Degradation of Rice Straw by Rumen Fungi and Cellulolytic Bacteria through Mono-, Co- or Sequential- Cultures

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ABSTRACT : Two strains of rumen fungi (*Piromyces rhizinflata* B157, *Orpinomyces joyonii* SG4) and three strains of rumen cellulolytic bacteria (*Ruminococcus albus* B199, *Ruminococcus flavefaciens* FD1 and *Fibrobacter succinogenes* S85) were used as mono-cultures or combinationally arranged as co- and sequential-cultures to assess the relative contributions and interactions between rumen fungi and cellulolytic bacteria on rice straw degradation. The rates of dry matter degradation of co-cultures were similar to those of corresponding bacterial mono-cultures. Compared to corresponding sequential-cultures, the degradation of rice straw was reduced in all co-cultures ($P<0.01$). Regardless of the microbial species, the cellulolytic bacteria seemed to inhibit the degradation of rice straw by rumen fungi. The high efficiency of fungal cellulolysis seems to affect bacterial degradation rates. (*Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 6 : 797-802*)

Key Words : Rumen Fungi, Cellulolytic Bacteria, Co-Culture, Sequential-Culture, Inhibition

INTRODUCTION

Rumen fungi are known to display strong cellulolytic activity, but the roles of anaerobic fungi in the rumen are not yet clear. Windham and Akin (1984) reported that cellulolysis in the rumen depended mainly on the bacteria due to their numeric predominance and metabolic diversity. The absence of fungi decreased feed consumption and fiber digestion in the rumen (Gordon and Phillips, 1992). A number of experimental data suggest that cellulolytic activity by fungi is dependent upon conditions within the rumen. When hydrogen, a fungal metabolic end product and inhibitor of fungal growth is removed by methanogenic or non-methanogenic H_2-utilizing bacteria, fungal growth and cellulolysis is accelerated (Bernalier et al., 1990, 1991; Joblin et al., 1990; Marvin-Sikkema et al., 1990; Joblin and Williams, 1991). However, interactions between rumen fungi and cellulolytic bacteria are known to depend upon the species and strains of bacteria involved. *Ruminococci*, in particular *R. flavefaciens* (Bernalier et al., 1993; Williams et al., 1994), inhibit fungal cellulolysis and xylanolysis, while *Fibrobacter succinogenes* (Bernalier et al., 1993; Roger et al., 1993) and some *R. albus* strains (Irvine and Stewart, 1991) do not seem to influence anaerobic fungi. As with the fungal populations in the rumen, the higher the fiber content of the feed, the higher the cellulolytic bacterial population (Gouws and Kistner, 1965). Therefore, there may be considerable opportunities for interactions between fungi and cellulolytic bacteria in the rumen. Most studies on interactions between fungi and cellulolytic bacteria and their ability to digest fiber were conducted by comparisons of cocultures with corresponding mono-cultures.

Experimental results on the degradation of rice straw by mono-, co- and sequential- cultures of monocentric or polycentric rumen fungi and cellulolytic bacterial species are reported here.

MATERIALS AND METHODS

Organisms and culture conditions

The microorganisms used in these experiments were *Piromyces rhizinflata* B157, *Orpinomyces joyonii* SG4, *Ruminococcus albus* B199, *Ruminococcus flavefaciens* FD1 and *Fibrobacter succinogenes* S85. All of the microorganisms were obtained from the Culture Center of the Lethbridge Research Station, Agriculture and Agri-Food Canada.

Fungal and bacterial cultures were prepared and grown under strictly anaerobic conditions according to the methods of Hungate (1969). The fungal inocula were composed of 1 or 0.5 mL volumes of a 72 hours old culture for mono- or co-culture experiments and bacterial inocula were from 24 hours old cultures. Co-cultures were made by inoculating the two microorganisms simultaneously. Sequential cultures were incubated for 6 days with 1 mL of the first inoculated microorganism and then for another 6 days with the second inocula (1 mL) on renewed medium (9 mL). Cultures were grown in tubes containing 9 mL of medium B (Lowe et al. 1985) in which glucose and cellobiose were replaced by pieces of rice

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straw internodes which were cut to 3 mm lengths (about 750 mg per tube) and used as a sole energy source. The NDF and ADF composition of rice straw was 75.20% and 52.12% (DM base). All incubations were carried out at 39℃ without shaking and all experiments were performed in triplicate.

