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Original scientific paper

FERMENTATIVE ACTIVITY OF FIVE STRAIN OF *NEOCALLIMASTIX FRONTALIS* CULTIVATED ON A DIFFERENT SUBSTRATES

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A b s t r a c t: The importance of the microbial ecology and diversity of microorganisms in the digestive tract of herbivora has gained increasing attention in response to recent trends in the global livestock production. The microorganisms in the digestive tracts of ruminants and non-ruminant herbivora have a profound influence on the conversion of feed into end-products, which can impact on the animals and environment. Anaerobic fungi are the significant constituent of rumen microbiota in livestock that rely on poor-quality fibrous diets. They produce the whole set of enzymes necessary for plant cell-wall degradation. These enzymes enable fungi to penetrate plant cell walls, access fermentable substrates not available to surface-acting bacteria, colonize the sturdy plant structures, weaken and degrade plant tissues and reduce the plant particles size. The present paper studies the influence of different carbohydrate substrates on fermentative activity of five strains of *Neocallimastix frontalis*. Experiments for fermentative activity were performed with carboxymethyl cellulose, Whatman N°1 and Avicel, and enzyme activity was detected extracellularly in culture supernatants, through reducing sugars, after vegetative growth. The enzymes CM-cellulase, FP-ase and avicelase secreted from *N. frontalis* strain J3 released the largest quantity of reducing sugars, compared to the other strains.

Key words: anaerobic fungi; *Neocallimastix frontalis;* herbivora; ruminant; enzymes

ФЕРМЕНТАТИВНА АКТИВНОСТ НА ПЕТ СОЈА НА *NEOCALLIMASTIX FRONTALIS* КУЛТИВИРАНИ НА РАЗЛИЧНИ СУПСТРАТИ

А п с т р а к т: Важноста на микробната екологија и диверзитетот на микроорганизмите од дигестивниот систем на хербиворите добива сè поголемо внимание како одговор на најновите трендови во глобалното производство на добиток. Микроорганизмите од дигестивниот систем на руминантните и неруминантните хербивори имаат длабоко влијание врз претворбата на храната во крајни продукти, кои можат да влијаат врз животните и животната средина. Анаеробните фунги претставуваат сигнификантен дел од микроорганизмите кои го населуваат руменот кај добитокот кој се храни со неквалитетна влакнеста храна. Тие произведуваат цел сет на ензими кои се неопходни за разградување на растителните клеточни ѕидови. Овие ензими им овозможуваат на фунгите да пенетрираат во растителните клеточни ѕидови, да ги колонизираат растителните структури, да ги ослабнат и разградат растителните ткива. Овој труд го проучува влијанието на различните јаглехидратни супстрати врз ферментативната активност на пет соја на *Neocallimastix frontalis*. Експериментите се изведувани со карбоксиметил целулоза, Whatman N°1 и Avicel, додека ензимската активност беше детектирана екстрацелуларно, во супернатантот, преку количеството на ослободените редуцирачки шеќери. Ензимите CM-целулаза, FP-аза и авицелаза секретирани од *N. frontalis* соj J3 ослободуваат најгололемо количество редуцирачки шеќери, споредено со другите соеви.

Клучни зборови: анаеробни фунги; *Neocallimastix frontalis*; хербивори; румен; ензими

INTRODUCTION

The life cycle and role of anaerobic fungi has been well characterized in the rumen, but not elsewhere in the ruminant alimentary tract. Their respective occurrence is dependent upon host and diet (Liggenstoffer et al., 2010).

The physiology of the microbial community is fundamental for understanding the processes of anaerobic decomposition of plant material, and has an economic relevance for mankind. The distribution of organisms within the rumen is essential for our understanding of the biochemistry of cellulose degradation.

Anaerobic fungi (phylum *Neocallimastigomycota*) inhabit the gastrointestinal tract of mammalian herbivores, where they play an important role in the degradation of plant material. The *Neocallimastigomycota* represent the earliest diverging lineage of the zoosporic fungi; however, understanding of the relationships of the different taxa (both genera and species) within this phylum is in need of revision. Issues exist with the current approaches used for their identification and classification, and recent evidence suggests the presence of several novel taxa (potential candidate genera) that remain to be characterized.

Greater understanding of the 'resistant' phase(s) of their life cycle is needed, as is study of their role and significance in other herbivores.

