Mycoscience 35: 199–211, 1994

**Review**

# The nature of anaerobic fungi and their polysaccharide degrading enzymes

**Scott Borneman[1] and D. E. Akin[2]**

[1] Genencor International, Inc. 180 Kimball Way, South San Francisco, California, 94080, U.S.A.
[2] Agricultural Research Service, U.S. Department of Agriculture, Athens, Georgia 30613, U.S.A.

Accepted for publication 19 March 1994

## I. Discovery and taxonomy

Before the discovery of anaerobic fungi, it was believed that cellulolytic bacteria were primarily responsible for the degradation of plant biomass in the rumen environment. Anaerobic fungi that colonize and ferment plant fiber have only recently been shown to be an indigenous part of the rumen ecosystem (Orpin, 1975, 1976, 1977b; Bauchop, 1979). However, the zoospores of the species had been observed many years previously by microbiogists who reported small flagellated microorganisms in rumen liquor. These flagellated organisms, smaller than the readily identified ciliate protozoa, were all believed to be flagellate protozoa, and descriptions were published by Liebetanz (1910) and Braune (1913) of several distinct morphological types. Liebetanz (1910) described four uniflagellate organisms and assigned them to four genera, *Cercomonas, Oikomonas, Sphaeromonas* and *Piromonas. Sphaeromonas* and *Piromonas* were similar except for the larger size and more elongate shape of *Piromonas* (Leibetanz, 1910). Braune (1913) described these genera in greater detail and suggested that the four genera were synonymous.

Weissenberg (1912) described a multiflagellate organism that was parasitic on freshwater copepods and named it *Callimastix cyclopis* Weissenberg. Subsequently, new species of multiflagellated organisms that were believed to be *Callimastix* were isolated. Braune (1913) isolated *C. frontais* from the rumen, and Hsuing (1930) isolated *C. equi* from horse cecum. In 1950 it was suggested that *C. cyclopsis* might be a zoospore of a primitive fungus (Weissenberg, 1950), but Kudo (1954) placed the genus *Callimastix* in the family Callimastigidae of the protozoan order Polymastigina.

Vavra and Joyon (1966) reported that *C. cyclopsis* flagellates were zoospores from the vegetative stage of a zoosporic fungus. Ultrastructural studies showed that these flagellates had characteristics similar to zoospores of the Blastocladiales. The genus *Callimastix*, with *C. cyclopsis* as the type species, was, therefore, transferred to the order Blastocladiales (Vavra and Joyon, 1966). A new genus, *Neocallimastix* Vavra & Joyon, with *N. frontalis* Vavra & Joyon and type species, was created to accommodate the three other species of the former genus *Callimastix* (Vavra and Joyon, 1966).

Little attention was given to these organisms until Warner (1966) measured diurnal fluctuations of the population densities of rumen microorganisms in sheep and observed that motile flagellated cells, similar to *Callimastix frontalis*, significantly increased in density one hour after feed ingestion by the animal. Orpin (1974) also examined this fluctuation in population and found a considerably greater variation than reported by Warner, with the peak population density of multiflagellated cells occurring 15-30 min after the animal had eaten. further studies showed that the high population density of flagellates could be duplicate in vitro by treating large digesta fragments, centrifuged from the rumen fluid, with an aqueous extract from oats. This population increase suggested that either the flagellates sequenstered on or within the plant fragments or that a multiple reproductive phase, such as a sporangium, was present in the life cycle. Both possibilities were novel for rumen microorganisms.

Orpin first succeeded in culturing one of these polyflagellated organisms under anaerobic conditions by initially following the culture methods of Jensen and Hammond (1964) for the flagellated protozoa. Further studies of cultures revealed that the motile flagellates were released from sporangia that resembled those of the zoosporic fungi. Microscopic evaluation revealed that the multiflagellated organisms, originally considered to be protozoa, were zoospores produced from the zoosporangium of *N. frontalis* (Orpin, 1975). Heath et al. (1983) validated the genus *Neocallimastix* and assigned it to a new family Neocallimasticaceae in the order Spizellomycetales of the class Chytridiomycetes. Since then, the original isolate that was used to establish *Neocallimastix* as a zoosporic fungus has been renamed *N. patriciarum* Orpin & Munn (Orpin and Munn, 1986).

The fungal nature of these organisms was strongly indicated by the morphology of the vegetative stage, and the life cycles were similar to known species of zoosporic fungi and particularly to Chytridiomycetes. The

demonstration that the cell walls of these species contained chitin (Orpin, 1977b) and that chitin synthetase was produced (Brownlee, 1986) gave further evidence that these organisms were true fungi, despite their being strict anaerobes. The discovery of obligately anaerobic fungi has raised questions about many accepted concepts of mycology and rumen microbiology. Until these fungi were discovered, fungi were regarded as being aerobes or facultative anaerobes, and studies on the rumen ecosystem did not take into account anaerobic fungi (Hungate, 1966).

The four major groups of zoosporic fungi are placed in the subdivision Mastigomycotina of the Kingdom of Fungi (Eumycota). Based on morphological grounds the anaerobic fungus *Neocallimastix* was assigned to the Chytridiomycetes (Heath et al., 1983). The Chytridiomycetes are thought to be the ancestral group for higher fungi which are subdivided into four orders: the Basocladiales, Monoblepharidiales, Chytridiales and Spizellomycetales (Barr, 1980). Based on ultrastructural characteristics of the zoospores anaerobic fungi were assigned to the order of the Spizellomycetales in a new family, the Neocallimasticaceae (Barr, 1988). Gold et al. (1988) suggested the subdivision of this family into three genera containing monocentric species, *Neocallimastix, Piromyces* (previously *Piromonas*) and *Caecomyces* (previously *Sphaeromonas*). Subsequently, three polycentric genera have been described namely, *Orpinomyces* (Barr et al., 1989) and *Ruminomyces* (Ho et al., 1990), and *Anaeromyces* (Breton et al., 1990). Recently, Munn and Orpin (1993) erected a new family, Anaeromycetales, to encompass the anaerobic fungi due to the evidence accumulated on the variable morphology, uniform possession of a hydrogenosomes, and novel fine structural features.

To date, sixteen species of anaerobic fungi have been described and classified (Table 1). *Neocallimastix joyonii* does not conform to the proposed generic description and can probably be assigned to the *Orpinomyces* genus. Furthermore, *Anaeromyces mucronatans* belongs probably to the *Ruminomyces* genus. For definitive classification of these fungi, additional comparative morphological research is needed.

## II. Distribution in nature and growth characteristics

Taxonomy of anaerobic fungi is still relatively primitive as only morphological and fementation characteristics have been used. More refined criteria (e.g. sRNA, isoenzyme pattern analysis) is required to supplement these characteristics for taxonomic definition.

The distribution of these strictly anaerobic fungi appears to be limited to the gut of herbivores. Anaerobic fungi have been found in gut contents of herbivorous mammals from wide-ranging geographical locations (Bauchop, 1979, 1980). They have been isolated from foregut fermenters, such as the ruminants cattle, red deer, impala (Bauchop, 1980, 1983; Kudo et al., 1990) as well as from ruminant-like animals such as grey kangaroo, wallaroo, swamp wallaby (Bauchop, 1980) and llama (Mile et al., 1989). Further, anaerobic fungi have been isolated from faecal samples of the ass (Breton et al., 1991), horse (Orpin, 1981), elephant, rhinoceros and zebra (Mile et al., 1989; Teunissen et al., 1991a), and mara (South-American rodent, Teunissen et al., 1991a) which are all hindgut fermenters. Orpin and Joblin (1988) have extensively sampled terrestrial and aquatic environments such as lake and river sediments and sewage treatment plants for anaerobic fungi of the type in herbivores and found none.

All rumen fungal species studied to date grow in a narrow temperature range (33-41 °C) and are strictly anaerobic. As a result of active fermentation, conditions in the rumen are highly anaerobic with a redox potential

Table 1. Anaerobic fungal species currently classified.

| Genus | Species | Host | Reference |
|---|---|---|---|
| *Neocallimastix* | *N. frontalis* | sheep | Health et al. 1983 |
| | *N. hurleyensis* | sheep | Webb & Theodorou 1992 |
| | *N. patriciarum* | sheep | Orpin & Munn 1986 |
| | *N. variabilis* | cattle | Ho et al. 1993 |
| *Caecomyces* | *C. Communis* | sheep | Orpin 1976 |
| | *C. equi* | horse | Gold et al. 1988 |
| *Piromyces* | *P. communis* | sheep | Orpin 1977 |
| | *P. dumbonica* | elephant | Li & Heath 1990 |
| | *P. mae* | horse | Li & Heath 1990 |
| | *P. munutus* | deer | Ho et al. 1993 |
| | *P. spiralis* | goat | Ho et al. 1993 |
| *Orpinomyces* | *O. bovis* | cattle | Barr et al. 1989 |
| | *o. joyonii*[1] | sheep | Breton et al. 1989 |
| *Ruminomyces* | *R. elegan* | cattle | Ho & Bauchop 1990 |
| | *R. muconatans*[2] | cattle | Breton et al. 1990 |

[1] Originally *Neocallimastix Joyonii.*
[2] Originally *Anaeromyces mucronatans.*

($E_h$) of between −300 to −350 m (Latham, 1980). The absence of cytochrome, menaquinone and mitochondria dictates that the fungi completely depend on fementation processes for energy production. Bicarbonate and phosphate salts in saliva of ruminants provide buffering capacity and maintain a pH of 6-7 for forage-fed animals.

