Mycoscience (2011) 52:31–38
DOI 10.1007/s10267-010-0071-6

FULL PAPER

In vitro fibrolytic potential of anaerobic rumen fungi from ruminants and non-ruminant herbivores

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Received: 24 February 2010 / Accepted: 23 August 2010 / Published online: 22 September 2010

Abstract In the present study, anaerobic fungi were isolated from different ruminants and non-ruminants; i.e., cattle, buffalo, sheep, goats, wild bluebulls, elephants, deer, and zebras; and were identified as *Anaeromyces, Orpinomyces, Caecomyces, Piromyces,* and *Neocallimastix* sp., based on their morphological characteristics. These isolates possessed significant in vitro hydrolytic enzyme activities; however, an isolate of *Caecomyces* sp. from elephant was found to exhibit maximum activity, i.e., filter paper cellulase (Fpase; 21.4 mIU/ml), carboxymethyl cellulose (CMCase; 15.1 mIU/ml), cellobiase (37.4 mIU/ml), and xylanase (26.0 mIU/ml). Besides, this isolate also showed the significantly highest ability to digest plant cell-wall contents in vitro. The in vitro dry matter digestibility increased from 45.1 to 48.9% after 48 h of incubation, and the plant cell-wall contents, in terms of neutral detergent fiber and acid detergent fiber, decreased from 64.2 to 61.3% and from 31.3 to 29.6%, respectively. These results indicate that such fibrolytic ruminal fungal strains are prevalent in wild herbivores such as elephants, as well as in other ruminants and non-ruminants, and could be exploited as microbial feed additives for improved nutrition and productivity in domesticated ruminants.

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Keywords Animal nutrition · Cellulolytic enzymes · Plant–fiber degradation · Rumen microflora

Introduction

Anaerobic fungi play a key role in plant–fiber degradation in the rumen, by releasing various enzymes such as cellulases, hemicellulases, proteases, and esterases, justifying their use as animal feed additives for improved ruminant nutrition (Lee et al. 2004; Nagpal et al. 2009). It is now well established that these ruminal fungi efficiently take part in fiber digestibility in ruminants, leading to a more rapid degradation of forage entering the rumen (Orpin and Joblin 1988; Lee et al. 2004). These fungi have been found in all of the geographic regions of the world, being ubiquitous in foregut fermenters and ruminants such as cattle, buffalo, and goats (Ho et al. 1993a, b; Thareja et al. 2006); red deer and impala (Bauchop 1979); and the wild bluebull (*Boselaphus tragocamelus*) (Paul et al. 2004a; Tripathi et al. 2007a); as well as in marsupials including kangaroos, wallaroos, and swamp wallabies (Breton et al. 1989). These fungi have also been isolated from fecal samples of horses, zebras, donkeys, rhinoceroses, and Indian elephants (Breton et al. 1990; Li et al. 1990; Nagpal et al. 2009), all of which are hindgut fermenters. Therefore, these fungi appear to be a standard part of the gut microflora in many herbivores that feed on a highly fibrous diet. Because there are considerable disparities in the fiber-degrading potential among the fungal isolates from domestic and wild animals, there exists immense scope to isolate efficient fibrolytic fungal strains that generate elevated levels of fibrolytic enzymes so that they can be inoculated in the rumen of domestic animals in order to improve feed utilization. Hence, the present

investigation aimed to isolate such fibrolytic chytrids from a wide range of domesticated and wild ruminants and non-ruminant herbivores, and to study their potential for the in vitro degradation of plant fibers such as wheat straw.

