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Gut anaerobic fungi: The extremophilic colonizers of plant fibres in the rumen

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ABSTRACT

Anaerobic rumen fungi are strict anaerobes that inhabit the rumen and hindgut of herbivores and play a catalytic role in microbe-dependent fibre digestion in the gut. Among different gut microbes, anaerobic rumen fungi possess the most potent varieties of complex plant cell-wall degrading enzymes having the unique ability to break and penetrate the cuticle of fibrous feed particles through penetration of rhizoids. These are the very first colonizers of fibrous feeds and act as a biological scissor to break fibre particles apart, which helps the rumen bacteria to access the secondary cell-wall of feed particles. Though, the rumen fungi make up only 5 to 10% of total microbial biomass but their exclusion from the rumen causes nearly 30% reduction in digestion of fibre that confirms their key role in the anaerobic digestion of fibre in the rumen. These can degrade non-lignified plant cell-wall completely and preferably colonize on lignified areas of fibre and release the phenolic monomers. Based on the ultra structural characteristics of zoospores, anaerobic rumen fungi were assigned to the order of *Spizellomycetales* in the family *Neocallimasticaceae*. Similarly based on the type of thallus development, anaerobic rumen fungi were classified into monocentric and polycentric groups. The life cycle of monocentric fungi consist of an alteration between a motile zoosporic stage and a vegetative zoosporangial stage. Polycentric fungi, on the other hand, have nucleated rhizoids with an indeterminate life cycle and are not dependent upon the formation of zoospores for their continued survival. Six genera and about 20 species of anaerobic rumen fungi have so far been isolated and described, but a few more have also been recently discovered which will be reported soon. Based on the analysis of ITS sequences available in public databases (including those from culture independent studies), the presence of many new genera and species have been suggested. The gut fungi are difficult to maintain and even brief exposure to traces of oxygen (>10ppm) can kill them making it tedious to work with them. These are also reported to have a resting oxygen resistant spore like stage in natural condition that helps them survive in faeces for longer and thus transfer from one animal to another. Developing such an oxygen resistant resting stage *in vitro* has not, however, been successful. Since relatively little is known about the physiology of this novel group of anaerobes, further efforts are called for increasing our understanding about their exact function, biology and genetic makeup, besides their ecological interaction with other microbes in the gut.

Keywords: Anaerobic fungi, rumen, fibre digestion, herbivores, extremophiles

INTRODUCTION

The microbial ecosystems in the gut of herbivores are quite complex both in terms of types of microbes and their interactions with each other and/or with the host. Orpin (1975) recognized that at least some of the flagellates thought to be protozoa were, in fact, the motile zoospore stages of a new class of microorganisms, the anaerobic chytridiomycetous fungi. Although fungi are the primary colonizers and degraders of plant fibres in terrestrial environments, prior to the Orpin's observations of fungi in the rumen, members of this ubiquitous group had never been reported in anaerobic environments. Anaerobic rumen fungi inhabit the gastrointestinal tract of ruminant and non-ruminant herbivores, where these constitute nearly 20% of the total microbial biomass (Rezaeian *et al.,* 2004). These are regarded as the primary colonizers and most active lignocellulose degraders in the biological world (Wood and Wilson, 1995; Bauchop, 1979; 1989). These contribute significantly to overall metabolism of the host with their high cellulolytic activity and play a greater role in the degradation of lignified plant tissues (Akin and Borneman, 1990) with the help of a wide range of hydrolytic enzymes (Thareja *et al*., 2006; Nagpal *et al.,* 2011; Shelke *et al.,* 2009; Tripathi *et al.,* 2007b) including cellulases (Barichievich and Calza, 1990), hemicellulases (Novotna *et al.,* 2010; Mountfort and Asher, 1989), proteases (Michel *et al.,* 1993), amylases, amyloglycosidases (Paul *et al.,* 2004a;b), feruloyl and p-coumaroylesterases (Borneman *et al.,* 1990), various disaccharidases (Chen *et al.,* 1995) and pectinases (Kopecný and Hodrová, 1995). Their role is very critical in the digestion of poor quality forages, especially in tropical regions where forages are generally fibrous and of low quality (Ho and Barr, 1995). Anaerobic rumen fungi have unique features to penetrate the fibrous feeds and colonize preferably on the highly lignified tissues of plants and solubilize lignin from cell walls, and thus, weakening the structure of plants and facilitating fermentation by other group of microbes especially bacteria (Grenet *et al.,* 1989). These fungi can also cleave tannin-protein complex and degrade the phenolic monomers, thus reducing toxic effects (Gordon *et al.*, 1995; Paul *et al.*, 2003; 2006). The rumen fungi also increase the digestibility of fibrous feeds (Tripathi *et al*., 2007a; Saxena *et al*., 2010; Sehgal *et al*., 2008; Paul *et al.*, 2011) and voluntary dry matter intake to