Dry matter degradation

The extent of dry matter degradation of rice straw was measured after 1, 2, 4 and 6 days of incubation. At the end of the period of incubation, pH was checked and then tubes were centrifuged at 1,600×g for 25 min. The supernatant was removed for the analysis of end-products and stored at -40℃. The pellets were filtered through Whatman no. 54 filter paper with a Buchner filter and vacuum system, and dried to a constant weight at 80℃ for 48 h.

Reducing sugar concentration and enzyme activities

Supernatants of cultures and glucose standard solutions were treated with DNS (dinitrosalicylic acid) reagent and 0.1*M* acetate buffer (pH 5.0) for determining reducing sugar concentration according to Miller et al. (1960).

Cellulase activity was determined by OBR-HEC (Sigma O-6879), a dye-labeled substrate (Biely *et al.*, 1985). A 0.1 mL volume of culture supernatants was added to 0.1 mL of 0.8% (w/v) OBR-HEC in 0.05*M* sodium phosphate buffer (pH 6.8) and 0.01 mL of 2% sodium azide and incubated for 2 h at 37℃. After treatment with acetone-ethanol, O.D. value was measured at 550 nm. Xylanase activity was determined using RBB-xylan (Biely et al., 1988) and the same method as with cellulase activity, but incubation time was 30 min and O.D. value was measured at 595 nm. Enzyme activity was presented as the change in O.D.

Statistical analysis

Analysis of variance was carried out and means were compared by Duncan's multiple range test using GLM (general linear model) procedures of SAS (1996).

RESULTS

Degradation by mono-cultures

P. rhizinflata degraded rice straw continuously to 51% after 6 days of incubation. With *O. joyonii*, the dry matter degradation rate was high initially, but very slow towards the end of incubation and ended with 31% degradation. *F. succinogenes* and *R. albus* degraded substrate very slowly up to the 6th day, but *R. flavefaciens* degraded substrate rapidly after the 2nd day and up to the 4th day. Up to the 2nd day, there was no difference in dry matter degradation among all of the mono-cultures, but after the second day, *P. Rhizinflata* and *R. flavefaciens* degraded the rice straw very effectively and showed the highest degradation at the 4th and 6th days ($p<0.01$). In contrast, *O. joyonii*, *F. succinogenes* and *R. albus* were slow degraders and degraded substrate to similar degrees (table 1).

In the cases of treatment groups showing dry matter digestibility(DMD) above 33%, there was a negative correlation ($r<-0.80$) between DMD and pH of culture. Growth of *P. rhizinflata* and *R. flavefaciens* led to the lowest culture pH values (data not shown).

Degradation by co-cultures

In the co-culture between *P. rhizinflata* and *F. succinogenes*, the greatest extent of dry matter degradation was obtained after 4 days of incubation. Degradation in the co-culture of *P. rhizinflata* with *R. albus* was very similar to that by the mono-culture of

Table 1. Rice Straw degradation by rumen fungi and rumen cellulolytic bacteria in mono- or in co-culture (Unit: %)

