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Effect of Glucose Levels and N Sources in Defined Media on Fibrolytic Activity Profiles of *Neocallimastix sp.* YQ1 Grown on Chinese Wildrye Grass Hay or Alfalfa Hay

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ABSTRACT : Ferulic acid esterase (FAE) and acetyl esterase (AE) cleave feruloyl groups substituted at the 5′-OH group of arabinosyl residues and acetyl groups substituted at O-2/O-3 of the xylan backbone, respectively, of arabinoxylans in the cell wall of grasses. In this study, the enzyme profiles of FAE, AE and polysaccharide hydrolases of the anaerobic rumen fungus *Neocallimastix* sp. YQ1 grown on Chinese wildrye grass hay (CW) or alfalfa hay (AH) were investigated by two 2×4 factorial experiments, each in 10-day pure cultures. The treatments consisted of two glucose levels (G^+: glucose at 1.0 g/L, G^-: no glucose) and four N sources (N1: 1.0 g/L yeast extract, 1.0 g/L tryptone and 0.5 g/L $(NH_4)_2SO_4$; N2: 2.8 g/L yeast extract and 0.5 g/L $(NH_4)_2SO_4$; N3: 1.6 g/L tryptone and 0.5 g/L $(NH_4)_2SO_4$; N4: 1.4 g/L tryptone and 1.7 g/L yeast extract) in defined media. The optimal combinations of glucose level and N source for the fungus on CW, instead of AH, were G^-N4 and G^-N3 for maximum production of FAE and AE, respectively. Xylanase activity peaked on day 4 and day 6 for the fungus grown on CW and AH, respectively. The activities of esterases were positively correlated with those of xylanase and carboxymethyl cellulase. The fungus grown on CW exhibited a greater volatile fatty acid production than on AH with a greater release of ferulic acid from plant cell wall. (**Key Words :** Rumen Fungi, Ferulic Acid Esterase, Acetyl Esterase, Polysaccharide Hydrolase, Volatile Fatty Acids)

INTRODUCTION

The plant cell wall is composed mainly of cellulose, hemicelluloses and lignin. Cellulose is the most abundant polysaccharide, and can be hydrolyzed via a complex process involving cellulases (Beauchemin et al., 2004). The xylan backbone of hemicelluloses can be degraded by xylanase (Wang and McAllister, 2002). Monocotyledonous plants, such as grasses, and dicotyledonous ones, such as alfalfa, have lignin-type compounds within the secondary walls and middle lamella of cell walls. Fungi account for only approximately 8% of the microbial biomass in the rumen (Orpin and Joblin, 1988). However, these rumen fungi play an important role in fibre digestion because they can penetrate both the cuticle and cell wall of lignified tissue (Akin, 1986). Detailed discussions of microbial enzymes have been presented elsewhere (Forsberg et al., 1986; White et al., 1993). Anaerobic fungi possess a broad range of fibrolytic polysaccharide hydrolases, including cellulases, xylanases, and others (Ushida et al., 1997; Ho and Abdulah, 1999). *Neocallimastix frontalis* has the highest cellulolytic activity of any organism ever reported in the literature (Varga and Kolver, 1997). Borneman et al. (1990, 1991) demonstrated strong activities of ferulic acid esterase (FAE) and *p*-coumaric acid esterase (CAE) of two monocentric and three polycentric fungi. The activities of these enzymes can aid in the utilization of low quality roughage by breaking ester bonds in the cross-linkages between lignin and hemicelluloses. These linkages between two ferulic acid molecules on adjacent chains provide cell wall integrity, and, in general, are resistant to enzymatic attack by microorganisms (Iiyama et al., 1994).

