Anaerobic Rumen Fungi: Their Characterization and Ecology in Plant Cell Wall Degradation in the Rumen

Heidi Mattai

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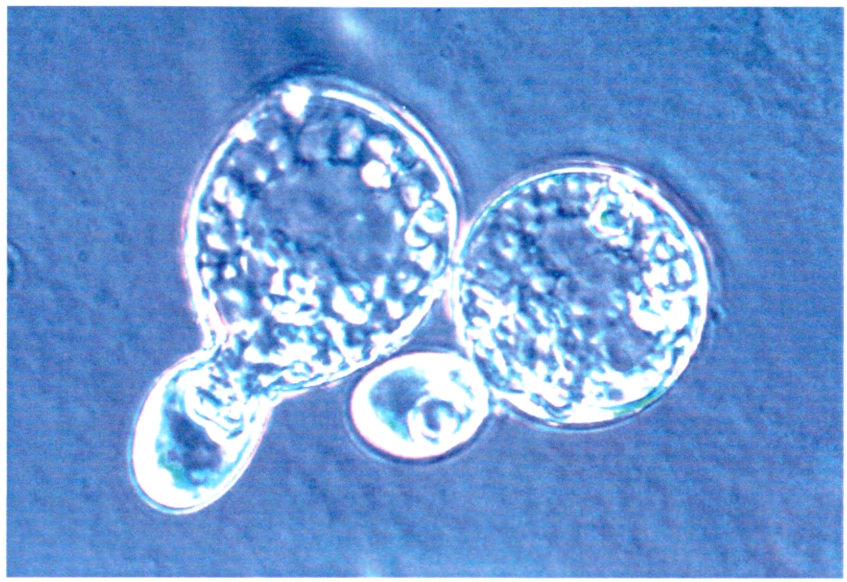

Hiroki Matsui

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Chapter 1: General Introduction

Taxonomy of anaerobic fungi

Interest in the anaerobic fungi have been growing in recent decades especially on their capacity for fiber digestion (Orpin and Joblin, 1988; Bauchop, 1989a; Joblin, 1990; Fonty and Joblin, 1991; Mountfort and Orpin, 1994). The gut anaerobic fungi are unique in that they are the only known strictly anaerobic true fungi in the biosphere. However, monoflagellated zoospores of anaerobic fungi were discovered long time ago by Lievetanz (1910) as monoflagellated protozoa and were given the names as *Piromonas communis* and *Sphaeromonas communis*. In 1913, Braune discovered a multiflagellated organism and named it *Callimastix frontalis*. From the ultrastructural studies on *Callimastix* sp., Vavra and Joyon (1966) concluded that this organism belonged not to the protozoa, but to the fungi. They created a novel genus *Neocallimastix*. About ten years later, Orpin (1975) was the first to succeed in culturing these organisms and he defined their life cycle. Later he has demonstrated the presence of chitin in their cell-wall (Orpin, 1977b). He concluded that *N. frontalis*, *P. communis* and *S. communis* belong to the class Chytridiomycetes in subdivision Mastigomycota. This conclusion was confirmed by 18S rRNA sequence analyses on the rumen fungi (Doré and Stahl, 1991). Barr *et al.* (1989) involved the anaerobic fungi into the order Spizellomycetales in sub-division Mastigomycota and separated anaerobic fungi from Chytridiomycetales on the basis of the ultrastructural differences in zoospores. Heath *et al.* (1983) had proposed a new family Neocallimastigaceae in the order of Spizellomycetales. However, from recent rRNA analyses, Li and Heath (1992) and Li *et al.* (1993) have proposed to elevate the family Neocallimastigaceae to the new order Neocallimasticales in the Chytridiomycota. Five genera have been established so far; *Neocallimastix, Piromyces* (formerly *Piromonas*), *Caecomyces* (formerly *Sphaeromonas*), *Orpinomyces*, *Anaeromyces* (synonym for *Ruminomyces*) and 17 species are known (Table 1). The change in nomenclature for *Piromonas* and *Sphaeromonas* was proposed by Gold *et al.*

(1988), because their former names were given as the protozoa. *Ruminomyces* and *Anaeromyces* have similar morphological features and considered to be synonymous (Li *et al.*, 1993). Three of the genera develop monocentric thalli and the latter two produce polycentric thalli (Table 1). Among the anaerobic fungi, species belonging to the genera *Neocallimastix* and *Orpinomyces* produce polyflagellated zoospores, and the remaining species produce monoflagellated zoospores.

Classification of fungi is principally based principally up on the morphological characteristics of the thallus. This is also true for the anaerobic fungi, but an analysis of the ultrastructural features of zoospores is often required for the species-level identification (Gold *et al.*, 1988). The traditional physiological tests such as sugar utilization or end products formation for classification do not seem to be efficient for identifying species (Gordon and Phillips, 1989). The G+C contents in fungal DNA has been measured in several strains (Brownlee, 1994). The G+C contents of these microorganisms are often as low as 13 to 20 %, indicating the uniqueness of anaerobic fungi as true fungi, although coding sequences have the usual G+C contents. The use of G+C contents for species level identification is still under investigation. Brownlee (1994) has proposed the use of DNA base sequence of the spacer non-coding region between 18S rRNA gene and 28S rRNA gene. Further molecular level analyses on the anaerobic fungi are required for the efficient identification of the species.

Life cycle of anaerobic fungi

A schematic life cycle of anaerobic fungi is shown in Figure 1. The life cycle consists of a motile, flagellate zoospore stage, free living in the liquid phase of contents of digestive tracts, and a nonmotile, vegetative, reproductive stage on solid phase of the contents. The zoospores of anaerobic fungi are released from mature sporangia into liquid phase of habitat. Anaerobic fungi in the rumen appear to release zoospores within 30 min after feeding (Orpin, 1977a). Free zoospores move in the liquid towards damaged area or stomata of plant fragments by chemotactic response to soluble

carbohydrates released from plant cells. After attachment of the zoospores to the plant fragments, flagella are detached from zoospores, then encystment and germination occur, followed by penetration of the plant tissues by the fungal rhizoid. Anaerobic fungi invade the plant tissue by developing the rhizoid and form the sporangia. Substances involved in the plant tissues such as haemes induce zoospore differentiation (Orpin and Greenwood, 1986b). Thus the mature sporangia release the zoospores and the next life cycle begins. The life cycle of anaerobic fungi lasts about 24-32 hours depending upon the nutritional condition (Joblin, 1981; Lowe *et al.*, 1987; Bauchop, 1989a). Anaerobic fungi are thought to form a resistant body which withstands drying process and exposure to air. Davies *et al.* (1993) compared viability of anaerobic fungi in digesta obtained from steer foregut, midgut, hindgut and in the faeces which had been dried at ambient temperature and stored in air for up to 9 month. Viable counts of anaerobic fungi in hindgut and faeces were higher than those of the foregut and midgut regions. They suggested that anaerobic fungi have aero-tolerant stage (cyst or resistant zoosporangium) in the life cycle so that fungi are able to survive in faeces until re-introduction into a new host animal.

Isolation of anaerobic fungi

Most anaerobic fungi have been isolated from the rumen, but some have been isolated from hind gut contents or feces of a range of herbivores, such as ass, horses, kangaroos, elephants, camel and rhinoceros (Orpin, 1994). Since Hungate (1969) provided the roll tube anaerobic culture technique, hundreds of anaerobic rumen bacteria were isolated by this conventional technique (Stewart and Bryant, 1988). Anaerobic fungi are not exceptions. The methods published by Joblin (1981) and Lowe *et al.* (1985) are routinely used in many laboratories. The selection of anaerobic fungi is facilitated by the addition of antibacterial agents. Pure-cultures are usually maintained by a frequent transfer to the fresh media. Cryopreservation of zoospores or mature thalli are also possible (Yarlett *et al.*, 1986b), but it is not certain that all strains do persist

during storage. However, long storage is sometimes possible at 39 ℃ under usual culture condition (Joblin, 1981). This suggests the presence of dormant spores or some kinds of resting cells.

Population sizes of anaerobic fungi

The quantification of biomass of anaerobic fungi is still difficult. Determination of only the relative population size is possible. The numbers of viable zoospores are usually estimated as colony forming units (CFU) by roll-tube techniques. Thallus forming units (TFU) based on the most probable number technique using ground wheat straw broth has been proposed by Theodorou *et al.* (1990). Enumeration of zoosporangia developed on agar strips containing carbohydrates after incubation within the rumen has also been used (Ushida *et al.*, 1989b). Recently, Kojima *et al.* (unpublished) have developed a novel *in vitro* culture of anaerobic fungi in the dual-phase medium, a solid medium containing carbohydrate overlaid by a liquid medium. Enumeration of zoosporangia under a binocular microscope were done after 6 h incubation. Analysis of chitin or protein provides the good estimation of biomass of pure-cultures. Since feeds contain various level of background chitin which comes from contamination of aerobic fungi, it does not work properly *in vivo*. Recent development of rDNA-targeting-oligonucleotide probes may give more precise estimation for fungal biomass.

Anaerobic fungi appear in the rumen about ten days after the birth (Fonty *et al.*, 1987). The development of the subsequent population depends on the nature of diet; forage based diets promote the development of fungal populations, but starch-based diets lower the population from the young animals. In general, diets rich in fiber promote a larger population of anaerobic fungi in the rumen of adult ruminants. Although their capacity for starch utilization has been stressed by certain researchers (McAllister *et al.*, 1993), their dependence on fibrous feed is obvious (Grenet *et al.*, 1989b).

Little information is available about generic composition of anaerobic fungi in the rumen. Monocentric species (mostly *Neocallimastix*) had seemed to be predominant in sheep rumen in earlier studies (Ushida *et al.*, 1992), and polycentric species (*Orpinomyces* and *Ruminomyces*) have been isolated almost entirely from cattle. However recent DNA probe works suggested the predominance of polycentric species even in the sheep rumen (Millet *et al.*, 1995). Since polycentric species often produce low numbers of zoospore in pure-culture, it is often difficult to determine by the usual roll-tube technique whether polycentric species are the predominant anaerobic fungi in the rumen.

Hydrolytic enzymes and carbohydrate fermentation

The anaerobic fungi have an array of polysaccharidases (*endo*-glucanase, *exo*-glucanase, xylanase, cellodextrinase, amylase), glycosidases (α- and β-glycosidase, β-fructosidase, β-xylosidase, α-L-arabinofuranosidase etc.), and esterase (acetylxylan esterase, *p*-coumaroyl esterase, feruroyl esterase) (Williams and Orpin, 1987a, b; Joblin *et al.*, 1990; Borneman *et al.*, 1991; this study). Several *endo*-glucanases, xylanases, β-glucosidases and β-xylosidases have been purified from strains belonging to genera *Neocallimastix*, *Piromyces* and *Orpinomyces* (Hébraud and Févre, 1990; Li and Calza, 1991; Teunissen *et al.*, 1992b; Wilson and Wood, 1992; Garcia-Campayo and Wood, 1993; Gomez de Segura and Févre, 1993; Chen *et al.*, 1994; Zhou *et al.*, 1994). These enzymes are all extracellular, and have acidic pH (5 to 6) and mesophillic temperature (50 to 55 ℃) optima. Anaerobic fungi secrete potent polysaccharidases into the medium (Wood *et al.*, 1986; Barichievich and Calza, 1990; Matsui *et al.*, 1992; Wilson and Wood, 1992), but the protein production is usually small.

More than ten genes encoding *endo*- glucanases and xylanase have been cloned and expressed in *Escherichia coli* so far (Xue *et al.*, 1992, 1993; Lee *et al.*, 1993; Gilbert *et al.*, 1992; Fujino *et al.*, 1995). The *endo*-glucanases of anaerobic fungi that have been sequenced so far were classified into *family* 5 - *subfamily* 4 - endoglucanase. Family 5

cellulases are also produced by aerobic true fungi such as *Trichoderma* spp., but these enzymes are classified into subfamily 5. Interestingly most of the *family* 5 *sub* 4 cellulases of anaerobic microbes are ruminal origin.

High homology between anaerobic fungal endoglucanase gene and those of *Clostridia* and of *Ruminococci* suggested the horizontal transfer of cellulase gene. The intron-less structure of *celB* gene of *N. patriciarum* and *celA* gene of *N. frontalis* (Zhou *et al.*, 1994; Fujino *et al.*, 1995) may support this hypothesis.

Cellulase genes of *Neocallimastix* have a reiterated sequence at the C-terminal region which is thought to be a binding structure with the scaffolding protein (Xue *et al.*, 1992; Fanutti *et al.*, 1995; Fujino *et al.*, 1995). The reiterated sequences strongly suggest the presence of cellulosomes in this group of fungi. Indeed, high molecular mass (HMM) complexes have been detected in the culture supernatant of *Neocallimastix* (Wood *et al.*, 1988) and *Piromyces* (Teunissen *et al.*, 1992b). Anaerobic fungi can ferment a wide range of carbohydrates in plant materials. Most species ferment plant polymers, such as cellulose, xylan and starch, but cannot ferment pectin and inulin. Certain species cannot grow on cellulose (this study).

Anaerobic fungi also have protease activities (Wallace and Joblin, 1985; Asao *et al.*, 1993). Proteases may have a role on cell-wall degradation, because the plant structural protein, such as extensin, increases the integrity of plant cell-wall (Fry, 1986). Possession of protease is a unique characteristic of anaerobic fungi as rumen cellulolytic microorganisms, because the major ruminal cellulolytic bacteria do not have protease activity.

Most of strains produce hydrogen, carbon dioxide, formate, lactate, succinate, acetate and ethanol as metabolic end products. Certain species do not produce succinate and ethanol. Glucose fermentation pathways of the four rumen fibrolytic bacteria and fungi used in this study are illustrated in Figure 2-6. Unlike bacteria, fermentation pathways of anaerobic fungi may not vary between genera since fermentation end

products are quite similar. Metabolic pathway has been defined in three strains of *Neocallimastix* (Yarlett *et al.*, 1986a; O'Fallon *et al.*, 1991; Marvin-Sikkema *et al.*, 1994). *N. patriciarum* metabolizes glucose via EMP pathway to phosphoenol pyruvate that is further metabolized to pyruvate with two intermediate products, oxaloacetate and malate (Figure 6). Redoxidation of NADH generated during glycolysis is coupled with hydrogen production, the reduction of pyruvate and acetaldehyde. Since this strain produces only trace amount of ethanol and formate, hydrogen and lactate are the major electron sink. *N. frontalis* possesses a similar carbon and electron flow system.

Role of anaerobic fungi in ruminal fiber degradation

As shown above, the life cycle of fungi suggests the important roles of fungi in fiber degradation in the rumen. Their colonization certainly weakens the integrity of plant tissues to increase the fragmentation of feed particles (Akin *et al.*, 1990). The anaerobic fungi appear to preferentially colonize on lignified tissues such as sclerenchyma and xylem that remain within the reticulo-rumen longer than other tissues (Bauchop, 1979; Grenet *et al.*, 1989b). This observation can be explained by the life cycle of the anaerobic fungi and because the maturation time is 24 to 32 h after encystment in the rumen environment (Lowe *et al.*, 1987).

Most strains tested so far degrade hay particles such as timothy or ryegrass by 40 to 70 % during 6 to 8 days under batch culture conditions. In a continuous culture system (*i.e.* RUSITEC), an addition of one strain of *Neocallimastix* to the mixed rumen bacteria increased degradation rate of wheat straw by 15 % (Hillaire and Jouany, 1989). Fonty and Gouet (1989) inoculated either *N. frontalis*, *P. communis* or mixed anaerobic fungi to the rumen of new born lambs which had been individually introduced into a sterile isolator before the establishment of cellulolytic flora in the rumen. They also prepared the lambs which harbored *Fibrobacter succinogenes* or *Ruminococcus flavefaciens* as the sole cellulolytic organisms. Then they determined the *in sacco* digestibility of wheat straw or ryegrass hay in the rumen of these lambs. The results indicated that lambs

harboring anaerobic fungi digested fiber less efficiently than those harboring cellulolytic bacteria. However, in the case of the mature rumen, elimination of anaerobic fungi from the rumen by the chemical means often decreases plant fiber degradation (Windham and Akin, 1984; Ford *et al.*, 1987; Calderon-Cortes *et al.*, 1989; Ushida *et al.*, 1989a). The overall role of anaerobic fungi in fiber degradation is still debatable.

Interaction between anaerobic fungi and other microorganisms in the rumen

The fiber degrading activity of anaerobic fungi is complicated by the interaction with other microorganisms. Co-culture studies have been done in several laboratories in order to define interactions between bacteria and anaerobic fungi. Co-culture with methanogenic bacteria enhanced fiber digestion by anaerobic fungi with few exception (Ushida, 1993). This kind of interaction is defined as a facultative inter-species hydrogen and formate transfer which improves metabolic activity of hydrogen producing organisms (Wolin and Miller, 1983). The presence of methanogens also improves the specific activity of fungal cellulases (Joblin and Williams, 1991). The negative effect on fungal fiber digestion by some strains of *Ruminococcus, Butyrivibrio*, and *Megasphaera* were evident (see Ushida, 1993). The mechanisms involved in this negative interaction have not been well defined except for *R. flavefaciens* 007. This bacterium produces the proteins that inhibit *endo*-glucanase of anaerobic fungi (Bernalier *et al.*, 1993), and these proteins may have chytinolytic activity that may release a cellulase complex from fungal cell-wall to reduce cellulolytic activity (Kopecny *et al.*, 1996). The significance of this inhibition under *in vivo* situation is not known.

Rumen ciliate protozoa predate anaerobic fungi (Williams *et al.*, 1994) and digest them (Morgavi *et al.*, 1993), therefore defaunation (elimination of protozoa) often increase the population size of anaerobic fungi (Ushida *et al.*, 1989a, 1991).

Anaerobic fungi fascinate many scientists in terms of their capacities, roles, and ecological relationships to other ruminal microorganisms in fiber degradation. The

present study aims to investigate characteristics and ecology of anaerobic fungi in relation to fiber digestion in the rumen.

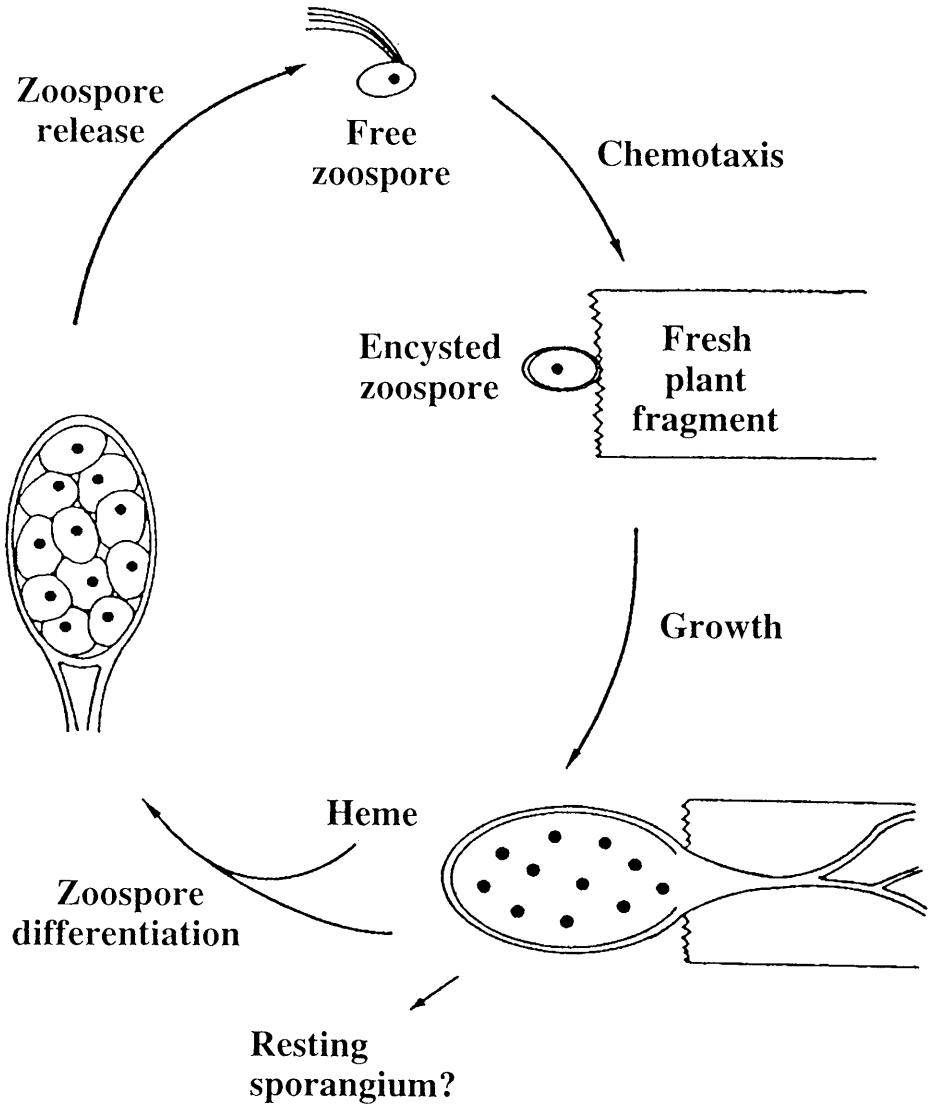

Figure 1. Schematic life cycle of anaerobic fungi (Orpin 1994).

