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Effects of Phenolic Monomers on the Degradation of ^{14}C-Cellulose by Rumen Fungi

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Abstract: Anaerobic fungi were isolated from the rumens of cattle, buffalo and goat. A total of 133 isolates were obtained. Three of these isolates namely *Neocallimastix frontalis* B9, *Piromyces mae* B6 and *Orpinomyces joyonii* C3 were studied for their cellulolytic activity by using ^{14}C-cellulose produced by *Acetobacter xylinum* as the substrate. The effects of phenolic acids on the cellulolytic activity of a fungal isolate *N. frontalis* B9 from buffalo was also determined. The results showed that among the fungal species, *N. frontalis* B9 had the highest cellulolytic activity, followed by *P. mae* B6 and *O. joyonii* C3. The cellulolytic activities of *N. frontalis* isolates from buffalo, cattle and goat were not significantly different. Both ρ-coumaric and ferulic acids reduced significantly ($p<0.05$) the celluloytic activity of *N. frontalis* B9.

Key words: *Neocallimastix frontalis*, phenolic monomers, ρ-coumaric acid, ferulic acid, rumen, fungus

INTRODUCTION

Studies have shown that certain phenolic monomers have effects on the biomass and hydrolytic enzyme activities of rumen fungi (Paul *et al*., 2003) and depress cellulose degradation (Varel and Jung, 1986). Direct studies on the effects of phenolics on cell wall degradation are complicated by the large variety of polysaccharides present in plant cell walls. An alternative experimental approach is to use pure cellulose that can be obtained from a cellulose-synthesizing bacterium,*Acetobacter xylinum*, grown in glucose. The cellulose can be labelled with ^{14}C by growing the bacterium in U-^{14}C glucose medium. The use of ^{14}C-labelled bacterial cellulose in digestibility studies provides a simple and versatile method to measure total cellulolytic activity. The digestion products can be easily monitored by measuring the release of radioactivity from the ^{14}C-labelled cellulose.

The present study was conducted to assess the degradation rates of ^{14}C-cellulose by various rumen fungi and to determine the effects of phenolic acids such as ρ-coumaric and ferulic acids on the degradation rate.

MATERIALS AND METHODS

Isolation and Maintenance of Anaerobic Rumen Fungi

Animals

Two male Kedah Kelantan cattle (*Bos indicus*), a male swamp buffalo (*Bubalus bubalis*) and a goat (*Capra hircus*) were kept indoors in single pens in the animal house at the Department of Animal

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Science, Universiti Putra Malaysia. The animals had free access to drinking water and mineral blocks. Each animal was fitted with a rumen cannula and fed once daily with guinea grass (*Panicum maximum*) *ad libitum*.

The agar growth medium used to isolate rumen fungi was prepared following the method of Bauchop (1979). Fungal identifications were based on the classification of anaerobic gut fungi by Ho and Barr (1995). The pure cultures obtained were maintained in ball-milled filter paper medium or rice straw medium at 39°C and subcultured every three days to ensure that the fungal isolates maintained their cellulolytic activity.

Growth and Culture Maintenance of *Acetobacter Xylinum*

Acetobacter xylinum ATCC 23770 was kindly donated by Dr. G.L.R. Gordon from CSIRO, Prospect, New South Wales, Australia. The culture was maintained in Wheaton tubes containing 5 mL of Glucose-Phosphate-Peptone-Yeast (GPPY) medium (5 g of yeast extract, 5 g of peptone, 6.8 g KH_2PO_4 and 10 g glucose/L, pH 6.3). Subculturing was done monthly by transferring the whole bacterial pellicle which developed on the surface of the medium into a tube with fresh GPPY medium. The tubes were incubated loosely capped at 30°C.