The study of anaerobic gut fungi is currently hampered by the lack of reliable methods for their accurate identification, differentiation and enumeration. Molecular biology may provide tools and approaches which will aid in the challenges faced by rumen ecologists and nutritionists. Since their recognition in 1975, the anaerobic gut fungus *Neocallimastix frontalis* from sheep has been classified as

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Chytridiomycetes, based on thallus morphology (Orpin, 1975) and the presence of chitin in the cell walls (Orpin 1976; Orpin, 1977). Fungi are more prevalent in the rumen of cattle and sheep fed high-fibre diets (Bauchop, 1979). Fibrolytic fungi isolated from rumen contents have been classified into two monocentric (e.g. *Neocallimastix* and *Piromyces*) and three polycentric genera (e.g. *Orpinomyces*, *Anaeromyces* and *Caecomyces*). All of these fungi have been shown to have strong fibrolytic activities (Ushida et al., 1997; Hodrova et al., 1998; Tripathi et al., 2007; Atanasova-Pancevska and Kungulovski, 2008). Gerbi et al. (1996) reported that production of cellulase and xylanase by *Caecomyces communis* was regulated by glucose and cellobiose in the growth medium. However, little attention has been paid to production of esterases that degrade cross-linkages, including FAE, CAE, acetyl esterase (AE) or acetyl xylan esterase. Although anaerobic fungi isolated from the rumen produce FAE, CAE and AE (Borneman et al., 1990; Borneman et al., 1991), there is little information about the effects of different fibrous substrates, different levels of soluble sugar, or different sources of N on the production of these esterases.

The objective of this study was to investigate fibrolytic activities profiles of FAE, AE, carboxymethyl cellulases, xylanase, avicelase and volatile fatty acids production of *Neocallimastix sp.* YQ1, grown on Chinese wildrye grass hay and alfalfa hay with comparable content of ferulic acid, in response to different glucose levels and N sources in defined anaerobic media.

MATERIALS AND METHODS

Microorganism

Neocallimastix sp. YQ1 was isolated from the rumen fluid of Holstein steers using a roll-tube technique as described by Hungate (1969). The steers were kept at the Experimental Farm of China Agricultural University in Beijing, and they were fed as previously described (Yang et al., 2009). The fungus was identified according to morphological characteristics of the thallus (Tripathi et al., 2007) and its partial 18S rDNA sequence (Brookman et al., 2000; Chen et al., 2003). The partial sequence of the 18S rDNA of *Neocallimastix sp.* YQ1 is deposited in GenBank (Accession no. FJ687481). The fungus was incubated anaerobically on autoclaved Hungate's basal culture media at 39°C with 8 g/L chopped wheat straw and transferred to fresh culture medium every 4 days.

Substrates and chemical analyses

Chinese wildrye grass hay (CW) (*Leymus chinensis*) and alfalfa hay (AH) at the late-bloom stage were harvested in Jinlin province of China and chopped into 2-5 mm lengths. Chemical analysis of ground samples (1.0 mm screen) of CW and AH was carried out using methods of the Association of Official Analytical Chemists (AOAC, 2005). The samples were dried at 105°C for 4 h and equilibrated in a desiccator to determine dry matter (DM) content (ID 930.5) (AOAC, 2005). Total nitrogen (N) was determined using a Kjeldahl method (ID 984.13) (AOAC, 2005) with a Kjelfoss apparatus (Kjeltec^{TM}, Hollgard, Denmark), and crude protein content was calculated as N×6.25. Ash was measured following the methods of AOAC (ID 942.05) (AOAC, 2005). Neutral detergent fibre (NDF) was analyzed by the method of Van Soest et al. (1991), without the use of sodium sulfite and amylase. Acid detergent fibre (ADF) was assayed using the method of AOAC (ID 973.18) (AOAC, 2005). Both NDF and ADF were not corrected with the ash content. The concentrations of alkali-extractable ferulic acid in CW and AH were determined by a high-performance liquid chromatography method (Yang et al., 2009).