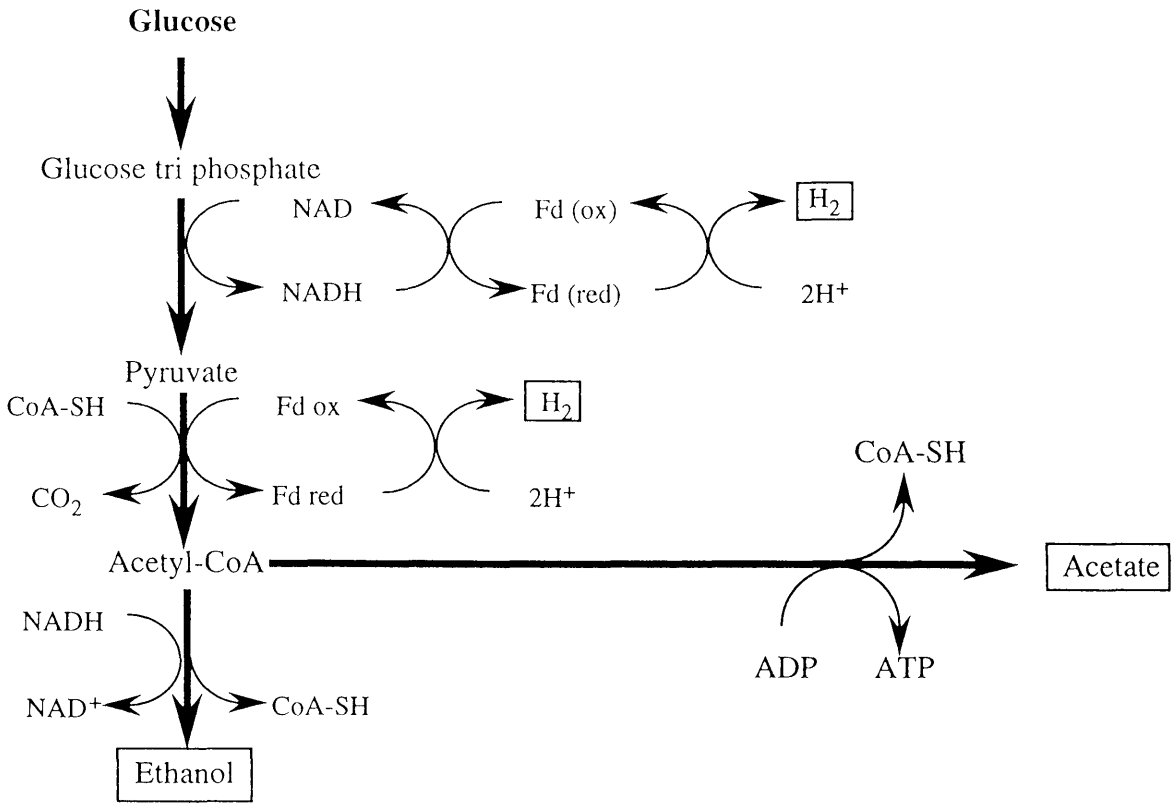

Figure 2. Glucose fermentation pathway by *Ruminococcus albus.*
Fd, Ferredoxin; NAD, nicotinamide adenine dinucleotide; CoA, coenzyme A.

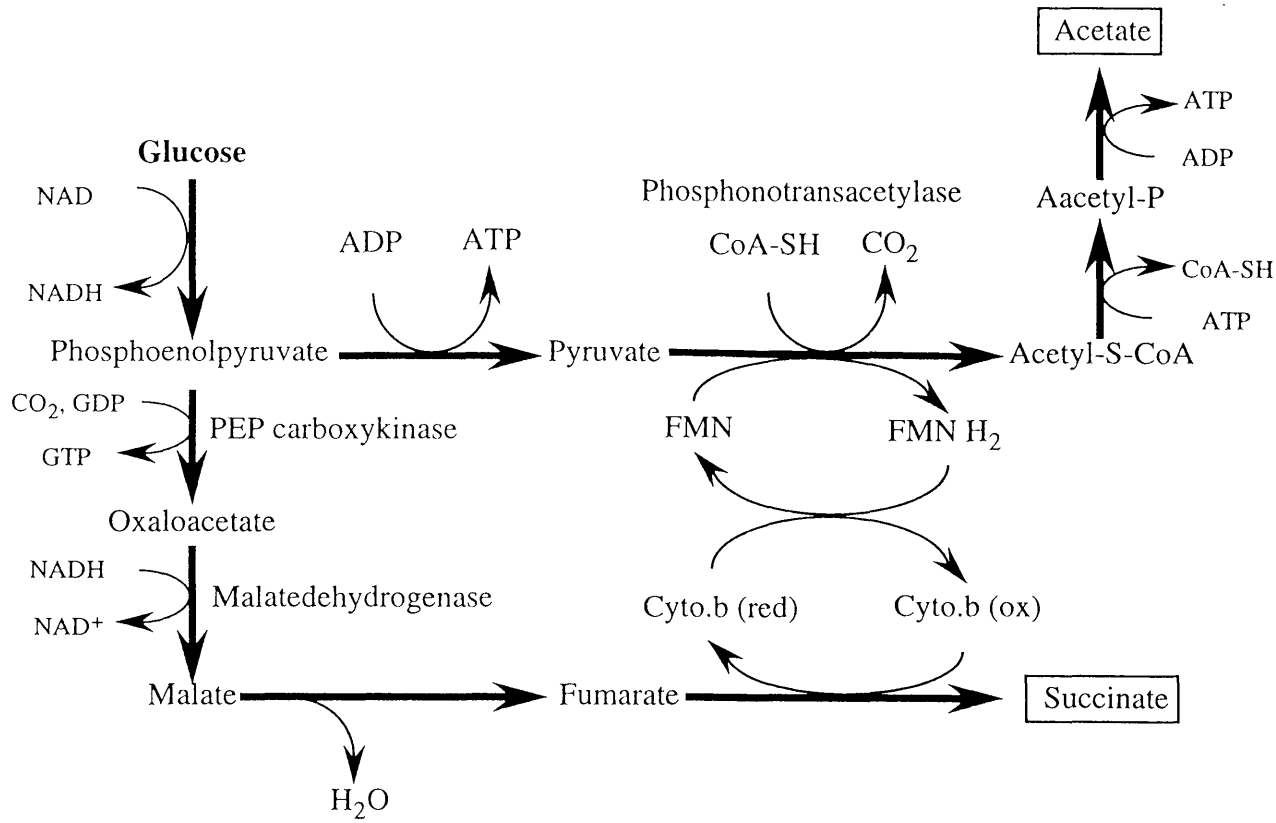

Figure 3. Pathway of glucose fermentation by *Fibrobacter succinogenes.*
FMN, Flavin mononucleotide; GDP, guanosine 5'-diphosphate; GTP, guanosine 5'-tiphosphate; Cyto. b, cytochrome b.

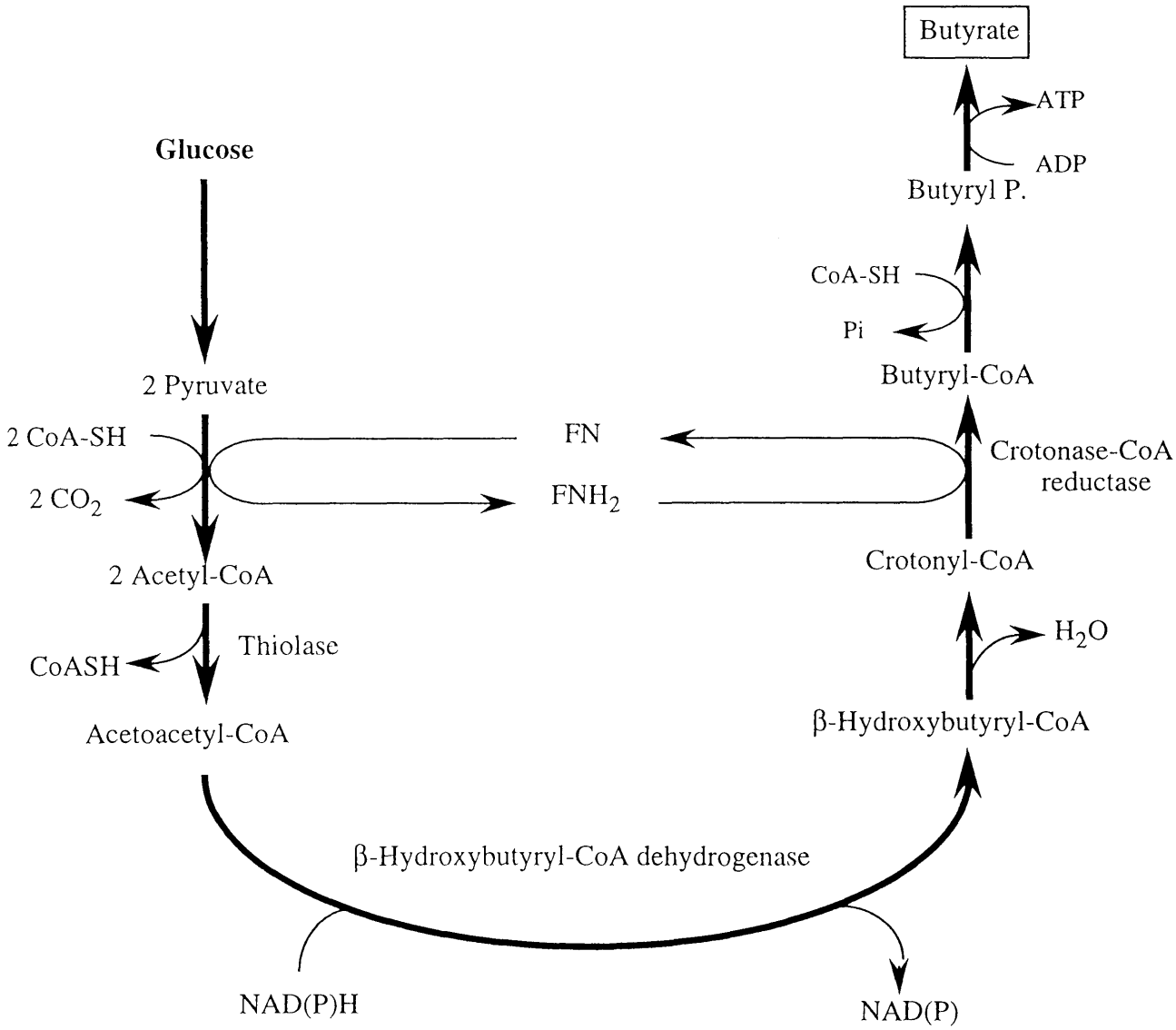

Figure 4. Pathway of glucose fermentation by *Butyrivibriio fibrisolvens.*
FN, Flavin nucleotide; NAD, nicotinamide adenine dinucleotide;.

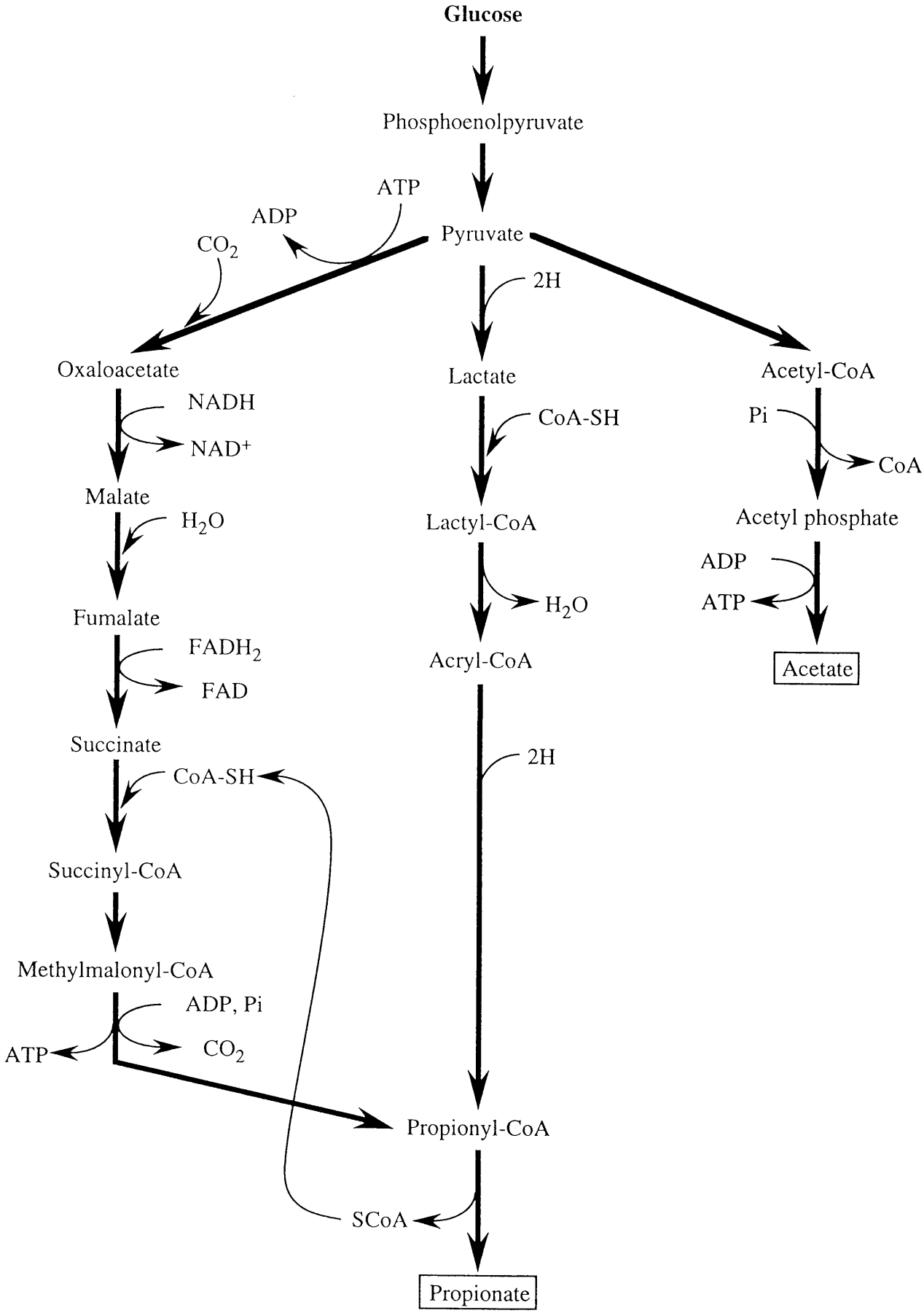

Figure 5. Pathway of glucose fermentation by *Prevotella ruminicola*.
NAD, nicotinamide adenine dinucleotide; CoA, coenzyme A; FAD, flavin adenine dinucleotide.

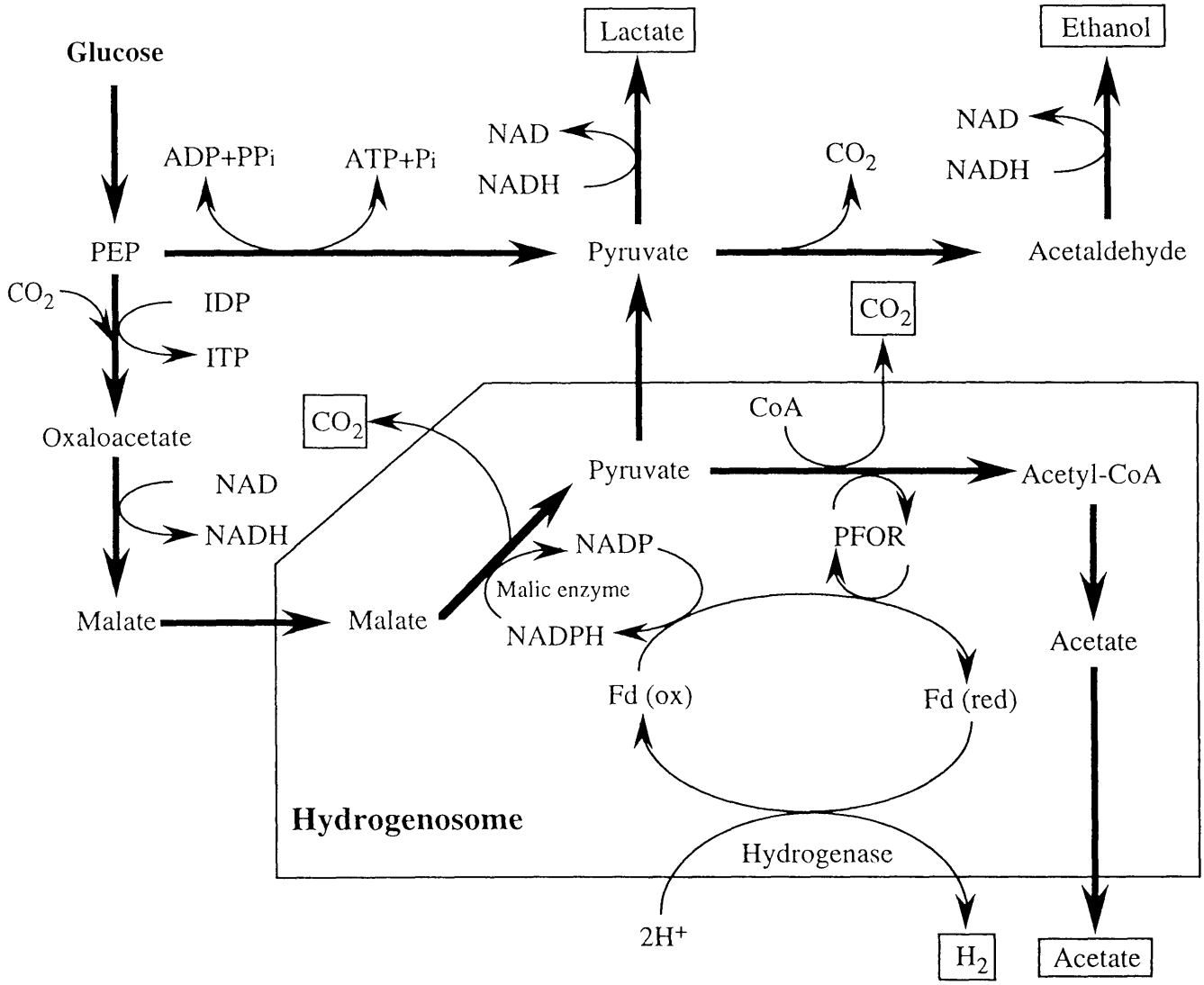

Figure 6. Pathway of glucose fermentation by anaerobic fungi *Neocallimastix particiarum*.
Fd, Ferredoxin; IDP, inosine 5'-diphosphate; ITP, inosine;5'-triphosphate PFOR, pyruvate : ferredoxin oxidoreductase; NAD, nicotinamide adenine dinucleotide.

Table 1. Classification of anaerobic fungi.

