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COMPARATIVE STUDY OF CELLULOLYTIC ACTIVITY OF THREE RUMEN FUNGI ON DIFFERENT SUBSTRATES

ABSTRACT: Anaerobic chytridiomycete fungi are found in the gastrointestinal tracts of many domesticated ruminant and nonruminant herbivores and of a wide variety of wild herbivorous mammals. They produce high levels of cellulases and hemicellulases; these enzymes are regulated by substrate (especially soluble sugars) available to the organisms.

The aim of this paper was to do a comparative study of cellulolytic activity of three rumen fungi on carboxymethyl cellulose and Avicel. The capacity of enzymes was determined by monitoring the growth on carboxymethyl cellulose (CMC) and Avicel. Enzyme activity was detected extracellularly in culture supernatants after vegetative growth. All of the isolates degraded CMC and avicel, and exhibited cellulolytic activities (carboxymethyl cellulose-(CMC-ase) and avicelase).

KEY WORDS: anaerobic fungi, cellulases, gas production, *Neocallimastix*, *Piromyces*

INTRODUCTION

All animals, including humans, need to consume food on a fairly regular basis in an effort to assimilate specific essential nutrients necessary to support body structure and functions. Fortunately, not all animals attempt to consume the same foods in order to obtain these essential nutrients. Otherwise, tremendous competition would occur for available food reserves resulting in minimization of species diversity. The greatest amounts of stored nutrients in the world are in the form of plant cell wall material, which is indigestible by all mammalian digestive enzymes. Only bacteria and fungi possess the capacity to degrade plant cell wall materials.

The rumen is a highly complex ecosystem that contains different microbial species. Ruminant's performance depends on the activities of their microorganisms to utilize dietary feeds. The rumen microbial ecosystem is comprised of at least 30 bacterial (10^{10} to 10^{11}/ml rumen fluid) (Stewart et al., 1997), 40 protozoa (10^5 to 10^7 (Williams and Coleman, 1997), and 6 fungal

species (<10^5) (O z k o s e et al., 2001; N a g p a l et al., 2009b). Bacteria, fungi, and protozoa are responsible for 50 to 82% of cell-wall degradation (L e e et al., 2000).

Anaerobic fungi inhabit the gastrointestinal tract of herbivores, especially ruminants, and make a significant contribution to rumen metabolism, particularly to the digestion of plant structural biomass. Rumen fungi can even colonize highly recalcitrant material, including wheat and rice straw, maize stems, soybean hulls, temperate and tropical grasses or palm press fiber (H o et al., 1991; R o g e r et al., 1992; L e e et al., 2000). These properties make anaerobic fungi interesting for the scientific community.

Besides digestive tract and faeces (D a v i e s et al., 1993), anaerobic fungi have also been isolated from saliva of a sheep (L o w e et al., 1987a).

Rumen fungi produce a wide range of polysaccharide degrading enzymes (such as cellulase and xylanase) that degrade lignin-containing plant cell walls and have the ability to degrade up to 65% of dry weight of plant tissues in pure cultures (O r p i n and J o b l i n , 1988).

MATERIALS AND METHODS

Isolates

The anaerobic fungi used were isolates OEM1, C1 and G1, isolated from faeces of *Cervus dama* (from the Skopje ZOO), domestic cow and domestic goat, respectively. The methods used for isolation from the faeces, as well as the maintenance of pure fungal cultures, have been already described (A t a n a s o v a – P a n c e v s k a, 2006). Strain OEM1 resembled *N. frontalis* (H e a t h et al., 1983; O r p i n, 1975), whereas strains C1 and G1, resembled *P. communis* (O r p i n, 1977) and *P. mae* (L i et al., 1990), respectively.

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 the bacterial colony formation.

Medium

Complex medium for growth and maintenance of fungi was medium 10 of C a l d w e l l and B r y a n t (1966), except that glucose (4 g/l) was the only sugar present, and 10% (v/v) of clarified rumen fluid was added. The pH was adjusted to 7.0-7.2. Medium was prepared anaerobically using cysteine – HCl (0.05%) as reducing agent.

Culture conditions

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 fungus culture. The cultures were examined in triplicate. Inoculated cultures were grown at 39°C for 4 days.

Assay of cellulolytic enzymes in culture supernatants

Avicel and carboxymethyl-cellulose (CMC) were used as growth substrates for the production of cellulolytic enzymes. The inoculated serum bottles were incubated at 39ºC for 12, 24, 36, 48, 72 and 96 hours. Enzyme activities 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 uninoculated 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.