Microorganisms are present in the rumen in the liquid phase of digesta contents associated with plant fragments, and as a lining on the rumen epithelium (Lathum, 1980). Bacteria in the rumen fluid may be present at concentrations of $10^9$-$10^{10}$/ml whereas protozoal populations range from $10^5$-$10^6$/ml (Hungate, 1966). A value for the population density of anaerobic fungi, within the range of $10^3$-$10^5$/ml, has been given by Orpin and Joblin

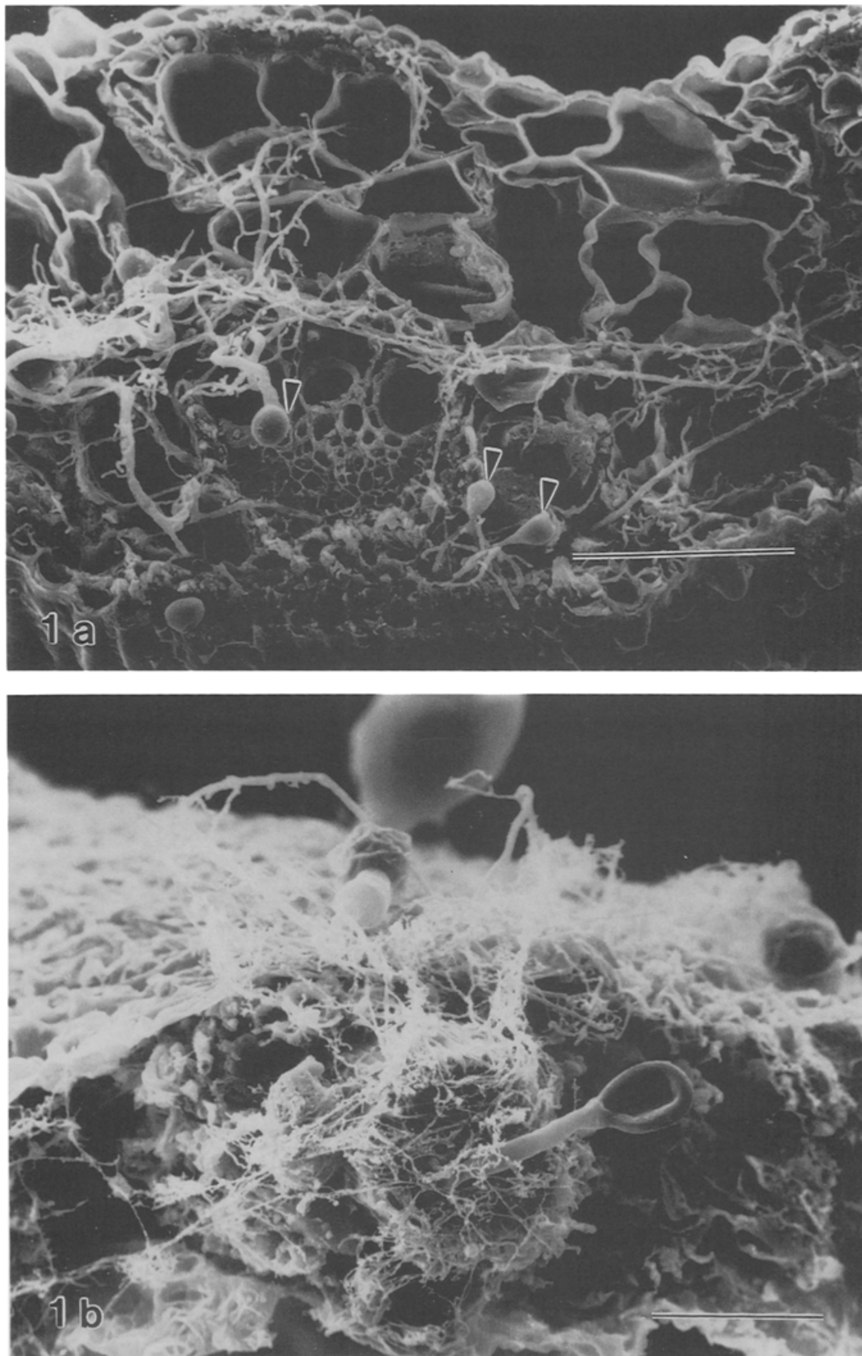


Fig. 1. Scanning electron micrographs of colonization of grass leaf blade by a *Neocallimastic* MC 2, a monocentric anaerobic fungus. Bar=50 $\mu$m. A. Initial colonization (24 h) with germinating zoopores (arrows) and developing rhhizoids is evident on mostly undegraded plant tissues. B. Colonization for 7 days results in extensive degradation of plant tissues and some sporangia and rhizoids still present.

(1988), although the conventional enumeration procedures used by these workers may not be suitable for estimating fungal populations (Joblin, 1981; Theodorou and Trinci, 1989).

## III. Life cycles

Monocentric anaerobic fungi have asexual life-cycles which occur both in vitro and in vivo. The posteriorly flagellated motile zoospore contacts a suitable substrate, typically a fragment of forage fiber, and sheds its flagella. A rhizoid develops, and a cell-wall is synthesized around the cell-body. The rhizoids develop on plant fiber and anchor the organism to cell walls; in this process of development, plant cell walls are degraded which provide energy for growing fungi (Fig. 1). The rhizoids penetrate the substrate and the cell body enlarges by synthesis of organelles and cytoplasm accompanied by multiple round of mitosis (Gold et al., 1988; Orpin, 1988). After a variable degree of cell body enlargement, zoosporogenesis can be induced. The cell body becomes the zoosporangium (Fig. 2) wherein all of the nuclei and cytoplasm are cleaved in 1 to 114 uninucleate zoospores (Heath et al., 1983; Orpin, 1976). Zoospores are released from the sporangium by dissolution of the sporangial wall (Fig. 3) and the cycle is complete (Heath et al., 1983). Some species of anaerobic fungi are known to produce oxygen-resistant forms that maintain viability in feces for several months (Wubah et al., 1991). Wubah further isolated species of *Neocallimasix, Piromyces, Orpinomyces* and *Caecomyces* from both fresh and dry faeces of a cow. Each of these isolates produced a melanized resting stage in vitro and similar melanized sporangia were observed in the faecal smears from which they were isolated. Dietary contamination by faeces in common and may be an important means for transfer of fungi between individual ruminants.

The polycentric fungi are not dependent on zoospore production for their propagation as their growth pattern resembles that of the higher fungi: fungal growth is propagated by hyphae (Ho et al., 1990). Often colonization of plant fiber occurs without development of sporangia (Fig. 4), and zoospores are produced infequently or zoosporogenesis is even absent (Phillips, 1989). Some polycentric anaerobic fungi form zoospores readily and abundantly in certain media, but the majority of the sporangia do not differentiate and release zoospores. The development of sporangia occurs laterally or terminally at hyphae. Sporangia of *Orpinomyces* can be branched (Barr, 1980), whereas those of *Ruminomyces* are unbranched.

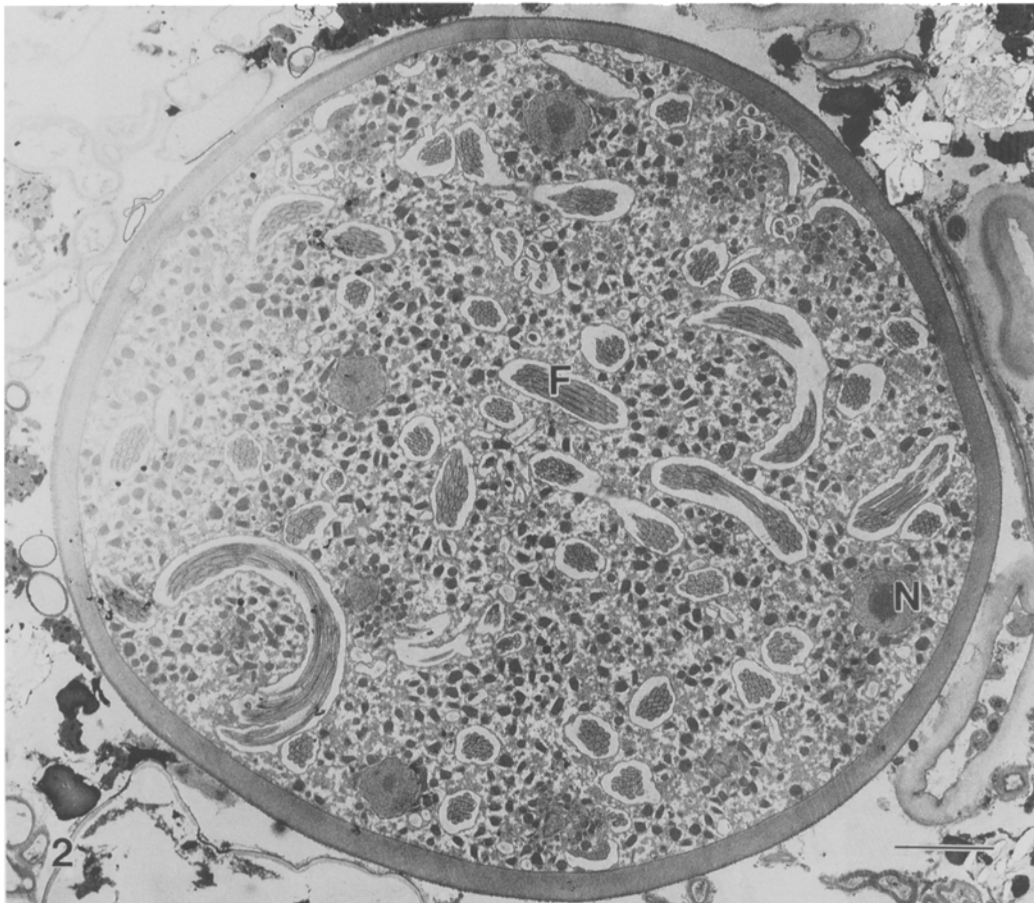


Fig. 2. Transmission electron micrograph of sporangium within a leaf blade with developing components of zoospores including flagellar bundles (F) and nuclei (N). Bar=0.5 $\mu$m. (From Akin et al., 1983).