A major part of organisms within the rumen fluid encompasses bacteria (10^{10} to 10^{11} / ml rumen fluid) and flagellates (10^5 to 10^7 / ml rumen fluid), but fresh and undigested plant material is rapidly colonized by anaerobic fungi ($<10^5$) (Ozkose et al., 2001; Nagpal et al., 2009b). It is now generally known that the degradation of herbal carbohydrates by rumen fungi accelerates the digestion by downsizing the plant tissue particles. Those particles are subsequently more easily decomposed by bacteria and protozoa. The effectiveness of digestion is an important contributor to the health of animals in husbandry (Wulff, 2001). Today, it is well known that bacteria, fungi, and protozoa are responsible for 50 to 82 % of cell-wall degradation (Lee et al., 2000).

Rumen fungi are now classified in a single order (*Neocallimastigales*) within the recently erected phylum *Neocallimastigomycota* (Hibbett et al., 2007). Currently, only 6 genera and 20 species have been described (Griffith et al., 2009), although multiple uncharacterized isolates have also been reported (Philips and Gordon, 1989; Ho and Barr, 1995).

Until now, only several species of gut fungi have been described, probably because of the problematic cultivation and maintenance of these organisms and high morphological variability depending on growth conditions.

Some of the cellulases of anaerobic fungi originated via horizontal gene transfer from bacteria, so these are the only fungi known to possess cellulosomes, cell-wall associated multienzyme complexes (Garcia-Vallve et al., 2000; Steenbakkers et al., 2001). Combined with their anaerobic metabolism and ability grow at elevated temperatures (39 ºC), they have great biotechnological potential.

Biotechnological application of anaerobic fungi, and their highly active cellulolytic and hemicellulolytic enzymes, has been a rapidly increasing area of research and development in the last decade.

The present paper studies the influence of different carbohydrate substrates on fermentative activity of five strains of *N. frontalis*.

MATERIAL AND METHODS

Fungal strains and culture conditions

Five strains of *N. frontalis*, isolated from animal feces by previously described method of Atanasova-Pančevska and Kungulovski (2002/2003, 2008a, 2008b, 2011a, 2011b, 2011c) were used in this study: EZ1, EZ2, Z2, J3 and MR1 (Table 1).

Fungal strains were taxonomically determined morphologically by light microscopic analysis, and were identified according to colony morphology, size of fungal rhizoids, and appearance of zoospores, according to Ho and Barr (1995). Inocula of fungal cultures were maintained anaerobically at 39 °C on the medium M10 (Caldwell and Bryant, 1966) enriched by 20 % (v/v) rumen fluid with either glucose or cellobiose (both 4 g/l) as the source of carbon. Incubations were carried out in 20 ml flasks closed by butyl rubber stoppers, under O_2-free CO_2 atmosphere. The medium (15 ml) was inoculated by 1 ml of 3 days-old culture of a fungus. Cultures were done in triplicate. Inoculated cultures were grown at 39 °C for 4 days.

Subculturing of isolates was performed every 4 days to maintain fungal viability.

Table 1

The origin of fungal strains of anaerobic fungi

Animal	Strain	Species
Fallow deer (*Cervus dama*)	EZ1	*Neocallimastix frontalis*
Fallow deer (*Cervus dama*)	EZ2	*Neocallimastix frontalis*
Zebu (*Bos indicus*)	Z2	*Neocallimastix frontalis*
Yak (*Bos gruniens*)	J3	*Neocallimastix frontalis*
Moufflon (*Ovis musimon*)	MR1	*Neocallimastix frontalis*

Culture purity

Fungal isolates were routinely checked for purity by examination of wet mounts, Gram staining and transfer of isolates from liquid culture to agar plates containing medium with 0.2 % cellobiose to check for bacterial colony formation.

Fermentation tests

Fermentation tests for each isolate were carried out in M10, substituting glucose with alternative carbon sources.

Whatman N°1, carboxymethyl cellulose (CMC) and Avicel were used as growth substrates for the production of cellulolytic enzymes. The inoculated serum bottles were incubated at 39 °C for 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120 hours. Enzyme activities and the amount of gas produced were measured at the end of each incubation period. The utilization of the substrates was assayed at each time point using three biological replicates per fungal isolate. Five un-inoculated serum bottles were used as negative controls.