the extent of 35-40% by facilitating the physical disruption of fibres in feed (Theodorou *et al.,* 1990; Gordon and Phillips, 1993). Extensive studies have shown that increasing fungal population in the rumen by dosing directly resulted in an increased fibre digestion (Paul *et al.*, 2004 a; b; Tripathi *et al.*, 2007a; Sirohi *et al.*, 2012). However, information is limited about the physiology of this novel and economically important group of anaerobes. Further studies are needed to increase understanding about their function, biology, genetic makeup and potential, besides their interaction with other microbes in the gastro intestinal tract of the animals. An attempt has been made to review the available literature on different aspects of anaerobic rumen fungi.

GROWTH PHASES

The rumen fungi possess a simple life cycle consisting of a motile flagellated zoospore stage alternating with a non-motile vegetative and reproductive stage attached to the digesta fragments. During the non-motile phase, fungi colonize and degrade fibrous plant materials, thus playing a role in the digestion of fibers in the rumen. The cycle starts with the differentiation of zoospores in the sporangia and their release into the rumen shortly after offering of the feed to animal. The reproductive sporangia are stimulated to differentiate and liberate zoospores in response to soluble carbohydrates. Flagellate zoospores may remain motile in the rumen fluid for hours before their attachment and encystment over plant fragments (Lowe *et al.,* 1987a), but usually zoospores get attached within 30 min to feed particles after release from the sporangium (Heath *et al.,* 1986). The zoospores after attachment increase in size, loose flagella and produce highly branched rhizoids. After 14 to 20 h, new zoospore formation commences (Lowe *et al.,* 1987a). In monocentric fungal group, one sporangium is formed per thallus. Nuclei are present and multiply within the zoosporangium developed endogenously. This causes the rhizoidal system to be devoid of nucleus. In monocentric fungi, after zoosporogenesis, the remaining thallus is autolyzed without further development (Lowe *et al.,* 1987a). In polycentric fungal group, following encystment, the zoospore forms rhizoids, where nucleus also gets migrated (Barr *et al.,* 1989). Newly formed rhizoid develops a new multiple sporangia.

TAXONOMIC STATUS AND CHARACTERIZATION

Even though more than three decades have elapsed since their discovery, and significant contribution has been made in understanding their physiology, enzymology and genetics, only limited information is available on their ecology, distribution, survival and interactions within and outside host (Griffith *et al.,* 2009). As the cultivation of anaerobic rumen fungi is very difficult and cumbersome, the area is relatively unexplored and several recent reports have even suggested the presence of many more uncultivated genera (Liggenstoffer *et al.,* 2010). Because of motile zoospore and obligatory anaerobic nature of these fungi, a separate family *Neocallimasticaceae* was created (Heath *et al.,* 1983). The current systematics of anaerobic rumen fungi is: Division, *Eumycota*; Subdivision, *Mastigomycotina*; Phylum, *Neocallimastigomycota*; Class, *Chytridiomycetes*; Order, *Spizellomycetales*; Family, *Neocallimasticacea*e; Genera, *Neocallimastix*, *Piromyces*, *Caecomyces*, *Anaeromyces, Orpinomyces* and *Cyllamyces* (Barr, 1988; Barr *et al.*, 1989; Hibbett *et al.,* 2007). Based on the traditional classification system of using morphological features like growth pattern (monocentric or polycentric), thallus morphology (filamentous or bulbous) and number of flagella per zoospore (monoflagellated or polyflagellated), 20 species included in 6 genera have been described (Griffith *et al.,* 2009). These morphological features are highly pleomorphic, varying with culture conditions, particularly carbon source, and hence, do not provide conspicuous resolution about their status (Brookman *et al.,* 2000). Many a time, the polycentric groups fail to produce sporangia or zoospores, making their identification and differentiation very difficult. Similarly the shapes and sizes of sporangia in monocentric isolates also tend to vary. Therefore, molecular approaches have been employed for the accurate identification and differentiation of various genera and species of anaerobic rumen fungi. For species level identification,18S rDNA (SSU) based identification appears to be obsolete because of highly conserved and less variable regions (Fliegerova *et al.,* 2006; Brookman *et al.,* 2000). The internal transcribed spacer (ITS) region was successfully used to resolve the taxonomy related problems and phylogenetic analyses (Tuckwell *et al.,* 2005; Fliegerova *et al.,* 2004).The variability of ITS region is sometimes not high enough to be able to differentiate at species level, thus making it inappropriate for diversity studies (Eckart *et al.,* 2010). Moreover, the abundance of intra-individual variations in ITS region also prevents conspicuous resolution of the sequences (Tuckwell *et al.,* 2005; Dagar *et al*., 2011). Hence, there is a need to explore other regions as well for better taxonomic identifications.