Preparation of ^{14}C-Cellulose from *Acetobacter Xylinum*

Pure ^{14}C-cellulose was prepared from *A. xylinum* cultures using the method described by Du Preez and Kistner (1986). One hundred and fifty milliliter of GPPY medium was aseptically dispensed into disposable plastic tissue culture flasks (180 mL) with a surface area of about 175 cm^2 (Cat. No. 1-56502, A/S Nunc, Kamstrup, DK 4000 Roskilde, Denmark) and then 1 mL of filter-sterlised D-[U-^{14}C]glucose (Amersham Biosciences: CFB96) was aseptically dispensed into the flask. A single pellicle from a 5 mL culture of *A. xylinum* that had been incubated for 7 to 10 days was immediately transferred into the flask. The flask was incubated in a flat position, loosely cap, at 30°C for 17 to 18 days.

Two batches of ^{14}C-cellulose were prepared. For Batch 1, 6 μCi of D-[U]-^{14}C-glucose was used for each flask, while for Batch 2, 38.5 μCi of D-[U-^{14}C] glucose was used to increase the specific activity of ^{14}C-cellulose.

Harvesting of ^{14}C-Cellulose

After 17 to 18 days of incubation, the pellicles formed on the surface of the medium in the flasks were removed and washed with distilled water until the washings were clear. The pellicles were boiled in 2 M KOH for 20 min and washed with distilled water until the washings were neutral. The pellicles were then macerated in small quantities in a blender at high speed for 30 sec. The ^{14}C-cellulose slurries were frozen overnight and freeze dried. The freeze-dried ^{14}C-cellulose was weighed and kept in a desiccator at ambient temperature. The initial specific activity of the ^{14}C-cellulose used was determined after treatment with the commercial cellulase from *Trichoderma viride*.

Counting of ^{14}C-Cellulose

A 1 mL sample was added to 9 mL of water-miscible β-scintillant solution (OptiPhase Hisafe 3; Wallac Oy, 20101 Turku, Finland) and the radioactivity was counted by standard liquid scintillation procedures with correction for counting efficiency by using an internal ^{14}C standard. Daily count of specific activity was determined. The amount of solubilized ^{14}C in each tube was calculated as dpm per mL. The counting of the radioactivity of solubilized ^{14}C-cellulose was done by using the liquid scintillation counter (Beckman Instrument Inc. LS Analyzer).

Degradation of ^{14}C-Cellulose by Three Rumen Fungal Species

Rumen fungal species namely *Neocallimastix frontalis* B9 and *Piromyces mae* B6, both isolated from buffalo and *Orpinomyces joyonii* C3 isolated from cattle were chosen for this study. The basal medium used was a pre-reduced anaerobic culture medium without rumen fluid (Akin, 1980). It was modified to contain 0.2% (v/v) ball-milled Batch 1 ^{14}C-cellulose.

The three fungal species used were first cultured in ball-milled filter paper medium for 3 days at 39°C and 0.5 mL of the fungal culture was inoculated into Hungate tubes containing 10 mL of ^{14}C-cellulose medium. Three replicate tubes were made for each fungal species. The tubes were incubated at 39°C for 96 h. The release of radioactivity from the ^{14}C-cellulose was determined by analysing 1 mL of the liquid culture withdrawn at 0 h (immediately following inoculation), 24, 48, 72 and 96 h of incubation. The contents of the tubes were mixed by a single inversion 1 h before the samples of culture liquid were withdrawn for analysis.

Degradation of ^{14}C-Cellulose by *N. frontalis* from Buffalo, Cattle and Goat

Neocallimastix frontalis B9 isolated from buffalo, *N. frontalis* C20 from cattle and *N. frontalis* G8 from goat were compared in their ability to digest ^{14}C-cellulose. Degradation of ^{14}C-cellulose by *N. frontalis* isolates was determined according to the method previously described. Batch 2 ^{14}C-cellulose was used.

Effects of ρ-Coumaric and Ferulic Acids on the Degradation of ^{14}C-Cellulose by *N. frontalis* from Buffalo

Neocallimastix frontalis B9 isolated from swamp buffalo was used in this study. The method was as previously described, but culture medium contained Batch 2 ^{14}C-cellulose and either ρ-coumaric or ferulic acids at 0.1% concentration.