Materials and methods

Fresh rumen fluid was collected in a pre-gassed thermos flask from the rumen of permanently fistulated adult cattle and buffalo, fed on a standard diet containing 10 kg green fodder maize, 1 kg concentrate mixture, and wheat straw available ad libitum, maintained at our institute's cattle yard; and, for goats and sheep, rumen fluid was collected from a slaughterhouse located in Karnal. It was immediately brought to the laboratory and strained through double layers of muslin cloth before being used as inoculum. Fresh feces from other ruminants and non-ruminant herbivores i.e., blue bulls, deer, elephants, and zebra were collected into pre-gassed plastic bags from the floor at Chattbir Zoological Park, Chandigarh. Fecal samples were first homogenized and incubated with anaerobic broth medium containing antibiotics (penicillin and streptomycin 0.10 mg/ml) at 39°C for 48 h for pre-enrichment, and this enriched culture medium was used as an inoculum for the isolation of fungi by the Hungate roll-tube method, which involves a series of dilutions of molten agar medium containing clarified rumen fluid and antibiotics (penicillin and streptomycin; 0.10 mg/ml) (Hungate 1969; Joblin 1981). All the media were prepared and dispensed under an atmosphere of CO_2. Rumen liquor was strained through a double layer of muslin cloth and clarified by centrifugation at 12,000 *g* for 20 min before being used as an ingredient in the media. The roll tubes were kept in a CO_2 incubator at 39°C and were regularly observed for the appearance of fungal colonies (Hungate 1969; Joblin 1981). Distinct anaerobic rumen fungal colonies, which appeared mostly on the third day, were subcultured in Medium C supplemented with wheat straw (5 g/l) and antibacterial antibiotics (Orpin 1975). Morphological identification of isolated fungal rhizoids was done microscopically up to genus level, based on the features of zoospores, types of sporangia, and nature of rhizomycelia (Trinci et al. 1994; Thareja et al. 2006; Tripathi et al. 2007a; Chen et al. 2007), using lactophenol cotton blue stain (Table 1). Genera of anaerobic fungi were characterized on the basis of the number of flagella per zoospore, thallus morphology (monocentric or polycentric), and rhizoid type (filamentous or vegetative cells).

The hydrolytic enzyme activities of the ruminal fungi were estimated after 96 h of incubation in Orpin's broth supplemented with 1% each of carboxymethyl cellulose (CMC), Whatman No. 1 filter paper (6 × 1 cm ≈ 50 mg), cellobiose, and xylan (from oat spelt; Sigma, Bangalore, India) separately, for assaying their carboxymethyl cellulase (CMCase), filter paper cellulase (FPase), cellobiase (Mandels et al. 1976; Srinivasan et al. 2001; Nagpal et al. 2009), and xylanase (Kawaminami and Izuka 1970; Srinivasan et al. 2001) activities, respectively, keeping one non-inoculated set as the control. Supernatants from the incubated cultures were analyzed for the estimation of reducing sugars (glucose and xylose), using a dinitrosalicylic acid method (Miller 1959). The reaction mixture, comprising 1.0 ml of 0.1 M phosphate buffer (pH 6.8), 0.5 ml of the respective substrate, and 0.5 ml of culture supernatant, was incubated at 39°C for 30 min for xylanase and 1 h for CMCase, FPase, and cellobiase. A similar reaction mixture was prepared for the control. The enzyme activities were calculated as IU, i.e., mmol of glucose or xylose released per hour per milliliter of culture filtrate. The protein concentration in the culture supernatants was also measured, at 595 nm, using a spectrophotometer (Jenway, Genova, UK), by the method of Bradford (1976). A 100-µl sample and 1 ml Bradford reagent [100 mg Coomassie Brilliant Blue (dissolved in 50 ml 95% ethanol) and 100 ml 85% phosphoric acid, brought up to 1 l] with bovine serum albumin was used as a standard.