MORPHOLOGICAL FEATURES

All anaerobic rumen fungi that have been described are classified in six genera (Ho and Barr, 1995; Ozkose *et al.*, 2001), but few have been more recently reported and will be available soon with more details. The already reported genera are *Neocallimastix* (Heath *et al.*, 1983), *Piromyces* (Barr *et al.*, 1989), *Caecomyces* (Gold *et al.,* 1988), *Orpinomyces* (Barr *et al.,* 1989), *Anaeromyces* (Breton *et al.,* 1990) and *Cyllamyces* (Ozkose *et al.*, 2001). The classification is mainly based on the morphological features of zoospores and thallus development pattern of these fungi. The fungal thallus is either mono- or poly-centric in nature. In the monocentric, the thallus usually develops a single sporangium, while in polycentric, the thallus develops a number of sporangia (Ho and Barr, 1995). *Neocallimastix*, *Piromyces* and *Caecomyces* are

monocentric, whereas *Anaeromyces*, *Orpinomyces* and *Cyllamyces* are polycentric. The main characteristic features of different genera are presented in **Table 1**. *Neocallimastix* and *Anaeromyces* are presented in **Fig. 1a** and **Fig.1b**.

Till date, only six genera and nearly 20 species of anaerobic rumen fungi have been described. Based on analysis of ITS sequences available in public databases, the presences of many new genera and species have been suggested (Liggenstoffer *et al.*, 2010).Only few species have been characterized morphologically based on culture studies. Morphological characteristics of *Neocallimastix frontalis* (Orpin, 1975) was: size of zoospore, 20.6 x 8.7 µm; shape, variable but usually ovoid; number of flagella, 14; flagella size, 36.6 x 2.5 µm; zoospore movement, erratic and gyratory; size of sporangia, 21 x 9 µm to 74 x 52 µm; number of zoospore produced per sporangium, 2-38; rhizoid, branched; type of growth, monocentric i.e. only one sporangium per rhizoid system. Due to some anomalies, the isolate referred as *N. frontalis* by Orpin was subsequently renamed as *N. patricarium* (Orpin and Munn, 1986). Later, the isolates like PN1 (Bauchop and Mountfort, 1981), L2 (Maruin- Sikkema *et al.,* 1993), CS36, CL16, XY6, CS4, XS 26 (Sijtsma and Tan, 1993) were also classified under the genus *Neocallimastix*.

Further, Orpin (1976) isolated another rumen fungus – *Sphaeromonas communis*. Assuming the similarity between the isolate and Liebetanz's protozoans, Orpin