RESULTS

Rumen Fungal Isolates

A total of 133 isolates were obtained from the rumens of cattle and buffalo and 115 isolates were identified as *N. frontalis* (Fig. 1), 15 isolates were *Piromyces mae* (Fig. 2) and 3 isolates were *Orpinomyces joyonii* (Fig. 3). A representative isolate of each species was selected based on their good growth in straw and ball-milled filter paper media and on their ability to maintain zoosporogenesis and viability in the subcultures for the studies on the degradation of ^{14}C-cellulose.

Cellulose Production from Glucose by *Acetobacter xylinum*

Static culture of *A. xylinum* formed a thick surface mat, called a pellicle in which the embedded cells of this obligate aerobe had direct contact with the liquid/air interface. The pellicle was a visible film of cellulose covering the surface of the growth medium. It was colourless and opaque. It was also jelly-like but strong and flexible. The specific activity of the ^{14}C-cellulose obtained was 1475.2 dpm mg^{-1} for Batch1 and 9394.8 dpm mg^{-1} for Batch 2.

Degradation of ^{14}C-cellulose by *N. frontalis* B9, *P. mae* B6 and *O. joyonii* C3

Percentage degradation of ^{14}C-cellulose by *N. frontalis* B9 was significantly ($p<0.05$) higher than that of *P. mae* B6 or *O. joyonii* C3 at 24, 48, 72 and 96 h (Table 1). Maximum percentage degradation was observed at 72 h for all the fungal species. At this incubation period, the radioactivity count for cultures grown with *N. frontalis* B9, *P. mae* B6 and *O. joyonii* C3 were 2429, 1494 and 1105 dpm mL^{-1}, respectively (Fig. 4) and the culture medium was clear due to the degradation of cellulose.

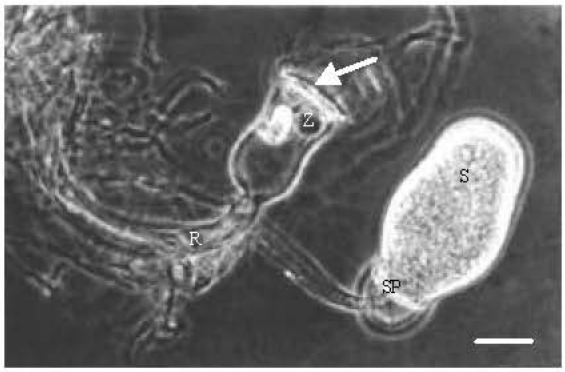

Fig. 1: *Neocallimastix frontalis* showing an exogenous sporangium (S) with a short, eggcup-shaped sporangiophore (SP) and a sporangium with a ruptured apical portion (arrow). Note a zoospore (Z) remaining in the sporangium. Bar = 20 µm

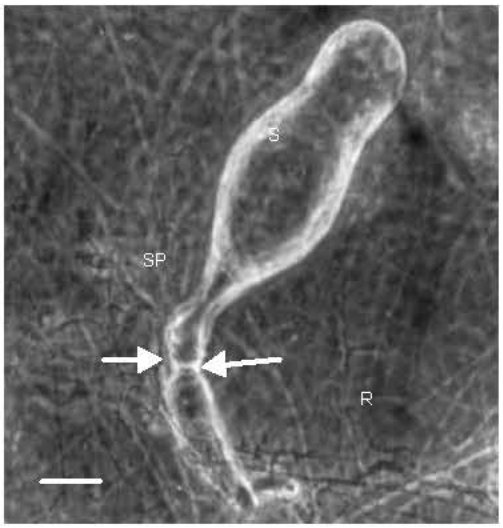

Fig. 2: *Piromyces mae* showing an exogenous sporangium with a sporangiophore (SP) and a constriction at the base of the sporangiophore (arrows) and rhizoids (R). Bar = 20 µm