Experimental design

To increase feruloyl and acetyl esterase production of *Neocallimastix sp.* YQ1, two simultaneous 2×4 factorial experiments were designed with two levels of glucose (G^+: glucose added at a level of 1.0 g/L, G^-: without glucose) and four soluble N sources (N1, N2, N3 and N4). In the liquid basal culture described by Yue et al. (2009), the fibrous substrate source of wheat straw was replaced by either CW or AH at an inclusion level of 8 g/L. The levels of glucose in G^+, yeast extract, tryptone and $(NH_4)_2SO_4$ in N1 were the same as those in Hungate's basal culture medium (Hungate, 1969). N sources with equal N content were arranged as four supplementations including N1: a mixture of 1.0 g/L yeast extract, 1.0 g/L tryptone and 0.5 g/L $(NH_4)_2SO_4$; N2: 2.8 g/L yeast extract and 0.5 g/L $(NH_4)_2SO_4$; N3: 1.6 g/L tryptone and 0.5 g/L $(NH_4)_2SO_4$; N4: 1.4 g/L yeast extract, 1.7 g/L tryptone.

For each combination of glucose, N and substrate indicated above, an aliquot of 0.1 ml of the inoculum described above was added to each of four Hungate tubes filled with 9.0 ml anaerobic medium supplemented with 1,600 IU/ml penicillin and 2,000 IU/ml streptomycin. The cultures were incubated at 39°C for 10 days. An aliquot of 0.7 ml culture was sampled daily using a 1.0 ml sterile syringe, and 0.7 ml fresh medium was immediately added to the tube to compensate for the withdrawn sample.

Samples of the liquid cultures were centrifuged for 10 min at 1,000×*g* at 4°C, and the supernatant was immediately analyzed for fibrolytic activity. The activities of FAE, AE, xylanase, carboxymethyl cellulase (CMCase) and avicelase were determined immediately as described previously (Yang et al., 2009). One enzyme activity unit (U) was defined as the amount of enzyme required to release 1 μmol of ferulic acid or *p*-nitrophenol or reducing sugar (e.g. xylose or glucose equivalent) per minute per millilitre of

culture. The liquid cultures (0.5 ml) were centrifuged at 10,000×g at 4°C for 30 min and the concentration of ferulic acid in the supernatants was determined by absorbance at 320 nm (Xie, 2007). The concentrations of volatile fatty acids (VFA), including acetate, propionate, butyrate, iso-butyrate, valerate and iso-valerate, were measured using a gas chromatograph as described by Yue et al. (2009). Individual VFAs were summed to obtain total VFA production. The ratio of non-glucogenic to glucogenic acids (NGR) (Ørskov, 1975) was calculated using Eq. (1).

$$NGR = (Acetate+2\times Butyrate+Valerate)/(Propionate+Valerate) \quad (1)$$

where VFAs were expressed in molar proportions of total VFA production at the end of the 10-day culture period.

Statistical analyses

For each feedstuff, fibrolytic enzyme activities, VFA production, and levels of reducing sugars and ferulic acid were analysed to compare differences among glucose levels, N source, and their interaction, by the PROC MIXED of SAS (1999). The day of sampling was treated as a repeated variable, and the random effect of two forage types was analysed by the RANDOM statement in the PROC MIXED procedure. Least square means for each combination of glucose and N source were listed using the LSMEANS statement of SAS (1999) and compared using a multiple comparison test (Tukey). The least square means were considered different at $p<0.05$. The model is represented by Eq. (2):

$$Y_{ijkl} = \mu+R_i+G_j+N_k+D_l+(G\times N)_{jk}+\varepsilon_{jkl} \quad (2)$$

Table 1. Chemical composition of forages (g/kg DM)

	Chinese wildrye grass	Alfalfa hay
Crude protein	145.1[a]	130.9[b]
Neutral detergent fibre	683.9[a]	638.9[b]
Acid detergent fibre	383.5[b]	478.9[a]
Ash	65.3[b]	93.4[a]
Ferulic acid	5.9	6.1

Means in a row without common superscripts differ ($p<0.05$).

where Y_{ijkl} is the dependent variable under examination, μ is the overall mean, R_i is the replicate effect (i = 4), G_j is the fixed effect of glucose levels (j = 2, G^+, G^-), N_k is the fixed effect of the N source (k = 4, N1, N2, N3, N4), D_l is the incubation day effect (l = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10). G×N is the fixed effect of the interaction between glucose and N source, and ε_{ijkl} is the error term. Least squares means are reported throughout the 10 day incubation, and significance was declared at $p<0.05$ unless otherwise noted.