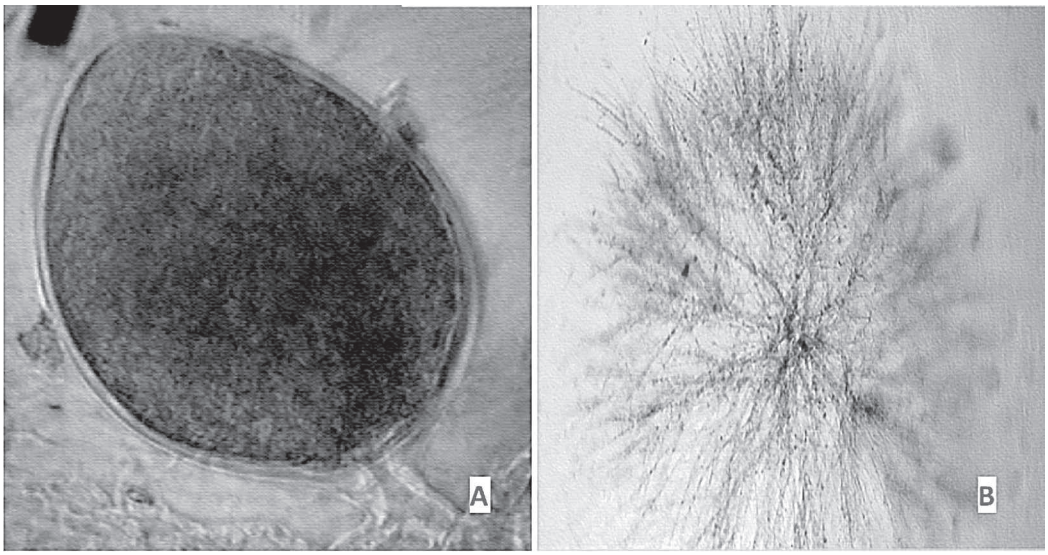

Fig.1. Vegetative stage of fungus **(a)** monocentric anaerobic fungus *Neocallimastix* sp. CF 17(X40), **(b)** anaerobic fungal isolate *Anaeromyces* sp. BRF1(x40)

Table 1. Key morphological features of anaerobic rumen fungi for identification.

Genus	Growth pattern/ Thallus	Type of rhizoid	Type of development	Flagella per zoospore	Colonial characterstic(s)
Caecomyces	Monocentric	Bulbous or sphaerical rhizoid; old culture may have few blunt radially developing rhizoid	Endogenous	Uniflagellate; 01 (Occasionally bi- or quadriflagellate)	Slow growing; 3-5 mm; fluffy, dotted, compact, circular
Neocallimastix	Monocentric	Filamentous	Endogenous/ Exogenous	Polyflagellate; >07 (up to 30)	2-4 mm; dark centre surrounded with radiating rhizoids, circular
Piromyces	Monocentric	Filamentous	Endogenous/ Exogenous	Usually 01 (Occasionally bi- or quadriflagellate)	1-3 mm; dark and dense colony, circular
Anaeromyces	Polycentric	Filamentous	Exogenous	Uniflagellate; 01	Radiating; yellow, dense, irregular; 5-7 mm
Cyllamyces	Polycentric	A bulbous rhizoid having presence of as many as 12 sporangia per thallus on up to 5 sporangiophores	Exogenous	Uniflagellate; 01 (Occasionally bi- or triflagellate)	Small; granular
Orpinomyces	Polycentric	Filamentous	Exogenous	Polyflagellate; 10 to 25	8-12 mm; dark centred, layered, matted, circular; sometimes cottony, irregular

retained the generic name used by Liebetanz (1910). However, later on to give emphasis on its fungal status, the genus *Sphaeromonas* was renamed as *Caecomyces* (Gold *et al.,* 1988). Type species were *Caecomyces communis* (isolated from the sheep rumen) and *C. equi* (isolated from horse caecum). The characteristics of *Caecomyces* were: zoospores usually irregular immediately after release, but most become sphaerical within 2-3 min after release; monoflagellated; flagellum, 24.9 µm long; bulbus rhizoid; 114 zoospore per sporangium, etc.

Additionally, *Piromonas communis*, isolated and characterized by Orpin (1977) was renamed as *Piromyces*. The type species are *P. communis* (Gold *et al.,* 1988), *P. mae*, *P. deembonica*, *P. rhizinflata* (Li *et al.,* 1990), *P. minutus* (Ho *et al.,* 1993a), *P. spiralis* (Ho *et al.,* 1993 b). The features of the *Piromyces* isolates were: monocentric; flagellates irregular in shape immediately after release, but became regular elongated after 2 to 3 min with average axial dimension of 7.1 x 14.6 µm (Orpin, 1977); monoflagellated zoospores; non septate highly branched rhizoid; 2 - 78 zoospores per sporangium.