With CMC as the substrate, 0.2 ml of supernatant was incubated with 1.8 ml of 50 mM citrate-phosphate buffer (pH 6.8) containing 10 mg of CMC for 30 minutes at 50°C. The reaction was terminated and reducing sugars were detected by the addition of 3 ml of dinitrosalicylic acid reagent (DNS). The A550 values were read with glucose representing the standard.

With Avicel, 0.25 ml of culture supernatant was incubated with 50 mg of substrate in 1.75 ml of 50 mM citrate-phosphate buffer (pH 6.6) at 40°C for 4 hours. The reaction was terminated by placing the reaction tubes in boiling water for 5 minutes. The samples were centrifuged to pellet the residual Avicel, and the reducing sugars that were liberated were analyzed with DNS.

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.

RESULTS

This is relatively new area of rumen microbiology in Macedonia because of lack of information about cellulolytic activity of rumen fungi (Atanasova-Pancevska and Kungulovski, 2003/2004; Atanasova-Pancevska, 2006; Atanasova-Pancevska and Kungulovski, 2008). In the present study, three monocentric type ruminal fungi were isolated from ruminant herbivores. These isolates were characterized by their morphologies, gas production, and production of cellulolytic enzymes.

Anaerobic fungi are obligate anaerobes and gain energy from the fermentation of carbohydrates (O r p i n, 1994).

The changes of pH in cultures of the three fungi are shown in Table 1 and Figure 1. The pH of OEM1 cultures grown on Avicel was the lowest, while C1 cultures grown on CMC had the highest peak after 24 h of incubation, followed by gradual reduction to the final pH value of 7. The pH of all other cultures was virtually identical.

Tab. 1 – pH of cultures of isolates OEM1, C1 and G1, grown on CMC and Avicel

pH	CMC			Avicel		
	OEM1	C1	G1	OEM1	C1	G1
0	7.1	7.1	7.1	7.1	7.1	7.1
12	7.15	7.19	7.12	7.13	7.15	7.14
24	7.2	7.47	7.27	7.15	7.21	7.23
36	7.23	7.39	7.2	7.15	7.18	7.18
48	7.2	7.4	7.16	7.12	7.16	7.16
72	7.1	7	7	7.1	6.98	6.99

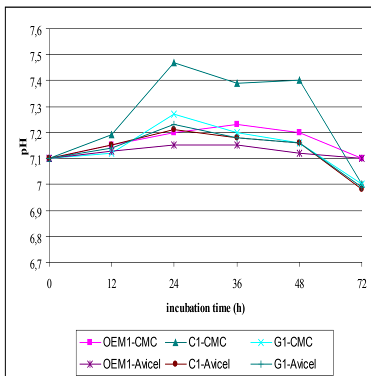

Fig. 1 – pH of cultures of isolates OEM1, C1 and G1, grown on CMC and Avicel

Fungal growth in cellulose cultures was measured by gas production during fermentation of CMC and Avicel. The amount of gas produced was determined after 12, 24, 36, 48, and 72 hours. The results are shown in Table 2 and Figure 2.

Tab. 2 – Gas production by OEM1, C1 and G1, grown on CMC and Avicel

gas (mL)	CMC			Avicel		
hours	OEM1	C1	G1	OEM1	C1	G1
0	0	0	0	0	0	0
12	5	4	3	6	5	5
24	32	28	25	29	27	26
36	30	27	24	27	28	26
48	15	13	13	15	12	13
72	9	7	6	8	7	8

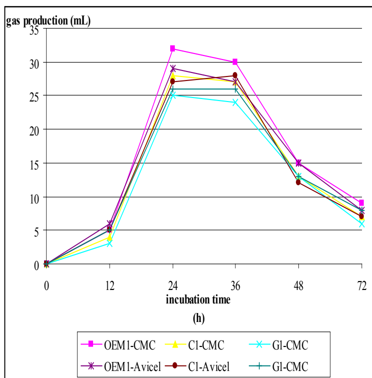

Fig. 2 – Gas production by OEM1, C1 and G1, grown on CMC and Avicel

The isolates were initially isolated on medium M10 with glucose as a sole carbohydrate source, and were then grown on Avicel (crystalline cellulose) and CMC, as a growth substrate. All isolates produced an array of enzymes that allowed them to hydrolyze plant cell walls. The enzymatic activity was simultaneous with the growth of the isolate (Table 3), as it was the case with other ruminal fungi (L o w e et al., 1987b; M o u n t f o r t and A s h e r , 1985).