RESULTS

Chemical compositions of CW and AH

Crude protein in the NDF was greater in CW than in AH (Table 1). The ADF and ash of CW were lower than those of AH. Ferulic acid content did not differ between CW and AH.

Fibrolytic enzyme activity profiles

The interaction between glucose and N had marked effects on the production of AE and CMCase for fungus grown on CW ($p<0.01$) (Table 2). G^+ depressed the production of FAE, AE, CMCase and avicelase ($p<0.01$).

Table 2. Effects of different glucose levels and N sources on fibrolytic activities, production of volatile fatty acids (VFA) and ferulic acid in cultures of *Neocallimastix sp.* YQ1 grown on chopped Chinese wildrye grass for 10 days

Items	Glucose levels		N sources				SEM	p values		
	G^-	G^+	N1	N2	N3	N4		Glucose	N	Interaction
Ferulic acid esterase (mU)	2.6[a]	1.9[b]	2.0[bc]	1.9[c]	2.5[a]	2.4[ab]	0.08	**	*	NS
Acetyl esterase (mU)	79[a]	49[b]	47[c]	49[c]	70[b]	85[a]	1.9	**	**	**
Xylanase (mU)	519	495	479[b]	480[b]	541[b]	526[a]	13.9	NS	*	NS
Carboxymethyl cellulase (mU)	63[a]	49[b]	51[c]	54[bc]	58[ab]	61[a]	2.8	**	*	**
Avicelase (mU)	83[a]	73[b]	45[c]	76[b]	94[a]	94[a]	3.9	*	*	**
Ferulic acid (μmol/L)	111	107	108[ab]	114[a]	104[b]	107[ab]	3.4	NS	*	NS
Total VFA (mM)	49.2	49.0	50.2	48.4	48.7	49.2	2.49	NS	NS	NS
Acetate (mmol/mol)	806[a]	761[b]	781	780	786	781	5.3	**	NS	**
Propionate (mmol/mol)	109[b]	116[a]	109	113	112	115	3.2	*	NS	**
Butyrate (mmol/mol)	68[b]	108[a]	95[a]	89[ab]	87[b]	89[ab]	3.8	**	*	**
NGR	8.1	8.0	8.4[a]	7.9[ab]	8.0[ab]	7.8[b]	0.23	NS	*	NS

Different superscripts indicate significant differences within values in a row in the same class. SEM represents standard error of least square means in a row. NGR represents ratio of non-glucogenic to glucogenic acids.
* $p<0.05$, ** $p<0.01$, NS $p>0.05$.

Production of FAE, AE, xylanase, CMCase and avicelase in N3 and N4 were greater than those in N1 ($p<0.05$). The correlation coefficients (r) of FAE with xylanase, CMCase and avicelase were 0.39, 0.34 and 0.14, respectively ($p<0.05$). The r values of AE with xylanase, CMCase and avicelase were 0.75 ($p<0.01$), 0.34 ($p<0.01$) and 0.08 ($p = 0.11$) respectively. The r value between FAE and AE was 0.56 ($p<0.01$).

When the fungus was grown in AH substrate (Table 3), there were interactions between glucose and N for FAE and AE ($p<0.01$). G^+ depressed AE production, but it did not alter the production of FAE, xylanase, CMCase and avicelase. The production of FAE, xylanase and CMCase did not differ among the different N sources. The production of AE in N4 was greater than that in N1 ($p<0.05$), and the production of avicelase in N1 was greater than in the other N sources ($p<0.01$). The r values of FAE with xylanase, CMCase and avicelase were 0.19 ($p<0.01$), 0.11 ($p = 0.06$) and 0.01 ($p = 0.76$), respectively. The r values of AE with xylanase, CMCase and avicelase were 0.66 ($p<0.01$), 0.32 ($p<0.01$) and 0.02 ($p = 0.64$) respectively. The r value between FAE and AE was 0.31 ($p<0.01$).