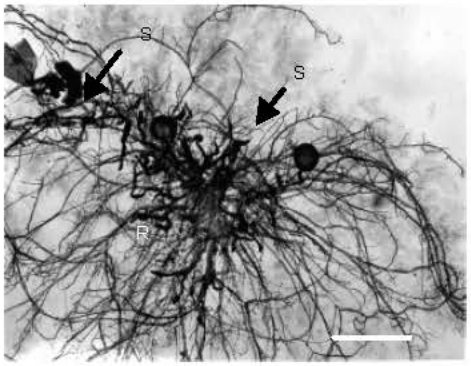

Fig. 3: *Orpinomyces joyonii* showing rhizomycellium complex (R) with sporangia (S). Bar = 20 µm

Degradation of ^{14}C-Cellulose by *N. frontalis* Isolates from Buffalo, Cattle and Goat

The isolates *N. frontalis* B9 from buffalo, *N. frontalis* C20 from cattle and *N. frontalis* G8 from goat were compared in their ability to degrade ^{14}C-cellulose. The percentage degradation of ^{14}C-cellulose were not significantly different among *N. frontalis* isolates at all incubation times (Table 2).

^{14}C degradation rate increased steadily with incubation time and the highest radioactivity counts were observed at 96 h for all the three isolates (Fig. 5). At 96 h of incubation, *N. frontalis* B9 from buffalo showed the highest percentage (83.5%) of ^{14}C-cellulose degradation.

Since there were no significant differences in the degradation rates of ^{14}C-cellulose among the *N. frontalis* isolates, *N. frontalis* B9 was selected for the experiment on the effect of phenolic acid on the degradation of ^{14}C-cellulose.

Effects of ρ-Coumaric and Ferulic Acids on the Degradation of ^{14}C-Cellulose by *N. frontalis* B9

As shown in Table 3, both ρ-coumaric acid and ferulic acid, at 0.1% concentration, had no significant effect on the ^{14}C-cellulose degrading activities of *N. frontalis* B9 at 24 h, but they

Table 1: Percentage degradation of ^{14}C-cellulose by *N. frontalis* B9, *P. mae* B6 and *O. joyonii* C3

Incubation time (h)	Degradation of ^{14}C-cellulose (%)		
	N. frontalis B9	*P. mae* B6	*O. joyonii* C3
0	0.0±0.00	0.0±0.00	0.0±0.00
24	40.4±0.47^{a}	33.8±0.51^{b}	33.8±0.51^{b}
48	60.2±0.31^{a}	41.8±0.25^{b}	33.9±0.36^{c}
72	86.4±0.47^{a}	63.1±0.46^{b}	39.3±0.15^{c}
96	85.5±0.44^{a}	63.0±0.26^{b}	35.6±0.58^{c}

Values presented are means±SD of three replicates. $^{a-c}$Means within the same row with different superscripts are significantly different ($p<0.05$). The initial specific activity of the ^{14}C-cellulose used was 1475 dpm mg^{-1} (Batch 1)

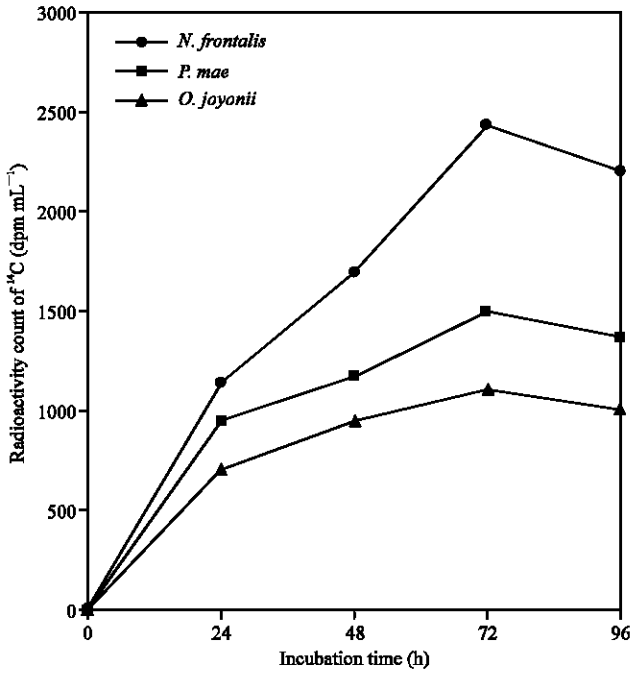

Fig. 4: Radioactivity counts of ^{14}C (dpm mL^{-1}) by *N. frontalis* B9, *P. mae* B6 and *O. joyonii* C3