The activities of CMC-ase and Avicelase of OEM1, C1 and G1 grown on both media proceeded in a similar manner (Table 3). After an initial lag phase of about 12-24 h, the activity of enzymes increased rapidly, reaching its maximum in 96th hour. The increase in the production of total gas for all strains was accompanied by an increase of enzyme activity.

Tab. 3 – Activities for carboxymethylcellulose (CMC-ase) and avicelase of OEM1, C1 and G1 grown on M10 medium with CMC and Avicel

Fungal isolate	Incubation period (h)	Enzyme (μmol/min/mL)			
		CMC-ase on CMC	Avicelase on CMC	CMC-ase on Avicel	Avicelase on Avicel
OEM1	12	0.0821	0	0	0.1910
	24	0.2356	0.0461	0.1966	0.2865
	36	0.4775	0.5730	0.2865	0.3820
	48	0.6685	0.6685	0.2865	0.3820
	72	0.7640	0.8596	0.3820	0.6685
	96	0.8893	0.8596	0.8596	0.6685
C1	12	0	0.2865	0	0
	24	0.0562	0.5730	0	0.0225
	36	0.2022	0.6685	0.2865	0.1461
	48	0.4775	0.6685	0.3427	0.3820
	72	0.6236	0.7640	0.3483	0.6685
	96	0.8202	0.7697	0.4775	0.7640
G1	12	0	0.3820	0	0
	24	0	0.5730	0	0.0225
	36	0.0506	0.5730	0.1517	0.2865
	48	0.4382	0.5730	0.2865	0.4775
	72	0.4775	0.6685	0.4382	0.7640
	96	0.5281	0.7191	0.6685	0.8596

pH	CMC			Avicel		
	OEM1	C1	G1	OEM1	C1	G1
0	7.1	7.1	7.1	7.1	7.1	7.1
12	7.15	7.19	7.12	7.13	7.15	7.14
24	7.2	7.47	7.27	7.15	7.21	7.23
36	7.23	7.39	7.2	7.15	7.18	7.18
48	7.2	7.4	7.16	7.12	7.16	7.16
72	7.1	7	7	7.1	6.98	6.99

DISCUSSION

The rumen provides an environment rich in nutrients and cofactors, including aminoacids, peptides, vitamins, and minerals, required by microorganisms for fermentation and growth (H u n g a t e, 1966).

The three fungal isolates from faeces used in this study belong to the groups of morphologically similar fungi.

The in vitro growth of the ruminal fungi followed a pattern typical of a wide variety of other fungi. When introduced into fresh media, these fungi proceed through a succession of phases beginning with a lag phase, continuing with a growth phase and then a stationary phase, and ending with a death phase (G r i f f e n, 1981). Often, the initial growth can be exponential, and it is usually followed by a longer period with a declining growth rate, which can appear as linear growth (Figures 1 and 2).

During the last 3 decades, measurement of *in vitro* microbial gas production (MGP) has received great impetus and become increasingly popular for determining the rate of fermentation (M e n k e et al., 1979; T h e o d o r o u et al., 1994; D a v i e s et al., 2000).

The syringe system is used widely to record the gas values at different times of incubation (D u a n et al., 2006).

This method for measuring gas production as an index of activity *in vitro* was first described by M e n k e et al. (M e n k e et al., 1979).

Changes in pH and total gas production of the culture were closely related to the extent of CMC and Avicel digestion. After an initial lag period of 12 hours, the fungal gas increased rapidly between 12 and 24 h and reached its maximum. After that, the stationary period of about 12 hours occurred, followed by a decrease to 6 mL gas at 72nd hour. Subsequently, the pH decreased rapidly between 48 and 72 h before stabilizing. The production of gas was identical, with slight differences (Figure 2).

The fermentation rate of various carbohydrates was present during the total gas production. As it was expected, gas production increased simultaneously with enzyme production in all the tested isolates (Figure 2). Isolates OEM1, C1 and G1 produced a maximum of 32, 28 and 25 ml gas, respectively, when incubated on M10 with CMC as the sole energy source, and 29, 27and 26 ml gas, respectively, when incubated on M10 with Avicel as the sole energy source.