For determination of the in vitro fiber-degrading ability of fungal isolates in terms of percentage in vitro dry matter digestibility (IVDMD), neutral detergent fiber (NDF), and acid detergent fiber (ADF), wheat straw (500 mg) was taken in a flask (100 ml) along with 40 ml McDougall's buffer, 10 ml strained rumen liquor (SRL) (freshly collected rumen liquor from fistulated cattle, strained through four layers of muslin cloth, stored at 7–8°C and thawed before adding to the medium), and 5 ml of selected cultures ($\approx 10^6$ thallus-forming units per milliliter) in triplicate. For this, five reaction treatments (one control and four reactions) were done, as follows:

Tc = Straw + buffer + strained rumen liquor + 5 ml anaerobic broth (control);
T1 = Straw + buffer + strained rumen liquor + 5 ml *Caecomyces* sp. (FE5);
T2 = Straw + buffer + strained rumen liquor + 5 ml *Orpinomyces* sp. (RB2);
T3 = Straw + buffer + strained rumen liquor + 5 ml *Orpinomyces* sp. (RC1);
T4 = Straw + buffer + strained rumen liquor + 5 ml *Neocallimastix* sp. (RG5)

The samples were analyzed for IVDMD (Tilley and Terry 1963), and for NDF and ADF (Goering and Vansoest 1970; Association of Official Analytical Chemists [AOAC] 1995). Total volatile fatty acid (TVFA) contents were estimated by the method of Barnett and Reid (1957). The data were expressed as the means (±standard deviation) of three replicates, using a randomized factorial design of

Table 1 Morphological characteristics of selected anaerobic fungal isolates from rumen liquor/feces of ruminant and non-ruminant herbivores

Isolate/source	Thallus morphology	Nature of growth	Shape of sporangium	Rhizoid type	Zoospore flagellation	Identified genus
RC1; cattle rumen	Polycentric	Exogenous	Many globose sporangia	Highly branched	Polyflagellated	*Orpinomyces*
RB2; buffalo rumen	Polycentric	Exogenous	Many globose sporangia	Highly branched	Polyflagellated	*Orpinomyces*
RG5; goat rumen	Monocentric	Endogenous	Globose/ellipsoid	Branched	Polyflagellated	*Neocallimastix*
RS3; sheep rumen	Monocentric	Endogenous	Globose/ellipsoid	Branched	Polyflagellated	*Neocallimastix*
FC3; cattle feces	Polycentric	Exogenous	Circular terminal	Highly branched segmented	Monoflagellated	*Anaeromyces*
FB1; buffalo feces	Polycentric	Exogenous	Many globose sporangia	Highly branched	Polyflagellated	*Orpinomyces*
FG2; goat feces	Monocentric	Endogenous	Globose/ellipsoid	Branched	Polyflagellated	*Neocallimastix*
FD1; deer feces	Monocentric	Endogenous	Globose/ellipsoid	Branched	Polyflagellated	*Neocallimastix*
FE5; elephant feces	Monocentric	Endogenous	Globose	Single spherical	Monoflagellated	*Caecomyces*
FBB3; wild bluebull feces	Monocentric	Endogenous	Irregular	Branched	Monoflagellated	*Piromyces*
FZ1; zebra feces	Monocentric	Endogenous	Irregular	Branched	Monoflagellated	*Piromyces*

analysis of variance (ANOVA) according to the General Linear Models procedure of SYSTAT Version 6.0.1 (1996, SPSS, IBM, USA) for test of significance ($P < 0.05$), as per the method of Snedecor and Cochran (1980).