Division	Eumycotina
Sub-division	Mastigomycetes
Class	Chytridiomycota
Order	Neocallimastigales
Family	Neocallimastigaceae

Genus: characteristics	Species	Source of isolate	Reference
Neocallimastix	*frontalis*	Sheep	Heath *et al.*, 1983
Monocentric; polyflagellate	*patriciarum*	Sheep	Orpin and Munn, 1986
zoospores; extensive,	*hurleyensis*	Sheep	Webb and Theodorou, 1991
filamentous rhizomycelium	*variabilis*	Sheep	Ho *et al.*, 1993b
Piromyces	*communis*	Sheep	Gold *et al.*, 1988
Monocentric; sporangia	*mae*	Horse	Li and Heath, 1990
mono flagellate zoospore;	*dumbonicus*	Elephant	Li and Heath 1990
filamentous rhizomycelium	*rhizinflatus*	Saharian ass	Breton *et al.*, 1991
	minutus	Deer	Ho *et al.*, 1993c
	spiralis	Goat	Ho *et al.*, 1993d
	citronii	Horse	Gaillard-Martinie *et al.*, 1995
Orpinomyces	*joyonii*	Sheep	Li *et al.*, 1991
Polycentric; polyflagellate zoospores; filamentous rhizomycelium	*intercalaris*	Cattle	Ho *et al.*, 1994
Caecomyces	*communis*	Sheep	Gold *et al.*, 1988
Monocentric or polycentric; uniflagellate zoospores; spherical holdfasts	*equi*	Horse	Gold *et al.*, 1988
Anaeromyces	*mucronatus*	Cow	Breton *et al.*, 1990
Polycentric; uniflagellate zoospores; filamentous rhizomycelium	*elegans*	Sheep	Ho *et al.*, 1993a

Chapter 2: Effect of Dietary Concentrate on Fungal Zoosporogenesis in Sheep Rumen

Introduction

Anaerobic rumen fungi have been isolated from the rumen and are considered to play important roles in plant cell wall digestion (Fonty and Joblin, 1991). In general, feeding of the high fiber diet resulted in larger fungal populations (Bauchop, 1979, 1989b; Grenet *et al.*, 1989a, b, Orpin, 1984), while feeding of the high concentrate diets resulted in smaller fungal populations (Bauchop, 1979, 1989b; Grenet *et al.*, 1989b). However, supplementation of maize to sorghum silage resulted in a larger fungal population and stimulated fungal fiber digestion in the rumen (Akin and Windham, 1989). Roles of fungi in starch degradation were also emphasized (McAllister *et al.*, 1993). Thus, effect of fibrous feed on fungal population size may be complicated by the presence of concentrate feed.

In this chapter, effects of concentrate diets on relative fungal population size in the rumen were examined by using a modified method of Ushida *et al.* (1989b).

Materials and Methods

Three rumen-fistulated wethers (BW; 55-63 kg, AV; 60 kg) were used in this experiment. Timothy (*Phleum pratense*) hay (NDF 59.2, ADF 34.1 %) and commercial concentrate (TDN 70, DCP 12 %, Coop Dairy 14. Kumiai-shiryo, Kobe, Japan) were fed to the animals. Three consecutive experimental periods were designed: first period, all forage (AF); second period, forage : concentrate = 2 : 1 (moderate concentrate, MC); third period, forage : concentrate = 1 : 2 (high concentrate, HC). Each period had a 14 d adaptation period followed by a 2 d sampling period. Equal amount of diets (1,200 g/d) was fed to animals at 9:00 and 21:00. The hay was substituted by concentrate at 100 g/d for 4 d in an adaptation period. Mineral block and water were fed to wethers *ad libitum*.

Agar strips were prepared according to Ushida *et al.* (1989b), but agar concentration was increased to 4 %. The agar strips were introduced into the rumen of sheep through fistula at 9:00 (0 h), 12:00 (3 h), 15:00 (6 h), 18:00 (9 h) and withdrawn after 3 h incubation. Fungal germinated zoospores developed on sections of the agar strips were counted after cotton-blue staining under appropriate magnification using a stereoscopic microscope (SMZ-U, Nikon, Tokyo, Japan) equipped with an eye piece grid. Because the zoospores survived for 2 - 3 h (Orpin, 1975, 1976) and the germinated zoospores could satisfactorily be observed after 3 h incubation time, measurements were made with 3 h intervals to estimate the number of viable zoospores in the rumen at the time of introduction.

Approximately 50 ml of rumen fluid was collected *via* fistula at the introduction or withdrawal of agar strip and measured pH immediately. The sample was strained through four layers of surgical gauze and stored at -20℃ until short chain fatty acid (SCFA) analysis. Five milliliters of rumen fluid were added 1 ml of 30 % metaphosphoric acid and 1 ml of crotonic acid (8 g/l), and analyzed for SCFA by gas chromatography (GC4BM-PF, Shimadzu, Kyoto, Japan). Separation of SCFA was made by 5 % polyethylene glycol (PEG) 6000 on Shimalite TPA (Wako Jun'yaku, Osaka, Japan) and quantified by C-R6A integrator (Shimadzu).

Results

In the method of Ushida *et al.* (1989b), agar strips were incubated for 24 h. In our preliminary experiment, germinated zoospores on agar strips could be satisfactory detected at the end of 3 h-incubation. This incubation period was employed to observe detailed fungal fluctuation in the rumen. The numbers of germinated zoospores were considered as relative fungal population size at the time of introduction of agar strips into the rumen.

The numbers of the germinated zoospores and pH in the rumen of sheep fed varying amount of concentrate feed are shown in Figure 1. The number of the zoospores on the strip was drastically decreased after morning feed with AF diet (Fig. 1-a). The number was the highest at 0 h (1.34×10^2 /cm^2), then declined to 2.0×10^3 /cm^2 at 9 h after feeding. The average ruminal pH of animals fed AF diet at 0 h was 7.1. Then the pH declined and was maintained at lower levels (6.8 - 6.9). The correlation between number of zoospores and pH was not apparent. In the rumen of animals fed MC diet, the number of zoospores decreased with time after feeding, although the rate of decrement was slower than that with AF diet. During 0-3 h after feeding, number of zoospores was 1.6×10^4 /cm^2. Although the number slightly decreased at 6 and 9h, the relatively high levels were maintained. It seems that the inducers for zoospore-release were maintained at relatively high concentration throughout incubation period. At 0 h, the average ruminal pH of animals fed MC diet was 6.7, then the values declined to 6.4. The fluctuation pattern of number of germinated zoospores was different in the rumen of animals fed HC diet from those of AF and MC diets. The number of zoospores was constantly maintained at lower level (1.0×10^3 /cm^2) than the other diets. The ruminal pH was also placed at lower level (6.3 - 6.4) than the other diets. The numbers of zoospores at 0 h reflected the fungal population in the rumen.

Discussion

Release of zoospores can be principally triggered by the introduction of hemes in plants into the rumen (Orpin and Greenwood, 1986b). For AF diet, high number of zoospores at 0 h was due to the release of zoospores from mature fungi induced by the hemes in timothy hay ingested by sheep. After that, other rumen microorganisms might utilize or destroy the hemes or the hemes might be washed out from the rumen, so that release of zoospore was decreased. Orpin and Greenwood (1986b) suggested that hemes acted on zoosporogenesis in synergism with other components of diet, or the responses of fungi were different according to the physiological state of fungi. The regulation of zoosporogenesis is indeed complex, since it could not be reproduced routinely *in vitro* (Orpin and Greenwood, 1986a). For MC diet, continuous high number of zoospores may be due to the continuous release of zoospores by hemes in timothy hay and concentrate feed, and by unknown mechanisms. Unlike AF diet which promoted relatively rapid decline of zoosporogenesis, supplementation of concentrate feed to the timothy hay did not promote such rapid decline of zoosporogenesis. It was suggested that production of inducers for zoosporogenesis from concentrate feed persisted longer time than from timothy hay.

Since life cycle of anaerobic fungi is relatively long (24 - 32 h) (Joblin, 1981; Lowe *et al.*, 1987), fungi appeared to preferentially colonize on the ligno-cellulosic tissue which is tolerant to microbial attack and has longer retention time in the rumen (Akin, 1987; Akin and Benner, 1988; Akin *et al.*, 1989; Bauchop, 1979; Bauchop, 1981; Bauchop, 1989b). Fungal population size was increased in the rumen, when animals were fed alfalfa hay or meadow hay (Bauchop, 1981; Grenet *et al.*, 1989a, b). On the other hand, the population size decreased when animals were fed sugar beat, soft leaf forage or grains (Bauchop, 1979; Bauchop, 1981; Gordon, 1985; Grenet *et al.*, 1989a). Therefore the dependence of fungal development on the forage is obvious. HC diet promoted the

lowest zoospore production, suggested the lowest fungal population size in this experiment. The present results were compatible with those earlier studies.

Concentrate feed usually promotes low ruminal pH. Low pH (less than 6.0) decreased release of zoospores in fresh rumen fluid (Orpin, 1975, 1976, 1977a, c). Indeed, the pH was the lowest with HC diet in this experiment. Concentrate feed also often promotes high molar ratio of propionate. Propionate is known to have antifungal activity and is used as a food preservative (Horiguchi, 1982). Ushida *et al.* (1993) suggested that high rumen propionate level (27 mM) affected the composition of fungal microflora but not total population size. Since concentration of propionate ranged between 10 to 15 mM in the rumen of animals fed HC diet, the propionate level did not affect fungal population. Accordingly the smaller number of germinated zoospores was due to smaller supply of ligno-cellulosic materials and probably to low pH in the rumen.

Since concentrate feed mixture contains proteins, microelements, vitamins and other nutrients, growth of fibrolytic microorganisms seems to be stimulated if physico-chemical condition such as pH is not critical. The larger number of zoospores that reflected larger fungal population size with MC diet might be caused by this mechanisms, because amino acids, branched short chain fatty acids and vitamins stimulated growth of *Neocallimastix patriciarum* in pure-culture, and acetate and soluble sugars stimulated zoosporic germination (Orpin and Greenwood, 1986a). Moreover, several isolates could not grow on cellulose without cellobiose suggesting the importance of soluble sugars (Matsui and Ushida, unpublished observation).

This result shows that an appropriate amount of concentrate may support fungal growth and stimulate zoosporogenesis in the rumen.

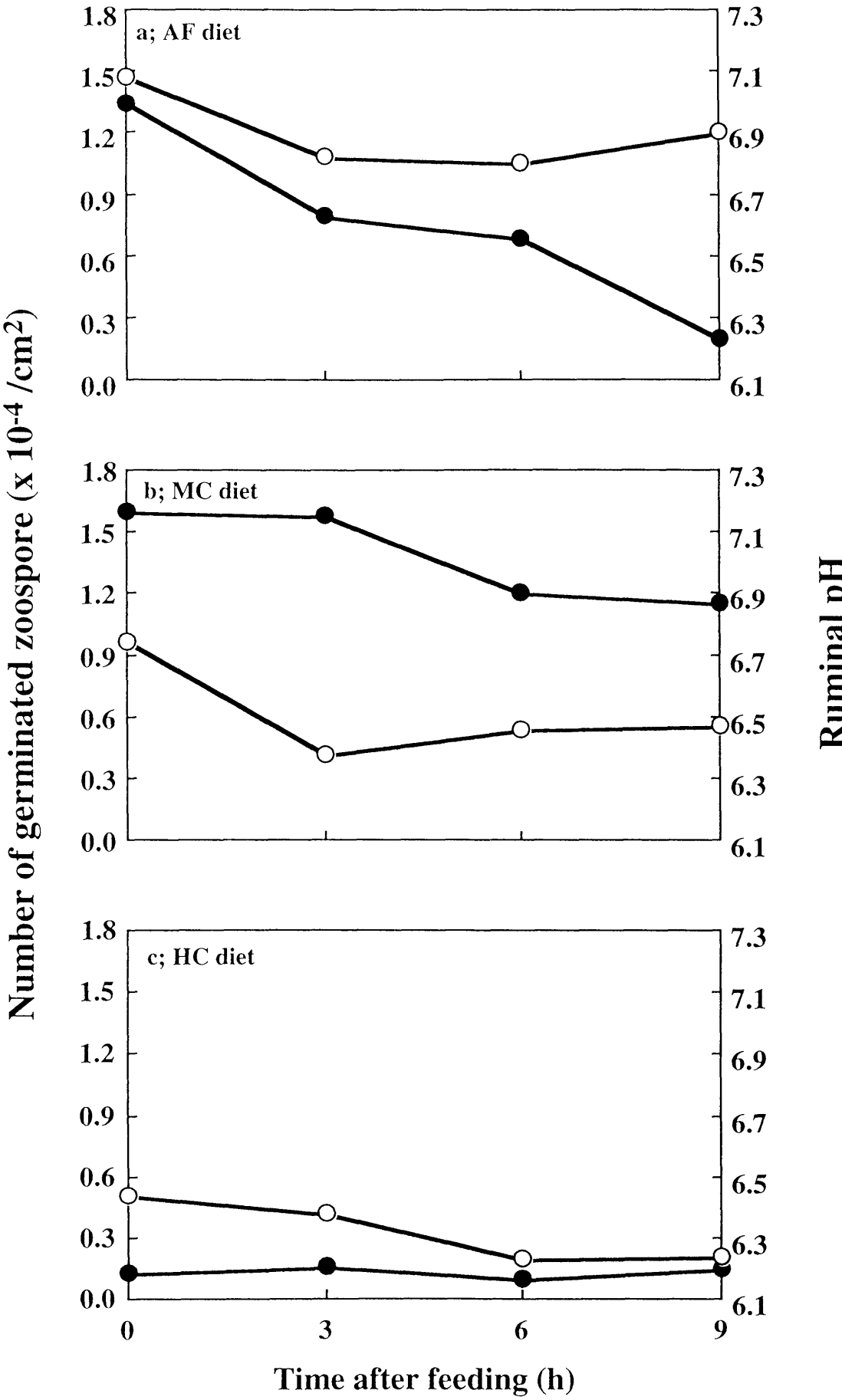

Figure 1. The effect of diet on number of germinated zoospores and ruminal pH.
O, Ruminal pH; ●, number of germinated zoospore. AF diet, All forage diet; MC diet, Forage : Concentrate = 2 : 1; HC diet, Forage : Concentrate =1 : 2.

Chapter 3: Profiles of Fiber degrading Enzymes produced by Anaerobic Fungi

Introduction

Cellulases (*endo*-glucanase, cellobiohydrolase and β-D-glucosidase), hemicellulases (*endo*- and *exo*- xylanase, β-D-xylosidase), pectinases, and aryl esterases (*p*-coumaroyl and feruroyl esterase, and acetyl xylan esterase) are involved in breakdown of structural carbohydrates and phenolic polymers (or lignins) in plant cell-walls in the rumen (Chesson and Forsberg, 1988). These enzymes produced by fibrolytic microorganisms act synergistically on plant cell-walls ingested by host animals.

Anaerobic fungi are now considered as one of principal components of fibrolytic microbial consortia in the rumen (Fonty and Joblin, 1991). Studies on fungal enzymes involved in fiber digestion have mainly focused on enzymes from *Neocallimastix* spp. (Mountfort and Asher, 1985; Pearce and Bauchop, 1985; Williams and Orpin, 1987a, b; Hébraud and Févre, 1988; Barichievich and Calza, 1990; Borneman *et al.* 1990; Hébraud and Févre, 1990). Moreover, these results so far have been obtained with a few exceptions from fungal cultures growing rumen fluid-containing media (Mountfort and Asher, 1985; Pearce and Bauchop, 1985; Williams and Orpin, 1987a, b; Hébraud and Févre, 1988). In the present study, the enzymatic profiles of *Neocallimastix* sp. N1 and *Piromyces* sp. P1 in relation to the growth substrates using a synthetic medium are described. Since the growth substrate usually affects enzyme production (induction and repression), this experiment has a particular importance in determining the enzyme production profiles of anaerobic fungi as affected by culture condition. This may lead to a better understanding of role of fungi in plant degradation in the rumen. Since most of fungal glycoside hydrolase and polysaccharide depolymerase activity is extracellular (Pearce and Bauchop, 1985; Hébraud and Févre, 1988), the activities of extracellular enzymes were assayed in this experiment. Because genera *Neocallimastix* and *Piromyces* are usually predominantly isolated from the rumen by roll tube method (Joblin,

1981), isolates belonging to these two genera were used.

Materials and Methods

Isolation and incubation of anaerobic fungi

Two fungal strains listed in appendix were used for enzyme assay. These fungi were isolated in this laboratory from sheep rumen according to Joblin (1981) and they had been cryopreserved at -70 ℃ according to Yarlett *et al.* (1986b) until required. Both isolates developed monocentric thalli. One isolate produced polyflagellate zoospores and the other produced monoflagellate zoospores. On the basis of these characteristics together with their fermentation end products, the organisms were identified as a genus *Neocallimastix* and a genus *Piromyces* and were given the trivial name *Neocallimastix* sp. N1 and *Piromyces* sp. P1, respectively (Tanaka *et al.*, 1992).

The fungi (5 % inoculum) were preliminary subcultured twice in 40 ml of medium B broth (Lowe *et al.*, 1985) containing either glucose, xylose, cellobiose, crystalline cellulose (Avicel SF, Asahi Kasei, Tokyo) or xylan (Oat spelt xylan, Aldrich Chemical Co. USA) (each 0.2 % wt/vol) as the sole carbon source. All incubations were carried out at 39 ℃ using 100 ml serum bottles equipped with butyl rubber septa and a gas phase of oxygen-free 100 % CO_2. The fungi were then inoculated into the experimental media under the same conditions as subculture. Anaerobic fungi produce hydrogen gas as an end product (Orpin and Joblin, 1988). Head space gas was analyzed daily by gas chromatography (Ushida *et al.*, 1982) and the growth of fungi was monitored by the accumulation of hydrogen gas in the head space of bottles. All incubation was run in triplicate. The incubation was terminated at the late exponential growth phase (6 to 9 d) by centrifugation at 10,000 x g for 20 min at 4 ℃. The supernatants were preserved as crude enzyme at -20 ℃ under N_2 until required.

Enzyme assay

Fungal extracellular glycosidase, esterase and xylanase activities were

chromogenically determined and glucanase activity was viscometrically assayed. All enzyme assays were done at 39 ℃. Glycoside hydrolase activities were determined by measuring the rate of *p*-nitrophenol release from appropriate *p*-nitrophenyl derivatives (Williams and Orpin, 1987b). Each of *p*-nitrophenyl derivative was dissolved in 0.05 mM sodium phosphate buffer (pH 6.5) and was used as a substrate. A portion (0.3 ml) of the substrate solution was added to a reaction mixture. Reaction was started by adding 0.1 ml of fungal enzyme solution. After 30 min, the reaction was terminated by the addition of 2.5 ml of sodium carbonate solution (125 mM). Optical density of reaction mixture was measured at 420 nm. The specific activities of glycoside hydrolase are expressed as nmol *p*-nitrophenol released/mg protein in 1 min. Esterase activities were determined by measuring α-naphthol release from α-naphthyl acetate and α-naphthyl propionate (Hespell and O'Bryan-Shah, 1988). The specific activities of esterase are expressed as mmol α-naphthol released/mg protein in 1 min. *Endo*-1,4-β-xylanase activities were determined by measuring the rate of remazol brilliant blue-R (RBB) release from RBB xylan (Biely *et al.*, 1985). The specific activities of *endo*-xylanase are expressed as mg RBB-xylan hydrolyzed/mg protein in 1 min. *Endo*-1,4-β-glucanase activities were determined by measuring the reduction in viscosity of carboxymethyl cellulose solution (CMC) using an Ostwald viscometer (Capillary I.D.=0.75 mm). Crude enzyme solution (1 ml) and 4 vol. of sodium phosphate buffer (0.05 M, pH 6.5) were mixed with 5 vol. of CMC solution (2 % wt/vol in 0.05 M sodium phosphate buffer, pH6.5). The specific activities of *endo*-glucanase are expressed as viscosity reducing unit (VRU). A unit of VRU corresponds to an enzyme activity which halves substrate viscosity in 10 min (Endo, 1986).

The protein concentration of crude enzyme solution was determined by dye-binding method (Bradford, 1976). Coomassie Brilliant Blue G-250 was purchased from Fluka Chemie AG (Switzerland). Substrates for enzyme assay were all obtained from Sigma Chemical Co. (USA). Other chemicals were all reagent grade and purchased from Wako

Pure Chem. Ind. Ltd. (Osaka) and Nacalai tesque (Kyoto) unless otherwise stated.

Results

Polysaccharidase and glycosidase

Neocallimastix sp. N1 did not grow on Avicel and *Piromyces* sp. P1 did not grow on xylose as sole carbon sources.