Cultures	Incubation time (days)			
	1	2	4	6
O. joyonii SG4	26.50±0.95	26.99±2.48	22.70±0.66^{a}	31.19±0.60^{cd}
P. rhizinflata B157	24.75±0.60	27.68±1.00	41.23±0.41^{a}	50.54±3.44^{a}
F. succinogenes S85	21.47±1.51	26.93±1.90	24.70±0.86^{cd}	33.56±0.51^{bc}
R. albus B199	21.01±0.78	26.08±0.79	28.58±1.76^{c}	30.03±1.03^{d}
R. flavefaciens FD1	22.95±0.39	24.49±0.29	45.96±1.66^{a}	47.78±0.65^{a}
P. rhizinflata+*F. succinogenes*	21.72±0.25	24.79±0.43	29.09±0.74^{bc}	31.65±1.55^{cd}
P. rhizinflata+*R. albus*	22.85±0.73	25.15±0.28	26.41±1.35^{cd}	29.54±1.14^{d}
P. rhizinflata+*R. flavefaciens*	22.71±1.07	24.57±0.92	33.82±0.94^{b}	44.05±2.32^{ab}
O. joyonii+*F. succinogenes*	22.49±1.13	24.53±0.97	28.25±0.74^{c}	31.76±0.83^{cd}
O. joyonii+*R. albus*	23.47±0.76	27.77±0.80	27.34±0.66^{cd}	31.33±1.36^{cd}
O. joyonii+*R. flavefaciens*	26.47±4.16	24.62±0.80	44.35±2.53^{a}	41.73±5.83^{abc}

DMD of "0" time is 15.67±0.54%.
Each value represents the mean±SEM of triplicates cultures.
Means in the same column with different superscripts are significantly different ($p<0.01$).

R. albus. Among the co-cultures, the *P. rhizinflata* and *R. flavefaciens* combination showed the highest extent of degradation, but was less effective than a mono-culture of *P. rhizinflata* or of *R. flavefaciens*. Co-cultures of *P. rhizinflata* with all other bacteria showed lower degradation than obtained by mono-cultures of *P. rhizinflata* and degradation was in all cases similar to that obtained by the corresponding bacterial mono-culture. Co-cultures of *O. joyonii* with bacteria degraded the substrate to a similar extent to that by co-cultures of *P. rhizinflata* with bacteria ($p<0.01$). Whatever the fungal species, when *R. flavefaciens* was associated with the fungus, the highest DMD of all co-cultures was detected ($p<0.01$). DMDs by co-cultures were similar to those of the corresponding bacterial mono-cultures.

Degradation by sequential-cultures

In the sequential-cultures of *P. rhizinflata* and bacteria, the treatments in which the fungus was the first inoculum, were more effective in dry matter degradation than when the fungus was the second inoculum. But sequential-cultures with *O. joyonii* displayed no such order-dependent trends (figure 1). Co-cultures with *R. flavefaciens* were cellulolytically more active than co-cultures with other bacteria and the sequential-culture of *P. rhizinflata* and *R. flavefaciens* was the best at dry matter degradation. Cultures of *P. rhizinflata* as the first inoculum were more effective than those with *O. joyonii* as the fungal inoculant. As with the mono-cultures, *P. rhizinflata* showed higher efficiency of dry matter degradation than did *O. joyonii*. Regardless of the treatments, DMDs in all sequential-cultures were higher than those of the corresponding groups of co-cultures ($p<0.01$).

Reducing sugar concentration

Mono-cultures of *R. flavefaciens* FD1 and *P. rhizinflata* B157 showed the highest concentration of reducing sugar in the supernatant (table 2). In the sequential cultures, reducing sugars started to accumulate in the initial medium and that corresponds with the high efficiency of DMD in the initial cultures. There was a positive correlation ($r=0.88$) between DMD and reducing sugar concentration. Replacement of glucose and cellobiose with rice straw may have induced enzyme expression, resulting in higher degradation of substrate and accumulation of reducing sugar in the initial culture.

Cellulase and xylanase activity

Cellulase activity in the *P. rhizinflata* culture was the highest among the treatment groups. Whereas cellulase activities of fungi cultures increased up to the 6th day of growth, those of bacterial cultures increased only up to the 4th day of growth. A consistent pattern of cellulase expression in the two strain co-culture treatments was not observed (table 3). Co-cultures had higher extracellular cellulase activities than did mono-cultures of bacteria. Cellulases in the co-cultures were therefore probably from fungi, which means that fungal growth continued until the end of incubation in the co-cultures.