Results and discussion

A total of 11 isolates of anaerobic fungi were isolated from the rumen fluid as well as from the fecal samples of different domestic and zoo ruminants and non-ruminant herbivores (Table 1). Generally, two types of fungal colonies were obtained on the agar surface after 48–72 h of incubation. Type A colonies were 8–10 mm in diameter with a dark inner zone and a scattered fiber-like translucent network surrounding the zone, and these colonies were observed mostly with polycentric fungi. Type B colonies were smaller, 2–4 mm in size, off-white-colored and pin-head-shaped; these were mostly encountered in monocentric fungi (Fig. 1). Thareja et al. (2006) and Tripathi et al. (2007a) also reported similar types of colonies for anaerobic fungi from small ruminants and wild bluebulls. Akin and Rigsby (1987) also observed three types of fungal colonies with a round extensive rhizomycelial network on the agar surface. Based on the further microscopic morphological characteristics such as the nature of growth, sporangia, rhizoids, and zoospore flagellation (Orpin 1975; Gold et al. 1988; Barr et al. 1989; Breton et al. 1990; Li et al. 1991; Ho et al. 1993a, b; Thareja et al. 2006; Tripathi et al. 2007a; Chen et al. 2007; Nagpal et al. 2009), these 11 isolates were tentatively identified as *Neocallimastix, Orpinomyces, Piromyces, Caecomyces*, and *Anaeromyces* species (Table 1; Fig. 1). Of these 11 isolates, 7 were monocentric and 4 isolates were polycentric. Trinci et al. (1994) also classified rumen anaerobic fungi broadly into two categories (i.e., monocentric and polycentric) based on the number of sporangia developed from the thallus. Such anaerobic fungi, being ubiquitous among foregut fermenters and ruminants, have been isolated, by various workers, from cattle, buffalo, goats (Ho et al. 1993a, b; Thareja et al. 2006), red deer, impala (Bauchop 1979), and wild bluebulls (*Boselaphus tragocamelus)* (Paul et al. 2004a; Tripathi et al. 2007a), as well as from marsupials, including kangaroos, wallaroos, and swamp wallabies (Breton et al. 1989). Therefore, these fungi appear to be a standard constituent of the gut microflora in many herbivores fed on a highly fibrous diet. In the present study, it was observed that the distribution of fungal genera varied from animal to animal and, further, more than one genus was found within one animal species, as in the cattle rumen and fecal samples (Table 1). Even though both polycentric and monocentric fungi were found in the rumen fluid of different animals, it was observed that monocentric fungi were prevalent in fecal samples. This could be attributed to the characteristic of polycentric fungi not being efficient spore-formers and therefore, they cannot survive for long in the feces, once outside the host (Davies et al. 1993; Brookman et al. 2000; Nagpal et al. 2009). However, some polycentric fungi were also found in the fecal samples of cattle and buffalo, and this may highlight the presence of some dormant stage in the life cycle of polycentric fungi, suggesting that they are able to survive for considerable periods without any need for subculturing as a resting stage within the anaerobic environment of an animal's gut (Brookman et al. 2000).

The ability of ruminants to digestplant structural polysaccharides, primarily cellulose and hemicelluloses,