A wide range of glycoside hydrolases was detected in the culture supernatant of both fungal isolates (Table 1). In addition to these enzyme activities listed in Table 1, both fungi produced extracellularly α-D-glucosidase (EC 3.2.1.20), α-D-galactosidase (EC 3.2.1.22), α-L-fucosidase (EC 3.2.1.51), β-D-glucuronidase (EC 3.2.1.31), *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) and α-D-mannosidase (EC 3.2.1.24) activities. β-D-Glucosidase and β-D-xylosidase activities were in general the higher among the tested extracellular glycosidase. α-L-Arabinofuranosidase and β-D-cellobiosidase activities were also moderately detectable in both fungi. The other glycoside hydrolase activities were detectable but generally low. When *Neocallimastix* sp. N1 was grown on xylan or xylose, most of the extracellular glycoside hydrolase activities were higher than those obtained from other growth substrates. While most of extracellular glycoside hydrolase activities was higher following the growth on cellobiose or Avicel, when *Piromyces* sp. P1 was examined. Growth substrates had similar effected on the specific activities of extracellular *endo*-glucanase and *endo*-xylanase. Xylan and xylose stimulated the specific activities of both these polysaccharide depolymerases of *Neocallimastix* sp. N1, while cellobiose and Avicel stimulated the specific activities of these enzymes from *Piromyces* sp. P1.

Esterase

Two esterase activities were detected in the culture supernatant of *Neocallimastix* sp. N1 and *Piromyces* sp. P1. Naphthyl propionate esterase activity of *Neocallimastix* sp. N1 was the highest following the growth on xylose. When *Piromyces* sp. P1 was grown on Avicel and cellobiose, both naphthyl acetate and naphthyl propionate esterase

activities were higher than those obtained from other growth substrates. Both esterase activities of *Piromyces* sp. P1 were higher than those of *Neocallimastix* sp. N1.

Specific activities of naphthyl propionate esterase were lower than those of naphthyl acetate esterase with the exception of the esterases from *Neocallimastix* sp. N1 grown on xylose.

Discussion

A wide range of enzyme activities involved in plant polysaccharide degradation were detected in the culture supernatants of both fungal isolates. Although the specific activities of enzymes were strongly affected by the growth substrates, in all cases where fungal growth occurred all enzyme activities were detectable.

The present results (Table 1) are basically consistent with previously published results (Williams and Orpin, 1987a, b; Barichievich and Calza, 1990) and demonstrate that the rumen fungi are capable of degrading wide variety of plant polysaccharides. *Neocallimastix* sp. N1 and *Piromyces* sp. P1 could not grow on Avicel and/or xylose, respectively. These fungal strains were isolated from the ovine rumen by Joblin's medium containing cellobiose as the sole energy source and maintained on a same medium in which agar was omitted and cellobiose was replaced by a milled timothy hay. These fungi were, therefore, not isolated as cellulolytic organisms. However, *Neocallimastix* sp. N1 was able to degrade Avicel in a medium when cellobiose was present (Tanaka *et al.*, 1991; Tanaka *et al.*, 1992). *Neocallimastix* sp. N1 is likely to require oligosaccharide(s) to achieve the growth on crystalline cellulose.

A wide range of glycoside hydrolases was detected in the culture supernatant of both fungal isolates (Table 1). Williams and Orpin (1987b) have shown that *N. patriciarum* and *P. communis* produce a wide range of extracellular glycoside hydrolases. The glycoside hydrolase profiles of isolates used in this experiment are similar to those obtained from their isolates. In their experiment, structurally related substrates

stimulated appropriate glycosidase activities. However in the present study, the higher activities of the appropriate glycosidase were not necessarily detected following growth on the structurally related substrates with the exception of β-D-glucosidase of *Piromyces* sp. P1 when grown on cellobiose (Table 1). Overall, it appears that there is a trend for the enzyme production profiles to be genus-dependent rather than substrate dependent. This is also in the case for rumen fibrolytic bacteria. Table 2 summarizes literature data on glycoside and polysaccharide degrading enzymes of rumen bacteria grown on various substrates (Williams and Withers, 1981, 1982). Although glucose tended to decrease the specific activities of glycosidases of both isolates *Neocallimastix* sp. N1 and *Piromyces* sp. P1, soluble sugars were not necessarily less effective in inducing these enzymes. Similar results have been obtained from rumen bacteria; the specific activity of cellulase produced by *R. albus* RUM5 was highest when the bacterium was grown on xylose (Table 2).

Several isolates of *Piromyces* spp. do not grow or grow poorly only on xylose (Phillips and Gordon, 1988; Gordon and Phillips, 1989). Generally, anaerobic fungi cannot utilize arabinose as the sole carbon source (Gordon and Phillips, 1989). Therefore, the enzymes which release pentoses may work not to provide pentoses as carbon sources but rather for removing pentoses from xyloglucan or arabinoxylan chain to enable easy access for hemicellulases.

Neocallimastix sp. N1 and *Piromyces* sp. P1 produced at least two esterase activities in the culture supernatant. Naphthyl acetate esterase activity was the highest, when *B. fibrisolvens* was cultured on xylan (Hespell and O'Bryan-Shah, 1988). For the rumen fibrolytic bacteria listed in Table 2, xylan was not a good inducer of acetate esterase activity. In the present experiment, xylan also did not promote high naphthyl acetate esterase activity in supernatants of anaerobic fungi.

The specific activities of naphthyl propionate esterase were lower than those of naphthyl acetate esterase with the exception of *Neocallimastix* sp. N1 grown on xylose.

Increasing carbon chain length of substrate fatty acids decreases the measured activity of esterases (Hespell and O'Bryan-Shah, 1988; McDermid *et al.*, 1990). The reasons are unknown; lower substrate turnovers with enzyme and decreased substrate solubility were pointed out (Hespell and O'Bryan-Shah, 1988). The results obtained in the present study agree with the published results.

In this study, the profiles of enzyme activities were apparently genus-dependent. Although the mode of regulation in fungal enzyme production has not been well established, two isolates used in this experiment are likely to have different enzyme induction systems. It would appear that enzyme profiles in relation to growth substrate provide further information about the role of fungi in the rumen fiber digestion. Isolate *Neocallimastix* sp. N1 may depend more upon hemicellulosic substrates, while isolate *Piromyces* sp. P1 may depend more upon cellulosic substrates for their growth.

The present experiment was conducted by using crude enzyme preparations and the detection of activity does not necessarily mean the presence of specific enzyme. Thus the use of purified enzyme system will be required in the further studies.

Table 1. Extracellular glycoside hydrolase, *endo*-glucanase, *endo*-xylanase and esterase activities of two anaerobic fungal isolates grown on five different carbohydrates†.

Enzyme	Fungal Isolate	Carbohydrate				
		Avicel	Cellobiose	Glucose	Xylan	Xylose
β-D-Glucosidase†	N1	NG *	511	695	2210	1170
(EC 3.2.1.21)	P1	358	2010	242	72.5	NG
β-D-Cellobiosidase†	N1	NG	66.5	79.0	310	225
(EC 3.2.1.91)	P1	147	203	20.7	7.70	NG
β-D-Xylosidase†	N1	NG	707	985	1580	772
(EC 3.2.1.37)	P1	855	1990	485	57.0	NG
α-L-Arabinofuranosidase†	N1	NG	143	212	733	335
(EC 3.2.1.55)	P1	394	300	98.2	66.0	NG
endo-Glucanase‡	N1	NG	6.00	11.6	24.3	45.5
(EC 3.2.1.4)	P1	28.5	37.5	7.80	2.20	NG
endo-Xylanase§	N1	NG	5.10	7.70	34.3	10.1
(EC 3.2.1.8)	P1	9.70	3.40	2.60	1.80	NG
Naphthyl Acetate Esterase#	N1	NG	6.20	1.10	1.20	1.10
(EC 3.1.1.2)	P1	21.5	19.8	5.30	4.90	NG
Naphthyl Propionate Esterase#	N1	NG	1.80	0.30	0.50	3.10
(EC 3.1.1.2)	P1	10.7	6.10	3.00	3.20	NG

All average values of duplicate assays varied < 10 %.

† Glycoside hydrolase activities were expressed as nmole *p*-nitrophenol released/mg protein/min.

‡ *endo*-Glucanase activity was expressed as viscosity reducing unit (VRU)/mg protein.

§ *endo*-Xylanase activity was expressed as mg RBB-xylan hydrolyzed/mg protein/min.

Esterase activity was expressed as μmole α-naphthol released/mg protein/min.

* No growth.

Table 2. Glycoside and polysaccharide degrading enzyme activities of bacterial isolates from the rumen after growth on a range of carbon sources.

Enzyme	Microorganism	Carbohydrate			
		Glucose	Cellobiose	Xylose	Xylan
β-D-Glucosidase†	*Ruminococcus flavefaciens* 123	1.2	18.0	18.3	12.3
	Fibrobacter succinogenes S85	3.6	5.9	10.5	6.4
	Prevotella ruminicola brevis GA33	ND §	10.5	6.4	37.4
	P. ruminicola H2b	22.8	19.5	9.3	47.1
	Butyrivibrio fibrisolvens H10b	264	529	185	218
α-L-Arabinofuranosidase†	*P. ruminicola* H2b	98.9	342	6199	5557
	B. fibrisolvens H10b	8.9	22.1	39.0	27.4
β-D-Xylosidase†	*F. succinogenes* S85	ND	2.3	2.5	31.7
	P. ruminicola brevis GA33	ND	25.1	ND	34.8
	P. ruminicola H2b	ND	0.5	9.3	14.8
	B. fibrisolvens H10b	25.6	34.4	351	261
Cellulase‡	*R. albus* RUM5	ND	ND	124	2.9
	R. flavefaciens 123	ND	ND	24.0	39.7
	F. succinogenes S85	9.0	22.2	21.2	68.8
	P. ruminicola brevis GA33	ND	33.0	41.0	76.0
	P. ruminicola H2b	ND	ND	38.4	ND
	B. fibrisolvens H10b	202	294	85.8	108
Xylanase‡	*R. albus* RUM5	ND	ND	65.1	5.23
	R. flavefaciens 123	ND	11.9	11.8	2.5
	F. succinogenes S85	ND	3.8	6.8	26.8
	P. ruminicola brevis GA33	ND	9.8	15.1	12.7
	P. ruminicola H2b	6.2	ND	19.3	1.1
	B. fibrisolvens H10b	48.4	32.8	10.4	13.7
Acetate Esterase†	*R. albus* RUM5	12.2	15.4	20.6	16.4
	R. flavefaciens 123	23.8	24.2	25.2	38.1
	F. succinogenes S85	13.4	16.2	16.1	15.0
	P. ruminicola brevis GA33	3.4	18.4	6.6	17.1
	P. ruminicola H2b	12.4	9.2	17.1	14.8
	B. fibrisolvens H10b	11.8	19.2	20.7	16.3

Adapted from Williams and Withers (1981 and 1982).

† Units: nmole *p*-nitrophenol released/mg protein/h.

‡ Units: nmole reducing sugars released/mg protein/h.

§ Not determined.

Chapter 4: Profiles of Cell-Wall Degradation by Anaerobic Fungi

Introduction

Information on the identity and population density of the fibrolytic microorganisms which take part the fiber breakdown in the rumen is important if rumen function is to be optimized. Fiber breakdown in the rumen is a result of complex microbial process involving bacteria, anaerobic fungi and ciliate protozoa. They produce a range of cellulolytic, hemicellulolytic and pectinolytic enzymes which are necessary for plant cell-wall degradation (Chapter 3). Lignification of and the fine structures of polysaccharides including the crystallinity of cellulose of plant cell-walls are limiting factors in cell-wall degradation in the rumen (Chesson and Forsberg, 1988). The ruminal degradability of plant cell-walls is also influenced by the hemicellulose/cellulose ratio (Buxton and Casler, 1993). In an earlier study Morris and Bacon (1977) found that the lower the xylose (xylan) to glucose (cellulose) ratio in plant cell-walls the higher digestibility of forage in the rumen. In terms of rumen microorganisms, it has been suggested that the synergism between cellulolytic and hemicellulolytic bacteria is required for efficient plant cell-wall degradation (Osborne and Dehority, 1989), other reports suggest that cellulolytic bacterium *Fibrobacter succinogenes* is the most important in terms of ruminal fiber degradation (Cheng *et al.*, 1983; Miron, 1991). A wide range of anaerobic fungi can be found in the rumens of grazing livestock (Orpin, 1994) and different fungal species show different abilities to degrade lignocellulosic tissues. However, little is known about the relative capacities of fungal species to degrade specific types of forage tissue.

In chapter 3, it is shown that anaerobic fungi produce a wide range of fiber-degrading enzymes and can be characterized by their enzyme production profiles, *i.e.* as cellulose degraders, xylan degraders. The present part of the study was conducted to examine the abilities of fungi from three genera to degrade fresh grass stem and leaf tissues by using three genera, and to identify the fiber digesting traits of anaerobic fungi

comparing to rumen fibrolytic bacteria. The patterns of xylan and cellulose removal from plant cell-wall by ruminal bacteria and fungi were determined to characterize microorgansisms in terms of their fiber degradation capacities. The values of xylan to cellulose (X/C) ratio in degraded tissues *in vitro* were compared with those found in the same tissue material incubated *in sacco*.

Materials and Methods

Microorganisms

Microorganisms used in this Chapter and their carbohydrate utilization are listed in appendix and Table 1, respectively. In fresh ryegrass degradation experiments, five fungal isolates, *Neocallimastix frontalis* PNK2, *Piromyces communis* B19, *Piromyces* sp. P1, *Caecomyces* sp. GT6 and B7 were co-cultured with a methanogen, *Methanobrevibacter smithii* PS, and maintained on sisal-containing medium. As *N. frontalis* PNK2, *P. communis* B19, *Piromyces* sp. P1, *Caecomyces* sp. GT6 and B7 were isolated from grazing animals in New Zealand, these fungi were used in experiment for fresh grass degradation. Fungus-methanogen co-cultures were used instead of fungal monocultures in order to better simulate the rumen environment (Bauchop and Mountfort, 1981; Joblin, 1990).

In experiments on timothy degradation, four rumen bacteria, *Butyrivibrio fibrisolvens* ATCC19171, *Fibrobacter succinogenes* ATCC19169, *Prevotella ruminicola* subsp. *brevis* 7-31, and *Ruminococcus albus* 7, and four anaerobic fungi, *Neocallimastix frontalis* MCH3, *Piromyces communis* 93-3, *Piromyces* sp. P1 and unidentified polycentric fungus KP1 (probably *Orpinomyces* sp.) were used.

Preparation of Medium and Incubation

Fresh ryegrass degradation

Sections (10 mm long) of leaf blade and internode portions of freshly-harvested perennial ryegrass were cut and used as substrates. Substrates (each 400 mg wet weight) placed in Hungate tubes were washed with distilled water to remove soluble

material and equilibrated overnight under O_2-free CO_2. Anaerobic basal medium (10 ml) (Table 2-a, b) was added aseptically to each tube. In order to retain ryegrass tissues in a natural state, substrates were not autoclaved. Instead antibiotics (penicillin and streptomycin (Joblin, 1981) were added to suppress any bacterial growth arising from the grass samples. Tubes were inoculated with 0.3 ml from each fungus-methanogen co-culture and cultures were incubated anaerobically together with uninoculated controls for 2, 3, 4, and 6 d at 39 ℃. After each incubation period, the weight loss from each of the two substrates were measured and CH_4 production and culture pH were measured as indicators of growth of the fungus-methanogen co-cultures.

Timothy hay degradation

Timothy (*Phleum pratense*) hay was used as a substrate throughout the experiment. Medium was prepared according to Joblin (1981), but agar and cellobiose were omitted. To obtain constant a physical quality of substrate, timothy hay was ground by a Wiley-mill to pass through a 1-mm sieve and further sieved through a Nylon mesh (100 μm pore) before the introduction into a medium at 2 % (w/v). Microorganisms were subcultured on the above mentioned medium for three times and inoculated to the test medium (4 % inoculum) at the late exponential growth phase. The inocula inevitably contained a few particles of timothy hay, but appeared not to distort measurements of degradability in the test medium. Indeed in the cases of fungi which do not release a large number of zoospores, an inoculation of colonized hay particles to a flesh media was required. Incubation was performed in Hungate-type culture tubes (Bellco Biotechnology, Vineland, NJ, USA) under 100 % carbon dioxide at 39 ℃. In the case of fungi, penicillin and streptomycin (Joblin, 1981) were supplemented to the medium to avoid eventual contamination of bacteria.

Analysis

In the fresh ryegrass experiment, after CH_4 determination by GC and pH of culture fluid to estimate fungal growth, substrate was washed twice with distilled water in the fresh ryegrass experiment. The substrate was dried at 70 ℃ for 2 d, then desiccated over P_2O_5 *in vacuo* for gravimetric measurement.

In the timothy hay experiment, fermentation was stopped by centrifugation (1,500 x g, 15 min, 4 ℃) at 6,12, 24, 48, 72, and 96 h (*F. succinogenes* and *P. ruminicola*), 24, 48, 72, 96 and 120 h (*B. fibrisolvens* and *R. albus*) after inoculation of bacteria and 24, 48, 72, 96, 120, 144 and 168 h after inoculation of fungi. Triplicate tubes were allotted to each incubation time. Pellets were exhaustively washed with distilled water and dried to a constant weight at 80 ℃. Dried samples were then ground in a mortar. A portion (10 mg) of ground samples was hydrolyzed and derivatized to alditol acetate for gas chromatographic determination of plant cell-wall sugar contents (Ushida *et al.*, 1991). Detected glucose and xylose were considered to originate from cellulose and xylan, respectively.

In sacco measurement

In sacco measurement for timothy hay was performed in two ruminally-fistulated wethers fed 400 g timothy hay and 200 g commercial concentrate (Coop Dairy 14, Kumiai Shiryo Co., Kobe) twice a day at 9:00 and 21:00. The concentrate feed contained maize (47 %), wheat bran (39 %), and soybean cake (8 %); the remainder consisted of molasses, calcium carbonate, calcium phosphate, sodium chloride and microelements. Water and mineral block (Green-Kouen, Nihon Zenyaku Kogyo, Fukushima, Japan) were available at all times. Nylon bags (5 x 10 cm, 100 μm pore) containing approximately 0.3 g ground timothy hay, treated as described above, were suspended in the rumen of sheep for 6, 12, 24, 48, and 72 h. Immediately after withdrawal from the rumen, bags were washed under gentle stream of water for 3 min. Bags were frozen at -15 ℃. Bags were thawed and washed again with water. The

bags were dried to the constant weight at 80 ℃. The residual timothy hay was analyzed for cell-wall sugar contents by the above mentioned method.

Results

Fresh ryegrass degradation by anaerobic fungi

Stem internode degradation

Figure 1 shows time course degradability of ryegrass leaf blade and stem internode, and CH_4 production by five fungus-methanogen co-cultures during 6 d incubation period. Stem tissue was more highly degraded than leaf blade for all fungus-methanogen co-cultures. For *N. frontalis* PNK2, *P. communis* B19, *Caecomyces* sp. GT6 and B7, degradation of stem tissue started after 2-3 d lag time (Figure 1- a, b, d and e). In *N. frontalis* PNK2-methanogen coculture, although CH_4 formation was detected, there was no significant stem degradation at 2 d after inoculation. For *Piromyces* sp. P1, shorter lag time (less than 2 d) was observed (Figure 1-c). After 6 d incubation period, *N. frontalis* PNK2, *P. communis* B19 and *Piromyces* sp. P1 degraded stem tissue at the extent of 35, 29 and 39 %, respectively. *Caecomyces* sp. GT6 and B7 showed lower degradability of stem tissue (14 and 19 %, respectively) than those of other isolates after 6 d incubation.

Leaf blade degradation

Leaf tissue was degraded more quickly than stem tissue. No significant fungal growth occurred on leaf tissue as evidenced by the lack of CH_4 formation. Lag time of degradation was shorter than that observed in stem tissue. In *N. frontalis* PNK2, *P. communis* B19, *Caecomyces* sp. GT6 and B7, there was slight leaf tissue degradation (3-7 %). *Piromyces* sp. P1 showed no activity against leaf tissue which contrasts with the other fungi yet this isolate was the most active against stem tissue.

Degradation of timothy hay by anaerobic fungi and rumen fibrolytic bacteria.