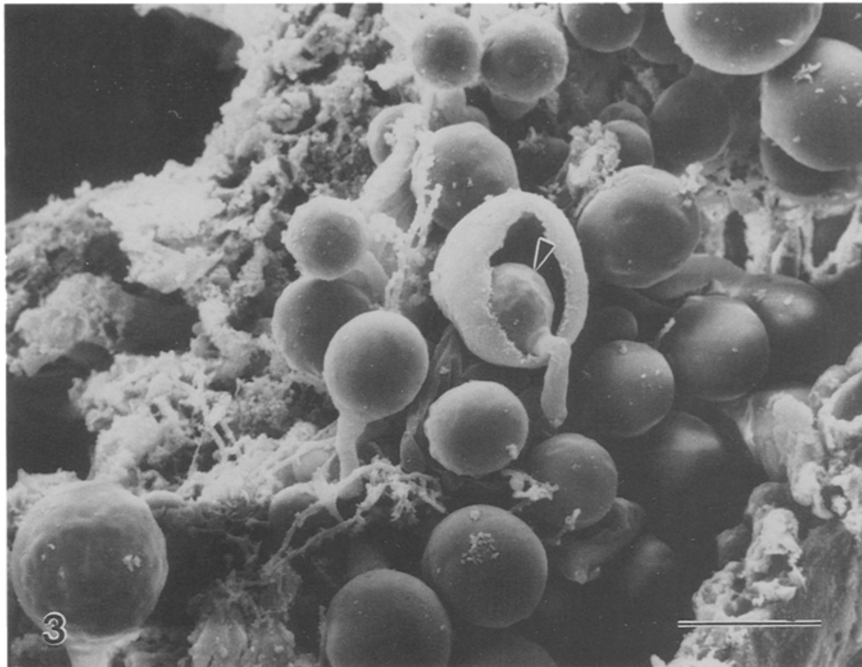

Fig. 3. Scanning electron micrograph of stem with sporangia, one of which appears to have partially dissolved with a germinating zoospore within (arrow). Bar = 25 μm.

In monocentric fungi zoosporogenesis and liberation of zoospores from the sporangia occur soon after the animal is fed. With *Neocallimastix*, a peak in the population density of zoospores was found 15–30 minutes after feeding (Orpin, 1974, 1975), but with *Caecomyces* and *Piromyces* the peak occurred about 1 h after feeding (Orpin, 1976). Zoosporogenesis in *Neocallimastix* was induced by a water-soluble component of the diet (Warner, 1966; Orpin, 1974) and, subsequently, hemes were shown to induce zoosporogenesis (Orpin and Greenwood, 1986a). Hemes in chemically oxidized or reduced form are present in all living plant tissues as enzyme prosthetic groups, and oxidized forms occur in dead plant tissues. Therefore whenever the animal eats, hemes enter the rumen. To our knowledge, other potential zoospogenic factors have not been elucidated.

Free zoospores of *Neocallimastix* showed a chemotactic response to soluble carbohydrates (Orpin and Bountiff, 1978). Soluble carbohydrates present in freshly ingested plant tissues diffuse into the liquor, and *Neocallimastix* zoospores possibly locate these plant fragments by migrating up soluble carbohydrate-gradients to damaged surface regions and plant stomata. Four chemoattractants identified were glucose, fructose, mannitol, and mannose, and low concentrations of mixtures of glucose, sucrose and fructose resulted in a synergistic response. This synergism could be responsible for specific tissue location by the zoospores. Chemotactic response was very sensitive, being elicited by as little as 1 μm sucrose. After zoospores located plant tissue, attachment, encystment and germination occurred, followed by the penetration of the plant tissues by the fungal rhizoidal or rhizomycelial system and subsequent growth of the sporangium. From in vitro studies, it appears that the zoospore, still bearing flagella, adopts an amoeboid stage and may move across the surface, presumably to locate exactly the right site for encystment. Microscopic studies have indicated that certain lignified tissues appear to be preferentially colonized by anaerobic fungi, but chemotactic factors related to this phenomenon are not known.

From in vivo and in vitro studies, the life cycle of *Neocallimastix* sp. has been determineed to be about 24–32 h (Joblin, 1981; Bauchop, 1983; Lowe et al., 1987a), although under appropriate conditions zoosporogenesis may occur as early as 8 h after encystment (Orpin, 1977b). In continuously fed animals the life cycle may, therefore, be as short as 8 h.

## IV. Nutritional requirements

Representative organisms of each genus are routinely grown in a semi-synthetic medium developed by Caldwell and Bryant (1966), consisting of the following: mineral solutions, $Na_2CO_3$, volatile fatty acids, hemin, trypticase, yeast extract, a carbon source, and cysteine-$Na_2S$ reducing agent. Precise nutritional requirements are, in general, undetermined for these fungi, and only *N. patriciarum* has been grown in a chemically defined medium (Orpin and Greenwood, 1986b). Minimal nutritional requirements for growth of *N. patriciarum* on cellobiose in a $CO_2$ atmosphere were satisfied by the provision of heme, D-biotin, thiamine or its precursors, ammonium ions, and reduced sulfur and trace elements. Growth was stimulated by amino acids, straight and branched short-chain volatile fatty acids, low concentrations of long-chain fat-

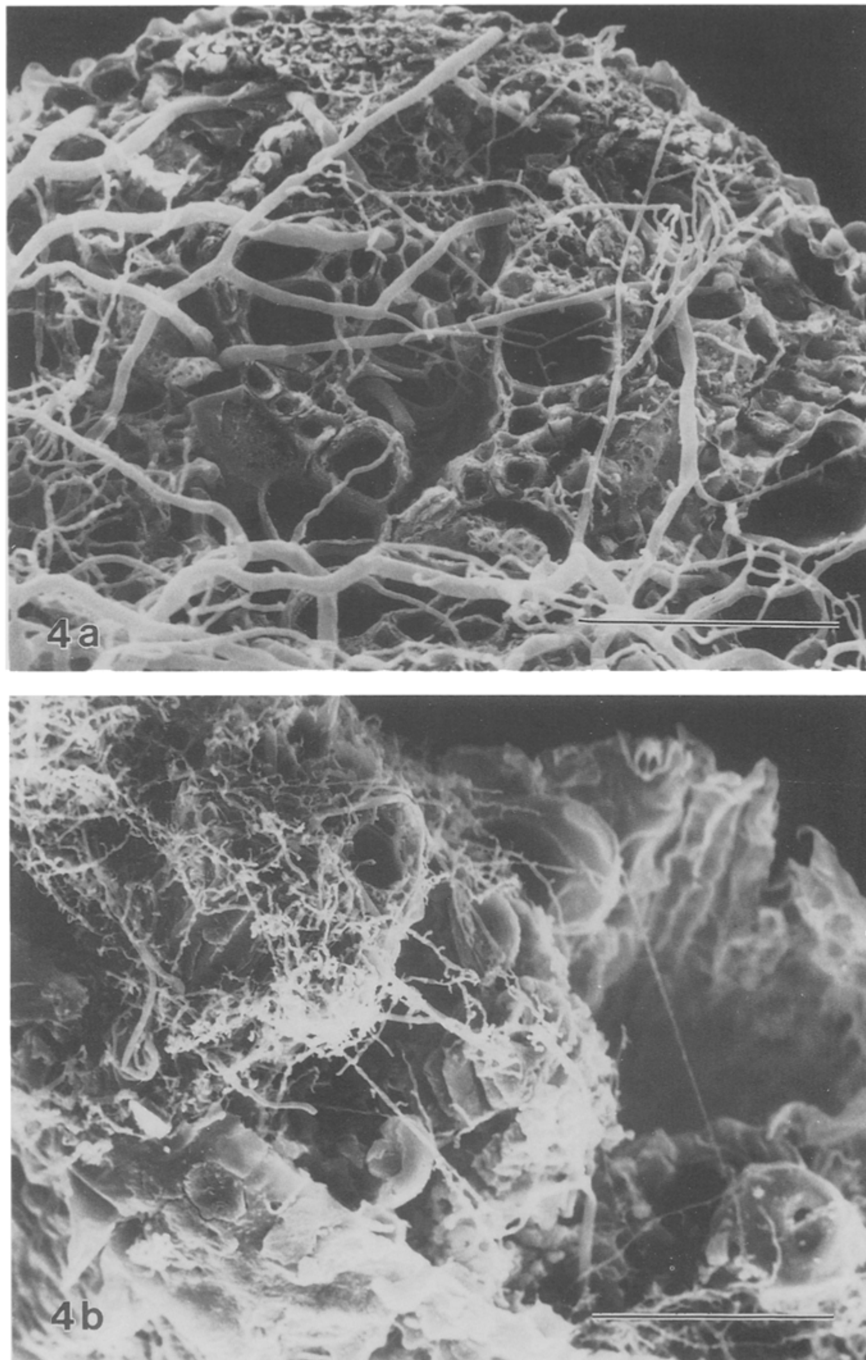


Fig. 4. Scanning electron micrographs of colonization of grass leaf blade by a *Orpinomyces* PC 3, a polycentric anaerobic fungus. Bar = 50 $\mu$m. A Initial colonization (24 h) with extensive rhizomycelium over mostly undegraded plant tissues and lack of sporangia. B. Colonization for 7 days results in extensive degradation of plant tissues and some rhizomycelium on a vascular bundle.

ty acids and a number of vitamins. Germination was stimulated by acetic acid and soluble, fementable carbohydrates. Hemes appear to play a major role in both the nutrition and zoosporogenesis of rumen fungi (Orpin and Greenwood, 1986a).