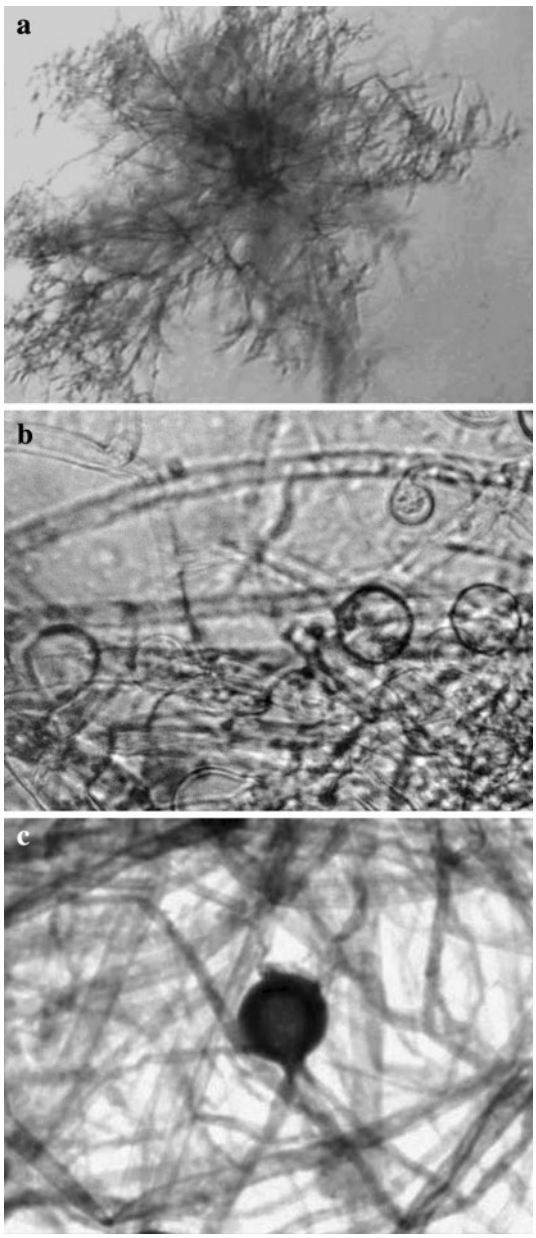

Fig. 1 Morphological features of different rumen fungal isolates. **a** A typical monocentric fungal colony, ×10. **b** Isolate FE5 (*Caecomyces* sp.), ×40. **c** Isolate RG5 (*Neocallimastix* sp.), ×40

depends on the capacity of microorganisms inhabiting the rumen, and rumen anaerobic fungi are well known to play a major role in the degradation of lignified plant tissues (Akin and Benner 1988). For plant cell-wall degradation, these fungi produce a wide range of hydrolytic enzymes, cellulases, hemicellulases, proteases, amylases, amyloglycosidases, feruloyl and *p*-coumaryl esterases, various disaccharidases, pectinases, and exonucleases (Mountfort and Asher 1989; Barichievich and Calza 1990; Borneman et al. 1992; Yanke et al. 1993; Chen et al. 1994; Nagpal et al. 2009). Therefore, in the present investigation, all the isolates were tested for their in vitro hydrolytic enzyme activities; i.e., CMCase, FPase, cellobiase, and xylanase (Table 1). All the 11 isolates possessed detectable in vitro hydrolytic enzyme activities; however, isolates RC1, RB2, RG5, and FE5 showed significantly higher activities ($P < 0.05$). Interestingly, isolate FE5 (*Caecomyces* sp.) from elephant was found to exhibit the maximum enzyme activity (mIU/ml) among all the isolates (Fig. 2). It not only showed the highest FPase activity (21.4), but also the highest CMCase, cellobiase, and xylanase activities, of 15.1, 37.4, and 26.0, respectively, indicating its possible potential in fiber degradation. Isolate RB2 (*Orpinomyces* sp.) also showed high FPase and CMCase activities, of 16.7 and 13.7, respectively, but its xylanase activity (23.6) was lower than that of *Orpinomyces* isolate RC1 (24.7) and that of *Neocallimastix* isolate RG5 (26.1), and its cellobiase activity (36.3) was lower than that of isolate RC1 (37.2) but higher than that of isolate RG5 (31.7). Although isolate RG5 showed xylanase activity similar to that of isolate FE5, it did not show high FPase, CMCase, and cellobiase activities, when compared with isolates RC1 and RB2 (Fig. 2). The enzyme activities were well supported by their supernatant protein values s (μg/ml), as shown in Fig. 3. Supernatant protein values were found to be highest in isolate FE5 (57.4 μg/ml), followed by isolates RB2 (49.3), RC1 (41.7), and RG5 (39.3). The values for supernatant proteins recorded for the other isolates were in the range of 26.4–37.3 (Fig. 3). Other workers have also observed a similar range of enzyme activities in fungi such as *Orpinomyces*, *Neocallimastix,* and *Piromyces* sp. from different animals such as cattle, small ruminants, wild bluebulls, and Indian elephants (Lee et al. 2004; Thareja et al. 2006; Tripathi et al. 2007a; Nagpal et al. 2009). The differences in enzyme activities among the isolates may be attributed to the source animals' diverse origins and habitats; i.e., domesticated and wild-grazing, and also to the different dietary regimes and varying quantities of fibers in their diets.