Degradation of timothy hay dry matter by ruminal bacteria, fungi and *in sacco*. Losses of dry matter during incubation of four ruminal bacteria are shown in Figure 2-a. Similar dry weight losses at the end of incubation (96 h) were observed with two bacteria,

R. albus (28.8 %) and *F. succinogenes* (27.7 %). Timothy hay was degraded to a lesser extent by *P. ruminicola* (16.4 %) and much lesser extent by *B. fibrisolvens* (7.6 %). Losses of dry matter during incubation of four ruminal fungi are shown in Figure 2-b. Dry matter losses at 168 h were the highest with *N. frontalis* MCH3 (59.4 %), followed by a polycentric fungus KP1 (27.6 %), *P. communis* 93-3 (23.0 %) and finally *Piromyces* sp. P1 (12.7 %). More than 35 % increase in dry matter degradation was observed in *N. frontalis* MCH3 cultures during 120 to 168 h incubation. Losses of dry matter of timothy hay during incubation within the rumen are shown in Figure 2-c. The degradation was nearly finished at 48h after incubation. The degradability of dry matter at 72 h was 39.9 %.

Degradation rate of cellulose and xylan in timothy hay by ruminal bacteria, fungi and *in sacco*

The amounts of xylose (xylan) and glucose (cellulose) in timothy hay degraded during incubation are shown in Table 3. At any incubation period (each 24 h), the amounts of degraded cellulose were greater than those of xylan in *in sacco* measurements as well as pure-culture experiments except for *N. frontalis* MCH3 and *P. communis* 93-3. During the first 24 h incubation, these fungi degraded more xylan than cellulose. The rates of degradation of xylan and cellulose in bacterial cultures were relatively constant over the period, this was most evident in *B. fibrisolvens* and *R. albus* cultures. However, the degradation rates of these cell-wall components were not constant and higher in the later incubation periods (72 h to 168 h) for most of fungi. The progressive increase in degradation rates of these cell-wall components was seen in *in sacco* measurements.

Traits in digested xylan to digested cellulose ratio (X/C ratio) during incubation

Ratios of digested xylan to digested cellulose of timothy hay during the each 24 h-incubation periods are shown in Figure 3. None of the other organisms produced a higher X/C ratio than 1 through incubation. Although *N. frontalis* MCH3 and *P. communis* 93-3 showed high X/C ratio at 24 h incubation, the ratio obtained from these organisms declined below 1 after 48 h incubation. These values (< 1) indicate that cellulose digested more intensively than xylan. There are three majors groups in the X/C ratios of 8 tested microorganisms. Those of *P. ruminicola* and *B. fibrisolvens* were placed in relatively high range (> 0.6). Those of *R. albus*, *P. communis* 93-3, *Piromyces* .sp. P1, and *N. frontalis* MCH3 were placed in middle range (0.3 to 0.5). T hose of *F. succinogenes* and polycentric fungus KP1 were placed in the lowest range (<0.3). Those observed in *in sacco* measurements were placed in the lowest range. The values were relatively constant or slightly decreasing with incubation time for the most of cultures, some of them (*B. fibrisolvens*, *P. ruminicola*) varied significantly.

Discussion

Many microscopic observations have shown colonization and attack on lignocellulosic tissues in forage by anaerobic fungi (Orpin, 1977a, b; Bauchop, 1979; Akin *et al.* 1983; Ho *et al.*, 1988; Orpin and Joblin, 1988). Anaerobic fungi colonize initially at damaged sites of forage fragments (Bauchop, 1979) and at stomata (Orpin, 1977a; Akin *et al.*, 1983). Lignified cell-walls are preferentially colonized, although non-lignified cell-walls are also attacked. In *in vitro* cultures, anaerobic fungi reduced the breaking strength of forage leaf blade (Akin *et al.*, 1983). The ability of rumen fungi to reduce the size of plant fragments (Orpin, 1983/84; Calderon-Cortes *et al.*, 1989) suggests that fungal activity could affect the residence time of particles in the rumen, for only the smallest plant particles pass out of the rumen (Ullyatt *et al.*, 1986).

Sijtsma and Tan (1996) used ground (approx. 1 mm of particle size) stem and leaf cell-walls of perennial ryegrass treated with neutral detergent (ND) as a substrate for *in vitro* experiment. They demonstrated that *Neocallimastix* sp. strain CS3b could remove nearly all glucose, xylose, and arabinose from treated leaf and stem cell-walls. There was no significant difference in removal of these sugars by strain CS3b between treated leaf and stem cell-walls. ND treatment removes lipids, sugars, organic acids, water-soluble matter, pectin, starch, non-protein N and soluble protein from plant tissues (Van Soest, 1982). Therefore, effects of those substances on microorganisms would be ignored when ND-treated forage is used as a substrate. Ryegrass tissues used in the present study were not treated with ND in order to maintain the tissue as intact as possible. The extent of degradation of stem tissue by five anaerobic fungi was higher than that of leaf blade tissue (Figure 1). This is notable and novel observation for anaerobic fungi. The results suggest that ND-soluble substances in leaf blade tissues affect fungal activity. Plant cell-wall contains 10 % of protein in higher plants (Lam *et al.*, 1990). Thionins is an example of proteins toxic to plant-pathogenic fungi. These are loosely bound to the cell-walls in dicot and monocot plants. Bohlmann (1988) reported barley leaf-specific thionins which inhibited to fungal growth at 5×10^{-4} M.

The present results suggest that rumen fungi belonging to *Neocallimastix*, *Piromyces* and *Caecomyces* genera have little activity against leaf tissue *in vivo* and that *Neocallimastix* and *Piromyces* species are likely to be more active than *Caecomyces* species at degrading fresh ryegrass stem tissues in the rumen.

The nature and number of fibrolytic microorganisms which take part in the fiber breakdown in the rumen has not been well established. The present study was conducted to characterize ruminal fiber digesting organisms by the ratio of digested xylan to cellulose (X/C ratio) in the pure-culture and then comparing the values to those obtained from *in sacco* measurements.

Among the organisms tested, *R. albus*, *F. succinogenes* and polycentric fungus KP1

degraded timothy hay better than other test organisms. The X/C ratios of these organisms are smaller than 0.5 throughout incubation. *B. fibrisolvens* and *P. ruminicola*, that degraded timothy hay less efficiently than former groups, showed X/C ratios higher than them. *P. ruminicola* (X/C > 0.6) is usually considered as a hemicellulolytic bacterium, while *R. albus* (X/C < 0.4) and *F. succinogenes* (X/C < 0.3) are cellulolytic organisms. Since none of the test organisms produced an X/C ratio higher than 1, it is apparent that species designated as hemicellulolytic are not necessarily absolute hemicellulose degraders. The organisms having X/C ratio higher than 0.6 are suggested to be more hemicellulolytic and those having the ratios lower than 0.5 are suggested to be more cellulolytic. Presently used anaerobic fungi are thus classified as cellulolytic, but *Neocallimastix* and *Piromyces* can be considered as "multi-function" fiber degrader.

The X/C ratios decreased with increasing incubation time except that from *P. ruminicola*. Such a decline in the ratio suggests that xylan degradation preceded to cellulose degradation in the fiber digestion by most of fiber digesting organisms. Indeed, the rates of cellulose degradation increased with incubation time. Removal of hemicellulose therefore appears to stimulate cellulose degradation. *P. ruminicola* showed a completely opposite trait to other organisms. The X/C ratio timothy hay degraded by this bacterium increased with time, suggesting cellulose was degraded most intensively in the early period of incubation. For *in sacco* measurement, the X/C ratio showed a similar trait to those observed with *F. succinogenes* and polycentric fungus KP1. This similarity of values and trends of the X/C ratios indicates that cellulolytic organisms, such as *F. succinogenes,* play a predominant role in fiber digestion in the rumen. Several workers have suggested that *F. succinogenes* plays a predominant role in determining the extent of cell-wall digestion in the rumen (Cheng *et al.*, 1983; Kudo and Cheng, 1994; Miron, 1991). Present results support this suggestion. However, synergistic relationships between cellulolytic, hemicellulolytic and pectinolytic bacteria

have been reported to be important in fiber degradation (Osborne and Dehority, 1989). There is little doubt that the removal of hemicellulose by hemicellulose degraders enhances cellulose degradation by cellulose degraders, because hemicellulose is limiting factor for fiber digestion (Buxton and Casler, 1993). This implies that role of hemicellulose degraders should not be ignored even it is considered as minor. Some ciliate protozoa, such as *Polyplastron multivesiculatum*, *Eudiplodinium maggii* and *Epidinium caudatum* are also important components of the fiber-digesting microbial population in the rumen (Williams and Coleman, 1992). These protozoa are hemicellulolytic rather than cellulolytic (Ushida *et al.*, 1991; Ushida and Jouany, 1993). They are directly involved in fiber digestion in the rumen, while other ciliate protozoa, such as *Entodinium* spp., not being fiber degraders, also are involved in fiber digestion through elimination of starch or ammonia supply to the cellulolytic bacteria (Kaneko *et al.*, 1989; Ushida and Kojima, 1991). The roles of protozoa in fiber digestion were not considered in the present experiments. Results from the study suggest that the *in sacco* experiments reflects exclusively the action of cellulolytic bacteria and fungi and that action of hemicellulose degraders such as protozoa may be ignored.

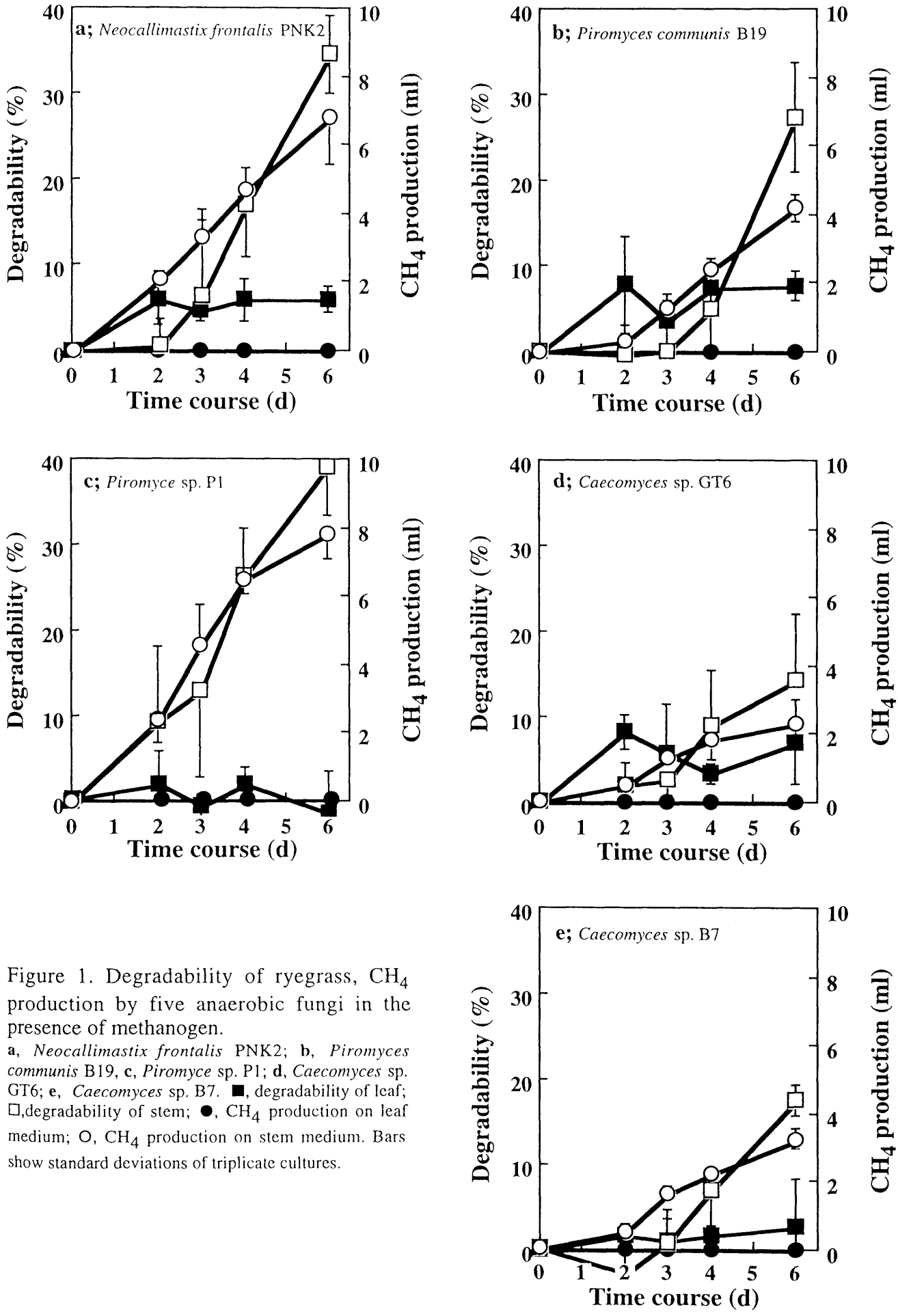

Figure 1. Degradability of ryegrass, CH_4 production by five anaerobic fungi in the presence of methanogen.

a, *Neocallimastix frontalis* PNK2; **b**, *Piromyces communis* B19, **c**, *Piromyce* sp. P1; **d**, *Caecomyces* sp. GT6; **e**, *Caecomyces* sp. B7. ■, degradability of leaf; □,degradability of stem; ●, CH_4 production on leaf medium; ○, CH_4 production on stem medium. Bars show standard deviations of triplicate cultures.

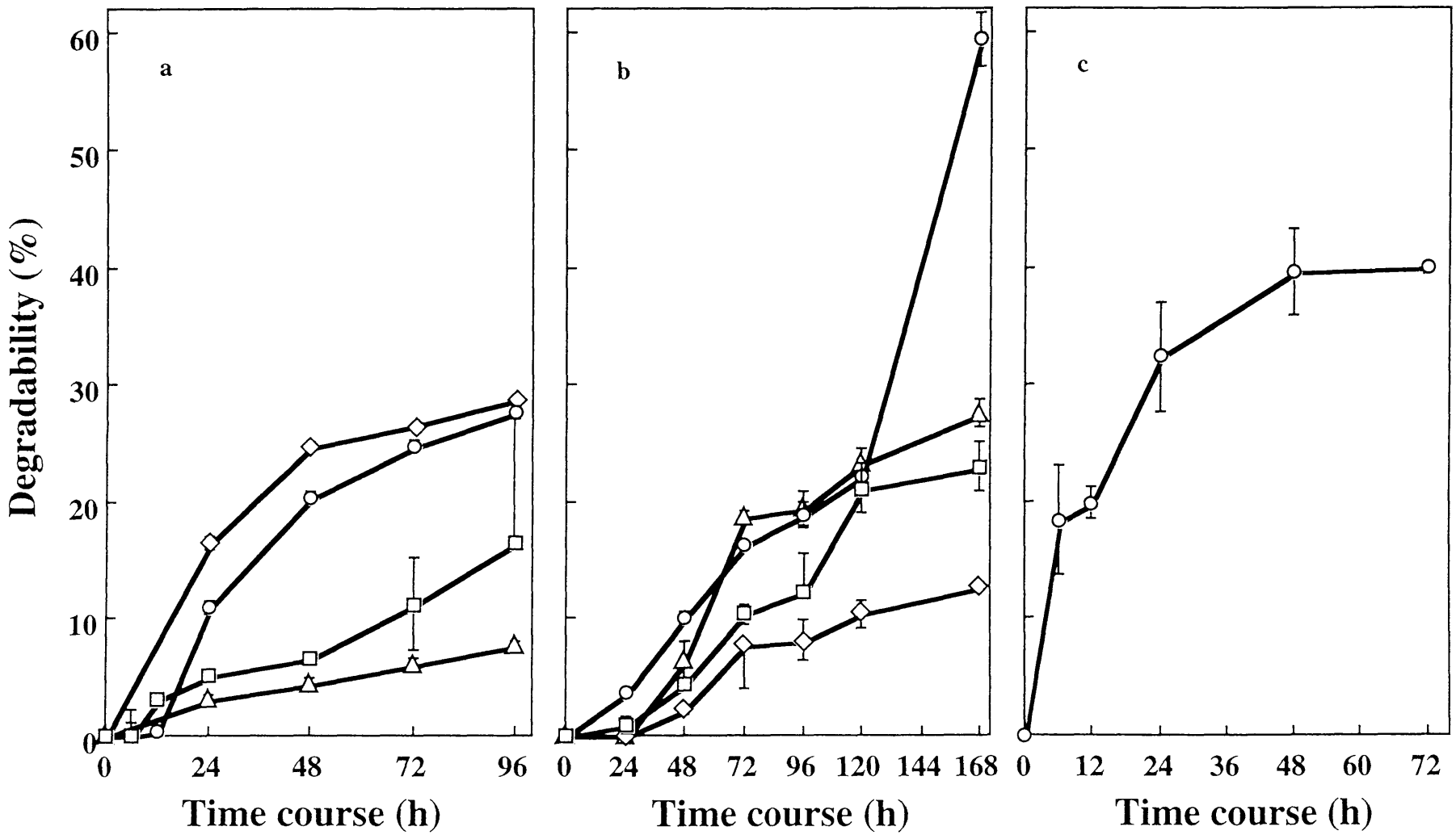

Figure 2. Losses of timothy hay dry matter by ruminal bacteria, anaerobic fungi and in sheep rumen. a: Rumen bacteria, O, *Fibrobacter succinogenes*19169; □, *Prevotella ruminicola* 7-31; △, *Buyrivibrio fibrisolvens* 19171; ◇, *Ruminococcus albus* 7. b: Anaerobic fungi, O, *Neocallimastix frontalis* MCH3; □, *Piromyces communis* 93-3; △, Polycentric fungus KP1; ◇, *Piromyces* sp. P1. c: *in sacco*. Bars show standard deviation of triplicate determination.

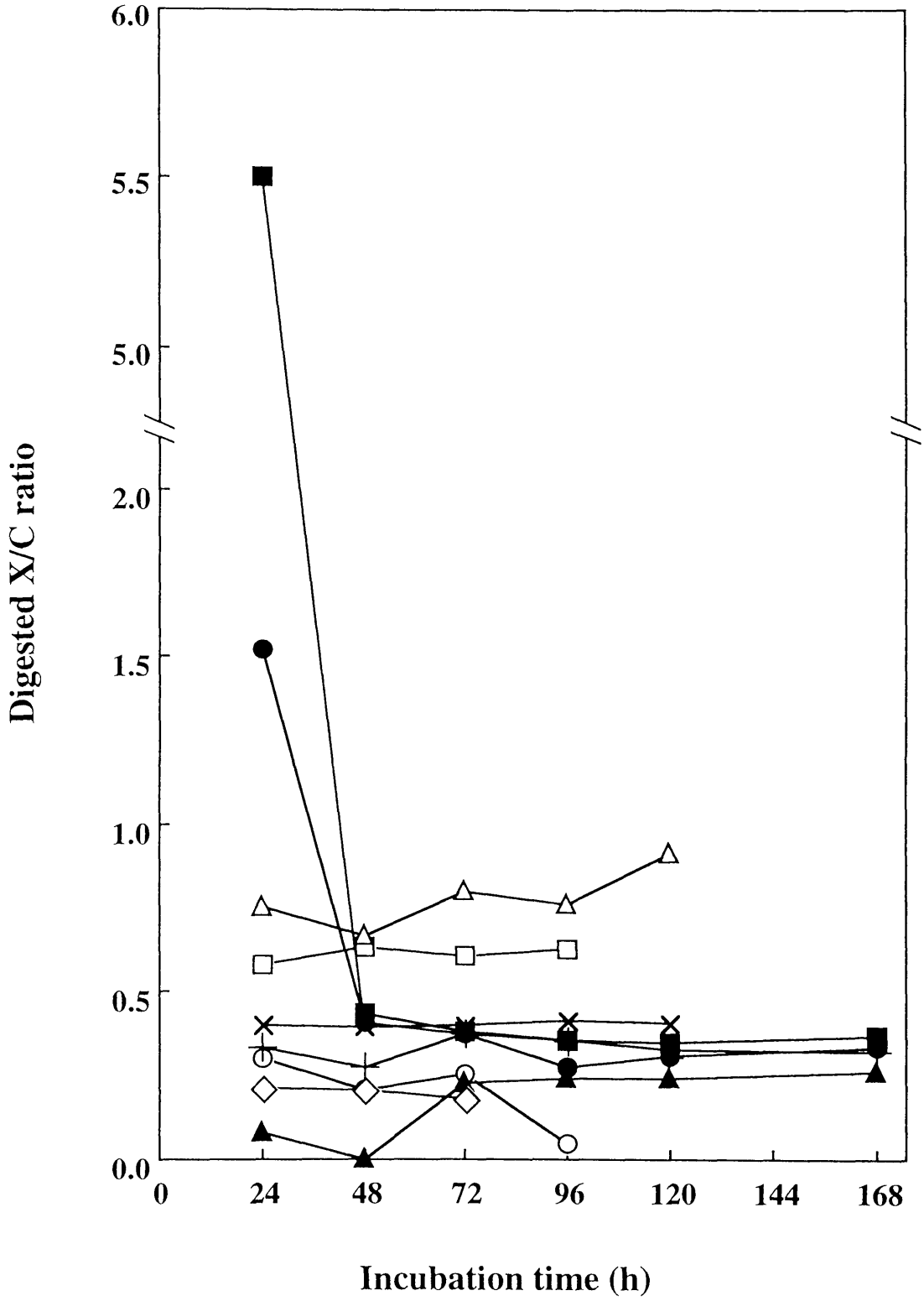

Figure 3. Digested xylan to cellulose ratio by rumen microorganisms during incubation and *in sacco*. ○, *Fibrobacter succinogenes* 19169; □, *Prevotella ruminicola* 7-31; △, *Butyrivibrio fibrisolvens* 19171; ×, *Ruminococcus albus* 7; ●, *Neocallimastix frontalis* MCH3; ■, *Piromyces communis* 93-3; ▲, polycentric fungus KP1; +, *Piromyces* sp. P1; ◇, *in sacco*.