After incubation, the medium was centrifuged at 1500 g for 15 minutes, and the supernatant was tested for the presence of active enzymes. Methods were described elsewhere (Atanasova-Pančevska and Kungulovski, 2002/2003, 2008a, 2008b, 2011a, 2011b, 2011c).

In vitro gas production

The total gas production during fermentation was measured with a 25-ml glass syringe connected to a needle, which pierced through the butyl stopper into the head-space of the flask. Effects of the fungal isolates on *in vitro* gas production (Menke and Steingass, 1988) were estimated using different carbohydrates as energy source.

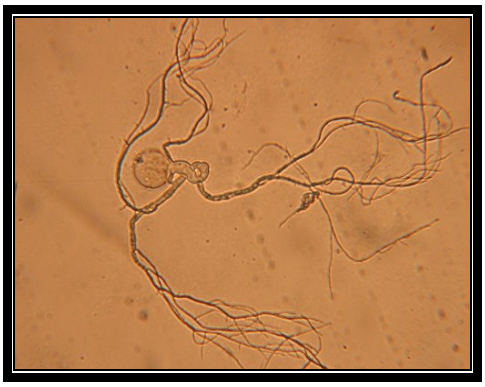
RESULTS AND DISCUSSION

Anaerobic fungi (phylum *Neocallimastigomycota*) inhabit the gastrointestinal tract of mammalian herbivores, where they play an important role in the degradation of plant material. Known adaptations of anaerobic fungi to their strict anaerobic lifestyle include the absence of mitochondria, cytochromes and other biochemical features of the oxidative phosphorylation pathway (Yarlett et al., 1986; Youssef et al., 2013). Instead, anaerobic fungi possess specialized organelles called hydrogenosomes, which couple the metabolism of glucose to cellular energy production without the need for oxygen.

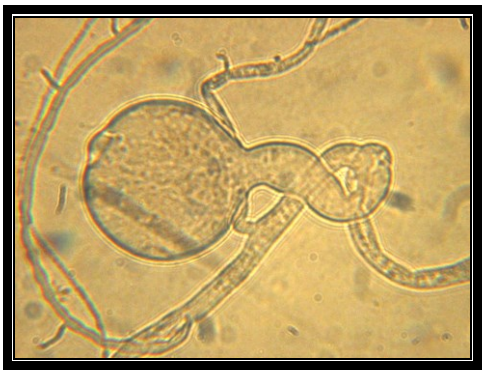
Description of strain EZ1

Endogenously developed zoosporangia, spheric with diameter of 65.71 μm; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 20 μm in diameter; main rhizoid is often coiled; rhizoids might have strongly narrowed places, rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore (Figures 1 and 2).

The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the ZOO, Skopje. Accordingly the key to determine the anaerobic fungi of Ho and Barr (1995), the description of the isolate corresponds completely to the description of *Neocallimastix frontalis*.



a)



b)

Fig. 1. Strain EZ1 – *N. frontalis*. Monocentric thallus with one sporangium, wide neck, main rhizoid coiled (narrow), a) magnification 10×; b) magnification 40×

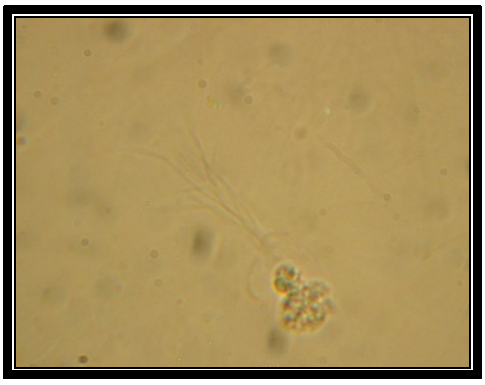
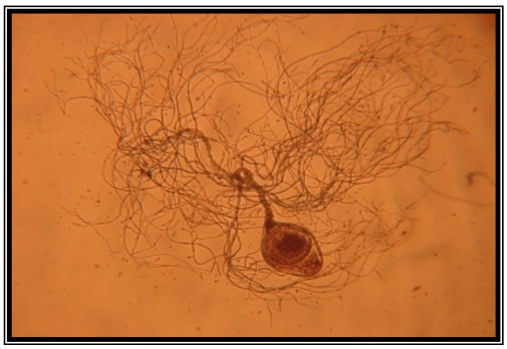


Fig. 2. Polyflagellate zoospore of *N. frontalis*.