Co-cultures of *P. rhizinflata* and *R. flavefaciens* showed the highest xylanase activity ($p<0.05$). Almost all the treatment groups showed the general tendency of an increase in xylanase activities as incubation time increased (table 4).

DISCUSSION

Co-cultures of *P. rhizinflata* and bacteria degraded the dry matter of rice straw less efficiently than mono-cultures of *P. rhizinflata*. With co-cultures of *O. joyonii* and bacteria, the amounts of degraded dry matter were only slightly more than that in the mono-culture of *O. joyonii*. Overall, rates of DM degradation in the co-cultures were similar to the rates of degradation by the corresponding bacterial mono-cultures. In co-cultures with *R. albus* B199 or *F. succinogenes* S85, *P. rhizinflata* didn't display the

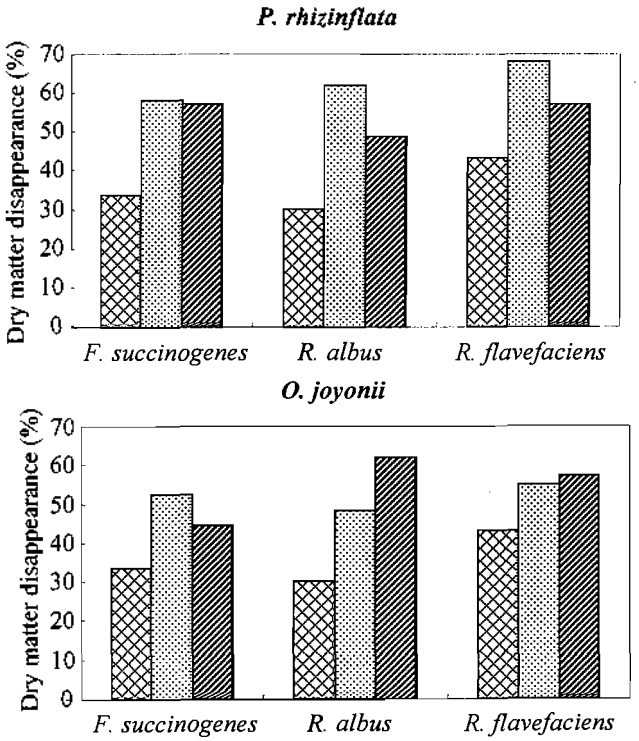

Figure 1. Degradation of rice straw by rumen fungi and cellulolytic bacteria in co-culture (▨) or sequential-culture (□, Fungi is the first inoculum; ▨, Bacteria is the first inoculum)

Table 2. Concentration of reducing sugars of supernatant of the medium incubated with rumen fungi and rumen bacteria in mono- or in co-culture (Unit: *mmole* · mL^{-1})

Cultures	Incubation time (days)			
	1	2	4	6
O. joyonii SG4	494.8 ± 4.6^{ab}	544.5 ± 24.4^{b}	1219.8 ± 8.1^{a}	730.8 ± 43.9^{cd}
P. rhizinflata B157	420.3 ± 6.7^{bcd}	504.1 ± 18.3^{bcd}	921.7 ± 20.9^{cd}	741.7 ± 24.3^{bc}
F. succinogenes S85	430.4 ± 41.2^{bc}	516.5 ± 46.5^{bc}	996.3 ± 22.3^{bc}	1019.5 ± 32.9^{a}
R. albus B199	555.4 ± 25.4^{a}	490.1 ± 16.5^{bcd}	735.4 ± 24.2^{e}	704.4 ± 24.5^{cd}
R. flavefaciens FD1	424.9 ± 24.1^{bcd}	650.1 ± 5.1^{a}	1017.2 ± 21.4^{b}	977.6 ± 62.6^{a}
P. rhizinflata+F. succinogenes	325.6 ± 5.8^{ef}	465.3 ± 19.1^{bcd}	682.7 ± 27.5^{e}	667.1 ± 66.2^{cd}
P. rhizinflata+R. albus	392.3 ± 32.0^{cde}	491.7 ± 8.3^{bcd}	591.1 ± 23.2^{f}	542.2 ± 11.5^{e}
P. rhizinflata+R. flavefaciens	362.8 ± 24.7^{cdef}	460.7 ± 34.5^{bcd}	890.7 ± 22.4^{d}	799.1 ± 5.1^{bc}
O. joyonii+F. succinogenes	338.0 ± 23.3^{def}	415.6 ± 22.2^{cd}	522.8 ± 19.8^{f}	626.0 ± 37.9^{cde}
O. joyonii+R. albus	397.0 ± 12.1^{cde}	510.3 ± 20.8^{bcd}	529.0 ± 15.4^{f}	563.1 ± 11.6^{de}
O. joyonii+R. flavefaciens	302.3 ± 6.6^{f}	409.4 ± 28.5^{d}	861.2 ± 26.3^{d}	904.7 ± 58.0^{ab}