## V. Intermediary metabolism

The major fementation products from carbohydrate catablism are acetate, formate, lactate, ethanol, hydrogen and carbon dioxide (Bauchop and Mountfort, 1981; Orpin and Munn, 1986; Lowe et al., 1987b; Borneman et al., 1989). Traces of succinate have also been detected in some cultures (Phillips and Gordon, 1988; Borneman

et al., 1989). Variations in the proportions of each product occurred for different strains and culture conditions.

In *N. patriciarum* the Embden-Meyerhof pathway appears to be the major glycolytic pathway for catabolism of carbohydrates (Yarlett et al., 1986). This fact is supported by the distribution of $^{14}CO_2$ produced by cultures fermenting glucose which had been labelled in the C-1, C-2 and C-6 positions (Orpin and Joblin, 1988).

Glycolysis generates phosphenolpyruvate, which is converted via oxaloacetate into malate. Hydrogenosomal enzymes generate energy from the oxidation of malate to pyruvate, which is coupled via NADPH: ferridoxin oxidoreductase, ferridoxin and hydrogenase to $H_2$ production. Some of the pyruvate generated is converted to acetyl-CoA via pyruvate: ferridoxin oxidoreductase and subsequently into acetate. Lactate dehydrogenase is responsible for lactate generation and, when cultures are grown in a $N_2$ rather than in a $CO_2$ atmosphere, low levels of alcohol dehydrogenase convert acetaldehyde into ethanol (Yarlett et al., 1986). Carbon dioxide appears to suppress acetate generation as well as ethanol production, perhaps by end-product inhibition of the pyruvate: ferridoxin oxidoreductase and pyruvate decarboxylase reactions, both which generate $CO_2$.

Studies with *N. frontalis* EB188 indicate a similar glucose metabolic pathway, but the metabolism of this organism differed in several important aspects from that of *N. patriciarum* (O'Fallon et al., 1991). Namely, the phosphoenol pyruvate carboxykinase and pyruvate kinase reactions reported by Yarlett et al. (1986) for *N. frontalis* are not likely to occur in the direction stated, the latter in fact constituting a futile cycle with its direction in glycolysis. In place of phosphoenolpyruvate carboxykinase, O'Fallon et al. (1991) found pyruvate carboxylase, which carboxylates pyruvate to form oxaloacetate.

Little information if available concerning formate metabolism. *Neocallimastix patriciarum*, which does not produce high levels of formate, lacks formate dehydrogenase and formate hydrogen lyase (Yarlett et al., 1986). Formate is the major end-product for *Neocallimastix* MC 2 (Borneman et al., 1989) and has been used as an index of growth in another strain of *Neocallimastix* sp. (Lowe et al., 1987).

The pattern of fermentation products is markedly affected by the presence of methanogenic bacteria (Mountfort et al., 1982; Bauchop and Mountfort, 1981; Williams et al., 1993) that can utilize the hydrogen and formate produced by the fungus. Removal of these fermentation products often results in increased substrate utilization and increased growth rate of the fungus with a concomitant increase in production of carbohydrate degrading enzymes. The close proximity of all microorganisms in vivo would ensure that much interspecies transfer of fermentation products would occur. Hydrogen and formate would be rapidly utilized by the surrounding microflora, as would the D(-)lactate, succinate, and ethanol. Culture of anaerobic fungi with methanogens often increases the rate of degradation of plant cell wall structural carbohydrates (Williams et al., 1993).

## VI. Carbohydrate utilization

A wide range of mono-, di-, and polysaccharides support growth of all species examined (Orpin, 1975, 1976, 1977b; Bauchop, 1980; Mountfort and Asher, 1983; Phillips and Gordon, 1988, Borneman et al., 1989), but differences among strains are evident with respect to the utilization of carbohydrates. Phillips and Gordon (1988) compared 17 isolates of rumen fungi for their ability to utilize a total of 40 carbohydrates as the sole source of fementable carbon. All the fungi utilized the monosaccharides D-fructose and D-glucose, and the disaccharides D-cellobiose, gentiobiose and lactose. None of the fungi utilized: i) monosaccharides, D- or L-arabinose, L-fucose, D-galactose, D-mannose, L-rhamnose, D-ribose, L-sorbose, ii) sugar alcohols, adonitol, dulcitol, *meso*-erythritol, *meso*-inositol, D- mannitol, D-sorbitol, xylitol, iii) sugar acids, sodium D-galacturonate, sodium D-glucuronate, iv) disaccharides, D-melibiose, D-trehalose, and v) polysaccharides, arabinogalactan, chitin, pectin or sodium polygalacturonate. The failure of any anaerobic fungus to ferment common plant carbohydrates such as arabinose, galactose, mannose and pectin is so far unexplained. A total of 11 sugars and polysaccharides (i.e., xylose, maltose, raffinose, sucrose, cellulose, glycogen, inulin, pullulan, pustulan, starch, and xylan) gave different patterns of utilization by isolates representative of *Neocallimastix* spp., *Sphaeromyces* spp. and *Piromyces* spp. (Phillips and Gordon, 1988). The patterns of utilization of sugars and polysaccharides by strains, classified on morphological grounds as *Neocallimastix* spp., were virtually identical (Gordon and Phillips, 1989a). Sugar and polysaccharide utilization were identical in three strains of *Sphaeromyces*, but isolates of *Piromyces* exhibited a variety of patterns for carbohydrate utilization (Gordon and Phillips, 1989a). *Neocallimastix frontalis* has been shown to grow actively on cellobiose, D-fructose, D-xylose, maltose, sucrose, and glucose, giving growth yields of 40-50 mg dry wt. mmol of hexose$^{-1}$ and 20 mg mmol pentose$^{-1}$ (Mountfort and Asher, 1983).

Studies on the utilization of paired substrates indicated that the uptake of fructose and xylose was inhibited by glucose by a catabolic regulatory mechanism (Mountfort and Asher, 1983). Chemotaxis of zoospores of *N. patriciarum* to soluble carbohydrates occured with compounds such as mannose, sorbose, sorbitol, and fucose; these carbohydrates were not utilized as carbon sources (Orpin and Bountiff, 1978). *Neocallimastix* sp. preferentially utilized cellobiose and sucrose over fructose and glucose, respectively (Mountfort and Asher, 1983).

## VII. Polysaccharide degrading enzymes

From the earliest studies of the anaerobic fungi, observers noted the marked attack on plant fiber (Orpin, 1977a; Bauchop, 1979; Akin et al., 1983). The anaerobic fungi produce a wide array of enzymes that can hydrolyse a range of glycosidic likages (Williams and Orpin, 1987b), digest the major structural poloysaccharides of plant cell

walls (Pearce and Bauchop, 1985; Wood et al., 1986; Williams and Orpin, 1987a, Lowe et al., 1987b, c; Borneman et al., 1989, 1990a), and enable the fungi to grow on a number of polysaccharides (Orpin and Letcher, 1979; Mountfort and Asher, 1983). Many of the polysaccharide hydrolysing enzymes are produced by rhizoids and rhizomycelia (vegetative stage) and are also excreted into the culture medium (Williams and Orpin, 1987b; Lowe et al., 1987b). It has also been reported that the zoospores of anaerobic fungi produce polysaccharide degrading enzymes (Williams and Orpin, 1987b).

All species studied to date utilize cellulose, xylan, starch, and hemicellulose (Orpin and Letcher, 1979; Bauchop, 1983; Orpin and Munn, 1986; Williams and Orpin, 1987a; Borneman et al., 1989). However, few isolates have been found to grow on pectin even though very low levels of pectin-hydrolysing enzymes were produced in culture media (Williams and Orpin, 1987a), and pectin was lost from plant fragments that were digested by isolates of rumen funti (Orpin, 1983/84). More recently, Gordon and Philips (1992) reported on the production of an endo-acting pectin lyase by *Neocallimastix* strain LI1 growing on pangola grass/pectin containing medium.

Rumen fungi degrade the lignin-containing plant walls and colonize such tissue preferentially (Bauchop, 1979; Akin and Rigsby, 1987; Akin and Benner, 1988). Consequently, studies have been undertaken to assess the ability of the fungi to attack the phenolic components of plant walls, with antibiotics included in the media to select microbial groups. These groups were tested for ability to release phenolics or carbohydrates in $^{14}C$ labelled lignocellulose of highly lignified cordgrass (Akin and Benner, 1988, Gordon and Philips, 1989). Results indicated that the mixed fungal population did not convert lignin to $CO_2$ but solubilized phenolics from the fiber. Similarly, in studies of specifically $^{14}C$ labelled oat plants degraded by 18 pure cultures of monocentric rumen fungi (Gordon and Philips, 1989a), 30 to 38% of the initial radioactivity in phenolics was solubilized, but negligible radioactive $CO_2$ was produced. Results so far obtained indicate that rumen fungal attack on lignocelulose results in solubilization but not in the mineralization of phenolic components comprising lignocellulose.

Both monocentric and polycentric species are able to extensively degrade plant cell walls (Figs. 1, 4). Lignified walls are entirely or partially degraded, depending on the particular lignocellulosic type (Akin and Rigsby, 1987). The result of this extensive attack on the lignocellulose is a substantial loss of textural strength (Akin et al., 1989). The anaerobic fungi are better able to weaken the physical structure of plants than are the fiber-digesting rumen bacteria (Borneman and Akin, 1990), and this phenemenon may be significant in feed intake and feeding characteristics of animals.