Description of strain EZ2

Endogenously developed zoosporangia, spherically elongated (egg-like); 157.14 μm in diameter; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 17.14 μm in diameter, coiled; main rhizoid is often coiled; rhizoids might have strongly narrowed places; rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape, globular zoospores with 10–25 μm in diameter, with 10 to 30 flagella, 35–50 μm long (Figure 3).

The isolate is taken from feces of a fallow deer (*Cervus dama*), kept in the ZOO, Skopje. Accordingly the key to determine the anaerobic fungi of Ho and Barr (1995), the description of the isolate corresponds completely to the description of *Neocallimastix frontalis*.



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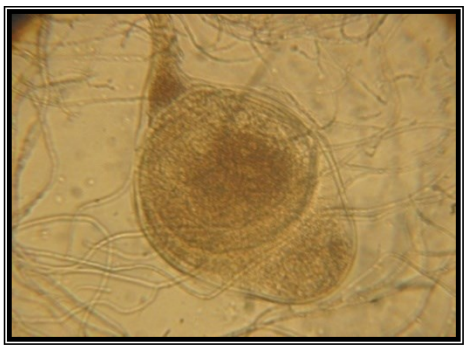


b

Fig. 3. Strain EZ2 – *N. frontalis*. Monocentric thallus with short egg-like sporangiophore.
a) magnification 10×; b) magnification 40×

Description of strain Z2

Endogenously developed zoosporangia, spheric, 83.61 μm in diameter; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 32 μm in diameter, coiled; main rhizoid is often coiled; rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape, spheric zoospores 7–22 μm in diameter, with 7 to around 30 flagella (Figures 4 and 5).

The isolate is taken from feces of a zebu (*Bos indicus*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of Ho and Barr (1995), the description of the isolate corresponds completely to the description of *Neocallimastix frontalis*.

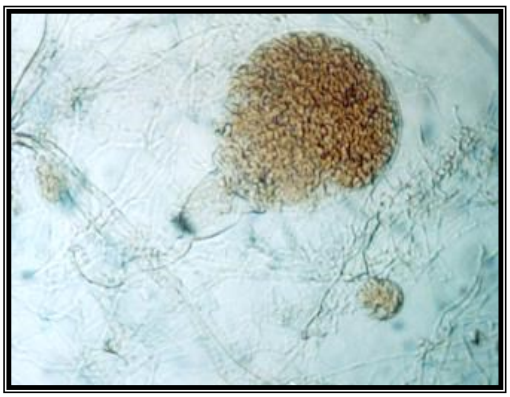


Fig. 4. Strain Z2 – *Neocallimastix frontalis,* exogenous sporangium with short, egg-like sporangiophore, full with spores. Magnification 900×

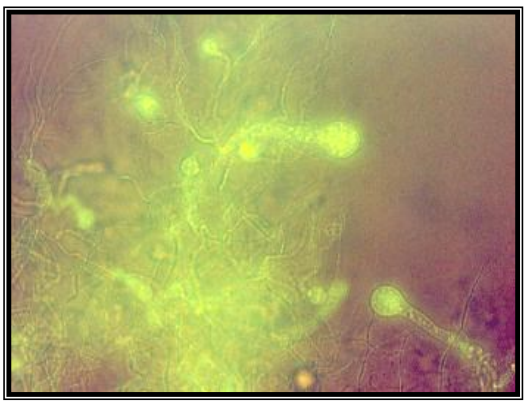


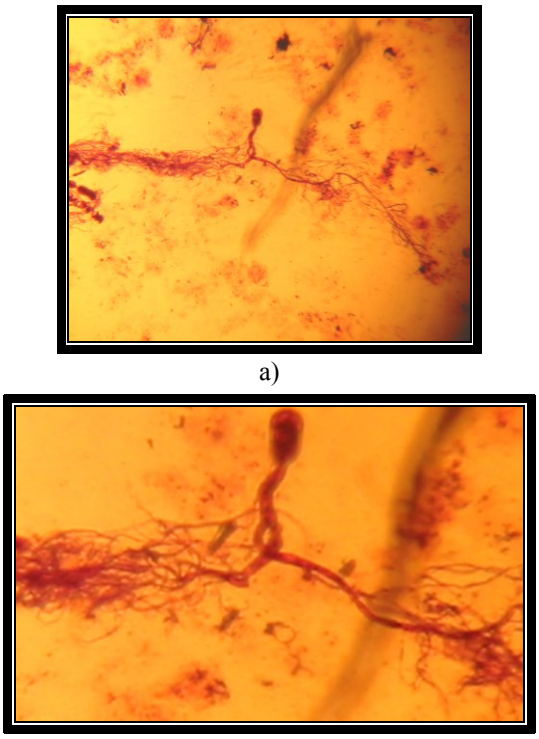
Fig. 5. Strain Z2 – *Neocallimastix frontalis,* under fluorescent microscopy. Magnification 400×. Fluorescence occur in nuclei