Each value represents the Mean ± SEM of triplicates cultures.
Means in the same column with different superscripts are significantly different ($p<0.01$).

Table 3. Cellulase activity (OBR-HEC) of the supernatant of the medium incubated with rumen fungi and rumen bacteria in mono- or in co-culture (Unit: OD value)

Cultures	Incubation time (days)			
	1	2	4	6
O. joyonii SG4	0.327 ± 0.006^{b}	0.399 ± 0.001^{ab}	0.554 ± 0.002^{a}	0.662 ± 0.003^{a}
P. rhizinflata B157	0.313 ± 0.023^{bc}	0.407 ± 0.008^{ab}	0.428 ± 0.004^{f}	0.566 ± 0.012^{b}
F. succinogenes S85	0.018 ± 0.000^{d}	0.088 ± 0.001^{e}	0.146 ± 0.000^{g}	0.120 ± 0.003^{f}
R. albus B199	0.031 ± 0.001^{d}	0.049 ± 0.001^{e}	0.086 ± 0.009^{h}	0.059 ± 0.002^{g}
R. flavefaciens FD1	0.030 ± 0.001^{d}	0.084 ± 0.002^{e}	0.160 ± 0.001^{g}	0.132 ± 0.001^{f}
P. rhizinflata+F. succinogenes	0.261 ± 0.020^{c}	0.353 ± 0.002^{bc}	0.507 ± 0.001^{c}	0.440 ± 0.003^{d}
P. rhizinflata+R. albus	0.299 ± 0.006^{bc}	0.256 ± 0.047^{d}	0.527 ± 0.003^{b}	0.496 ± 0.018^{c}
P. rhizinflata+R. flavefaciens	0.287 ± 0.006^{bc}	0.358 ± 0.003^{bc}	0.555 ± 0.003^{a}	0.463 ± 0.019^{cd}
O. joyonii+F. succinogenes	0.281 ± 0.003^{bc}	0.307 ± 0.001^{cd}	0.464 ± 0.001^{de}	0.324 ± 0.008^{e}
O. joyonii+R. albus	0.402 ± 0.002^{a}	0.423 ± 0.001^{a}	0.458 ± 0.003^{e}	0.462 ± 0.021^{cd}
O. joyonii+R. flavefaciens	0.391 ± 0.027^{a}	0.450 ± 0.002^{a}	0.477 ± 0.003^{d}	0.612 ± 0.013^{b}

Each value represents the Mean ± SEM of triplicates cultures.
Means in the same column with different superscripts are significantly different ($p<0.01$).

effective cellulolysis exhibited by the *P. rhizinflata* mono-culture and this fungus seemed to be inhibited by the bacteria. Cellulolysis by *O. joyonii* was similar whether in mono- or in co-culture and seemed not to be affected by bacteria.