Table 1. Carbohydrate utilization of rumen microorganisms used in this study.

Microorganisms	Cellulose	CMC	Cellobiose	Glucose	Xylan	Xylose	Arabinose
Anaerobic fungi							
Neocallimastix frontalis MCH3	+	+	+	+	+	+	-
N. frontalis PNK2	+	ND	+	+	+	+	-
Piromyces communis 12A	+	ND	+	+	+	ND	-
P. communis 93-3	+	ND	+	+	+	ND	-
P. communis B19							
Piromyces sp. P1	+	+	+	+	+	-	-
Caecomyces . sp. B7	+	ND	+	+	+	ND	-
Caecomyces . sp. GT6	+	ND	+	+	+	ND	-
Polycentric fungus KP1	+	+	+	+	+	+	-
Rumen bacteria							
Butyrivibrio fibrisolvens WV1	-	ND	+	+	ND	ND	ND
Butyrivibrio fibrisolvens ATCC19171	+	ND	+	+	+	+	+
Fibrobacter succinogenes ATCC19169	+	ND	+	+	+	+	-
Prevotella ruminicola subsp. *brevis* 7-31	+	ND	+	+	+	+	+
Ruminococcus albus 7	+	ND	+	+	+	+	+

Table 2-a. Components of basal medium.

Component		
Salt solution A	170	*
Salt solution B	170	*
Rumen fluid	300	*
Distilled water	360	*
0.1% Resazurin	1.0	*
$NaHCO_3$	5.0	**
L-Cystein HCl	0.5	**

* ml/L, ** g/L

Table 2-b. Components salt solutions in basal medium.

Component	g/L
Salt solution A	
NaCl	6.0
KH_2PO_4	3.0
$(NH_4)_2SO_4$	1.5
$CaCl_2$ anhydorous	0.6
$MgSO_4$	1.2
Salt solution B	
K_2HPO_4	6.0

Table 2. The amount (mg) of xylose (xylan) and glucose (cellulose) in timothy hay digested during incubation.

Microorganisms	Incubation time (h)											
	0 - 24		24 - 48		48 - 72		72 - 96		96 - 120		120 - 168	
	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose
Fibrobacter succinogenes 19169	3.3 ± 0.6	11.0 ± 1.6	2.9 ± 0.2	14.2 ± 0.6	4.5 ± 0.4	18.0 ± 1.0	4.2 ± 0.2	19.1 ± 0.5	ND †	ND	ND	ND
Prevotella ruminicola 7-31	4.4 ± 0.3	7.6 ± 1.4	3.6 ± 0.6	5.7 ± 2.3	3.4 ± 0.5	5.6 ± 1.6	4.3 ± 2.4	6.9 ± 7.5	ND	ND	ND	ND
Butyribivrio fibrisolvens 19171	1.2 ± 0.5	1.6 ± 1.1	1.4 ± 0.6	2.1 ± 1.4	1.6 ± 0.3	2.0 ± 0.4	1.9 ± 0.3	2.5 ± 1.5	2.0 ± 0.4	2.2 ± 0.9	ND	ND
Ruminococcus albus 7	10.2 ± 0.3	25.4 ± 1.4	10.8 ± 0.4	27.7 ± 1.2	11.4 ± 0.2	28.4 ± 2.0	10.7 ± 1.0	26.0 ± 4.2	11.3 ± 0.4	28.0 ± 2.9	ND	ND
Neocallimastix frontalis MCH3	3.8 ± 0.4	2.5 ± 7.1	4.3 ± 0.4	10.6 ± 0.4	5.3 ± 1.1	14.3 ± 1.7	5.4 ± 1.2	20.0 ± 4.0	5.4 ± 1.4	17.8 ± 2.3	11.7 ± 0.7	35.4 ± 1.7
Piromyces communis 93-3	3.3 ± 0.4	0.6 ± 7.6	3.4 ± 0.5	7.9 ± 0.3	4.1 ± 1.3	10.8 ± 2.3	6.2 ± 0.5	17.5 ± 1.4	10.1 ± 4.5	29.5 ± 13.2	7.1 ± 0.8	19.4 ± 2.5
Polycentric fungus KP1	0.2 ± 1.2	2.5 ± 2.1	0.0 ± 0.0	3.2 ± 0.2	3.6 ± 0.4	16.1 ± 0.9	4.1 ± 1.4	17.0 ± 1.7	4.9 ± 1.3	20.6 ± 2.3	5.8 ± 1.1	22.6 ± 1.9
Piromyces sp. P1	0.9 ± 1.1	2.7 ± 0.4	0.7 ± 2.3	2.6 ± 2.9	8.8 ± 5.5	23.8 ± 17.3	2.0 ± 1.4	5.7 ± 1.2	3.3 ± 0.2	10.1 ± 4.5	2.8 ± 1.3	8.8 ± 2.5
in sacco	6.4 ± 0.8	30.3 ± 6.0	7.9 ± 1.0	38.5 ± 3.8	7.3 ± 0.2	40.9 ± 1.3	ND	ND	ND	ND	ND	ND

Values are averege ± standard deviation of triplicate determination.

† not determined.

Chapter 5: Interactions between Anaerobic Fungi and Ruminal Bacteria.

Introduction

The rumen microbial ecosystem containing populations of bacteria, protozoa and anaerobic fungi (Hungate, 1988) are able to breakdown the structural polysaccharides in plant cell-walls ingested by host animals. The degradative process enables the microbial populations to convert the insoluble plant cell-walls into volatile fatty acids (VFAs). Host animals absorb VFAs as energy sources.

It is evident that microbial populations interact with each other during the degradation of plant cell-walls (Ushida *et al.*, 1991; Bernalier *et al.*, 1993; Miron and Ben-Ghedalia, 1993). This interaction is further complicated by the interaction within the population (Odenyo *et al.*, 1994). Between anaerobic fungi and bacteria, positive and negative effects of cocultivation have been reported. Synergistic interactions between anaerobic fungi and hydrogenotrophic bacteria in fiber degradation are well known (Bauchop and Mountfort, 1981; Mountfort *et al.*, 1982; Joblin *et al.*, 1989; Marvin-Sikkema *et al.*, 1990; Joblin and Williams, 1991; Tanaka *et al.*, 1991; Tanaka *et al.*, 1992; Hodrova, 1995). Enhancement of fungal fiber degradation in co-culture with heterotrophic bacteria is also known (Williams *et al.*, 1991; Williams *et al.*, 1994). More recently, inhibition of fungal cellulolysis by fiber-degrading bacteria has been reported (Stewart, *et al.*, 1992; Bernalier *et al.*, 1993; Joblin and Naylor, 1993; Kopecny *et al.*, 1996). However, in general little is known of the interactions between microbial populations in the complexity of its rumen microbial ecosystem.

In Chapter 3, the degradation of fresh ryegrass by anaerobic fungi growing in the presence of a methanogen and degradation of timothy hay by anaerobic fungi and rumen fibrolytic bacteria in pure-culture were discussed. Each microorganisms showed different capacities in terms of the forage degradation. In this chapter, experiments were conducted to investigate interactions between anaerobic fungi and rumen bacteria in fiber

degradation. Some fibrolytic bacteria were selected for study, because both fibrolytic bacteria and fungi bind to the plant particles during the degradation process. Phenolic monomers are released from plant cell-walls during degradation and may affect fungal-bacterial interaction. This point was also examined in the fungal-methanogen co-culture.

Materials and Methods

Microorganisms

Microorganisms used in this Chapter are listed in appendix. Investigations into fungus-*F. succinogenes* and fungus-methanogen co-cultivations involved the followings, *N. frontalis* PNK2, polycentric fungus KP1 and *Caecomyces* sp. B7, with *M. smithii* co-culture were used.

Media

Rumen fungi and fungus-methanogen co-culture were maintained on sisal-containing medium with rumen fluid. Rumen bacteria *B. fibrisolvens* WV1 and *F. succinogenes* ATCC19169 were maintained on 0.1 % glucose-containing basal medium (Chapter 4, Table 1-a, b) and rumen fluid-containing maltose-starch-xylan (RMSX) medium (Table 1-a, b), respectively. Paper strip (approx. 100 mg Whatman No. 1 filter paper) was added to 10 ml of basal medium (Chapter 4, Table 1-a, b) as a sole carbon source in Hungate tubes. For fungus-methanogen interaction, 10 ml of M10 medium (Caldwell and Bryant, 1966) with cellobiose (1 g/L) and Avicel (1 g/L) as the substrate was used. The medium contained different initial concentration (0, 1, 2, 4, 8 and 16 mmoles/L) of *p*-coumaric acid.

All media were prepared under O_2 free-100 % CO_2.

Incubation

To examine the inhibition of *B. fibrisolvens* WV1 on fungal cellulolysis, 20 h old WV1 culture fluid (O. D. > 1.0 at 600 nm) was lightly centrifuged for 10 min at 39 ℃ and the supernatant was stored at -20 ℃ until required. A portion (0.3 ml) of 5 day-old

fungal culture was inoculated into paper strip medium. The bacterial culture supernatant was thawed just before inoculation, then 1.0 ml of the supernatant or fresh basal medium were inoculated on the paper strip medium and incubated for 6 d at 39 ℃.

Culture supernatant of *B. fibrisolvens* WV1 was treated in various ways in order to obtain information on the nature of the inhibitory activity. A flow chart of the treatments of bacterial supernatant is provided in figure 1. The supernatant was 1) filtrated through cartridge filter (German Sciences, Supor® Acrodisc® 32, pore size = 0.2 μm) anaerobically and axenically (cell-free supernatant), 2) autoclaved at 121 ℃ for 20 min (autoclaved supernatant), and 3) frozen at -20 ℃ (frozen supernatant). The sedimented cells of *B. fibrisolvens* WV1 were washed twice with 10 ml of basal medium to remove residual inhibitor in the culture fluid and then resuspended in 10 ml of basal medium. Each supernatant, basal medium and 0.1 % glucose medium (1.0 ml each) were added to paper strip medium just after fungal inoculation. At the end of incubation with supernatant, frozen supernatant or cell suspension, the survival of *B. fibrisolvens* was checked by inoculating tubes of glucose medium. Growth of the bacterium was determined by measuring optical density at 600 nm.

In the experiment for fungal inhibition of rumen bacterium, *F. succinogenes* was pre-incubated on paper strip medium. Monocultures of bacteria and fungus (0.3 ml), or combination of both, were inoculated into paper strip medium. In experiments to investigate the effect of fungal supernatant on bacterial cellulose degradation, *N. frontalis* PNK2 culture fluid on sisal medium was harvested with following procedure. The fungal culture was sedimented by centrifugation, supernatant was removed anaerobically and frozen at -85 ℃ to terminate the fungus. The thawed supernatant (1.0 ml) was inoculated into an *F. succinogenes* culture immediately after bacterial inoculation.

Neocallimastix sp. N1 and *Piromyces* sp. P1 (5 % inoculum) with methanogen were inoculated on M10 medium to observe fungus-methanogen interaction. These fungi was isolated from sheep rumen with sole contamination of methanogen. In the co-culture

with methanogen, streptomycin and penicillin potassium were added. While sodium ampicillin, chloramphenicol and streptomycin sulfate were added (each 45 mg/L) to achieve the methanogen-free culture. The each culture was incubated at 39 ℃ for 7 d.

For fungus-*F. succinogenes* interaction experiment, the liquid portion of fresh-stem medium described in Chapter 3 was replaced with 10 ml of fresh basal medium to remove antibiotics already present in the medium. After the replacement, the medium was left for 2 h at 39 ℃ and replaced again with fresh basal medium. The replaced medium was shaken gently with rotary shaker overnight to release antibiotics from plant cells, then final replacement of basal medium was done. *F. succinogenes* was inoculated on the medium to confirm removal of antibiotics and its growth was observed. Fungus (either *N. frontalis* PNK2 or *Caecomyces* sp. B7) - methanogen (*Methanobrevibacter smithii*) co-culture and *F. succinogenes* pure-culture (0.3 ml for fungus and 0.5 ml for bacterium inoculum) were inoculated into the stem medium. *F. succinogenes* pure-culture and each fungus in the presence of methanogen were co-cultured on the same medium. These cultures were incubated at 39 ℃ for 6 d. All incubation and preparation were done anaerobically.

Measurement

Hydrogen and methane gas production (Joblin *et al.*, 1990; Tanaka *et al.*, 1992) and pH of culture fluid were measured at the end of incubation as indicators of growth of the microorganisms. Following this, sedimented solid substrates were washed twice with distilled water and dried at 70 ℃ for 48 h to measure the constant weight.

Statistics

Results in Table 2 and 5 were statistically analyzed by student *t*-test. Results in Table 3, 4, 6 were statistically analyzed by Tukey's procedure.

Results

Inhibitory interaction between anaerobic fungi and rumen bacteria.

Table 2 shows cellulose degradability, hydrogen gas production and pH of culture fluid after 6 d incubation by four rumen fungi with or without *B. fibrisolvens* WV1. Inhibition of *B. fibrisolvens* on fungal cellulose degradation was observed for all the fungal isolates tested. For *N. frontalis* PNK2, *Piromyces* sp. 12A and polycentric fungus KP1, the presence of *B. fibrisolvens* WV1 decreased cellulose degradation over 90 % in comparisons. The extent of inhibition to *Piromyces* sp. P1 was the lowest. The extent of cellulolysis by fungi and fungal H_2 production was significantly correlated (r^2=0.71, p<0.05). As *N. frontalis* PNK2 degraded cellulose more extensively for other fungal isolates, *N. frontalis* was used in a further examination of the inhibitory factor(s) produced by *B. fibrisolvens*. Inoculation of bacterial cell suspension extensively inhibited fungal cellulose degradation and growth (Table 3). Freezing of bacterial supernatant was aimed to destroy *B. fibrisolvens*, however, *B. fibrisolvens* survived after incubation. Therefore, inhibition by treatment with the frozen supernatant resembled to which observed in cell suspension treatment. Autoclaving of the supernatant completely inactivate the bacterial inhibition. Cell-free supernatant had little effect on fungal cellulolysis and the extent of inhibition was similar to addition of fresh glucose medium.

To observe inhibitory effects of fungus on *F. succinogenes* degradation of cellulose, *N. frontalis* PNK2 and its culture supernatant were inoculated into *F. succinogenes*-cultures. Cellulose degradation by *F. succinogenes*-culture was significantly lower than that of *N. frontalis* PNK2 (Table 4). When *F. succinogenes* was co-cultured with the fungus, extent of cellulose degradation did not differ from that of the fungal pure-culture. The fungal supernatant inhibited cellulolysis by *F. succinogenes* significantly.

Synergism between anaerobic fungi and rumen bacteria

Table 5 shows the effect of *p*-coumaric acid (PCA) and methanogen on fungal

cellulose degradation. PCA decreased fungal cellulose degradation at the concentration higher than 1 mM. Fungal cellulolysis was decreased with increasing concentration of PCA. Although the presence of methanogen significantly enhanced cellulose degradation at PCA concentrations lower than 4 and 2 mM, respectively for *Neocallimastix* sp. N1 and *Piromyces* sp. P1, over all the methanogen did not improve the degradabilities at higher concentration. Decrease in methane concentration induced by PCA in the co-culture with methanogen was somewhat greater than that of hydrogen in fungal pure-culture. Indeed, hydrogen concentration was not significantly decreased (from 8 % to 6 % in fungal pure-culture), while methane concentration was decreased from 20 to 6 % in the co-culture of each fungus and methanogen.

There was significant synergism between *Caecomyces* sp. B7 and *F. succinogenes* in ryegrass stem degradation (Table 6). Stem degradability by a triculture of *Caecomyces* sp. B7-methanogen and *F. succinogenes* was twice as high as those of the individual cellulolytic microbes. In contrast, *F. succinogenes* did not significantly affect the extent of stem degradation by a *N. frontalis* PNK2-methanogen co-culture. Similarly Table 4 showed little synergy in the degradation of cellulose this fungus and this bacterium.

Discussion

In the ruminal ecosystem, various kinds of microorganisms degrade and utilize structural polysaccharides in plant cell-walls as growth substrates. This study showed synergistic and inhibitory interactions between anaerobic fungi and rumen fibrolytic bacteria on fiber degradation.

B. fibrisolvens WV1 inhibited *in vitro* fungal cellulose degradation. The extent of inhibition differed with fungal isolates (Table 2) in agreement with observations by Joblin and Naylor (1994). This suggests that different anaerobic fungi have different resistance to this inhibition. Since the bacterial supernatant did not inhibit cellulose

degradation but the cell suspension strongly inhibited, the factor(s) would be cell associated. Fungal cellulolysis correlated significantly with fungal H_2 production suggesting that the inhibitor(s) acted on fungal growth. Some reports on the inhibitory activities of several rumen bacteria on fungal cellulolysis showed that the activities were present in the culture supernatants (Stewart *et al.*, 1992; Bernalier *et al.*, 1993; Kopecny *et al.*, 1996). Kopecny *et al.* (1996) demonstrated that culture supernatant of chitinolytic bacterium *Clostridium tertium* ChK5 inhibited extracellular *endo*-glucanase activity of *Orpinomyces joyonii* strain A4. They suggested that chitinase produced by *C. tertium* ChK5 disrupted the association of cellulase with components of fungal wall. However, the mechanisms of these inhibitors are still not clear. As the bacterial inhibitory factor(s) reduced cellulase activities of fungi but did not depress fungal growth, the following hypothesis is proposed; the inhibitors may attach for cellulases or cellulase complexes thereby reduce affinity of cellulases to substrate or prevent formation of cellulase complexes or they may release cellulases from fungal cell-wall to destroy cellulase complex (Stewart *et al.*, 1992; Bernalier *et al.*, 1993; Kopecny *et al.*, 1996). Odenyo *et al.* (1994) showed production of a bacteriocin-like activity in culture supernatant of *Ruminococcus albus* 8 against *R. flavefaciens* FD-1 in *in vitro*. In the paper, the activity had narrow spectrum of action toward bacteria. Since inhibitory factor(s) produced by *B. fibrisolvens* WV1 affects fungal growth to different extent (Joblin and Naylor, 1994), the inhibitory factor(s) appear to have specificities toward a certain fungal strains. For rumen bacteria, rapid elimination of introduced bacterium from the rumen has been attributed to antibacterial agent. Attwood *et al.* (1988) suggested that the half-life of *Prevotella ruminicola* subsp. *brevis* introduced in the rumen was less than 30 min not due to ruminal dilution or to bacteriophage attack but was possibly the results of a specific bacteriocin-like activity. The presence of antifungal substances in the rumen would not be surprising.