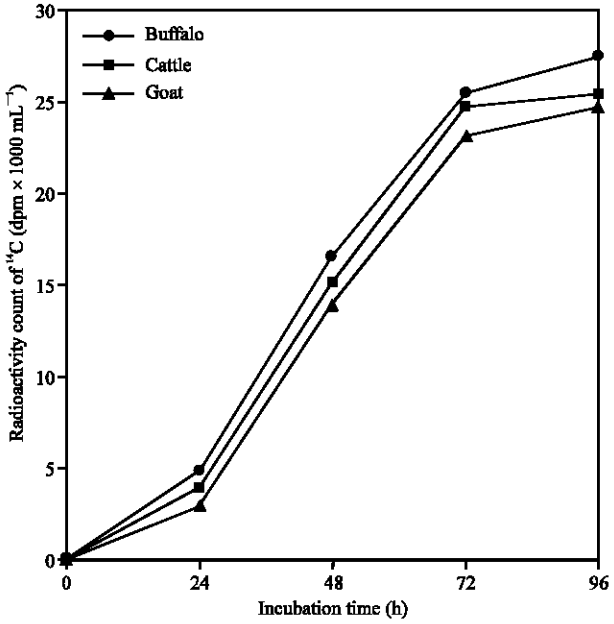

Fig. 5: Radioactivity counts of ^{14}C (dpm mL^{-1}) by *N. frontalis* strains from buffalo, cattle and goat

Table 2: Percentage of degradation of ^{14}C-cellulose by *N. frontalis* isolates from buffalo, cattle and goat

Incubation time (h)	Degradation of ^{14}C-cellulose (%)		
	N. frontalis B9 (from Buffalo)	*N. frontalis* C20 (from Cattle)	*N. frontalis* G8 (from Goat)
0	0.0±0.00	0.0±0.00	0.0±0.00
24	12.4±0.26	11.7±0.21	10.8±0.21
48	23.0±0.10	20.4±0.50	17.2±0.30
72	72.7±0.26	73.7±0.21	64.5±0.55
96	83.5±0.35	77.3±0.32	72.9±0.15

Values presented are means±SD of three replicates. Means within the same row are not significantly different. The initial specific activity of the ^{14}C-cellulose used was 9395 dpm mg^{-1} (Batch 2)

Table 3: Effects of ρ-coumaric and ferulic acids on the degradation of ^{14}C-cellulose by *N. frontalis* B9 from buffalo

Incubation time (h)	Degradation of ^{14}C-cellulose		
	Control	ρ-coumaric acid	Ferulic acid
0	0.0±0.00	0.0±0.00	0.0±0.00
24	21.9±0.95^{a}	24.1±0.62^{a}	24.9±0.95^{a}
48	34.6±0.91^{a}	33.8±0.80^{a}	29.6±0.96^{a}
72	80.7±0.72^{a}	40.6±0.66^{b}	34.7±0.23^{b}
96	83.7±0.40^{a}	51.9±0.30^{b}	50.5±0.51^{b}

Values presented are means±SD of three replicates. ^{a,b}Means within the same row with different superscripts are significantly different ($p<0.05$). The initial specific activity of the ^{14}C-cellulose used was 9395 dpm mg^{-1} (Batch 2)

significantly ($p<0.05$) reduced the activities at 72 and 96 h when compared to the control. At 72 h, the degradation percentage of ^{14}C-cellulose for the control culture was twice of that with the phenolic acids. During this period, *N. frontalis* B9 solubilized 80.7% of ^{14}C-cellulose in the control culture, but only 40.6 and 34.7% in cultures with ρ-coumaric acid and ferulic acid, respectively. Degradation rate increased in the first 24 h for all cultures, but the rate began to decrease for cultures treated with phenolic monomers at further incubation periods. However, degradation of ^{14}C-cellulose continued to increase for the control culture (Fig. 6).