Some polycentric fungi isolated and reported by Ho and Bauchop (1991) viz., LL, LC2 and *Ruminomyces elegans* (C2) from the rumen of cattle were renamed as *Anaeromyces* (Trinci *et al.,* 1994). The zoospores were polyflagellated in case of LL and LC2, but monoflagellated in case of *A. elegans*. Both LL and LC2 produced large rhizomycelia, comprising of extensively branched hyphae, which could be tubular and uniform in diameter. Other species was *A. mucronatus* from the sheep rumen (Breton *et al.,* 1990). Later, the polycentric *Neocallimastix joyonii* was renamed as *Orpinomyces joyonii* (Trinci *et al.,* 1994). Ozkose *et al.,* (2001), isolated a polycentric fungus, *Cyllamyces aberensis*.

TRANSMISSION AMONG ANIMALS

The fungal transfer among animals can occur in two phases; the initial acquisition followed by the addition (Saxena *et al.,* 2010)/ replacement of this population. Anaerobic rumen fungi have been consistently isolated from both fresh and dried faeces (Lowe *et al.,* 1987b; Trinci *et al.,* 1994). Saliva contains viable fungi (Lowe *et al.,* 1987b) and aerosols have been indicated as possible means of dissemination between animals (Orpin, 1989).

Fonty *et al. (*1987) reported that the rumen of lambs were colonized by anaerobic rumen fungi within the first two weeks of life, even when these were separated from other sheep soon after birth suggesting that initial transfer of fungi occur between juveniles and their dam. Saliva can be a mean for transfer through close mouth-to-mouth contact. Ingestion of faecal matter from pasture could be another important mean of transfer within and between flocks and herds.

Faeces contained substantial number of anaerobic rumen fungi, which declined slowly after drying and organisms could be cultured up to 10 months (Theodorou *et al.,* 1990). Motile zoospores were not detected in faeces, although fungal sporangium was observed. Theodorou *et al. (*1996) proposed that anaerobic rumen fungi consisted of three different stages: motile zoospore, vegetative thallus and aerotolerant survival stage. Orpin (1981) reported an appearance of thick walled resistant sporangia in caecal content of horse. Additionally, Milne *et al.* (1989) showed that anaerobic rumen fungi can be isolated from sheep saliva stored in air at 39 °C for up to 8 h and from sheep faeces dried in air at 20 °C or 39 °C for up to 128 days. They also observed that zoospores of *Neocallimastix* R1 on exposure to air remain motile for at least 3.5 h and the same fungus could be isolated from stationary phase cultures 14 h after they had been aerated for 1 min and then subsequently stored in air at 39 °C. Trinci *et al.* (1988) observed that when faecal pellets were allowed to dry out anaerobic rumen fungi survived longer than stored under moist condition in a polythene bag. They hypothesised that drying of faecal pellets stimulated resistant body formation, which protected the fungi. Secondly, continual microbial activity in moist faecal pellets caused loss of fungal viability. Wubah *et al.* (1991) described the development, pigmentation and nuclear condition of thalli of resting stage or resistant body of *Neocallimastix* sp. in aged cultures. A survival stage in anaerobic rumen fungi would thus explain their transfer through the entire gastrointestinal tract.

ISOLATION TECHNIQUES

To isolate anaerobic rumen fungi the sampling of the rumen liquor (usually from fistulated animals) is done in tightly capped, O_2 free CO_2 flushed and pre-autoclaved reagent bottles. Samples are immediately processed within 30 min of collection and flushed with O_2 free CO_2 for 1 min to separate fungal zoospores from the surface of feed particles followed by isolation. The first dilution is used for isolation by transferring 10 mL of sample into 90 mL of anaerobic diluent containing antibiotics (McSweeney *et al.,* 1994). Roll tubes are prepared by injecting 0.5 mL of inoculum from 10^{-1} dilution of the rumen liquor samples or enriched faecal into 50 mL serum bottles containing 5.0 mL cellobiose agar medium with antibiotics (Miller and Wolin, 1974). Care is taken to add the antibiotics and inoculums only after cooling of media to ≈45 °C. Inoculated bottles were incubated at 39±1 °C for 2-3 days for the development of colonies. The process of roll tube preparation and colony picking was repeated twice to get the axenic cultures (Dagar *et al.,* 2011). Since, it is difficult to maintain these fungi, Nagpal *et al.* (2012) reported the survival of anaerobic fungus *Caecomyces* sp. in various preservation methods.