The enzymes assayed in the present study are the major ones that play a part in plant fiber degradation; hence, the higher activity of these enzymes was anticipated to increase the fiber digestibility, as these high fibrolytic enzyme activities are considered to be one of the primary mechanisms of improved fiber breakdown in the rumen (Lee et al. 2004; Paul et al. 2004a, b; Tripathi et al. 2007a, b; Sehgal et al. 2008; Nagpal et al. 2009). Hence, the four isolates (i.e., isolates RC1, RB2, RG5, and FE5) showing maximum hydrolytic enzyme activities were further tested for their effect on IVDMD and the cell-wall contents of wheat straw, to observe their potential for fiber degradation (Fig. 4). The maximum digestibility was observed in treatment T1 (*Caecomyces* sp. FE5). The IVDMD of treatment T1 increased to 45.1% at 48 h and 48.9% at 72 h,

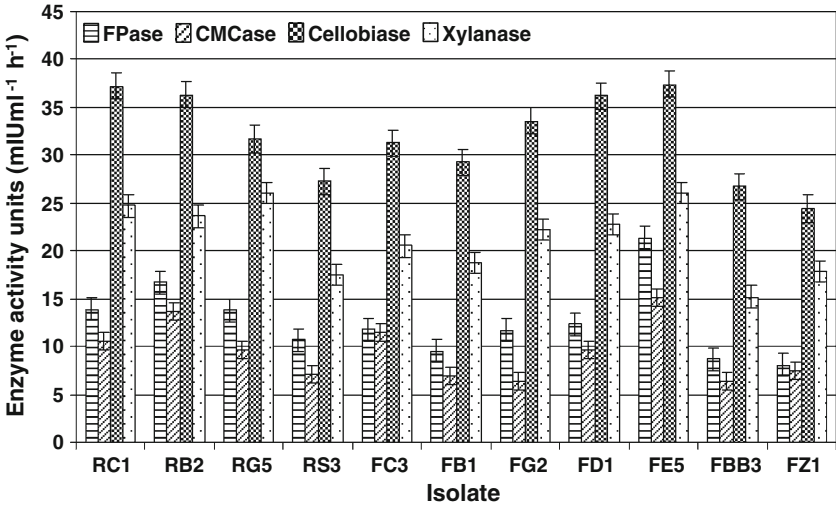

Fig. 2 Hydrolytic enzyme activities (mIU ml^{-1} h^{-1}) of fungal isolates using different substrates [data recorded as mean ± SD ($n = 3$); isolates as per Table 1]

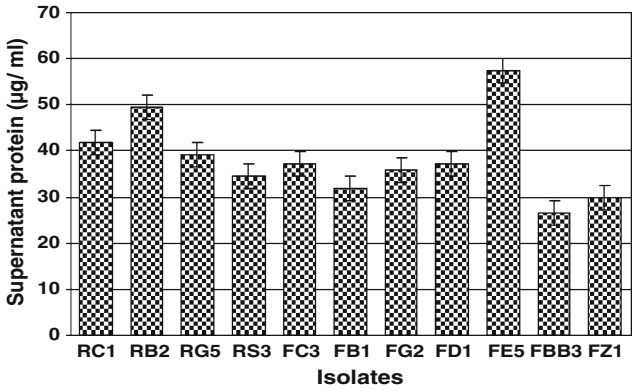

Fig. 3 Supernatant protein values (µg/ml) recorded after 96 h of incubation [data recorded as mean ± SD ($n = 3$); isolates as per Table 1]