Description of strain J3

Endogenously developed zoosporangia, ovally elongated; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis

close to the sporangium, up to 18 μm in diameter, coiled; main rhizoid is often twirld; rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape, 5-18 μm in diameter, with 7 to around 30 flagella (Figure 6).

The isolate is taken from the feces of a yak (*Bos gruniens*), kept in the ZOO in Skopje. Accordingly the key to determine the anaerobic fungi of Ho and Barr (1995), the description of the isolate corresponds completely to the description of *Neocallimastix frontalis*.



a)

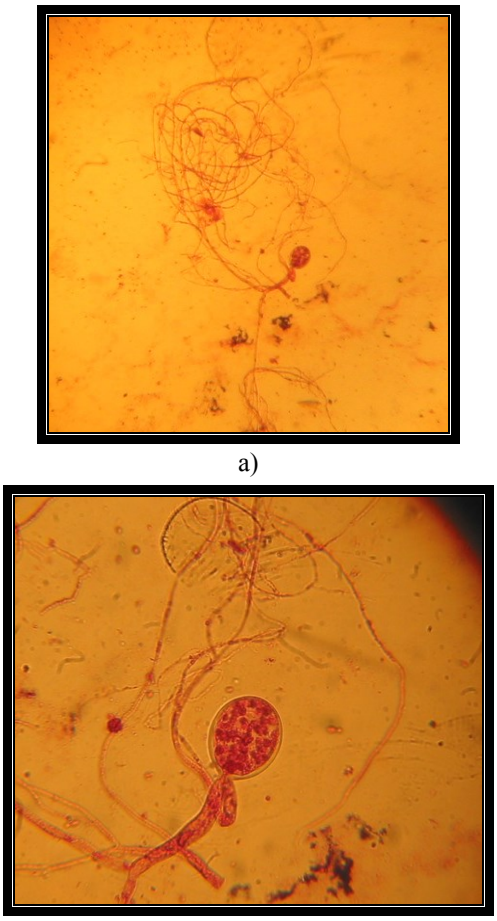
b)

Fig. 6. Strain J3 – *Neocallimastix frontalis,* sporangium on long sporangiophore, full with spores, a) magnification 10×; b) magnification 40×

Description of strain MR1

Endogenously developed zoosporangia, spheric with diameter of 42 μm; rhizoids mainly come over from one axis, occasionally two or three at the same side of the sporangium, the neck is wide; the main axis close to the sporangium, up to 15 μm in diameter, coiled; main rhizoid is often coiled; rhizoid system is spread up to 1 mm in diameter, zoospores are liberated through apical pore, accompanied by fast decomposition and cracking of the sporangium wall; zoospores are variable in length and shape, often spheric, with 7 to around 30 flagella (Figure 7).

The isolate is taken from the feces of a moufflon (*Ovis musimon*), kept in the Protected Nature Reserve Jasen, Skopje. Accordingly the key to determine the anaerobic fungi of Ho and Barr (1995), the description of the isolate corresponds completely to the description of *Neocallimastix frontalis*.



a)

b)

Fig. 7. Strain MR1 – *Neocallimastix frontalis,* sporangium with long sporangiophore, with spores, a) magnification 10×; b) magnification 40×

Enzyme activity

In this paper we study fermentative activity of five strains of *N. frontalis*, grown on different substrates. Growth pattern of isolated anaerobic fungi on various growth substrates are shown on Table 2.

Fungal growth in anaerobic cultures were measured by gas production during fermentation of CMC, Whatman N°1 and Avicel. The results are shown on Table 3 and Figures 8, 9 and 10.