The effectiveness of anaerobic fungi in ruminal cellulolysis depends on their ability to degrade complex polysaccharides which occur in plant cell walls. In addition to utilizing cellulose, *N. patriciarum* utilizes xylan and other grass hemicelluloses (O r p i n and L e t c h e r, 1979). Cultures of *N. patriciarum*, *Piromyces communis*, and *Sphaeromonas communis* (O r p i n, 1984), as well as several unnamed isolates of ruminal fungi which morphologically resemble *Neocallimastix* spp. or *S. communis* (G o r d o n, 1985; G o r d o n and A s h e s, 1984), all degrade various polysaccharide components of wheat straw cell walls. About half of the total cell walls, including about half of the cellulose and hemicellulose components, were lost from 4 to 5 days old cultures of *Neocallimastix* and *Piromonas* spp., whereas only smaller proportions

of these cell wall components disappeared from *Sphaeromonas* cultures grown for the same period of time (G o r d o n, 1985; O r p i n, 1984).

The extent to which ruminal fungi digest substrate depends on both the strain of fungi and the type of substrate. Cellulolytic enzymes were produced by our isolates after growth on CMC and Avicel. The effect of growth substrate on enzyme production by isolates OEM1, C1 and G1 was examined (Table 3). Although, cellulose was expected to be better inducer of cellulolytic enzymes, avicelase and CMC-ase activities were almost identical. Enzyme production was substrate dependent but differences were less obvious than in the case of ruminal *Piromyces* species (W i l l i a m s and O r p i n, 1987a, b).

The anaerobic fungi produce a wide range of polysaccharide degrading enzymes. Enzymes have been found associated with the rhizomycelium and many were also secreted into the surrounding environment (W i l l i a m s and O r p i n, 1987; L o w e et al., 1987d; B r e t o n et al., 1995; G e r b i et al., 1996a). The presence and activity of some surface associated enzymes fluctuated according to the stage of the life cycle (B r e t o n et al. 1995; G e r b i et al. 1996a). Also, the growth conditions greatly influence enzyme production, with three times the level of fibrolytic enzymes being produced in a stirred fermenter compared with static batch cultures in bottles (D i j k e r m a n et al., 1996a), whereas other continuous flow cultures produced up to twenty times the level of enzymes of batch cultures (Z h u et al., 1996). Fibrolytic enzymes were generally repressed by the presence of the sugar monomers resulting from degradation of polysaccharide: glucose for cellulases and xylose, and arabinose for xylanases (M o u n t f o r t, 1994).

CONCLUSION

Increased interest and research activities in the anaerobic gut fungi in the last decade or so have provided much information on their biology, taxonomy, physiology and enzymology. However, at present, there is still very little information on the range and diversity of fungal species inhabiting different host species, different substrates and different parts of the alimentary tract. It is not known whether the gut fungi are host or substrate specific. Further work is needed to elucidate this and some other aspects of their life, but in order to achieve these more specific techniques, involving molecular biology and molecular biotechnology, will be required.

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ЦЕЛУЛИТИЧКА АКТИВНОСТ ТРИ ВРСТЕ РУМЕНСКИХ ПЛЕСНИ КУЛТИВИРАНИХ НА РАЗЛИЧИТИМ СУПСТРАТИМА – КОМПАРАТИВНА СТУДИЈА

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Резиме

Румен представља изразито комплексан екосистем који садржи различите врсте микроба. Перформансе румена зависе од активности и способности његових микроорганизама у утилизацији нутритијената. Руменски микробни екосистем садржи бар 30 врста бактерија (10^{10} до 10^{11}/ml руменске течности) (Stewart et al., 1997), 40 врста протозоа (10^5 до 10^7 (Williams and Coleman, 1997), и 6 врста плесни ($<10^5$) (Ozkose et al., 2001; Nagpal et al., 2009б). Бактерије, плесни и протозоиски организми су одговорни за деградацију од 50 до 82% ћелијског зида (Lee et al., 2000).

Анаеробне хитридиомицетне плесни су пронађене у гастро-интестиналном тракту великог броја домаћих руминентних и неруминентних хербиворних животиња како и код великог броју дивљих хербиворних сисара. Ове плесни производе велике количине ензима целулазе и хемицелулазе, регулисаних од стране супстрата (посебно од стране растворљивих шећера) доступних организму.

Циљ овог рада је да се одреди целулолитичка активност код три врсте руменских плесни култивираних на карбоксиметилцелулазној (CMC) и Avicel-ској подлози. Ензимска активност је детектована екстрацелуларно у супернатанту култура након вегетативног раста. Сви изолати деградирају CMC и Avicel и показују целулолитичку активност (карбоксиметил целулаза и авицелаза).