The activities of esterases and polysaccharide hydrolases showed different trends and different peaks during the 10-day experimental period (Figures not shown). The activity of FAE in CW increased over time, reaching a peak on day 10, whereas activity of FAE in AH reached a peak on day 9. The highest mean AE activity occurred on day 4 in CW, but increased over time in AH. The peaks of xylanase activity occurred in CW on day 4 and in AH on day 6. CMCase activity in both CW and AH increased linearly over time. No distinctive profiles of avicelase activity were observed in either substrate.

Release of ferulic acid

Glucose addition did not alter the release of ferulic acid from CW (Table 2). However, more ferulic acid was released in N3 than in N2. The level of ferulic acid peaked after 3 days. During the first 3 days, the r values of ferulic acid with FAE, AE, xylanase, CMCase and avicelase were 0.37 ($p<0.01$), 0.66 ($p<0.01$), 0.62 ($p<0.01$), 0.45 ($p<0.01$) and 0.24 ($p<0.05$), respectively.

As shown in Table 3, G^+ depressed the release ferulic acid from AH ($p<0.01$) and N2 in cultures significantly enhanced the release of ferulic acid ($p<0.01$). The level of ferulic acid peaked after 3 days. During the first 3 days, the r values of ferulic acid with FAE, AE, xylanase, CMCase and avicelase were 0.03 ($p>0.05$), 0.28 ($p<0.01$), 0.52 ($p<0.01$), 0.45 ($p<0.01$) and -0.34 ($p<0.01$), respectively.

Production and patterns of VFA

There were interactions between glucose and N for acetate, butyrate, and propionate in the CW substrate ($p<0.05$) (Table 2). Neither glucose nor N affected the total VFA concentration. G^+ depressed the molar proportion of acetate ($p<0.01$) and increased the molar proportions of propionate ($p<0.05$) and butyrate ($p<0.01$). The N sources did not alter the molar proportions of acetate and butyrate. Glucose did not alter NGR, but a significant difference was observed between N1 and N4 (Table 2).

Total VFA and acetate in the AH substrate did not differ between G^- and G^+. G^+ depressed the production of acetate and enhanced that of butyrate ($p<0.01$) (Table 3). Difference in VFA production was not significant between N2 and N4. Compared with N1, N3 significantly decreased

Table 3. Effects of different glucose levels and N sources on fibrolytic activities, production of volatile fatty acids (VFA) and ferulic acid in cultures of *Neocallimastix sp.* YQ1 grown on chopped alfalfa hay for 10 days

Items	Glucose levels		N sources				SEM	p values		
	G^-	G^+	N1	N2	N3	N4		Glucose	N	Interaction
Ferulic acid esterase (mU)	1.1	1.0	1.0	0.8	1.2	1.1	0.12	NS	NS	**
Acetyl esterase (mU)	71^a	60^b	60^b	66^{ab}	64^{ab}	72^a	1.6	**	*	**
Xylanase (mU)	322	338	328	311	326	350	14.2	NS	NS	NS
Carboxymethyl cellulase (mU)	46	53	57	51	46	42	3.2	NS	NS	NS
Avicelase (mU)	53	58	76^a	57^b	43^c	44^c	3.6	NS	*	NS
Ferulic acid (μmol/L)	74^a	65^b	70^b	78^a	61^c	69^b	2.0	**	*	**
Total VFA (mM)	43.5	45.6	42.4^b	45.0^{ab}	41^b	49^a	2.81	NS	*	NS
Acetate (mmol/mol)	746^a	702^b	741^a	724^{ab}	710^b	726^{ab}	8.4	**	*	NS
Propionate (mmol/mol)	158	161	137^c	173^a	172^{ab}	158^b	6.7	NS	**	NS
Butyrate (mmol/mol)	73^b	118^a	99^a	81^b	97^a	98^a	6.3	**	*	NS
NGR	5.4	5.7	6.5^a	4.9^c	5.0^{bc}	5.6^b	0.53	NS	*	NS

Different superscripts indicate significant differences within values in a row in the same class. SEM represents standard error of least square means in a row. NGR represents ratio of non-glucogenic to glucogenic acids.