respectively, when compared with the control (38.2 and 38.6%). The IVDMD for isolate RB2 increased from 43.1% at 48 h to 45.8% at 72 h, and for isolate RC1, the values were 41.3% at 48 h and 45.7% at 72 h. Isolate RG5 showed IVDMD values of 38.8 and 40.6% after 48 and 72 h (Fig. 4a). The higher IVDMD values for isolate FE5 and other isolates indicate their capacity for better utilization of the fibrous material of wheat straw. Kostyukovsky et al. (1990) also reported that these fungi could utilize a wide spectrum of mono-, oligo-, and polysaccharides. Manikumar et al. (2004) also reported increased IVDMD of wheat straw after 48 h incubation with strained rumen liquor and *Orpinomyces* sp. The rhizoids of vegetative fungal thalli are better at penetrating plant tissue than are bacteria and protozoa, so they gain access to the plant material that is not accessible to other rumen microorganisms (Orpin and Joblin 1988). Bauchop and Mountfort (1981) also suggested that such penetrations lead to a faster and more complete degradation of forage entering the rumen. The weakening of plant tissues by fungal enzymes accelerates digestion, making rumination more effective in reducing particle size and increasing protozoal and bacterial digestion in the rumen. Because of the ability of cellulases to rapidly attack crystalline cellulose, there has been considerable interest in the fiber-degrading enzymes of anaerobic fungi (Teunissen et al. 1993).

Isolate FE5 (*Caecomyces* sp.) showed significantly high reduction in NDF and ADF (followed by isolates RB2, RC1, and RG5 [Fig. 4b, c]), which is attributable to its high hydrolytic enzyme activities. The NDF contents were found to be minimum, i.e., 64.2 and 61.3% in treatment T1 (*Caecomyces* sp. FE5) after 48 and 72 h, respectively, when compared to control and other treatments. In isolates RB2, RC1, and RG5, these values were reduced from 66.1 to 62.9, 68.5 to 64.4, and 72.7 to 70.8%, respectively (Fig. 4b). A similar pattern was observed in the in vitro degradation of ADF (Fig. 4c). The values for the TVFA concentration also followed the same pattern (Fig. 4d). There was a maximum TVFA concentration (mM/100 ml) of 12.7 at 48 h and 13.6 at 72 h for isolate FE5, when compared with control values of 8.3 and 8.5 at 48 and 72 h, respectively. In isolates RB2, RC1, and RG5, the values for TVFA concentrations at 48 and 72 h were 11.8 and 12.4, 11.3 and 12.0, and 9.8 and 10.8, respectively. The increased concentration of TVFAs in vitro is indicative of increased fermentation, which would possibly be beneficial in providing more energy for microbial growth and for maintenance and production activity in the host animal in vivo. The results for the enzyme activities and in vitro fiber degradation in the present study are quite consistent with those observed in *Orpinomyces* sp., *Neocallimastix* sp., *Piromyces* sp., and Caecomyces sp. (Lee et al. 2004; Thareja et al. 2006; Tripathi et al. 2007a, b; Dayanand et al. 2007; Sehgal et al. 2008; Nagpal et al. 2009). Similar results have also been reported for rumen fungi isolated from cattle and buffalo (Paul et al. 2004a, b; Dey et al. 2004). The digestibility