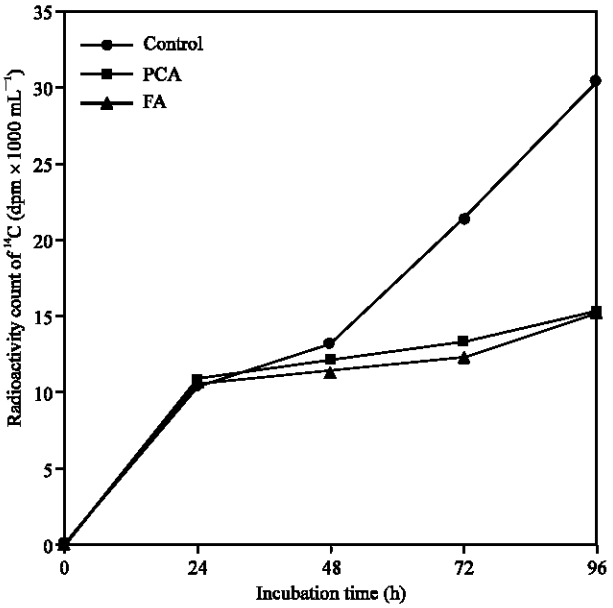

Fig. 6: Effects of ρ-coumaric (PCA) and ferulic acids (FA) on the degradation of ^{14}C-cellulose by *N. frontalis* B9 from buffalo

DISCUSSION

The cellulose produced by *A. xylinum* is a native (Type 1) with a crytallinity index considerably higher than that of the much-used substrates, filter paper and Avicel (Hestrin and Schramm, 1954). It is of exceptionally high purity, free of lignin and hemicellulose. This property, together with the ease with which it can be radio-labelled to a desired specific activity, make it a highly suitable substrate for studies of cellulose degradation.

In the present study, ^{14}C-labelled cellulose from *A. xylinum* was produced and used to determine the cellulolytic activities of three rumen fungal species, viz., *N. frontalis* B9, *P. mae* B6 and *O. joyonii* C3. The results showed that *N. frontalis* B9 exhibited the highest percentage degradation of ^{14}C-cellulose (86.4%) followed by *P. mae* (53.2%) and *O. joyonii* (39.3%). It was also found that the cellulolytic activities of *N. frontalis* isolates from three different ruminant host species were not significantly different. Cellulolytic activity of *O. joyonii* was comparatively low probably due to the rudimentary rhizoid produced by *O. joyonii* limited the ability of the fungus to attach to cellulose particles prior to digestion of the substrate.

Orpin and Letcher (1979) first demonstrated the cellulolytic activity of *N. frontalis* when purified cellulose was degraded by the organism. Later, Pearce and Bauchop (1985) reported that most of the cellulose in the medium was digested by *N. frontalis* after six days of growth. Gordon (1985) found that about half of the cellulose and hemicellulose components in the media were lost from 4 to 5 day-old cultures of *Neocallimastix* and *Piromyces* spp.. In the present study, more than 80% of the pure cellulose was degraded by *N. frontalis* by 96 h of incubation.

Phenolic compounds occur naturally in plants, particularly as secondary metabolites and are generally involved in plant defense mechanisms. Ferulic and ρ-coumaric acids in forage cell walls may act as crosslinking agents between polysaccharides and lignin components (Scalbert *et al*., 1985). Akin and Rigsby (1985) studied the effects of three phenolic acids, ρ-coumaric acid, ferulic acid and

sinapic acid on cellulolytic activity of anaerobic rumen fungi. They found that at relatively low concentration (0.1% w/v), these compounds decreased rumen fungal populations *in vitro* and the dry weight loss of plant cell walls (the substrate) was reduced by 25.5%.

