

## Effects of LCFA on the Gas Production, Cellulose Digestion and Cellulase Activities by the Rumen Anaerobic Fungus, *Neocallimastix frontalis* RE1

S. S. Lee\*, J. K. Ha<sup>1</sup> and K. J. Cheng<sup>2</sup>

National Livestock Research Institute, Rural Development Administration, Suweon 441-350, Korea

**ABSTRACT** : Responses of the rumen fungus, *Neocallimastix frontalis* RE1, to long chain fatty acid (LCFA) were evaluated by measuring gas production, filter paper (FP) cellulose digestion and polysaccharidase enzyme activities. LCFA (stearic acid, C<sub>18:0</sub>; oleic acid, C<sub>18:1</sub>; linoleic acid, C<sub>18:2</sub> and linolenic acid, C<sub>18:3</sub>) were emulsified by ultrasonication under anaerobic condition, and added to the medium. When *N. frontalis* RE1 was grown in culture with stearic, oleic and linoleic acid, the cumulative gas production, gas pool size, FP cellulose digestion and enzymes activities significantly ( $p < 0.05$ ) increased at some incubation times (especially, exponential phases of fungal growth, 48–120 h of incubation) relative to that for control cultures. However, the addition of linolenic acid strongly inhibited all of the investigated parameters up to 120 h incubation, but not after 168 and 216 h of incubation. These results indicated that stearic, oleic and linoleic acids tended to have great stimulatory effects on fungal cellulolysis, whereas linolenic acid caused a significant ( $p < 0.05$ ) inhibitory effects on the cellulolysis by the rumen fungus. These results are the first report of the effect of LCFAs on the ruminal fungi. Further research is needed to identify the mode of action of LCFAs on fungal strains and to verify whether or not ruminal fungi have ability to hydrate unsaturated LCFAs to saturated FAs. There was high correlation between cumulative *in vitro* gas production and fungal growth (94.78%), FP cellulose degradation (96.34%), CMCase activity (90.86%) or xylanase activity (87.67%). Thus measuring of cumulative gas production could be a useful tool for evaluating fungal growth and/or enzyme production by ruminal fungi. (*Asian-Aust. J. Anim. Sci.* 2001, Vol 14, No. 8 : 1110-1117)

**Key Words** : LCFA, Stearic Acid, Oleic Acid, Linoleic Acid, Linolenic Acid, Gas Production, Rumen Fungus, Fungal Cellulolysis

### INTRODUCTION

Nieman (1954) reviewed that unsaturated fatty acids (USFAs) with C<sub>18</sub> chain lengths have the most widespread inhibitory effects on pure cultures of rumen bacteria and oleic acid has, at lower concentrations, the most widespread growth-promoting effect. The addition of fat to diets often depresses cellulolysis and methanogenesis in the rumen. Fatty acids are also toxic to bacteria (Henderson, 1973) and protozoa (Maczulak et al., 1981) in pure culture experiments. Although ruminal fungi are recognized as a major component of the fibre degrading microbiota of ruminants (Gordon and Phillips, 1989), to our knowledge, no reports have been documented of the effect of FAs on such organisms. Cellulolysis had great effect on FAs by inhibiting growth of bacteria and protozoa but the effects of FAs on the fungal growth and their ability to degrade cell-wall polysaccharides are not completely understood.

Hydrogen (H<sub>2</sub>) is an intermediate produced particularly during plant cell wall breakdown by cellulolytic microorganisms, such as *Ruminococcus albus*, *R. flavefaciens*, and anaerobic fungi (Fonty and Joblin, 1991).

Hydrogen never accumulates in the rumen because it is rapidly used by methanogens. In pure culture studies, H<sub>2</sub>-utilizing microorganisms such as methanogens (Mountfort et al., 1982), acetogens (Bernalier et al., 1990) and *Selenomonas ruminantium* (Marvin-Sikkema et al., 1990) enhanced cellulose digestion and cellulolysis by ruminal fungi by preventing the accumulation of H<sub>2</sub> which is the main end-products of fungal fermentation. Unsaturated FAs are also able to capture H<sub>2</sub> and become saturated in the process. Therefore, if ruminal fungi have the ability to hydrate USFAs, then cellulolysis by fungi should be enhanced by the addition of USFAs to the medium.