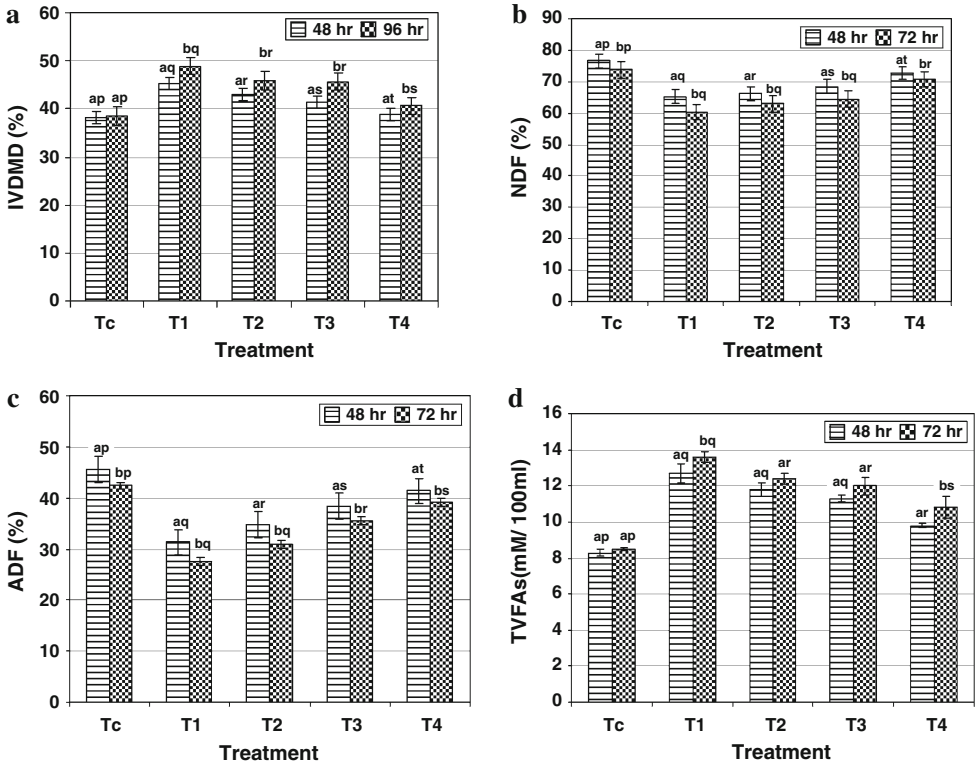

Fig. 4 Effect of ruminal fungal isolates on in vitro digestibility (%) of dry matter and cell-wall contents of wheat straw. Tc = straw + buffer + strained rumen liquor + 5 ml anaerobic broth (control); T1 = straw + buffer + strained rumen liquor + 5 ml *Caecomyces* sp. (FE5); T2 = straw + buffer + strained rumen liquor + 5 ml *Orpinomyces* sp. (RB2); T3 = straw + buffer + strained rumen liquor + 5 ml *Orpinomyces* sp. (RC1); T4 = straw + buffer + strained rumen liquor + 5 ml *Neocallimastix* sp. (RG5). *Error bars* indicate the standard deviation of the mean ($n = 3$). *Different letters* (*a–b*) in *bars* showing means for the same treatment at different incubation periods show significant differences ($P < 0.05$). *Different letters* (*p–t*) in *bars* showing means for the same incubation period with different treatments show significant differences ($P < 0.05$). *IVDMD*, In vitro dry matter digestibility; *NDF*, neutral detergent fiber; *ADF*, acid detergent fiber; *TVFA*, total volatile fatty acid

patterns of the control and treatments in the present study indicated that the presence of ruminal fungi in rumen liquor accelerated the rate of rumen fermentation in vitro and, hence, the digestibility of wheat straw increased significantly. Thus, anaerobic fungi from ruminants as well as those from non-ruminants possess significant fibrolytic activities and similar abilities to degrade plant fibers such as wheat straw, as is evident from Figs. 2 and 4.

Conclusion

In the present study, fungal strains from various host animals showed significant in vitro fibrolytic activities, clearly suggesting that the introduction of such fibrolytic fungal strains into the rumen could possibly improve nutrient utilization in ruminants. Based on the results obtained, it can be inferred that fiber-degrading strains of anaerobic fungi are present in wild herbivores as well as in other ruminants and non-ruminant herbivores, and the fibrolytic potential of these strains could also be exploited through their administration to domestic ruminants for improved nutrient utilization and animal nutrition. However, more in vitro as well as in vivo trials are needed to verify the possibility of the successful interspecies transfer of these fungal strains and their establishment in the rumen before these fungi can be exploited as directly fed microbials for domesticated ruminants.

Acknowledgments The authors express thanks for the research fellowships and funding provided by the National Dairy Research Institute, Karnal, and the Indian Council of Agricultural Research, New Delhi, India.

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