**A. Cellulases** Anaerobic fungi produce cellulases necessary for solubilizing both amorphous and highly ordered celluloses present in plant fiber. High levels of endo-β-1,4-glucanase (CMCase) were released into culture supernatants by *N. frontalis* (Mountfort and Asher, 1985; Wood et al., 1986) *Piromyces* sp., *Sphaeromyces* sp. (Williams and Orpin, 1987a), and *Orpinomyces* sp. (Borneman et al., 1989). Exoglucanase active against microcrystalline cellulose (Avicel) was also detected but at lower levels than CMCase (Mountfort and Asher, 1985; Borneman et al., 1989). To date, the fungi are the only rumen microorganisms reported than can solubilize the most resistant form of cellulose known i.e., cotton fiber. A *N. frontalis* strain produced extracellular exoglucanase(s) capable of solubilizing highly resistant cotton fiber as well as Avicel (Wood et al., 1986). Partial hydrolysis of celulose yields cellodextrins, and culture supernatants of *N. frontalis* have been shown to contain a cellodextrinase with a greater specific activity than CMCase. Avicelase, endoglucanase, and β-glucosidase activities of *N. frontalis* had pH and temperature optima of 5-7 and 37-50°C, respectively (Mountfort and Asher, 1985; Williams and Orpin, 1987a). Endoglucanase production was maximum when the fungus was grown on cellulose while synthesis was totally repressed by the addition of glucose, indicating that the enzyme was subject to regulation (Mountfort and Asher, 1985). The combination of extracellular exocellulase, endocellulase, and cellodextrinase gives fungi the capacity to degrade cellulose into cellobiose, which can then be fermented.

Studies by Wood et al. (1988) on *N. frontalis* suggested that the endoglucanse, β-glucosidase, and another factor (thought to be responsible for crystalline cellulose hydrolysis, i.e., exoglucanase) exist in solution as a multicomponent enzyme complex. Activity towards Avicel was associated almost entirely with the high molecular weight enzyme complex (Wood et al., 1988). The complex had an exceptional capacity for solubilizing cotton fiber, i.e., 80% in only 48 h. In comparison, a sample of the complete cellulase system of *Trichoderma reesei* C30 containing the same amount of endoglucanase solubilized only about 20% of the cotton fiber in the same time (Wood et al., 1988). Recently, Wilson and Wood (1992b) isolated a crystalline cellulose solubilizing factor from *Neocallimastix frontalis*, which resided in a high molecular mass complex of 670 kDa, that comprised a number of subunits ranging in size from 68 to 135 kDa. The isolated component showed endoglucanase and β-glucosidase activity and hydrolyzed crystalline cellulose to glucose as the sole product.

Seven carboxymethyl cellulases and six avicelases have been identified in supernatant fluids of cultures of *Neocallmastix frontalis* EB188 (Li and Calza, 1991a). Molecular weights ranged from 116 kDa to 19 kDa. The relationship between these cellulases is not clear since differing degrees of glycosylation (Li and Calza, 1991b) and possible proteolytic action by proteases secreted into the medium (Wallace and Joblin, 1985) may indicate a higher number of cellulases than are actually synthesized.

A majority of the anaerobic fungal enzymes studies are glycosylated; although glycosylation is not necessary for activity, it is apparently important for excretion (Orpin, 1993). Nonglycosylated enzymes may have modified substrate specificties (Li and Calza, 1991b).

The ability of *Neocallimastix* cellulases to retain activity when not glycosylated has enabled a range of plant cell-wall hydrolyzing genes to be isolated from a cDNA library prepared from *N. patriciarum* mRNA (Xue et al., 1992, 1993a, 1993b; Gilbert et al., 1992). Some of the recombinant genes isolated from this library encoded cellulases and xylanases containing more than one active site and some have active sites with multiple substrate specificities (Xue et al., 1992, 1993, 1993b; Gilbert et al., 1992).

Five different cellulase genes have been cloned from *N. patriciarum* (Xue et al., 1992; Gilbert et al., 1992). All of these genes express proteins in *Escherichia coli* that have cellodextrinase and endo-1,4-$\beta$-glucanase activities. Three of them (*CelA, CelD* and *CelE*) also have significant cellobiohydrolase activity and release cellobiose from crystalline cellulose, while *CelD* also has xylanase and xylo-oligosaccharase activity. Interestingly, while *CelA, CelB* and *CelC* are inducible by the presence of cellulose, *CelD* is produced constitutively. Constitutive cellulases are rare, and the enzyme expressed by *CelD* may be of major importance to the fungus during initial colonization of a plant fragment, which contains a mixture of structural carbohydrates, in the competitive environment of the rumen (Orpin, 1993).

**B. Xylanases** Xylan structure is more heterogeneous than that of cellulose. Xylan is a linear chain of $(1\rightarrow4)$-linked $\beta$-D-xylopyranosyl residues, which is highly substituted by various groups including arabinosyl, *O*-acetyl, uronyl, and arabinosyl-containing side chains (Biely, 1985). The arabinosyl substituents are sometimes esterified with ferulic and *p*-coumaric acids (Mueller-Harvey et al., 1986). Therefore, complete hydrolysis of xylan reqiures an assortment of enzymes.

Rumen fungi produce an array of hemicellulolytic enzymes that allow for complete degradation of the xylan heteropolymer. Xylanase is produced by both mono- and polycentric rumen fungi (Borneman et al., 1989) and is the most active of all endo-acting polysaccharide hydrolases studied from the anaerobic fungi (Mountfort and Asher, 1989). *Neocallimastix frontalis* released xylanase mainly into the culture medium, and only a small proportion ($<10\%$) of the enzyme was associated with the fungal rhizoid (Mountfort and Asher, 1989). *Neocallimastix frontalis* xylanase is endo hydrolytic and releases xylobiose and lesser quantities of xylooligosaccharides as products. One strain of *N. frontalis* has been shown to subsequently convert the xylooligomers to xylose (Pearce and Bauchop, 1985), indicative of a hydrolysis proceeding first via xylanase and then $\beta$-xylosidase. Another strain did not produce $\beta$-xylosidase (Mountfort and Asher, 1989). The production of xylanase was greatest when the fungus was grown on xylan-rich substrates, but the enzyme was also present when the organism was grown on cellobiose (Lowe et al., 1987b, Mountfort and Asher, 1989). That the enzyme is also inducible was evident from considerably elevated activites after growth of the organism on xylan (Mountfort and Asher, 1989). Xylose and arabinose were involved in the regulation of xylanase production in *N. frontalis* (Mountfort and Asher, 1989). The temperature and pH optima for 30 min assays were 55°C and 5–6.5 for *N. frontalis* xylanase and 50°C and 5–6.5 for $\beta$-xylosidase (Lowe et al., 1987b; Mountfort and Asher, 1989). Chen and Ljungdahl (1993) isolated a 29kDa endo-xylanase from *Orpinomyces* PC-2 with a pI above 8.0. The purified xylanase displayed no activity toward carboxymethyl or crystalline cellulose and had a high activity on oat spelt xylan ($V_{max}=1700$ IU mg$^{-1}$). A endoxylanase has also been purified from *Piromyces* strain E2 (Teunissen et al., 1992). The *Piromyces* endoxylanase was shown to be present in a high molecular mass complex protein and as a free protein in nearly equal amounts. It is a monomer with a molecular mass of 12.5 kDa and with a high activity on oat spelt xylan ($V_{max}=2600$ IU mg$^{-1}$). The products of the enzyme were xylo-oligosaccharides and no xylose was produced. The enzyme showed no cellulase activity.

Two different xylanase genes have been identified, *XylA* and *XylB* (Gilbert et al, 1992, Xue and Orpin, unpublished). While both enzymes also have xylo-oligosaccharase activities, neither has cellulase, but *XylB* has some cellobiosidase activity. *XylA* has substantial homology with prokaryote xylanases, suggesting horizontal gene transfer from prokaryotes at some time during the evolution of the fungus. *XylA* is a highly active endoxylanase, hydrolyzing about 90% of oat spelt xylan (Gilbert et al., 1992).

**C. Accessory enzymes for xylan hydrolysis** Rumen fungi produce enzymes required to hydrolyze xylan substituents such as *O*-acetyl and arabinosyl groups. Acetyl xylan esterase activity was greater than xylanase activity in both *Neocallimastix* MC-2 and *Orpinomyces* PC-2 (Borneman and Akin, 1990). Esterases capable of releasing ferulic and *p*-coumaric acids from plant cell walls (Borneman et al., 1990b) and from *O*-[5-*O*-((*E*)-*p*-coumaroyl)-$\alpha$-L-arabinofuranosyl]-$(1\rightarrow3)$-*O*-$\beta$-D-xylopyranosyl-$(1\rightarrow4)$-D-xylopyranose (PAXX) and *O*-[5-*O*-((*E*)-feruloyl)-$\alpha$-L-arabinofuranosyl]-$(1\rightarrow3)$-*O*-$\beta$-D-xylopyranosyl-$(1\rightarrow4)$-D-xylopyranose (FAXX) isolation from cell walls of Coastal bermudagrass (*Cynodon dactylon*) (CBG) have been demonstrated (Borneman et al., 1990). Two feruloyl (FAE-I and FAE-II) esterases and one *p*-coumaroyl (CAE) esterase have been purified from *Neocallimastix* strain MC-2 (Borneman et al., 1991; Borneman et al., 1992). FAE-I is a monomeric protein of molecular mass 68 kDa, FAE-II is a monomer of molecular mass 24 kDa, and CAE is a dimer of two 5.8 kDa subunits. FAE-I exhibited substantial activity toward both FAXX and PAXX, whereas FAE-I and CAE were specific for FAXX and PAXX, respectively. *Neocallimastix* MC-2 also exhibited $\alpha$-(4-*O*-methyl)-glucuronidase activity (Borneman, unpublished data).