Orpin (1975) isolated *Neocallimastix frontalis* from the sheep rumen by overlaying the sloppy agar medium containing antibiotics with particulate fraction of the rumen fluid, followed by gassing with oxygen free carbon dioxide

and incubating at 39 °C. Within 2 days of incubation, fungal growth was observed. The top 5 cm medium was then removed by aspiration and drops of the underlying sloppy agar containing the fungus were overlayed on fresh cultures. Successive subcultures eliminated bacteria from fungal isolate.

The method suggested by Joblin (1981) involved straining the rumen fluid through muslin cloth, mixing the filtrate with molten agar medium containing antibiotics and preparing roll tubes. Bauchop and Mountfort (1981) used the strained rumen fluid to make enrichment cultures in sloppy agar medium containing antibiotics. After three successive subculturing, the culture was transferred to liquid medium and single colony was picked up with a syringe and washed in buffer. In another method, Lowe *et al. (*1985) isolated anaerobic rumen fungi using a plate culture technique. Fungal enrichment was achieved by incubating 1 ml rumen digesta with 0.1 g milled barley straw and 10 ml of synthetic medium containing, minerals, vitamins and volatile fatty acids, antibiotics and lysozyme but no rumen fluid. Fungal colonies appeared after few days of incubation of plates at 39 °C. Sijtsma and Tan (1993) isolated fungi from the rumen of sheep using M1 medium containing cellobiose (0.5%) in presence of penicillin G (100 mg/ml), chloramphenicol (50 mg per ml) and streptomycin sulphate (200 mg per ml). After 8 days of incubation at 39 °C the growth was visible.

RUMEN FERMENTATION

a) Fibre degradation

Anaerobic rumen fungi have the unique ability to penetrate fibrous feeds and colonize preferably on highly lignified tissues of tropical roughages and solubilize part of lignin of plant cell walls (Akin *et al.,* 1983; Grenet *et al.,* 1989), thus weakening the integrity of forage tissues and facilitating colonization and fermentation by other group of microbes especially bacteria. Compared to the rumen bacteria, anaerobic rumen fungi are able to degrade sclerenchyma walls (most recalcitrant parts of cell wall), to a greater extent because of penetration of rhizoids. The rumen bacteria which are attached to the wall surface could only degrade the peripheral areas, resulting in only slight to moderate digestion of cell walls (Akin, 1994). Large population of fungi occur in animals fed stalky, hard stem herbage diets but these occur in low numbers or are absent, when animals are fed soft leafy diets (Grenet *et al.,* 1989). Elimination of fungi from the sheep rumen decreased straw digestion from 53.8 to 44.6% (Calderon-Cortes *et al.,* 1989), which was reversed, when fungi were re-introduced. The *in vivo* digestibility increased/decreased by 3-8% in presence or absence of anaerobic rumen fungi (Elliot *et al.,* 1987; Gordon and Phillips, 1993; Manikumar *et al.,* 2004; Tripathi *et al.,* 2007a; Dayanand *et al.,* 2007). In a continuous culturing, addition of *Neocallimastix* to the mixed rumen bacteria increased degradation rate of wheat straw by 15% (Hillaire and Jouany, 1989).

Neocallimastix sp. and *Piromonas* sp. were better than *Sphaeromonas* sp. in degrading fragments of plant tissues possibly because filamentous rhizoids are more effective than bulbous rhizoids at penetrating harder tissues (Orpin, 1989). In addition to the greater degradation of lignified tissues by anaerobic rumen fungi compared to bacteria, another unique attribute of fungi is their ability to penetrate the cuticle of grass leaf blades, which allow greater penetration and access to leaf substrates to the rumen microbes and not be limited to damaged sites (Akin and Rigsby, 1987).

b) Fermentation

Anaerobic rumen fungi gain energy from the fermentation of carbohydrates (Orpin, 1994). The common plant monosaccharides, fructose, glucose, xylose, cellobiose and gentiobiose were used by all the isolates (Phillips and Gordon, 1988; Stewart *et al.,* 1995; Dijkerman *et al.,* 1997), while galactose and mannose were utilized by some and L-arabinose was not used. When glucose was used, the major fermentation end products were acetate, ethanol, formate, lactate, succinate, CO_2 and H_2 (Lowe *et al.,* 1987c), though there was variation in the amounts among different genera. Generally, polycentric anaerobic rumen fungi produced less lactate than monocentric fungi (Borneman *et al.,* 1989; Phillips and Gordon, 1995), although *Piromyces* from the rumen did not produce any lactate (Ho *et al.,* 1996). Anaerobic rumen fungi use only the glycolysis (Embden-Meyerhof-Parnas) pathway for the catabolism of glucose to pyruvate or phosphoenolpyruvate (O'Fallon *et al.,* 1991; Maruin-Sikkema *et al.,* 1993).