Table 2

Growth pattern of isolated anaerobic fungi on various growth substrates

Isolated anaerobic fungi	*Neocallimastix frontalis*				
Growth substrate	MR1	Z2	J3	EZ2	EZ1
Whatman No1	+	+	+	+	+
CMC	+	+	+	+	+
Avicel	-	+	+	-	-

Table 3

Gas production when isolated strains are incubating on M10 with different carbohydrates as energy source

Incubation (h)	Whatman N°1					CMC					Avicel	
	EZ1	EZ2	Z2	J3	MR1	EZ1	EZ2	Z2	J3	MR1	Z2	J3
0	0	0	0	0	0	0	0	0	0	0	0	0
12	1	2	2	2	2	3	5	5	7	3	3	3.6
24	3	4	3.6	5	3.6	7.5	9	9	12	4.5	4	6.9
36	5.2	5.5	4.9	8	4.3	13.9	15	15	19	6.2	5.2	11
48	5.8	5.9	5.9	12	5.6	20	25	25	25	7.8	6.1	13.9
60	8	9	7.6	18	6.9	23	26	26	30	9.5	8	20.4
72	10	11	9.6	22	7.5	25	28	28	30	10.9	10	25.3
84	11.9	13	12.3	22	8.9	31	35	35	35	11.6	14	27
96	13.8	15	14	22	9.9	37	40	40	40	13.8	16	28
108	14.2	17	14	23	11	40	43	41	42	14.5	16	29
120	15.8	20	15	23	13	43	48	41	45	15	18	29

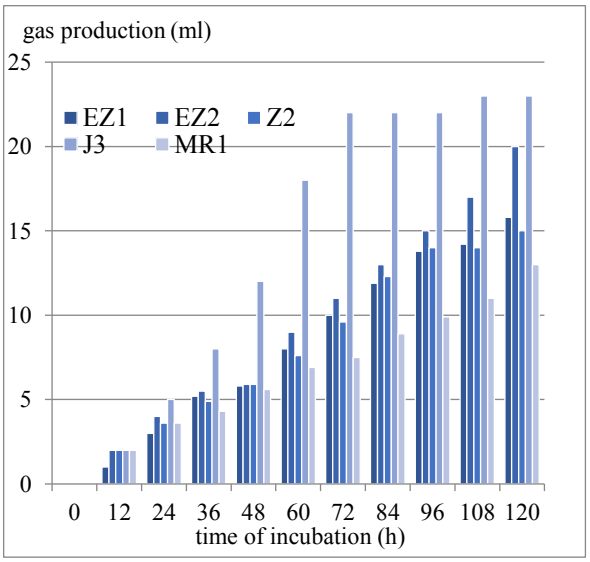


Fig. 8. Gas production when isolated strains were incubated on M10 with Whatman N°1 as an energy source (mean values, $n = 3$)

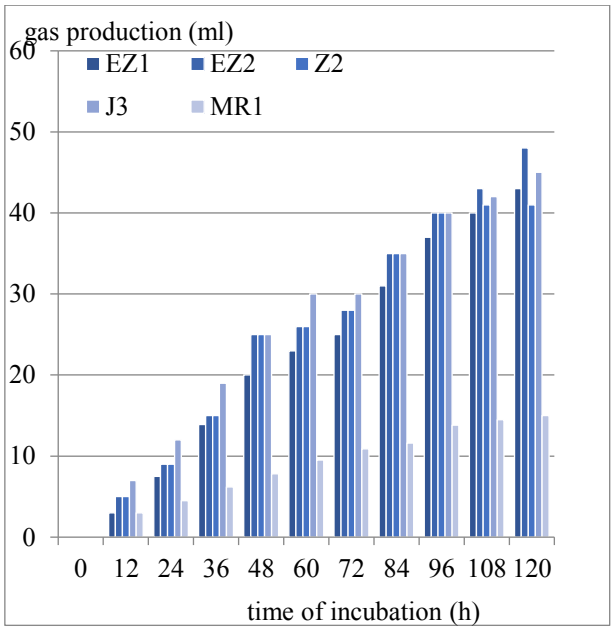


Fig. 9. Gas production when isolated strains were incubated on M10 with CMC as an energy source (mean values, $n = 3$)