**D. Proteases** Proteolytic activity has been determined in culture fluid of a strain of *N. frontalis*, and the enzyme appeared to be a metalloprotease since activity was inhibited by metal chelators such as phenanthroline and ethylenediaminetetraacetic acid (EDTA) (Wallace and Joblin, 1985). Inhibition by EDTA could be reversed by addition of $Zn^{2+}$, $Cu^{2+}$ or $Co^{2+}$. It was suggested that

the protease(s) functioned as follows: i) to provide amino acid for growth, ii) to modify the activities of other enzymes (i.e., cellulases), or iii) to degrade plant structural protein thereby facilitating invasion of plant tissue by fungal rhizoids (Mountfort, 1987). *Neocallimastix* sp. strain N1 and *Piromyces* sp. strain P1 were recently examined and shown to have high proteolytic activity (Asao et al. 1993). The cultures exhibited extracellular metalloprotease, cysteine protease, and serine protease activity. The temperature optima for enzymatic activity in both fungal strains was around 50°C with substantial activity remaining at 60°C. During a three hour hydrolysis of azocasein at 60°C, approximately 30% and 68% of maximal activity remained for strains N1 and P1, respectively. The proteases were active between pH 6.5 and 9.0 with a maximum of 7.9 for isolate N1 and between pH 6.5 and 10.5 with a maximum of 8.8 for isolate P1. In vitro studies in which *N. frontalis* was added to a mixed rumen bacterial culture (including the important proteolytic strains) fermenting solid substrate indicated that the fungi potentially could contribute up to 50% of the total proteolytic activity in the rumen (Wallace and Munro, 1986). It is of interest that the major rumen cellulolytic bacteria are not actively proteolytic, while the rumen fungi posses high activities of both proteases and cellulases.

**E. Amylases** *Neocallimastix frontalis* released $\alpha$-amylase into the culture medium, but less than 20% was associated with the fungal rhizoid (Mountfort and Asher, 1988). The amylase was endo-hydrolytic as demonstrated by the release of maltose, maltotriose, and maltotetraose as major end-products from soluble starch. The $\alpha$-amylase apparent $K_m$ for starch was 0.8 mg $ml^{-1}$ Amylase production was regulated by glucose concentration. The enzyme was stable and active at moderately elevated temperature (55–65°C) (Mountfort and Asher, 1988). Starch grains in grain endosperm have been shown to undergo digestion by rumen fungi, indicating a potential role for fungal amylases (as well as proteases) in vivo (McAllister et al., 1993). However, fungal degradation of cereal grains varied with fungal strain and with type of grain.

**F. Glycosidases** For the complete degradation of plant cell wall polysaccharides, glycosidases are necessary. A range of glycosidase activites has been detected in culture supernatants and whole cells of several species of anaerobic fungi. To completely degrade cellulose to glucose, glucosidase is required. This enzyme has been detected in culture supernatant fluids of *N. patriciarum* (Williams and Orpin, 1987b), *N. frontalis* (Pearce and Bauchop, 1985; Hebraud and Fever, 1988; Li and Calza, 1991a) and other rumen fungal cultures. Four different glucosidases, differentiated by molecular weight, were found in *N. frontalis* culture supernatant fluids, but their relationships were not determined. During the examination of five cloned cellulases from *N. patriciarum*, no glucosidase activity was detected, but cellobiosidase activity was present in two clones; one of these lacked cellobiohydrolase activity (Xue et al., 1993a). Further, a $\beta$-glucosidase was purified from the culture filtrate of *Piromyces* sp. strain E2 which was a monomer with a molecular mass of 45 kDa (Teunissen et al., 1992). The enzyme obtained from *Piromyces* also exhibited activity towards cellodextrins. This enzyme showed virtually no inhibition by glucose (up to 100 mM) in contrast to the $\beta$-glucosidase of the *Neocallimastix* strains, which were completely inhibited at a concentration of 100 mM glucose (Li and Calza, 1991a; Wilson and Wood, 1992a).

Xylosidase activity is also common in culture supernatants of anaerobic fungi, and the enzyme has been examined in more detail by Hebraud and Fever (1990). The $\beta$-xylosidase obtained from *Neocallimastix frontalis* was shown to be a dimer with a molecular mass of 180 kDa and had a pI of 4.35. The enzyme had also a small activity towards xylan indicating that it acts as an exoxylanase.

A non-specific glycoside which exhibited both $\beta$-glucosidase and $\beta$-fucosidase activities was purified from a strain of *N. frontalis* (Hebraud and Fever, 1990). The purified enzyme had a molecular mass of 120 kDa by gel filtration and pI of 3.85. The majority of the enzyme activity (>85%) was secreted into the culture fluid. The optimum pH and temperature under the assay conditions were 6.5 and 50°C, respectively (Hebraud and Fever, 1990). The optimum pH of the glycoside and polysaccharide hydrolases from rumen fungi (i.e., pH 6.2–6.8) are within the range of the rumen pH ($6.0<pH<7.0$) (Mountfort and Asher, 1985; Lowe et al., 1987b; Borneman et al., 1989).

## VIII. Conclusion

The discovery of anaerobic fungi has added a new member to the indigenous microorganisms that inhabit the rumen ecosystem. Anaerobic fungi do not appear essential for the survival of ruminants due to their presence in very low numbers, and sometimes absence, in ruminants fed low fiber diets, but their presence may likely be very important in the digestion of fibrous diets. The anaerobic fungi have adapted well to the rumen environment. They are able to ferment a large array of soluble carbohydrates and can synthesize cellular components in an anaerobic environment. The fungi posses hydrogenosomes for the removal of reducing equivalents in the form of molecular hydrogen and the removal of trace oxygen is a accomplished via removal by NADH oxidase. Their positive synergistic interaction with methanogenic bacteria eludes to their highly evolved role in the rumen environment. The fungi also produce resistant sporangia that allows for transfer of species to a new host in an oxygen environment. The anaerobic fungi posses a highly active array of polysaccharide degrading enzymes that may provide an advantage in the highly competitive rumen ecosystem. The production of specific enzymes that hydrolyze the lignocellulosic fraction of plant walls is unique in rumen microorganisms and allows for their attachment and growth on fibrous plant particles that are not available to the rumen bacteria.

## Literature cited


Akin, D. E. and Benner, R. 1988. Degradation of polysaccharides and lignin by ruminal bacteria and fungi. Appl. Environ. Microbiol. **54**: 1117-1125.

Akin, D. E., Gordon, G. L. R. and Hogan, J. P. 1983. Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. Appl. Environ. Microbiol. **46**: 738-748.

Akin, D. E., Lyon, C. E., Windham, W. R. and Rigsby, L. L. 1989. Physical degradation of lignified stem tissues by ruminal fungi. Appl. Environ. Microbiol. **55**: 611-616.

Akin, D. E. and Rigsby, L. L. 1987. Mixed fungal populations and lignocellulosic tissues degradation in the bovine rumen. Appl. Environ. Microbiol. **53**: 1987-1995.

Asao, N., Ushida, K. and Kojima, Y. 1993. Proteolytic activity of rumen fungi belonging to the genera *Neocallimastix* and *Piromyces*. Lett. Appl. Microbiol. **16**: 247-250.

Barr, D. J. S. 1980. An outline for the reclassification of the Chytridiales and for a new order, the Spizellomycetales. Can. J. Bot. **58**: 2830-2934.

Barr, D. J. S., 1988. How modern systematics relates to the rumen fungi. Biosystems **21**: 381-386.

Barr, D. J. S., Kudo, H., Jakober, K. D. and Cheng, K. J. 1989. Morphology and development of rumen fungi: *Neocallimasix* sp., *Piromyces communis* and *Orpinomyces* gen. nov sp. nov. Can. J. Bot. **67**: 2815-2824.

Bauchop, T. 1979. Rumen anaerobic fungi of cattle and sheep. Appl. Environ. Microbiol. **38**: 148-158.

Bauchop, T. 1980. Scanning electron microscopy in the study of microbial digestion of plant fragments in the gut. In: "Contemporary microbial ecology," (ed, by Ellwood, D. C., Edger, J. N., Lithium, M. J., Lynch, M. J. and Slater, J. H.), pp. 309-326. Academic Press, London.

Bauchop, T. and Mounfort, D. O. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Appl. Environ. Microbiol. **42**: 1103-1110.

Bauchop, T. 1983. The gut anaerobic fungi colonizers of dietary fiber. In: "Fiber in human and animal nutrition," (ed. by Wallace, G. and Bell, L.), pp. 143-148. Royal Society of New Zealand, Wellington.

Biely, P. 1985. Microbial xylanolytic systems, Trends Biotechnol. **3**: 286-290.

Borneman, W. S., Akin, D. E. and Ljungdahl, L. G. 1989. Fermentation products and plant cell wall degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. Appl. Environ. Microbiol. **55**: 1066-1073.

Borneman, W. S. and Akin, D. E. 1990a. Lignocellulose degradation by rumen fungi and bacteria: ultrastructure and cell wall enzymes. In: "Microbial and plant opportunities to improve lignocellulose utilization by ruminants," (ed. by Akin, D. E., Ljungdahl, L. G., Wilson, J. R. and Harris, P. J.), pp. 325-339. Elsevier, New York.

Borneman, W. S., Hartley, R. D., Himmelsbach, D. S. and Ljungdahl, L. G. 1990b. Assay for trans-*p*-coumaroyl esterase using a specific substrate from plant cell walls, Anal. Biochem. **190**: 129-133.

Borneman, W. S., Hartley, R. D., Morrison, W., Akin, D. E. and Ljungdahl, L. G. 1990c. Feruloyl and *p*-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation, Appl. Microbiol. Biotechnol. **33**: 345-351.

Borneman, W. S., Ljungdahl, L. G., Hartley, R. D. and Akin, D. E. 1991. Isolation and characterization of *p*-coumaroyl esterase from the anaerobic fungus *Neocallimastix* strain MC-2. Appl. Environ. Microbiol. **57**: 2337-2344.

Borneman, W. S., Ljungdahl, L. G., Hartley, R. D. and Akin, D. E. 1992. Purification and partial chracterization of two feruloyl esterases from the anaerobic fungus *Neocallimastix* strain MC-2. Appl. Environ. Microbiol. **58**: 3762-3766.