c) Protein digestion

Anaerobic rumen fungi contribute to the protein supply to the host animal, both through the production of proteolytic enzymes in the rumen and a portion of the microbial protein synthesized in the rumen that pass to abomasum and intestines for digestion and absorption. Unlike cellulolytic bacteria, fungi are protease positive, and are therefore, able to penetrate the proteinaceous layer of the feed particles through rhizoids, which help in accessing cell wall of plants. Fungi play an important role in degrading fibre associated protein or tannin-protein complex (Wallace and Munro, 1986; Gordon *et al.,* 1995). The extent of possible fungal contribution to proteolysis in the rumen still remains to be determined, since the only study to examine ruminal proteolysis both in the presence and absence of anaerobic rumen fungi used seven strains of anaerobic rumen fungi, of which only one was weakly proteolytic (Bonnemoy *et al.,* 1993).

d) Protein supply to ruminants

Gulati *et al. (*1989a) showed that fungal cells are composed of proteins with a well balanced combination of amino acids that will be available to the ruminant host. A high proportion of protein component of three monocentric

anaerobic rumen fungi (*Neocallimastix* sp., LMI, *Piromyces* sp. SMI and *Caecomyces* sp. NMI) was digested and absorbed in the intestine of sheep, with digestibility factors of 0.91-0.98 (Gulati *et al.,* 1988; 1989b). The contribution of anaerobic rumen fungi in supplying microbial protein to host is minor, as this comprised only 1.6% of the microbial nitrogen in digesta flowing to the duodenum. This microbial protein has been found to be of high quality and readily available to the animal (Faichney *et al.,* 1991). With possible manipulation of fungal populations in the rumen, either by inoculating the efficient strains or stimulating the existing biomass through dietary supplementation, it is likely that the supply of high quality microbial protein to the host ruminant can be enhanced.

e) Role in voluntary feed intake

The removal of anaerobic fungi from the rumen has permitted quantification of the contribution of fungi to feed intake. The removal of fungi from the sheep rumen reduced voluntary feed intake by about 70% (Gordon and Phillips, 1998) with little effect on bacteria and protozoa. Forage intake by early weaned calves was increased by 35% with dosing of anaerobic rumen fungi (Theodorou *et al.,* 1990), and dosing of fungus free sheep with fungi produced 40% increase in intake of straw based diets (Gordon and Phillips, 1998).

FERMENTATION END PRODUCTS

Fermentation of anaerobic rumen fungi was governed by the nature of substrate, and type of the fungus and the presence of other microbes (Theodorou *et al.,* 1996; Sirohi *et al.,* 2012). On glucose and xylose media, *Neocallimastix* sp. produced formate, acetate, lactate and ethanol (Lowe *et al.,* 1987c). Borneman *et al.* (1989) reported that the accumulation of fermentation products was concomitant with substrate utilization. The major fermentation products were formate, acetate, D (-) lactate, ethanol, carbon-dioxide and hydrogen. Propionate, butyrate, valerate or any branched chain volatile fatty acids were not produced. Monocentric isolates produced a high ratio (1.5 vs. 0.94-1.02) of oxidised (acetate) to reduced products (formate), when grown on glucose or coastal Bermuda grasses compared to polycentric ones, which produced a nearly equal ratio of these products. All fungal strains irrespective of their origin produce lactate except *Piromyces citron,* isolated from the caecum of donkey, which did not produce lactate (Julliand *et al*., 1998).

When grown in the presence of methanogens, the fermentation profile of anaerobic fungus was shifted from electron sink products such as ethanol and lactate and towards more reduced products such as acetate and formate (Theodorou *et al.*, 1996). In methanogenic co-culture of anaerobic rumen fungi, acetate was the major product and CO_2 production increased, whereas lactate and ethanol decreased (Bauchop and Mountfort, 1981). In addition to changes in fermentation, methanogenic co-culture showed significant increase in fungal biomass because of the removal of fermentation inhibitory intermediates (ethanol, formate and lactate).