Dry matter degradation rates in all of the sequential-cultures were higher than those in the corresponding groups of co-cultures ($p<0.01$). It seems that in sequential-cultures, there were no interactions such as synergism or inhibition because the medium was renewed for each inoculum in an effort to exclude the effects of metabolites from organisms involved. In a previous report, microorganisms were incubated alone with the same substrates after sterilization of the first inoculum and this did not affect cellulose digestion (Fondevila and Dehority, 1996). Therefore, differences of DMDs between co-cultures and sequential-cultures could be due to interactions between co-cultured microorganisms. In co-cultures, degradation activity by both *P. rhizinflata* B157 and *O. joyonii* SG4 were inhibited. *R. flavefaciens* and *R. albus* seemed to inhibit fungal degradation of DM in agreement with results reports by others (Richardson et al., 1986; Bernalier et al., 1992). *F. succinogenes* seemed to inhibit fungal cellulolysis in contrast to the reports of others (Roger et al., 1992; Bernalier et al., 1993). Discrepancies may result from using different strains of fungi or by making different comparisons using mono-cultures, co-cultures and sequential-cultures.

Extracellular cellulase activities in co-cultures of fungi with bacteria were lower than those of mono-cultures of fungi and much higher than those of bacterial mono-cultures ($p<0.01$). This suggests that

Table 4. Xylanase activity (RBB-Xylan) of the supernatant of the medium incubated with rumen fungi and rumen bacteria in mono-or in co-culture (Unit: OD value)

Cultures	Incubation time (days)			
	1	2	4	6
O. joyonii SG4	0.009±0.001	0.008±0.002^{c}	0.072±0.001^{b}	0.057±0.010^{a}
P. rhizinflata B157	0.009±0.002	0.010±0.000^{c}	0.014±0.001^{b}	0.018±0.001^{b}
F. succinogenes S85	0.013±0.001	0.011±0.000^{c}	0.012±0.004^{b}	0.024±0.009^{b}
R. albus B199	0.005±0.001	0.005±0.001^{c}	0.006±0.002^{b}	0.009±0.001^{b}
R. flavefaciens FD1	0.011±0.001	0.093±0.007^{a}	0.071±0.007^{b}	0.361±0.200^{ab}
P. rhizinflata + *F. succinogenes*	0.017±0.005	0.012±0.001^{c}	0.013±0.001^{b}	0.015±0.001^{b}
P. rhizinflata + *R. albus*	0.007±0.000	0.007±0.000^{c}	0.007±0.001^{b}	0.006±0.002^{b}
P. rhizinflata + *R. flavefaciens*	0.005±0.001	0.010±0.002^{c}	0.138±0.041^{b}	0.441±0.030^{a}
O. joyonii + *F. succinogenes*	0.005±0.000	0.011±0.001^{c}	0.031±0.007^{b}	0.047±0.002^{b}
O. joyonii + *R. albus*	0.126±0.084	0.011±0.002^{c}	0.012±0.001^{b}	0.010±0.001^{b}
O. joyonii + *R. flavefaciens*	0.011±0.001	0.025±0.004^{b}	0.340±0.155^{a}	0.319±0.199^{ab}

Each value represents the mean±SEM of triplicates cultures.
Means in the same column with different superscripts are significantly different($P<0.05$).

fungal enzyme activities were reduced in co-cultures. This could be the result of bacterial inhibitory factors (Bernalier et al., 1993) that, while not affecting fungal growth, inhibited the activity of the fungal cellulase.

The sequential-cultures of *P. rhizinflata* and bacteria, in which fungi were the first inocula, were more effective in dry matter degradation than when bacteria were the first inocula. In the sequential-cultures using *O. joyonii,* which degraded the rice straw with low efficiency, there was no difference in DMD regardless of the order of inoculation.

Previous experimental results suggest that fungal growth on plant fragments causes physical damage to plant tissue (Akin et al., 1983, 1989). Polycentric fungi can be more effective in physically disrupting plant tissues by rhizoidal development compared to monocentric fungi. In our experiments, however, *O. joyonii* was less effective in degradation of rice straw than *P. rhizinflata*. Possibly, high degradation of rice straw by *P. rhizinflata* might accelerate the attachment and therefore, more digestion by bacteria.

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