Borneman, W. S., Ljungdahl, L. G., Hartley, R. D. and Akin, D. E. 1993. Feruloyl and *p*-coumaroyl esterases from the anaerobic fungus *Neocallimastix* strain MC-2. In: "Hemicelluloses and hemicellulases," (ed. by Coughlan, M. P. and hazelwood, G. P.), pp. 85-102. Portland Press, London.

Braune, R. 1913. Untersuchungen über die im Widerkauermagen vorkommenden Protozoen. Arch. Protisenk. **32**: 111-170.

Breton, A., Bernalier, A., Bonnemoy, F., Fonty, G., Gaillard, B. and Gouet, Ph. 1989. Morphological and metabolic characterization of new species of strictly anaerobic rumen fungus: *Neocallimastix joyonni*. FEMS Microbiol. Lett. **58**: 309-314.

Breton, A., Bernalier, A., Dusse, M., Fonty, G., Gaillard-Martinie, B. and Guillot, J. 1990. *Anaeromyces nucronatons* nov. gen., nov. sp. A new strictly anaerobic rumen fungus with polycentric thallus. FEMS Microbiol. Lett. **70**: 177-182.

Breton, A., Dusse, M., Gaillard-Martinie, B., Guillot, J., Millet, J. and Prensier, G. 1991. *Piromyces rhizinflata* nov. sp. a strictly anaerobic fungus from faeces of the Saharan ass: a morphological, metabolic and ultrastructural study. FEMS Microbiol. Lett. **82**: 1-8.

Brownlee, A. G. 1986. Properties of chitin synthetase from a rumen anaerobic fungus. Abstr. 14th Int. Congr. Microbiol. p. 4.

Caldwell, D. R. and Bryant, M. P. 1966. Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. Appl. Environ. Microbiol. **14**: 794-801.

Chen, H. Z. and Ljungdahl, L. G. 1993. Purification and partial characterization of an endoxylanase from the anaerobic polycentric rumen fungus *Orpinomyces* PC-2. Abstr. Gen Meet. Am. Soc. Microbiol. **93**: 238.

Gilbert, H. J., Hazlewood, G. P., laurie, J., Orpin, C. G. and Xue, G. P. 1992. Homologous catalytic domains in a rumen fungal xylanase: evidence for gene duplication and prokaryotic origin. Mol. Microbiol. **6**: 2065-2072.

Gold, J. J., Heath, B. and Bauchop, T. 1988. Ultrastructural description of a new chytrid genus of caecum anaerobe. *Caecomyces equis* gen. nov., sp. nov., assigned to the Neoeallimasticaceae. Biosystems **21**: 403-415.

Gordon, G. L. R. and Philips, M. W. 1989a. Comparative fermentation properties of anaerobic fungi from the rumen. In: "The role of protozoa and fungi in ruminant digestion," (ed. by Nolan, J. V., Leng, R. A. and Demeyer, D. I.), pp. 127-128. Penambul Books, Armidale, Australia.

Gordon, G. L. R. and Philips, M. W. 1989b. Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine rumen. Appl. Environ. Microbiol. **55**: 1703-1710.

Gordon, G. L. R. and Philips, M. W. 1992. Extracellular pectin lyase produced by *Neocallimastix* sp. LM1, a rumen anaerobic fungus. Lett. Appl. Microbiol. **15**: 113-115.

Heath, I. B., Bauchop, T. and Skipp, R. A. 1983. Assignment of the rumen anaerobe *Neocallimastix frontalis* to the Spizellomycetales (Chytridiomycetes) on the basis of its polyflagellate zoospore ultrastructure. Can. J. Bot. **61**: 295-307.

Hebraud, M. and Fever, M. 1988. Characterization of glycosides and polysaccharide hydrolases secreted by the rumen anaerobic fungi *Neocallimastix frontalis, Sphaeromonas communis*, and *Piromonas communis*. J. Gen. Microbiol.

134: 1123–1129.

Hebraud, M. and Fever, M. 1990. Purification and characterization of an extracellular xylosidase from the rumen anaerobic fungus *neocallimastix frontalis*, FEMS Microbiol. Lett. **72**: 11–16.

Ho, Y. W., Bauchop, T., Abdullah, N. and Jalaludin, N. 1990. Morphology of three polycentric rumen fungi and description of a procedure for the induction of zoosporogenesis and release of zoospores in cultures. J. Gen. Microbiol. **137**: 213–217.

Ho, Y. W., Bauchop, T., Abdullah, N. and Jalaludin, S. 1990. *Ruminomyces elegans* gen. et sp. nov., a polycentric anaerobic rumen fungus from cattle. Mycotaxon **38**: 397–405.

Ho, Y. W., Barr, D. J. S., Abdullah, N., Jalaludin, S. and Kudo, H. 1993a. *Piromyces spiralis* a new species of anaerobic fungus from the rumen of goat. Mycotaxon **48**: 59–68.

Ho, Y. W., Barr, D. J. S., Abdullah, N., Jalaludin, S. and Kudo, H. 1993b. A new species of *Piromyces* from the rumen of deer in Malaysia. Mycotaxon **47**: 285–293.

Ho, Y. W., Barr, D. J. S., Abdullah, N., Jalaludin, S. and Kudo, H. 1993c. *Neocallimastix variablis* a new species of anaerobic fungus from the rumen of cattle. Mycotaxon **46**: 241–258.

Hsuing, T. S. 1930. A monograph on the protozoa of large intestine in the horse. Iowa State Coll. J. Sci. **4**: 359–363.

Hungate, R. E. 1966. The rumen and its microbes. Academic Press, New York, 533 p.

Jensen, E. H. C. and Hammond, D. M. 1964. A morphological study of trichomonads and related flagellates from the bovine digestive tract. J. Protozool. **11**: 386–391.

Joblin, K. N. 1981. Isolation, enumeration and maintenance of rumen anaerobic fungi in roll tubes. Appl. Environ. Microbiol. **42**: 1119–1122.

Kudo, R. R. 1954. "Protozoology, 4th ed.," Charles, C. Thomas, Springfield., IL. p.

Kudo, H., Jakober, K. D., Phillippe, R. C., Cheng, K.-J., Barr, D. J. S. and Costerton, J. W. 1990. Isolation and characterization of cellulolytic anaerobic fungi and associated mycoplasmas from the rumen of a steer fed a roughage diet. Can. J. Microbiol. **36**: 513–517.

Latham, M. J. 1980. Adhesion of rumen bacteria to plant cell walls. In: "Microbial adhesion to surfaces," (ed. by Berkeley, R. C. W., Lynch, J. M., Melling, J., Rutter, P. R. and Vincent, B. V.), pp. 339–350. Ellis Horwood, Chichester, UK.

Liebetanz, E. 1910. Die parasitischen Protozoen der Wiederkauermagens. Arch. Protist. **19**: 19–23.

Li, X. and Calza, R. E. 1991a. Fractionation of cellulases from the ruminal fungus *Neocallimastix frontalis*, Appl. Env. Microbiol. **57**: 3331–3336.

Li, X. and Calza, R. E. 1991b. Cellulases from *Neocallimastix frontalis* EB188 synthesized in the presence of glycosylation inhibitors: measurement of pH and temperature optima, protease and ion sensitivities. Appl. Microbiol. Biotechnol. **35**: 741–747.

Lowe S. E., Griffth C. G., Mile A., Theodorou M. K. and Trinci A. P. J. 1987a. The life cycle and growth kinetics of an anaerobic rumen fungus. J. Gen. Microbiol. **133**: 1815–182.

Lowe, S. E., Theodorou, M. K. and Trinci, A. P. J. 1987b. Cellulases and xylanase of an anaerobic rumen fungus grown on wheat-straw lignocellulose, wheat-straw holocellulose cellulose and xylan. Appl. Environ. Microbiol. **53**: 1216–122.

Lowe, S. E., Theodorou, M. K. and Trinci, A. P. J. 1987c. Growth and fermentation of anaerobic rumen fungus on various carbon sources and the effects of temperature on development. Appl. Environ. Microbiol. **53**: 1210–1215.

McAllister, T. M., Dong, Y., Yanke, L. J., Bae, H. D. and Cheng, K.-J. 1993. Cereal grain digestion by selected strains of ruminal fungi. Can. J. Microbiol. **39**: 367–376.

Mile, A., Theodorou, M. K., Jordan, S., KingSpooner, C. and Trinci, A. P. J. 1989. Survival of anaerobic fungi in feces, in saliva and in pure culture. Exp. Mycol. **13**: 27–31.

Mountfort, D. O. 1987. The rumen ynaerobic fungi. FEMS Microbiol. Rev. **46**: 401.

Mountfort, D. O. and Asher, R. A. 1983. Role of catabolite regulatory mechanism in control of carbohydrate utilization by the rumen anaerobic fungus *Neocallimastix frontalis*. Appl. Environ. Microbiol. **46**: 1331–1338.

Mountfort, D. O. and Asher, R. A. and 1985. Production and regulation of cellulase by two strains of the rumen anaerobic fungus *Neocallimastix frontalis*. Appl. Environ. Microbiol. **49**: 1314–1322.

Mountfort, D. O. and Asher, R. A. 1988. Production of alpha-amylase by the ruminal anaerobic fungus *Neocallimasix frontalis*. Appl. Environ. Microbiol. **54**: 2293–2299.

Mountfort, D. O. and Asher, R. A. 1989. Production of xylanase by the ruminal anaerobic fungus *Neocallimastix frontalis*. Appl. Environ. Microbiol. **55**: 1016–1022.

Mountfort, D. O., Asher, R. A. and Bauchop, T. 1982. Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. strain RA1 and *Methanosarcina barkeri*. Appl. Environ. Microbiol. **44**: 128–134.

Mueller-Harvey, I., Hartley, R. D., Harris, P. J. and Curzon, E. H. 1986. Linkage of *p*-coumaroyl and feruloyl groups to cell-wall polysaccharides of barley straw. Carbohydr. Res. **148**: 71–85.