* $p<0.05$, ** $p<0.01$, NS $p>0.05$.

the production of acetate ($p<0.05$), and N2 significantly decreased the production of butyrate ($p<0.05$). The highest production of propionate was observed in N2 ($p<0.01$) and the highest NGR was observed in N1 ($p<0.05$).

DISCUSSION

Effect of carbohydrate and N sources on fibrolytic enzyme activity profiles

In this study, different fibrous substrates and glucose levels resulted in different fibrolytic enzyme activity profiles. These results agree with those reported by Chungool et al. (2008), who reported that AE activity differed between the aerobic fungus *Streptomyces sp.* PC22 grown on birchwood xylan and corn husks. Furthermore, Yue et al. (2009) showed that not only the carbohydrate source altered activities of fibrolytic enzymes of a rumen fungus, but also the N source. The role of N source in the production of fungal enzymes has almost been ignored in the literature. In our study, we also observed that the N source affected the production of various enzymes. Because the structure of plant cell walls in fibrous feedstuffs with higher ferulic acid and *p*-coumaric acid contents (e.g. corn stalk, wheat straw, rice straw) is very complex, complete degradation of these complex substrates for animal production requires cooperative actions of fibrolytic enzymes. Therefore, it is necessary to screen for the optimal combination of carbohydrate source and N source to obtain maximal enzyme production. In CW substrate, the optimal combination of glucose level and N source was G^-N4 and G^-N3 for FAE and AE production, respectively. Activity of FAE increased until day 3 of incubation and remained at a high level thereafter, whereas the specific activity of AE peaked on day 4 (data not shown). In AH substrate, the optimal combination of glucose level and N source was G^-N3 for FAE production and G^-N4 for AE production. In this substrate, FAE and AE activities peaked on day 9 and day 10, respectively (data not shown).

Both FAE and AE activities in this study were greater than those produced by *Neocallimastix sp.* YQ2 previously isolated from the same host animals (Yue et al., 2009). However, FAE activity was lower than that reported for the aerobic fungus *Penicillium brasilianum* grown on sugar beet pulp or oat spelt xylan (maximum mean production of 225 mU) (Panagiotou et al., 2007). Similarly, the maximum AE activity observed in this study was lower than reported for the bacteria *Streptomyces sp.* PC22 grown on birchwood xylan or corn husks (Chungool et al., 2008). The CMCase activities determined in our study were comparable to those reported for the anaerobic rumen fungus *Neocallimastix frontalis* EB188, which was originally incubated in glucose medium and switched to glucose, cellobiose, or cellulose medium (Barchievich and Calza, 1990). However, the CMCase activities in our study were lower than the highest value (231 mU) obtained from 12 anaerobic fungal strains identified as species of *Neocallimastix*, *Piromyces*, *Orpinomyces*, *Anaeromyces* isolated from faecal samples of blue bull, as reported by Tripathi et al. (2007). Those authors also reported relatively low xylanase activities (127 mU) in comparison to the values in our study and an earlier study on *Neocallimastix sp.* (Lowe et al., 1987).

In this study, the activities of fibrolytic enzymes of YQ1 varied over the incubation period. Most showed irregular patterns of activity. Similar trends were reported by Hodrová et al. (1998) for enzymes produced by cultures of *Orpinomyces joyonii* and *C. communis* grown on microcrystalline cellulose and AH (Hodrova et al., 1998). However, depending on the growth substrates, some enzyme activities of YQ1 (*i.e.,* FAE, AE, xylanase and CMCase) increased during the 10-day incubation period. This is consistent with the results of Saad et al. (2008) and Yue et al. (2009). The variations might be related to the structure of different carbohydrate sources (e.g. the relative proportions of water soluble sugars, starch and structural carbohydrates). Alternatively, such variations could reflect the characteristics of the degradation process, in which activities of different fibrolytic enzymes are required at different stages of degradation to provide sufficient nutrients for fungal growth. These differences could also explain the variations among the correlations between esterase and polysaccharide hydrolase activities that were observed for this fungus.

