incubation time (h)		
	24	48	96
Rumen mixed microorganism	3.966	4.113	4.283
Ruminal anaerobic bacteria			
Fibrobacter succinogenes	0.170	0.791	1.401
Ruminococcus albus	0.305	0.487	0.857
Ruminococcus flavefaciens	0.302	0.842	1.067
Ruminal anaerobic fungi			
Piromyces rhizinflata	3.272	3.990	4.692
Orpinomyces joyonii	3.128	4.067	4.278
Neocallimastix frontalis	4.633	4.656	7.435
Commercial aerobic microorganisms			
Aspergillus oryzae	4.034	3.910	3.902
Saccharomyces cerevisiae	4.000	3.899	3.944

(Sources; Modified from Ha et al. (1995) and Lee et al. (1996))

woven into a matrix of other biopolymers) and by the nature of the ruminal environment (a continuously flowing system with a dense and diverse microflora that includes grazing protozoa, proteolytic bacteria and cellodextrin-utilizing, noncellulolytic opportunists). However, these bacteria have evolved to digest cellulose relatively rapidly (as shown in table 6); in fact, the only organism shown to digest cellulose more rapidly is the thermophilic, anaerobic bacterium *Clostridium thermocellum*, an organism that displays many nutritional and adaptive similarities to the predominant cellulolytics, but this also benefits from enhanced catalytic rates at its 60℃ growth optimum (Lynd et al., 1986).

Interestingly, the first-order rate constant for the digestion of highly ordered cellulose by the three ruminal species under optimal growth conditions appear to be fixed within a rather narrow range of 0.05 to 0.08 h^{-1} (Pavlostathis et al., 1986; Shi and Weimer, 1992; Weimer, 1996). These findings suggest that these species have adapted to some sort of upper limit of cellulose digestion within the constraints of digesting a structurally ordered, insoluble polymer.

On the other hand, anaerobic rumen fungi have a diverse enzyme excretion system of cellulase (Wood et al., 1986), xylanase (Mountfort and Asher, 1989a, b), hemicellulase, pectinase (Gordon and Phillips, 1992), protease (Wallace and Joblin, 1985), amylase and amyloglycosidases (Pearce and Bauchop, 1985) etc., and the activities of cellulase and xylanase are much higher than those of other predominant rumen cellulolytics and protozoa and anaerobic microorganisms used for commercial enzyme production such as *Trichoderma harzianum, Trichoderma reesei, Thermoascus aurantiacus, Aspergillus oryzae* and *Saccharomyces cerevisiae* (as shown in table 7).

Moreover, the rhizoidal system of rumen fungi has a function of taking feed particles to pieces or soften them physically (Ho et al., 1988). Akin et al. (1990) reported that rumen fungi are charge of over 70% of the total cellulose digestion in rumen *in vivo*.

Cellulolytic enzyme activities of rumen fungi is known to be 5 times higher than those of predominant ruminal cellulolytic bacteria (Ha et al., 1995; Lee et al., 1996; as summarized in table 7). Bernalier et al. (1992) also reported that the cellulase activities of *N. frontalis, P. communis* and *C. communis* are 3 to 4 times higher than those of predominant cellulolytics such as *F. succinogenes* and *R. flavefaciens*. Wood et al. (1986) reported that the cellulase activity of *N. patriciarum* was higher than that of *T. reesei* used widely as industrial process. Lee et al. (1995) reported that the cellulase and xylanase activities of ruminal fungi were higher than those of *A. oryzae* and *S. cerevisiae*. Teunissen et al. (1992) also showed that the CMCase activities of rumen fungi (*Neocallimastix* spp. and *Piromyces* spp.) were much higher approximately 3 to 6 times than those of *T. reesei* and *T. viridae*. The enzymes excreted by rumen fungi have a high activity and stability, besides substrate utilization of these enzymes is very wide, and enzyme excretion type is extracellular. Thus, there is a great potential to use these enzymes industrially. Teunissen and Op den Camp (1993) presented industrial application scheme of rumen fungi and the enzyme excreted by rumen fungi. Anaerobic fungi and their enzymes could be interesting for many industrial applications, including livestock industry, environmental industry, production of useful products as secondary metabolites such as antibiotics, chemicals, and zearalenone and so forth.

CONCLUSIONS

The rumen microbial population represents a rich source of novel enzymes with tremendous potential for industrial application. The enzymes of which activities confirmed to exist in the rumen are diverse. Such enzymes include plant cell wall polymer-degrading enzymes (e.g. cellulases, xylanase, β-glucanase, pectinase), amylases, proteases, phytases and specific plant toxin-degrading enzymes (e.g. tannases). The variety of enzymes present in the rumen arises not only from the diversity of the microbial community, but from the multiplicity of specific enzymes produced by individual microbial species (Flint et al., 1994; Ali et al., 1995). The rumen is increasingly being recognized as a promising source of superior fibrolytic enzymes. Cellulases and xylanases produced by ruminal fungi are among the most active fibrolytic enzymes described to date (Trinci et al. 1994). Thus, these anaerobic fungi and/or their enzymes have sparked interest for a number of biotechnological applications. These include development as probiotics and as feed additives for silages and total mixed rations, for saccharification of lignocellulosic residues, and for production of polysaccharide-hydrolysing enzymes.

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