In the present work, the culture fluid of *B. fibrisolvens* WV1 was fractionated and

treated in various way to determine location of inhibitory factor(s) and to characterize the nature of the factor(s). Since the cell-free supernatant did not show inhibitory activity and the cell suspension depressed fungal cellulolysis, the inhibitory factor(s) should be cell-associated (Table 3). The significance of the inhibition *in vivo* is a puzzle, because *B. fibrisolvens* WV1 has no cellulolytic activity and must rely on the fungal cellulolytic activity when cultured on cellulose. Because *B. fibrisolvens* complement relation *F. succinogenes* activity in forage cell-wall degradation (Miron and Ben-Ghedalia, 1993). It may be that *B. fibrisolvens* can help *F. succinogenes* compete with anaerobic fungi. *F. succinogenes* was inhibited by the culture supernatant of *N. frontalis* as discussed below.

In general little is known about fungal inhibition of fibrolytic bacteria *R. flavefaciens* can be inhibited by fungi (Joblin and Naylor, 1996) but the present results showed that addition of *N. frontalis* PNK2 culture supernatant depressed cellulolysis by *F. succinogenes* (Table 4) significantly. The extent of inhibition was lower than these observed for *B. fibrisolvens*. It is still unknown whether the inhibition was caused by fermentation end product(s) (such as formate, acetate, lactate or ethanol) or by fungal inhibitory factor(s). Production of antibiotics by *Penicillium chrysogenum* is well known (Brown and Peterson, 1950; Suzuki and Nakao, 1979) . In general, anaerobes do not produce antibiotics. Yet, it seems to be natural that anaerobic fungi are able to produce such inhibitory substances against rumen bacteria. Characteristics and mechanisms of the fungal inhibition toward nutritionally important bacteria should be cleared in future.

Synergistic interaction between fungi and methanogens and their effects on plant fiber degradation has been reviewed (Williams *et al.*, 1994). Anaerobic fungi produce hydrogen as a major electron sink product during fermentation. High partial pressure of hydrogen reduces reoxidation of fungal electron carrier, therefore, intermediary metabolites accumulate in fungal cells and reduce the metabolic rate. When

hydrogenotrophic microbes are co-cultured with fungi, removal of hydrogen results in reoxidation of the electron carriers enhancing fungal growth and fibrolytic activity. Synergism between the methanogen and the fungi is clear in this context. Moreover anaerobic fungi produce a wide range of enzymes which involved in plant structural polysaccharide breakdown (Williams and Orpin, 1987a, b; Joblin *et al.*, 1990; Matsui *et al.*, 1992) and the specific activities of these enzymes were markedly enhanced by cocultivation with *M. smithii* (Joblin *et al.*, 1990). These also explain enhancement of polysaccharide and plant degradation by fungus-methanogen co-cultures. Fungal-methanogen synergism obviously occur on the plant particles not in the rumen liquor, because interspecies hydrogen transfer occurs between juxtapositioned microorganisms (Vogels *et al.*, 1980; Thiele *et al.*, 1988). Substances generated from plant cell-wall by fungal degradation may affect this synergistic consortium. PCA is the most abundant material of plant cell-wall origin that affect microbial activity (Akin, 1986). Methanogens enhanced fungal cellulolytic activity at low PCA concentration (Table 5). Chesson *et al.* (1982) measured concentrations of phenolic acids in the rumen fluid ranging from 0.03 to 0.8 mM and found that these concentrations were not toxic to rumen bacteria. The attachment of fibrolytic microorganisms to plant material in the rumen has been reported (Minato *et al.*, 1966; Cheng *et al.*, 1993) and this association is most evident in the case of the anaerobic fungi. In the microenvironment surrounding attached microorganisms, the organisms should encounter phenolic acids at much higher concentrations than those found in rumen fluid. In discussions on the actual interaction between rumen microorganisms, the local concentrations of phenolic acids should be considered. In the present study, the accumulation of hydrogen containing increasing levels of PCA was not decreased but methane concentration was decreased in fungus-methanogen co-culture (Table 5). This suggests that PCA inhibited not only fungal cellulolysis but also methanogenic activity. Cellulolytic bacteria were not sensitive to phenolic acids up to 5-10 mM (Chesson *et al.*, 1982). Sensitivity of methanogens were

not reported so far, they may be sensitive to at least 2 mM PCA from present results. Associated methanogens seem to be less tolerant to PCA than tested fungi, PCA-release from plant degradation by fungi possibly affects the fungi-methanogen consortium.

When *N. frontalis* PNK2 was co-cultured with *F. succinogenes*, there was no significant increase in stem degradation (Table 6). This may be explained by the competition for the same energy source (Chapter 4, Table 1). On the other hand, *Caecomyces* sp. B7-*F. succinogenes* co-culture showed a complementary interaction. This result suggests that these microbes have different substrate utilization (Chapter 4, Table 1) of plant cell-wall components thereby avoiding competition. *Caecomyces* species form bulbous rhizoid that is distinctive of this genus. Orpin and Joblin (1988) indicated that plant cell-walls were degraded not only by enzymic action but also by physical pressure produced by the growing rhizoid. This mode of action might allow *F. succinogenes* to access substrates within plant cell-walls.

In this chapter different types of interaction between rumen microorganisms on fiber degradation have been investigated. The combinations tested in the present study are part of a complex microbial ecosystem. To improve plant cell-wall degradation in the rumen, these events need to be better understood.

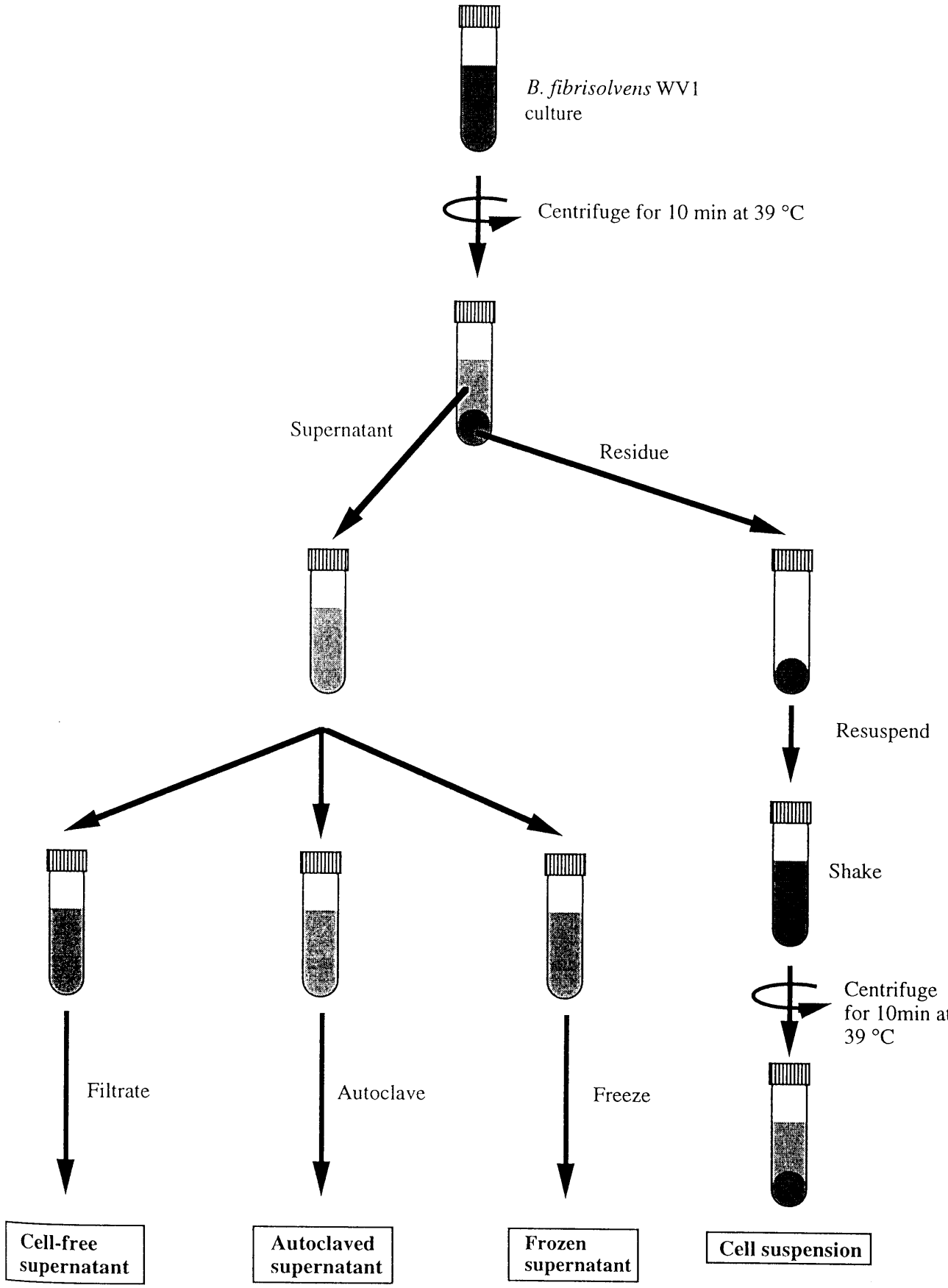

Figure 1. Fractionation and treatment of bacterial supernatant.

Table 1. Components of rumen fluid containing maltose-starch-xylan (RMSX) medium.

Component		
Salt solution A	170	*
Salt solution B	170	*
Rumen fluid	300	*
Distilled water	360	*
0.1% Resazurin	1.0	*
Maltose	2.0	**
Starch	2.0	**
Xylan from oat spelt	2.0	**
$NaHCO_3$	5.0	**
L-Cystein HCl	0.5	**

* ml/L

** g/L

Table 2. Degradability of cellulose, pH of culture fluid and H_2 production by 4 anaerobic fungi with or without supernatant (SN) of *Butyrivibrio fibrisolvens* WV1*.

Fungus		Degradability (%)	H_2 production (ml)	pH of culture fluid†
Neocallimastix frontalis PNK2	Control	37.1 ± 0.9**	1.5 ± 0.1**	5.80± 0.03
	+SN	2.7 ± 0.9**	0.9 ± 0.1**	6.44± 0.01
Piromyces communis 12A	Control	31.5 ± 1.5**	1.5 ± 0.1**	6.12± 0.02
	+SN	1.1 ± 1.0**	0.6 ± 0.0**	6.49± 0.02
Piromyces sp. P1	Control	27.1 ± 2.2**	1.4 ± 0.1**	6.08± 0.03
	+SN	6.7 ± 1.9**	1.1 ± 0.1**	6.39± 0.02
Polycentric fungus KP1	Control	25.5 ± 14.8	1.0 ± 0.2	5.93± 0.32
	+SN	2.7 ± 3.4	0.7 ± 0.4	6.50± 0.00

* Means ± standard deviation (n=3).

† pH of blank medium was 6.55 ± 0.01.

** Mean values between control and +SN within same isolate differed significantly ($p<0.01$)

Table 3. Inhibition of fungal cellulose degradation by rumen bacterium *Butyrivibrio fibrisolvens* WV1*.

Additive	Relative inhibition (%)	H_2 production (ml)	pH of culture fluid†
Basal medium	0	1.3 ± 0.3	5.93 ± 0.10
Glucose medium	8.8 ± 6.8	1.2 ± 0.1	6.00 ± 0.02
Cell-free supernatant	8.0 ± 5.6	1.4 ± 0.1	5.90 ± 0.04
Frozen supernatant	79.3 ± 7.6	1.1 ± 0.5	6.50 ± 0.04
Autoclaved supernatant	2.0 ± 2.8	1.1 ± 0.2	5.94 ± 0.07
Bacterial cell suspension	92.8 ± 1.4	0.2 ± 0.0	6.61 ± 0.01

* Means ± standard deviation (n=3).

† pH of blank medium was 6.60 ± 0.01.

Table 4. Paper strip degradability (%) and pH of culture fluid by *Fibrobacter succinogenes**.

Microorganism	Degradability (%)	pH of culture fluid†
Fibrobacter succinogenes	35.4 ± 0.3[a]	6.00 ± 0.01
Neocallimastix frontalis PNK2	40.6 ± 1.4[b]	5.89 ± 0.03
F. succinogenes + *N. frontalis*	41.2 ± 2.6[b]	5.94 ± 0.02
F. succinogenes + Fungal SN	30.2 ± 1.5[c]	6.10 ± 0.02

* Means ± standard deviation (n=3).

† pH of blank medium was 6.55 ± 0.01.

[a, b, c] Means of the degradability followed by different letters differ significantly ($P<0.01$).

Table 5. Effect of *p*-coumaric acid (PCA) on cellulose degradation by fungi with or without methanogen†.

Initial PCA (mM)	Methanogen‡	Degradability (%)	Products§		
			H_2		CH_4
		Neocallimastix sp. N1			
0	-	57.7 ± 6.2[a]	8.46 ± 0.16[a]		ND ‖
	+	88.0 ± 3.8[A*]	0.05 ± 0.02[A*]	22.33 ±	1.67[A]
1	-	47.6 ± 0.5[a]	6.63 ± 1.13[ab]		ND
	+	88.8 ± 1.8[A*]	0.38 ± 0.14[A*]	16.10 ±	1.28[B]
2	-	34.1 ± 12.4[b]	7.84 ± 0.19[b]		ND
	+	58.4 ± 29.4[B*]	0.33 ± 0.14[A*]	14.12 ±	2.01[B]
4	-	31.5 ± 9.7[b]	6.21 ± 0.28[C]		ND
	+	54.7 ± 16.4[B*]	1.69 ± 0.77[A*]	6.58 ±	2.67[C]
8	-	4.1 ± 1.4[c]	0.13 ± 0.09[c]		ND
	+	2.6 ± 10.6[c]	0.41 ± 0.04[A]	0.41 ±	0.04[C]
16	-	9.7 ± 14.9[c]	0.03 ± 0.04[c]		ND
	+	4.9 ± 4.6[c]	0.23 ± 0.17[A]	0.23 ±	0.17[C]
		Piromyces sp. P1			
0	-	62.1 ± 9.3[a]	8.00 ± 1.14[a]		ND
	+	90.9 ± 3.3[A*]	0.03 ± 0.01[A*]	21.74 ±	0.64[A]
1	-	53.5 ± 6.9[a]	6.54 ± 1.76[a]		ND
	+	66.7 ± 8.9[B*]	1.32 ± 0.22[B*]	8.07 ±	1.03[B]
2	-	38.9 ± 1.0[b]	7.00 ± 0.74[a]		ND
	+	68.2 ± 4.9[B*]	1.51 ± 0.35[B*]	5.69 ±	0.39[C]
4	-	7.1 ± 6.1[cd]	0.24 ± 0.02[b]		ND
	+	11.1 ± 1.9[C]	0.61 ± 0.14[A]	0.61 ±	0.14[D]
8	-	10.6 ± 12.8[c]	0.15 ± 0.02[b]		ND
	+	13.6 ± 3.3[C]	0.13 ± 0.00[A]	0.47 ±	0.09[D]
16	-	-1.0 ± 15.6[c]	0.12 ± 0.02[b]		ND
	+	5.1 ± 16.5[C]	0.12 ± 0.05[A]	0.32 ±	0.05[D]

† Means ± standard deviation (n=3).

‡ +, Fungi co-culture with methanogen; -, fungi pure-culture.

§ Concentration (%) in the head space gases.

‖ Not detected.

[a, b, c.] Values obtained from fungal pure-culture within a same column followed by different superscripts differ significantly ($P<0.05$).

[A, B, C.] Values obtained from fungal-methanogen co-cultures within a same column followed by different superscripts differ significantly ($P<0.05$).

* Asterisks denote the significant difference ($P<0.05$) between - and + at the same initial PCA concentration.

Table 6. Degradability of stem tissue, CH_4 production and pH of culture fluid by two rumen fungal strains in the presence of *Methanobrevibacter smithii* with or without *Fibrobacter succinogenes**.

Microorganism	Degradability (%)	CH_4 production (ml)	pH of culture fluid†
Neocallimastix frontalis PNK2	53.7 ± 2.6[a]	4.3 ± 0.2	6.34 ± 0.01
PNK2 + *Fibrobacter succinogenes*	58.3 ± 2.7[a]	4.4 ± 0.4	6.20 ± 0.01
Caecomyces sp. B7	23.1 ± 8.2[b]	1.9 ± 0.1	6.36 ± 0.01
B7 + *F. succinogenes*	40.4 ± 5.6[c]	2.3 ± 0.4	6.28 ± 0.07
F. succinogenes	22.5 ± 9.1[b]	0.0 ± 0.0	6.34 ± 0.03

* Means ± standard deviation (n=3).

† pH of blank medium was 6.41 ± 0.01.

[a, b, c] Values differ significantly between different superscripts ($P<0.01$).

Chapter 6: General Discussion

Ruminants depend on the plant fiber degradation within rumen microbial ecosystem. Since anaerobic fungi were accurately identified as true fungi (Orpin, 1977a, b, c; Orpin and Bountiff, 1978) and known to have high cellulolytic and xylanolytic activity, rumen microbiologists have been interested in those unique properties. Research into the role of these fungi in fiber degradation, and their interaction with other microorganisms and the application of anaerobic fungi are still developing (Mountfort, 1994). In the present study, the characteristics and ecology of anaerobic fungi are discussed in relation to plant fiber degradation.

As zoospore concentration in rumen fluid is not a reliable marker of the biomass and metabolic activity of anaerobic fungi in the rumen (Bauchop, 1979), the extent of fungal colonization on the solid substrate was determined to estimate relative fungal biomass by method of Ushida *et al.* (1989b) in the present study. In early, fluctuations of flagellates in the rumen were studied (Orpin, 1976, 1977a). Fluctuation of germinated fungal zoospore in the rumen of animal fed AF diet (Chapter 2, figure 1-a) was similar to the population density curve of flagellates for the ruminal fluid reported by Orpin (1976). Kostyukovsky *et al.* (1991) reported influence of four different diets on the fungal population density in the calf rumen. High proportion of hay in the diet resulted in high density of fungal zoospore in their experiments. And also, low density of zoospore was maintained in the rumen of animals fed wheat green diet which proportion of crude fiber is low. As supplementation of suitable amount of dietary concentrate supported high zoospore population size in the present study, appropriate proportion of crude fiber is needed if high population density of zoospore should be maintained. Because anaerobic fungi have two stage in their life cycle, relationships between fungal population and fiber degradation of plant cell-wall are not clear. Therefore, such relationship should be understood for evaluating fungal contribution on fiber degradation in the rumen. And more, shorter sampling interval should be used to observe detail of zoospore fluctuation.

N. patriciarum and *P. communis* and *C. communis* are known to produce a wide range of extracellular polysaccharide degrading enzymes and glycosidases involved in plant cell-wall degradation (Williams and Orpin, 1987 a, b; Hébraud and Févre, 1988). Both fungal isolates *Neocallimastix* sp. N1 and *Piromyces* sp. P1 used in this study also showed a capability of producing a wide range of polysaccharide and glycoside hydrolases involved in cell-wall degradation (Chapter 3, Table 1). In addition to those enzymes, pectinase, *p*-coumaroyl and feruroyl esterase, protease are present in fungal culture supernatant (Wallace and Joblin, 1985; Borneman *et al.*, 1990; Borneman *et al.*, 1991; Asao *et al.*, 1993; Yanke *et al.*, 1993; Kopecny and Hodrova, 1995). Growth substrates affected the specific activities but the production of these enzymes were maintained on all the substrates tested in this study. Mountfort (1994) reviewing fungal degradation and fermentation of carbohydrates, has noted that cellulase production is constitutive in anaerobic fungi. The present results are agreement with this. Constitutive production of these enzymes may provide fungi with a competitive advantage in situations where inducible cellulases might otherwise be repressed, because soluble sugars, especially glucose, may be present at high concentrations in the rumen immediately after ingestion of feed. Stimulation of fungal development by small amount of soluble sugar (*i.e.* 0.1 %) has often seen in pure-cultures (Tanaka and Ushida, unpublished). Although anaerobic fungi produce relatively small amount of enzymes into medium, the specific activities of fibrolytic enzymes are extremely high (Wood *et al.*, 1986). The capacity of fungi to produce a wide range of highly active enzymes may give fungi advantages over other rumen fibrolytic microorganisms.