Release of ferulic acid from CW and AH

In non-lignified tissues of young grasses, phenolic acids prevent microbial degradation and appear to be a major barrier to biodegradation (Akin, 2008). In our study, ferulic acid concentrations peaked during the first 3 days in both CW and AH substrates. The correlation analysis indicates that the extent of release of ferulic acid from substrate mainly depended on activities of FAE and AE, as well as those of xylanase and CMCase. Although there were no differences in the ferulic acid contents between CW and AH (Table 1), more ferulic acid was released from CW than from AH. The reason why ferulic acid levels decreased and remained at a stable, low level after day 3 is unclear. Biotransformation of ferulic acid into vanillin was reported in the *Streptomyces* isolate S10 (Sarangi et al., 2009) and *Aspergillus niger* (Baqueiro-pena et al., 2010). The fact that ferulic acid did not accumulate in this study suggests that it could be partly metabolized or transformed in the cultures, *e.g,* into vanillin. Some researchers have used high-performance liquid chromatography methods instead of spectrophotometric methods to measure ferulic acid in cultures (Sancho et al., 2001; Panagiotou et al., 2007). However, there are no reports of biotransformation of

ferulic acid into vanillin in the rumen by rumen fungi. Our results suggest that quantitative analyses of both vanillin and ferulic acid should be considered in later studies.

Responses of VFA end-product fibrolytic enzyme activities

In ruminant animals, microorganisms in the rumen ferment a wide range of endogenous and exogenous substrate to produce VFA, methane, carbon dioxide and hydrogen. The production of VFA could indirectly reflect fungal growth performance and the potential of the fungus to degrade the substrate (Phillips and Gordan, 1998). In our study, the addition of glucose to the media did not stimulate fungal growth during the 10-day incubation period. However, nitrogen source appears to be important for growth, because N4 (1.7 g/L yeast extract and 1.4 g/L tryptone) culture enhanced growth of the fungus. Bergman (1990) reported that typical total VFA concentrations in the rumen range from 60 to 120 mM. Specific molar proportions from a total rumen VFA concentration of 106 mM reported for sheep consuming hay were 690 mmol/mol acetate, 200 mmol/mol propionate, and 110 mmol/mol butyrate (Bergman et al., 1965). In this study, the molar proportion of acetate was greater than figures for mixed rumen microorganisms (Bergman, 1990), but lower than that (930 mmol/mol) reported previously for *Neocallimastix frontalis B9* grown in ball-milled filter paper and guinea grass media for 96 h (Saad et al., 2008). VFA production can account for over two-thirds of energy intake to the host ruminant animal (Bergman, 1973), and the balance between supply of glucogenic relative to non-glucogenic fatty acids influences the efficiency of VFA utilization for productive purposes (Ørskov, 1975). The high NGR in this study suggested that the fungi of *Neocallimastix* family predominantly produced non-glucogenic acids, especially acetate, in the rumen as a lipogenic nutrient for the host animal.

CONCLUSION

In the cell walls of forage grasses, ferulic acid is esterified to arabinoxylans and participates with lignin monomers in oxidative coupling pathways to generate ferulate-polysaccharide-lignin complexes that cross-link the cell wall. Such cross-links hinder cell wall degradation by ruminant microbes, reducing plant digestibility. Beside the optimal combinations of glucose levels and N sources in the defined Hungate's medium, the maximal production of side-chain degrading esterases and polysaccharide hydrolases was forage-specific for the anaerobic fungus *Neocallimastix sp.* YQ1. The activities of esterases were positively correlated with those of polysaccharide hydrolases, including xylanase and CMCase. Although there were no differences in ferulic acid contents between the two forages, the fungus grown on CW exhibited a greater enzyme production than on AH with a greater release of ferulic acid. Prospective applications of ruminal anaerobic fungi could be exploited for large scale production of fungal enzymes in the biotechnology industry.

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