In the present study, the addition of ρ-coumaric acid and ferulic acid in the media significantly ($p<0.05$) reduced the degradation of cellulose by *N. frontalis* B9. At 96 h, 83.7% of ^{14}C-cellulose was solubilized in the control culture without the phenolic acids, but in cultures with ρ-coumaric acid or ferulic acid, only 51.9 and 50.5% of ^{14}C-cellulose were degraded, respectively. The negative effects of these phenolic acids on cellulose degradation by *N. frontalis* B9 found in the present study agree with the findings of other workers. Jung and Sahlu (1986) reported that ρ-coumaric acid and ferulic acid reduced filter paper degradation by rumen fungi. The reduced degradation rate could be attributed to the inhibition of cellulolytic enzyme production as Paul *et al.* (2003) had observed that ρ-coumaric acid inhibited carboxymethylcellulase activity of *Piromyces* sp. It was also possible that phenolic monomers similar to those in plant cell walls inhibited the ability of rumen fungi to colonize and degrade the substrates (Akin and Rigsby, 1985). These results suggest that plant phenolics have a detrimental effect on the growth and cellulolytic activity of anaerobic rumen fungi.

REFERENCES

Akin, D.E., 1980. Evaluation by electron microscopy and anaerobic cultures of types of rumen bacteria associated with digestion of forage cell walls. Applied Environ. Microbiol., 39: 242-252.

Akin, D.E. and L.L. Rigsby, 1985. Influence of phenolic acids on rumen fungi. Agron. J., 77: 180-182.

Bauchop, T., 1979. Rumen anaerobic fungi of cattle and sheep. Applied Environ. Microbiol., 38: 148-158.

Du Preez, P. and A. Kistner, 1986. A versatile assay for total cellulase activity using U-[^{14}C]-labelled bacterial cellulose. Biotechnol. Lett., 8: 581-586.

Gordon, G.L.R., 1985. The Potential for Manipulation of Rumen Fungi. In: Reviews: Rural Sciences. Leng, R.A., J.S.F. Baker, D.B. Adams and K.J. Hutchinson (Eds.), Vol. 6, Armidale: University of New England Publications Office, pp: 124-128.

Hestrin, S. and M. Schramm, 1954. Synthesis of cellulose by *Acetobacter xylinum* II. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. Biochem. J., 58: 345-352.

Ho, Y.W. and D.J.S. Barr, 1995. Classification of anaerobic gut fungi from herbivores with emphasis on rumen fungi from Malaysia. Mycologia, 87: 655-677.

Jung, H.G. and T. Sahlu, 1986. Depression of cellulose digestion by esterified cinnamic acids. J. Sci. Food Agric., 37: 659-665.

Orpin, G.C. and A.J. Letcher, 1979. Utilization of cellulose, starch, xylan and other hemicelluloses for growth by the rumen phycomycete *Neocallimastix frontalis*. Curr. Microbiol., 3: 121-124.

Paul, S.S., D.N. Kamra, V.R. Sastry, N.P. Sahu and A. Kumar, 2003. Effect of phenolic monomers on biomass and hydrolytic enzyme activities of an anaerobic fungus isolated from wild nil gai (*Baselophus tragocamelus*). Lett. Applied Microbiol., 36: 377-381.

Pearce, P.D. and T. Bauchop, 1985. Glycosidases of the rumen anaerobic fungus *Neocallimastix frontalis* grown on cellulosic substrates. Applied Environ. Microbiol., 49: 1265-1269.

Scalbert A., B. Monties, J.Y. Lallemand, E. Guittet and C. Rolando, 1985. Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry, 24: 1359-1362.

Varel, V.H. and H.G. Jung, 1986. Influence of forage phenolics on ruminal fibrolytic bacteria and *in vitro* fiber degradation. Applied Environ. Microbiol., 52: 275-280.