In terms of the contribution of anaerobic fungi to plant cell-wall degradation, contrasting results have been reported. Gordon and Phillips (1993) showed a significant contribution from anaerobic fungi to acid-detergent fiber degradation in sheep rumen which were fed barley straw and lucerne. This contribution also improved feed consumption by the sheep. The removal of anaerobic fungi from the sheep rumen

reduced feed consumption and re-inoculation of fungi recovered it significantly. Sheep with fungi ingested 40 % more of a straw-based diet (high in fiber) than they ingested when without fungi (Gordon and Phillips, 1993). Since the increase in feed consumption is the most important factor to the ruminant production (Minson, 1990), the presence of fungi may directly relate to the ruminant production. However, opposite effects have been shown by Windham and Akin (1984) in *in vitro* studies. They assessed the *in vitro* degradabilities of Coastal bermuda grass, alfalfa and cord grass challenged by mixed population of anaerobic fungi obtained from sheep rumen. By adding appropriate combinations of biocides to whole rumen fluid, they selected for a fungal population, and a bacterial population. Suppression of the bacterial population resulted in a significant decrease of degradabilities of plant cell-walls. No significant decrease was observed when the fungal population was suppressed. Their conclusion was that fungi have only an insignificant role in plant cell wall degradation. However, the use of finely ground tissues as substrates may underestimate the fungal role in decreasing the physical strength of the plant. *Neocallimastix* sp. N1 and *Piromyces* sp. P1 produced major plant cell-wall degrading enzymes (Chapter 3, Table 2), and five fungal isolates, *N. frontalis* PNK2, *P. communis* B19, *Piromyces* sp. P1, *Caecomyces* sp. GT6 and *Caecomyces* sp. B7 showed a higher capacity to degrade stem tissue degradation rather than leaf tissue (Chapter 4, Figure 1). Stem tissue is a major component of forage and is much less digestible than leaf in the rumen (Van Soest *et al.*, 1995). The effective degradation of stem is crucial to reduce the physical limit for feed intake. The physical strength of plant is substantially weakened following the colonization by fungi (Akin *et al.*, 1990). This may enhance feed breakdown during rumination. Moreover, plant particle size distribution is affected by the presence of fungi (Calderon-Cortes *et al.*, 1989). Interpretation of experiments in which the chemical treatments are used to remove fungi from the rumen need to be interpreted with caution because treatments may affect other microbial populations and the physiological

condition of the host animal.

In the case of anaerobic fungi, synergism with methanogens (Bauchop and Mountfort, 1981; Joblin *et al.*, 1989; Marvin-Sikkema *et al.*, 1990; Mountfort *et al.*, 1982; Tanaka *et al.*, 1991; Tanaka *et al.*, 1992), inhibitory interaction with rumen fibrolytic bacteria (Stewart *et al.*, 1992; Bernalier *et al.*, 1993; Kopecny *et al.*, 1996) and decrease of fungal population (Ushida *et al.*, 1989a, 1991) by protozoal engulfment (Joblin, 1990, Widyastuti *et al.*, 1995) are known. In chapter 5, positive and negative interactions between anaerobic fungi and rumen bacteria were observed in fiber degradation. *B. fibrisolvens* WV1 inhibited fungal growth which was reflected by decreased H_2 production, *i.e.* fungal cellulolysis (Chapter 5, Table 2,3) by possibly competing with the uptake of soluble sugars. Soluble sugars stimulate the development of fungi especially in the early stage of growth. Although the extent of inhibition (approx. 5 %) was not high, supernatant of *N. frontalis* PNK2 inhibited cellulose degradation by *F. succinogenes* significantly (Chapter 5, Table 4). This result suggests that anaerobic fungi produce inhibitory factor(s) against bacterial growth or cellulolytic activity. Joblin and Naylor (1996) showed that supernatant of *P. communis* B19 inhibited *R. flavefaciens* FD1 growth on xylan but not on xylose. This suggests that the fungal inhibitory factors affect on xylanolytic enzymes. Weather the inhibitory factors produced by PNK2 are same as those of B19 or not, similar mechanism may be considered. Although the mechanisms have not been studied in this study, this kind of negative relations often occur on strain-specific basis (Miron and Ben-Ghedalia, 1993). Protein and peptides are often involved in such strain-specific negative interaction. Further research is needed to determine the mechanisms for the inhibitory interactions.

Although the potential for fungi to play a role in the energy nutrition of ruminants is clear, the fungal contribution to ruminant protein nutrition is less well known. The amino acid (AA) composition of anaerobic fungi have been determined (Gulati *et al.*, 1989a, b; Kemp *et al.*, 1985). The AA profiles of isolates were similar for fungi of different

genera with alanine and aspartic acid rich in fungal isolates *N. frontalis* and *P. communis* (Kemp *et al.*, 1985) and aspartic acid and glutamic acid rich in *Caecomyces* sp. NM1 and *Piromyces* sp. SM1 (Gulati *et al.*, 1989a). Gulati *et al.* (1989b) indicated that true digestibility of fungal proteins in sheep was *ca.* 0.9 which is higher than the value of 0.74 reported for total microbial protein and equivalent to that of high-quality milk protein (0.94). They suggested that an increased biomass of rumen fungi in sheep may result in increased quantities of essential and sulfur amino acids supply to the small intestine. No estimation is available the out flow rate of fungal mass from the rumen. This limits further discussion.

The present study has focused on some of the characteristics and ecological interestings of anaerobic fungi in concern with fiber degradation in the rumen. Anaerobic fungi play an important role for plant cell-wall degradation especially in the stem components. In arid areas in the world where arable land is limited, most farms should be used for production of human food (Sundstol, 1991). Crop by-products, for example rice or wheat straw, are a major component of the diets for ruminants in such areas. Such straws are high in fiber content and resistant to microbial degradation. The capacity of fungi in colonizing recalcitrant sclerenchyma portion of plant suggests that fungi have the potential to improve production in animals fed a low quality fibrous diet and their activities might be improved through modification of fungal population by inoculating highly cellulolytic species or strains as probiotics (Ha *et al.*, 1994), or by inoculating fungi that are not negatively affected by rumen bacteria.

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Appendix

List of microorganisms used in the present study.

Strain	Source	Obtained from	Chapter
Butyrivibrio fibrisolvens ATCC19171	Cattle rumen	NIAH *	3
Butyrivibrio fibrisolvens WV1	Cow rumen	AgR †	4
Fibrobacter succinogenes ATCC19169	Cattle rumen	NIAH	3,4
Prevotella ruminicola subsp. *brevis* 7-31	Cattle rumen	NIAH	3
Ruminococcus albus 7	Cattle rumen	NIAH	3
Methanobrevibacter smithii PS	Human gut	Dr. Bryant‖	4
Neocallimastix frontalis MCH3	Sheep rumen	INRA ‡	3
Neocallimastix frontalis PNK2	Sheep rumen	AgR	4
Neocallimastix sp. N1	Sheep rumen	KPU §	2
Piromyces communis 93-3	Sheep rumen	INRA	3
Piromyces communis B19	Cattle faeces	AgR	4
Piromyces communis 12A	Cattle faeces	AgR	4
Piromyces sp. P1	Sheep rumen	KPU	2, 3, 4
Caecomyces sp. B7	Bull faeces	AgR	4
Caecomyces sp. GT6	Goat faeces	AgR	4
Polycentric fungus KP1 (probably *Orpinomyces* sp.)	Sheep rumen	KPU	3, 4

* National Institute of Animal Health, Japan.
† AgResearch, New Zealand.
‡ Institut National de la Recherche Agronomique, France.
§ Kyoto Prefectural University, Japan.
‖ University of Illinois, USA.

Summary

Anaerobic fungi are now considered as one of principal components of fibrolytic microbial consortia in the rumen. Since forage based diets promote the development of zoosporic populations in the rumen, and anaerobic fungi are able to produce extracellular cellulase with high specific activity, anaerobic fungi are considered to have important roles in fiber digestion in the rumen. Indeed, removal of anaerobic fungi from the rumen ecosystem resulted in a significant decrease of acid-detergent fiber degradation. However, contribution of anaerobic fungi toward fiber degradation and their ecological significance in the rumen are still unclear. In the present study, experiments were conducted to understand physiological characteristics and ecology of anaerobic fungi in terms of fiber degradation in the rumen.

In chapter 2, fluctuation of fungal zoospores on agar strips were observed in the sheep rumen of animals fed three different level of dietary concentrate. The number of the zoospores on the strip was drastically decreased after morning feed with AF diet (Fig. 1-a). The number was the highest at 0 h (1.34×10^4 /cm^2), then declined to 2.0×10^3 /cm^2 at 9 h after feeding. The average ruminal pH of animals fed AF diet at 0 h was 7.1. Then the pH declined and was maintained at lower levels (6.8 - 6.9). The correlation between number of zoospores and pH was not apparent. In the rumen of animals fed MC diet, the number of zoospores decreased with time after feeding, although the rate of decrement was slower than that with AF diet. During 0 - 3 h after feeding, number of zoospores was 1.6×10^4 /cm^2. Although the number slightly decreased at 6 and 9h, the relatively high levels were maintained. It seems that the inducers for zoospore-release were maintained at relatively high concentration throughout incubation period. At 0 h, the average ruminal pH of animals fed MC diet was 6.7, then the values declined to 6.4. The fluctuation pattern of number of germinated zoospores was different in the rumen of animals fed HC diet from those of AF and MC diets. The number of zoospores was constantly maintained at lower level (1.0×10^3 /cm^2) than the other diets. The ruminal

pH was also placed at lower level (6.3 - 6.4) than the other diets. The numbers of zoospores at 0 h reflected the fungal population in the rumen.

For MC diet, continuous high number of zoospores may be due to the continuous release of zoospores by hemes in timothy hay and concentrate feed, and by unknown mechanisms. Unlike AF diet which promoted relatively rapid decline of zoosporogenesis, supplementation of concentrate feed to the timothy hay did not promote such rapid decline of zoosporogenesis. It was suggested that production of inducers for zoosporogenesis from concentrate feed persisted longer time than from timothy hay.

HC diet promoted the lowest zoospore production, suggested the lowest fungal population size in this experiment. The present results were compatible with those earlier studies.

Since concentration of propionate ranged between 10 to 15 mM in the rumen of animals fed HC diet, the propionate level did not affect fungal population. Accordingly the smaller number of germinated zoospores was due to smaller supply of ligno-cellulosic materials and probably to low pH in the rumen.

The larger number of zoospores that reflected larger fungal population size with MC diet might be caused by higher concentrate of proteins, microelements, vitamins and other nutrient. Moreover, several isolates could not grow on cellulose without cellobiose suggesting the importance of soluble sugars (Matsui and Ushida, unpublished observation).

In chapter 2, results show that an appropriate amount of concentrate may support fungal growth and stimulate zoosporogenesis in the rumen.

In chapter 3, activities of a range of plant cell-wall degrading enzymes produced by two anaerobic fungal isolates, *Neocallimastix* sp. N1 and *Piromyces* sp. P1, grown on five different carbohydrate substrates were determined fungal contribution on plant cell-wall degradation in the rumen.

Both fungal isolates were incubated on each Avicel, cellobiose, glucose, xylan and

xylose as a sole carbon source. *Neocallimastix* sp. N1 did not grow on Avicel and *Piromyces* sp. P1 did not grow on xylose as a sole carbohydrate growth substrate, respectively. These isolates produced extracellularly β-D-Glucosidase (EC 3.2.1.21), β-D-cellobiosidase (EC 3.2.1.91), β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), *endo*-glucanase (EC. 3.2.1.4), *endo*-xylanase (EC 3.2.1.8), naphthyl acetate esterase (EC 3.1.1.2) and naphthyl propionate esterase (EC 3.1.1.2) activities.

The production of cell-wall degrading enzymes were affected by carbohydrate growth substrates. When *Neocallimastix* sp. N1 was grown on xylan or xylose, most of the extracellular glycoside hydrolase activities were higher than those obtained from other growth substrates. While most of extracellular glycoside hydrolase activities was higher following the growth on cellobiose or Avicel, when *Piromyces* sp. P1 was examined. Xylan and xylose stimulated the specific activities of both these polysaccharide depolymerases of *Neocallimastix* sp. N1, while cellobiose and Avicel stimulated the specific activities of these enzymes from *Piromyces* sp. P1. When *Neocallimastix* sp. N1 was grown on cellobiose, both the extracellular naphthyl acetate esterase and naphthyl propionate activities were higher than those obtained from other growth substrates. While these esterase activities were higher following the growth on Avicel and cellobiose when *Piromyces* sp. P1 was examined.

Isolate *Neocallimastix* sp. N1 may depend more upon hemicellulosic substrates, while isolate *Piromyces* sp. P1 may depend more upon cellulosic substrates for their growth. Because no rumen fibrolytic bacteria are able to produce such a wide range of enzyme activities, anaerobic fungi may take advantages over the fibrolytic bacteria on cell-wall degradation in the rumen.

In chapter 4, the capacities of five rumen anaerobic fungi to degrade fresh perennial ryegrass (*Lollium perenne*) stem and leaf tissues in the presence of methanogen were studied to understand their roles of plant cell-wall degradation in the rumen of grazing animals. Traits in digestion of plant cell-wall components of timothy (*Phleum pratense*)

hay were also studied in pure cultures of ruminal fibrolytic microorganisms by comparing *in sacco* data to know fiber degrading characteristics of fibrolytic microorganisms.

Anaerobic fungi belonging to 3 genera, *Neocallimastix frontalis* PNK2, *Piromyces communis* B19, *Piromyces* sp. P1, *Caecomyces* sp. GT6 and B7, were co-cultured with *Methanobrevibacter smithii* PS on ryegrass internode and leaf. Internode tissue was more highly degraded than leaf blade for all fungus-methanogen co-cultures. Leaf tissue was degraded more quickly than stem tissue. No significant fungal growth occurred on leaf tissue as evidenced by the lack of CH_4 formation. *Piromyces* sp. P1 showed no activity against leaf tissue which contrasts with the other fungi yet this isolate was the most active against stem tissue. Present results suggest that rumen fungi belonging to *Neocallimastix*, *Piromyces* and *Caecomyces* genera have little activity against leaf tissue *in vivo* and that *Neocallimastix* and *Piromyces* species are likely to be more active than *Caecomyces* species at degrading fresh ryegrass stem tissues in the rumen.

Four strains of rumen bacteria, *Butyrivibrio fibrisolvens* ATCC 19171, *Fibrobacter succinogenes* ATCC 19169, *Prevotella ruminicola* subsp. *brevis* 7-31, and *Ruminococcus albus* 7, and four strains of anaerobic fungi, *Neocallimastix frontalis* MCH3, *Piromyces communis* 93-3, *Piromyces* sp. P1 and polycentric fungus KP1, were used in the experiment for plant cell-wall component degradation. Each microorganism was inoculated on ground timothy hay in an anaerobic medium and incubated at 39 ℃. Dry matter loss of timothy hay and digested xylan to digested cellulose (X/C) ratio of the substrate during incubation were determined. Degradabilities of timothy hay by *B. fibrisolvens*, *F. succinogenes*, *P. ruminicola*, *R. albus*, *N. frontalis*, *P. communis*, *Piromyces* sp. P1 and polycentric fungus were 8, 28, 16, 29, 60, 23, 13, and 28 % respectively. *In sacco* degradability of timothy hay was 40 % in the rumen. The X/C ratios in the cultures of *P. ruminicola* and *B. fibrisolvens* were greater than 0.5. Those of *R. albus*, *P. communis*, *Piromyces* sp. P1 and *N. frontalis* were between 0.3 and 0.5. Those of *F. succinogenes* and polycentric fungus KP1 were smaller than 0.3. The X/C

ratios observed *in sacco* were around 0.2 and close to those of *F. succinogenes*. The similarity of the digested X/C ratios between *F. succinogenes* and *in sacco* measurements suggests that *F. succinogenes* plays a predominant role in fiber digestion in the rumen. The present results suggest that the X/C ratio can be used as an index to characterize fibrolytic microorganisms.

The rumen microbial ecosystem containing populations of bacteria, protozoa and anaerobic fungi are able to breakdown the structural polysaccharides in plant cell-walls ingested by host animals. The degradative process enables the microbial populations to convert the insoluble plant cell-walls into volatile fatty acids (VFAs). Host animals absorb VFAs as energy sources. It is evident that microbial populations interact with each other during the degradation of plant cell-walls. However, the interactions are not completely understood. In chapter 5, interactions between anaerobic fungi and rumen bacteria on fiber degradation were examined.

When four anaerobic fungi, *N. frontalis* PNK2, *P. communis* 12A, *Piromyces* sp. P1 and polycentric fungus KP1 were co-cultured with *B. fibrisolvens* WV1, degradability of cellulose by those fungi were significantly inhibited ($p < 0.01$). Since addition of supernatant of *B. fibrisolvens* WV1 culture into *N. frontalis* PNK2 culture had no inhibitory effect on cellulose degradation and the presence of *B. fibrisolvens* WV1 cells inhibited fungal cellulolysis, inhibitory factor(s) is (are) shown to be cell-bound. To observe inhibitory effects of fungus on *F. succinogenes* degradation of cellulose, *N. frontalis* PNK2 and its culture supernatant were inoculated into *F. succinogenes*-cultures. The fungal supernatant inhibited cellulolysis by *F. succinogenes* significantly. This is the first observation of fungal inhibition of fibrolytic bacteria.

Since the presence of methanogen significantly ($p < 0.05$) enhanced cellulose degradation at *p*-coumaric acid (PCA) concentrations lower than 4 and 2 mM, respectively for *Neocallimastix* sp. N1 and *Piromyces* sp. P1, it is showed that the presence of methanogen increases fungal tolerance to PCA. There was significant

synergism between *Caecomyces* sp. B7 and *F. succinogenes* in ryegrass stem degradation. Stem degradability by a tri-culture of *Caecomyces* sp. B7-methanogen and *F. succinogenes* was twice as high as those of the individual cellulolytic microbes. In contrast, *F. succinogenes* did not significantly affect the extent of stem degradation by a *N. frontalis* PNK2-methanogen co-culture.

Thus, those results demonstrate that anaerobic fungi are able to produce a wide range of plant cell-wall degrading enzymes, and degrade plant cell-wall by interacting with rumen fibrolytic bacteria. And also, it is suggested that anaerobic fungi have important roles in stem degradation of fresh ryegrass.

Acknowledgments

I would like to express my sincere gratitude to Dr. Yoichi Kojima, Professor of Kyoto Prefectural University, for his valuable advice in preparation of this thesis.

I am indebted to Dr. Kazunari Ushida, Associate Professor of Kyoto Prefectural University, for his scientific advice, discussion and encouragement throughout complete my thesis. I also appreciate Dr. Ushida for advice to research overseas.

I am grateful to Dr. Kouji Miyazaki, for his helpful advice and encouragement on my research.

I should like to thank all students in Laboratory of Animal Science, Kyoto Prefectural University for their help.

I would like to express great appreciation to Dr. Keith N. Joblin, Senior Scientist at AgResearch in New Zealand, for his admission to research at AgResearch, encouragement at work, critical advice for this thesis.

I would like to appreciate Mr. Graham Naylor, technician at AgResearch, for his technical advice in experiments.

Dr. Graeme Jarvis, Dr. Andrew Hudson, Dr. Graeme Attwood, Dr. Ming Huang, Ms. Kerri Reilly, Ms. Dianna Chang, other staffs of Laboratory of Rumen Microbiology at AgResearch, are appreciated for their scientific advice and help in research during my stay in New Zealand.

I would like to thank Ms. Ann Ainscough, a librarian at AgResearch, for her kind help for searching books and her advices for New Zealand life. Other staffs at AgResearch are also appreciated for their kind advice and help. Because life in New Zealand is my first experience living in overseas, I suffered a lot of cultural differences. I could not complete my stay in New Zealand without their help. I would like to appreciate every person who helped me again.

I would like to thank Dr. Hajime Minato (Ibaraki University, Ibaraki, Japan. Formerly in National Institute of Animal Health, Tsukuba, Japan), Dr. Gerald Fonty and Ms Liliane Millet (INRA, Clermont-Ferrand-Theix, St.Genès-Champanelle, France) for their kind supply of microorganisms. I thank Dr. Tsugio Watanabe, Yakult Central Institute for Microbiological Research, Kunitachi, for his valuable suggestions on the glycosidase assay.

Part of work in chapter 2 was financially supported by the Ito Foundation and traveling expense for New Zealand was supported by the Nagai foundation. I would like to express my appreciation to both foundation.