Munn, E. A. and Orpin, C. G. 1993. Anaeromycetales: a new family to contain the anaerobic fungi. Mycol. Res. (in press).

O'Fallon J. V., Wright, R. W. and Calza, R. E. 1991. Glucose metabolic pathways in the rumen fungus *Neocallimastix frontalis* EB188. Biochem. J. **274**: 594–599.

Orpin, C. G. 1974. The rumen flagellate *Callimastix frontalis*:-does sequentration occur? J. Gen. Microbiol. **84**: 395–402.

Orpin, C. G. 1975. Studies on the rumen flagellate *Neocallimastix frontalis*. J. Gen. Microbiol. **91**: 249–262.

Orpin, C. G. 1976. Studies on the rumen flagellate *Sphaeromonas communis*. J. Gen. Microbiol. **94**: 270–280.

Orpin, C. G. 1977a. The rumen flagellate *Piromonas communis*: its life-history and invasion of plant material in rumen. J. Gen. Microbiol. **99**: 107–117.

Orpin, C. G. 1977b. Studies on the defaunation of the ovine rumen using dioctyl sodium sulphosuccinate. J. Appl. Bacteril. **43**: 309–318.

Orpin, C. G. 1981. Isolation of cellulolytic phycomycete fungi from the caecum of the horse. J. Gen. Microbiol. **123**: 287–296.

Orpin, C. G. 1983/84. The role of the ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim. Feed. Sci. Technol. **10**: 121–143.

Orpin, C. G. 1988. Nutrition and biochemistry of anaerobic Chytridiomycetes. Biosystems **21**: 365–370.

Orpin, C. G. 1993. Anaerobic fungi. In: "Stress tolerance of fungi. Mycology series, vol. 10," (ed. by Jennings, D. H.), pp. 257–273. Marcel Dekker, New York.

Orpin, C. G. and Bountiff, L. 1978. Zoospore chemotaxis in the rumen phycomycete *Neocallimastix frontalis*. J. Gen. Microbiol. **104**: 113–122.

Orpin, C. G. and Greenwood, Y. 1986a. The role of haems and

related compounds in the nutrition and zoosporogenesis of the rumen chytridiomycete *Neocallimastix frontalis* H8. Gen. Microbiol. **132**: 2179–2185.

Orpin, C. G. and Greenwood, Y. 1986b. Nutritional and germination requirements of the rumen chytridiomycete *Neocallimastix patriciarum*. Trans. Br. Mycol. Soc. **86**: 103–105.

Orpin, C. G. and Letcher, A. J. 1979. Utilization of cellulose, starch, xylan and other hemicelluloses for growth by the rumen phycomycete *Neocallimastix frontalis*. Cur. Microbiol. **3**: 121–124.

Orpin, C. G. and Joblin, K. N. 1988. The rumen anaerobic fungi. In: "The rumen microbial ecosystem," (ed. by Hobson, P. N.), pp 129–150. Elsevier, London.

Orpin, C. G. and Munn, E. A. 1986. *Neocallimastix patriciarum* sp. nov., a new member of the Neocallimasticaceae inhabiting the rumen of sheep. Trans. Br. Myc. Soc. **86**: 178–181.

Pearce, P. D. and Bauchop, T. 1985. Glycosidases of the rumen anaerobic fungus *Neocallimastix frontalis* grown on cellulosic substrates. Appl. Environ. Microbiol. **49**: 1269–1269.

Phillips, M. W. 1989. Unusual rumen fungi isolated from northern Australian cattle and water buffalo. In: "The role of protozoa and fungi in ruminant digestion," (ed. by Nolan, J. V., Leng, R. A. and Demeyer, D.), pp. 247–249. Penambul Books, Armidale, Australia.

Phillips, M. W. and Gordon, G. L. R. 1988. Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. Biosystems **21**: 377–38 .

Phillips, M. W. and Gordon, G. L. R. 1989. Growth characteristics on cellobiose of three different anaerobic fungi isolated from the ovine rumen. Appl. Environ. Microbiol. **55**: 1691–1702.

Teunissen, M. J., Op den Camp, H. J. M., Orpin, G. C., Huis in't Veld, J. H. J. and Voges, G. D. 1991a. Comparison of growth characteristics of anaerobic fungi isolated from ruminant and non-ruminant herbivores during cultivation in a defined medium. J. Gen. Microbiol. **137**: 1401–1408.

Teunissen, M. J., Smits, A. A. M., Op den Camp, H. J. M., Huis in't Veld, J. H. J. and Vogels, G. D. 1991b. Fermentation of cellulose and production of cellulolytic and xylanolytic enzymes by anaerobic fungi from ruminant and non-ruminant herbivores. Arch. Microbiol. **156**: 290–296.

Teunissen, M. J., Hermans, J. M. H., Huis in't Veld, J. H. J. and Vogels, G. D. 1992. Purification and characterization of a complex bound & a free $\beta$-1,4-endoxylanase from the culture fluid of the anaerobic fungus *Piromyces* sp. strain E2. Arch. Microbiol. **38**: 28–33.

Theodorou, M. J. and Trinci, A. P. J. 1989. Procedures of the isolation and culture of anaerobic fungi. In: "The roles of protozoa and fungi in ruminant digestion," (ed. by Nolan, J. R. Leng, R. A. and Demeyer, D. I. pp. 145–152. Penambul Books, Armidale, Australia.

Vavra, J. and Joyon, L. 1966. Etude sur la morphologie, le cycle evolutif et la possition systematique de *Callimastix cyclopis* Weisenberg 1912. Protistologica **2**: 2–15.

Wallace, R. J. and Joblin, K. N. 1985. Proteolytic activity of a rumen anaerobic fungus. FEMS Microbiol. Lett. **29**: 19–25.

Wallace, R. J. and Munro, C. A. 1986. Influence of the rumen anaerobic fungus *Neocallimastix frontalis* on the proteolytic activity of a defined mixture of rumen bacteria growing on a solid substrate. Lett. Appl. Microbiol. **3**: 23–26.

Warner, A. C. S. 1966. Diurnal changes in the concentrations of microorganisms in the rumens of sheep fed limited diets once daily. J. Gen. Microbiol. **45**: 213–235.

Weissenberg, R. 1912. *Callimastix cyclopis* gen. n, sp. n ein geisseltragendes Protozoon aus dem Serum von Cyyclops, S.-B. Ges. Naturforsch.-Fruende Berlin **5**: 299–311.

Weissenberg, R. 1950. The development and affinities of *Callimastix cyclopis* Weissenberg, a parasitic microorganism from the serum of cyclops. Proc. Am. Soc. Protozool. **1**: 4–13.

Williams, A. G., Joblin, K. N. and Fonty, G. 1993. Interactions between the rumen chytrid fungi and other microorganisms. In: "Anaerobic Fungi: biology, ecology and function," (ed. by Mountfort, D. O. and Orpin, C. G.), Marcel Dekker, New York. (in press).

Williams, A. G. and Orpin, C. G. 1987a. Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi on a range of carbohydrate substrates. Can. J. Microbiol. **33**: 418426.

Williams, A. G. and Orpin, C. G. 1987b. Glycoside hydrolase enzymes present in the zoospore and vegetative growth stages of the rumen fungi *Neocallimastix patriciarum, Piromonas communis* and identified isolate, grown on a range of carbohydrates. Can. J. Microbiol. **33**: 427–434.

Wilson, C. A. and Wood, T. M. 1992a. Studies of the cellulase of the rumen anaerobic fungus *Neocallimastix frontalis*, with special reference to the capacity to degrade crystalline cellulose. Enzyme Microbiol. Technol. **14**: 258–264.

Wilson, C. A. and Wood, T. M. 1992b. The anaerobic fungus *Neocallimastix frontalis*: Isolation and properties of a cellulosome-type enzyme fraction with the capacity to solubilize hydrogen bond-ordered cellulose. Appl. Microbiol. Biotechnol. **37**: 125–129.

Wood, T. M., Wilson, C. A., McCrae S. I. and Joblin, K. N. 1986. A highly active extracellular cellulase from the anaerobic rumen fungus *Neocallimastix frontalis*. FEMS Microbiol. Lett. **34**: 3740.

Wood, T. M., McCrae, S. I., Wilson, C. A., Bhat, K. M. and Grow, L. A. 1988. Aerobic and anaerobic fungal cellulases, with special reference to their mode of attack on crystalline cellulose. In: "Biochemistry and genetics of cellulose degradation," (ed. by Aubert, J.-P., Beguin, P. and Millet, J.), pp. 31–52. Academic Press, London.

Wubah, D. A., Fuller, M. S. and Akin, D. E. 1991. Resistant body formation in *Neocallimastix* sp., an anaerobic fungus from the rumen of a cow. Mycologia **83**: 4–47.

Xue, G-P., Orpin, C. G., Gobius, K. S., Aylward, J. H. and Simpson, G. D. 1992. Cloning and expression of multiple cellulase cDNAs from the anaerobic rumen fungus *Neocallimastix particiarum* in *Escherichia coli*. J. Gen. Microbiol. **138**: 1413–1420.

Xue, G. P., Gobius, K. S., and Orpin, C. G. 1993a. Isolation of a multifunctional cellulase (celE) from the rumen fungus *Neocallimastix patriciarum*. Proceedings of the XVII International Grassland Congress. (in press).

Xue, G. P., Gobius, K. S., and Orpin, C. G. 1993b. A novel polysaccharide hydrolase CDNA (celD) from *Neocallimastix patriciarum* encoding three multifunctional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities. J. Gen. Microbiol. **138**: 2397–2403.

Yarlett, N., Orpin, C. G., Munn, E. A., Yarlett, N. C. and Greenwood, C. A. 1986. Evidence for hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. Biochem. J. **236**: 729–739.