ENZYME ACTIVITIES

Lignocellulose, consisting of lignin, hemicellulose and cellulose, is the major structural component of plants and represents a major source of organic matter (Lynd *et al.,* 2002). The chemical properties of its constituents make it a substrate of huge biotechnological value. Of the three components, lignin is the most resistant to degradation whereas cellulose, because of its highly ordered crystalline structure, is more resistant to hydrolysis than hemicellulose. Unfortunately, most lignocellulosics are considered as wastes, and therefore, are disposed of by burning that causes environmental pollution. The enzymes degrading lignocellulosics can play a significant role in exploiting lignocellulosics for generating products that find applications in various industries. Anaerobic rumen fungi produce numerous lignocellulolytic enzymes that can play central role in unleashing the hidden potential of lignocellulosic residues. Most of the fungal enzymes have been found to be extracellular and/or cell-associated (Breton *et al.,* 1995; Williams and Orpin, 1987).

All the strains of anaerobic rumen fungi isolated from the rumen or caecum of herbivorous animals, are capable of hydrolyzing lingnocellulosics. The enzymes responsible for the hydrolysis of polysaccharides like starch, hemicellulose, cellulose, lignocellulose or ligno-hemicellulose are reported in anaerobic fungal isolates. The enzymes produced by anaerobic rumen fungi are: carboxymethylcellulase or endoglucanase (Lowe *et al.,* 1987d; Tripathi *et al.,* 2007a; Paul *et al.*, 2010a;b), avicelase or exoglucanase (Pearce and Bauchop, 1985), β-1,4 glucosidase (Li and Calza, 1991), hemicellulases (xylanase and xylosidase) [Hebraud and Fevre, 1988, 1990], pectin lyase (Gordon and Phillips, 1992), feruloyl esterase, p-coumaroyl esterase, amylase and protease (Borneman *et al.,* 1990; 1991 and 1992). Crystalline cellulose is the most recalcitrant and resistant form of cellulose for microbial attack in the rumen. The extracellular cellulase of *N. frontalis* has been shown to be very active on crystalline cellulose like cotton fibre (Wood *et al.,* 1986).

The sequencing of the genome of *Orpinomyces* sp. strain C1A revealed that this anaerobic fungus has a large genome (100.95 Mb) with 16,347 genes (Youssef *et al.,* 2013) and extremely low G+C content (17%). The genome has large noncoding intergenic regions (73.1%), proliferation of microsatellite repeats (4.9%) and multiple gene duplications. Multiple genes and pathways have been identified by comparative genomic analysis. The genes are encoded for posttranslational fucosylation, the production of specific intramembrane proteases and extracellular protease inhibitors, the formation of a complete axoneme and intraflagellar trafficking machinery, and near-complete focal adhesion machinery. The analysis of the lignocellulolytic

machinery in the genome revealed an extremely rich repertoire with the evidence of horizontal gene acquisition from multiple bacterial lineages. The strain C1A is a remarkable biomass degrader, capable of simultaneous saccharification and fermentation of the cellulosic and hemicellulosic fractions in grasses and crop residues.

APPLICATIONS

Anaerobic rumen fungi have been extensively studied in the past few decades for their potential biotechnological applications (Nagpal *et al.,* 2009). In their natural habitat, these act as most potent fibre degraders among all microbial groups because of their abilities to degrade lignified tissues extensively to partially degrade and weaken the more resistant tissues and to penetrate the cuticle barrier in forages (Akin and Borneman, 1990; Akin *et al.,* 1988). These fungi possess extensively branched rhizoidal system, and thus, are better than the rumen bacteria to degrade the structural barriers in plants. Their ability to produce a wide array of hydrolytic enzymes makes them the microbes of choice. In addition to enzymes, some of their other metabolites can also find various applications.

CONCLUSIONS

Anaerobic rumen fungi are an under explored group of the rumen microbes which have very potent fibre degrading abilities. The rumen is a natural ecosystem, where microbes especially anaerobic rumen fungi, effectively digest lignocellulosic biomass, and the fermented products can be further converted to a variety of products with numerous applications. In comparison with other microbial inoculums, the rumen fungi show a better hydrolytic activity when high fibrous lignocellulosics are the substrates. Further studies are called for understanding the diversity, ecology, physiology, metabolic pathways and underlying genetic makeup of such an important group of fungi, so that they can be utilized more effectively for improving ruminal fibre digestion and/or industrial enzyme production.

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