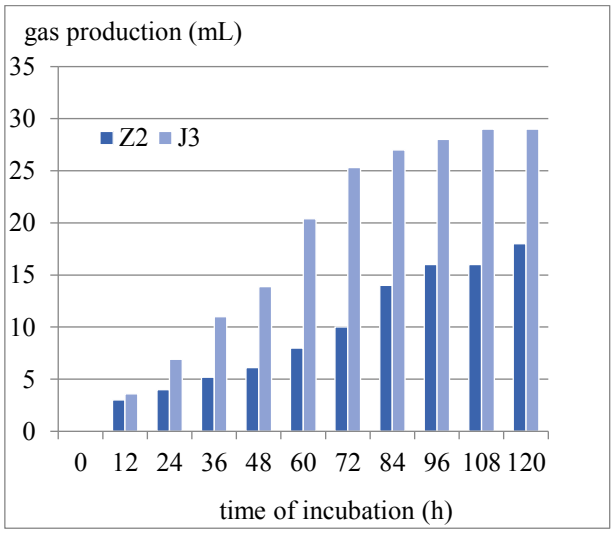


Fig. 10. Gas production when isolated strains were incubated on M10 with Avicel as an energy source (mean values, $n = 3$)

Our isolates, tested in this study released cellulolytic enzymes mainly into the culture fluid, which efficiently degrades highly ordered crystalline cellulose, like other examined anaerobic fungi (Mountfort and Asher, 1988; Wood et al., 1988, 1986). When cultures of anaerobic fungi were grown on CMC, Avicel and Whatman N°1, maximal activities obtained corresponded to maximal growth. This is also observed in the paper of Mountfort and Asher (1988), with *Neocallimastix frontalis* as test culture. Anaerobic fungi metabolize carbohydrates *via* mixed acid-type fermentation.

The isolates were initially isolated on medium M10 with glucose as a sole carbohydrate source, and then were grown on Whatman N°1, carboxymethyl cellulose (CMC) and Avicel, as a growth substrate. All isolates produced an array of enzymes that allowed them to hydrolyze plant cell walls. The enzymatic activity paralleled the growth of the isolate, as has been the case with other ruminal fungi (Mountfort and Asher, 1985; Lowe et al., 1987).

Table 4

Concentration of reducing sugars present in supernatants of five ruminal fungal isolates actively growing on Whatman No1, CMC and Avicel.

Reducing sugars (g/l)	EZ1	EZ2	Z2	J3	MR1
Whatman No1	12.9	14.6	26	42	6.8
CMC	15.4	17	22	51	6.9
Avicel	/	/	21	27	/

Our results showed that only isolate J3 and Z2 can grow on three tested carbohydrates as energy source. From Tables 3 and 4 it is clear that isolate J3 is the most active isolate from all tested anaerobic fungi. The enzymes CM-cellulose, FP-ase and avicelase secreted from J3 released the largest quantity of reducing sugars, compared to the other isolates. The results strongly suggest that anaerobic fungi found in the rumen possess common characteristics for carbohydrate utilization and fermentation end-products. However, other isolates need to be studied for comparison between monocentric and polycentric fungi. The efficiency of ruminants to utilize such a wide variety of feeds is due to highly diversified rumen microbial ecosystem consisting of bacteria (10^{10}–10^{11}cells/ml, representing more than 50 genera), ciliate protozoa (10^4–10^6/ml, from 25 genera), anaerobic fungi (10^3–10^5 zoospores/ml, representing five genera) and bacteriophages (10^8–10^9 /ml) (Kamra, 2005).

CONCLUSION

The anaerobic fungi produce a superior set of hemi/cellulolytic enzymes which they excrete separately or combined in cellulosomes. Additionally they are able to attack the plant material mechanically by their rhizoidal growth and open up the tissue for further digestion by bacteria.

Based on the overall results obtained, it could be concluded that all the examined isolates give glucose as a final product of fermentation of carbohydrates, followed with gas production in all the tested isolates. Also, the enzymes CM-cellulose, FP-ase and avicelase secreted from J3 released the largest quantity of reducing sugars, compared to the other isolates.

As a conclusion, based on all of these observations, anaerobic fungi, isolated from ruminant herbivores have highly active fibrolytic enzymes and these enzymes are attractive for scientific research.

The move towards understanding of anaerobic fungi using -omics based (genomic, transcriptomic and proteomic) approaches is starting to yield valuable insights into the unique cellular processes, evolutionary history, metabolic capabilities and adaptations that exist within the *Neocallimastigomycota*.

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