There is also a high correlation between biogas pressure and culture dry weight during the exponential phase of fungal growth on insoluble substrates (Theodorou et al., 1992). Thus measurement of gas production as developed by Theodorou and co-workers (Merry et al., 1991; Theodorou et al., 1991) is a simple procedure to measure the specific growth rate and yield of fungi in batch culture, which doesn't require syringes like the comparable Menke gas production method (Menke et al., 1979). There is no information about the effect of FAs on ruminal fungi. Therefore, the aim of this study was the first to determine the effects of saturated (C<sub>18:0</sub>) and unsaturated (C<sub>18:1</sub>, C<sub>18:2</sub> and C<sub>18:3</sub>) LCFAs on gas production by *Neocallimastix frontalis* in *in vitro* culture.

\* Address reprint request to S. S. Lee. Tel: +82-31-290-1618, Fax: +82-31-290-1665, E-mail: leess618@rda.go.kr.

<sup>1</sup> School of Agricultural Biotechnology, Seoul National University, Suweon, 441-744, Korea.

<sup>2</sup> Institute of BioAgricultural Resources, Academia Sinica, Taipei 115, Taiwan ROC.

## MATERIALS AND METHODS

### Sources and preparation of fatty acids

Long chain FAs including stearic acid (octadecanoic acid, C<sub>18:0</sub>), oleic acid (*cis*-9-octadecenoic acid, C<sub>18:1</sub>), linoleic acid (*cis*-9, *cis*-12-octadecadienoic acid, C<sub>18:2</sub>) and linolenic acid (*cis*-9, *cis*-12, *cis*-15-octadecatrienoic acid, C<sub>18:3</sub>) were obtained from Sigma Chemical Co., St. Louis, Mo. They were reported by the manufacturer to be >99.9% pure. Emulsions of FAs in autoclaved reducing H<sub>2</sub>O were prepared by ultrasonication for 4 min using a Vibra Cell™ sonicator (Sonics & Materials Inc., USA) under anaerobic conditions to prevent oxidation of FAs.

### Fungal strain

The monocentric strain *Neocallimastix frontalis* RE1, was used in the experiment and was obtained from the Lethbridge Research Centre Culture Collection. The fungus was maintained anaerobically in the liquid semi-defined medium B of Lowe et al. (1985) with Whatman no. 1 filter paper as the sole source of carbon and energy. Subtransfers were made every three days to maintain viability.

### Culture techniques and media

The anaerobic culture techniques of Hungate (1950) with modifications (Bryant and Burkey, 1953) were used for all incubations. About 75 mg of punched FP (0.60 mm diameter) was added to 30-mL serum vials in triplicate (Miller and Wolin, 1974). Anaerobic liquid medium without a carbon source was transferred in 14 mL quantities to each serum vial and the vials were purged with oxygen-free CO<sub>2</sub>. The vials were sterilized by autoclaving at 123°C for 20 min. The pH after autoclaving was between 6.65±0.02. Incubations were performed anaerobically at 39°C without shaking for 0, 12, 24, 48, 72, 120, 168 and 216 h. When emulsified FAs were included in the medium, it was added to yield final concentration of 0.001% (w/v for stearic acid, v/v for other fatty acids). The control medium contained no FAs. To study the effects of different FA on the growth of the fungus, 2.0 mL of mid-exponential-phase cultures grown in the rumen fluid medium was inoculated into 14mL of the appropriate test medium.

### Determination of enzyme activities

Extracellular enzyme activity against carboxymethylcellulose (CMC) was determined by incubating 0.5 mL aliquots of supernatant from the growing cultures (grown under different conditions) with 0.5 mL of 2% (w/v) CMC in 0.1 M Na acetate buffer (pH 5.0). After a 2 h incubation, the reaction was stopped by the addition of 0.25 mL of 8% Na<sub>2</sub>CO<sub>3</sub>. Samples of 1.25 mL were centrifuged at 12,000×g for 5 min and reducing sugars in the supernatants were assayed colorimetrically by using the DNS (dinitrosalicylic

acid) method of Miller (1959). Xylanase activities were determined by measuring the liberated reducing sugars from oat spelt xylan using the method described above. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of glucose (for CMCase) and xylose (for xylanase) equivalent per min.

### Gas production method

At the end of each incubation, gas production was determined by using a water displacement apparatus (Ferorak and Hrwdey, 1983). To give a more precise estimate of the gas production throughout the duration of fermentation, the following calculations were used to analyze the kinetic data by the method described by Merry et al. (1991) and Herrero et al. (1996). In this negative exponential curve model, the G<sub>p</sub> is a gas production (mL/0.1g DM of substrate) of time *t* according to the following formula:

$$G_p = a + b[1 - \exp^{-k \times (\text{time} - \text{lag})}]$$

where, constants *a*, *b*, *a+b* and *k* are a scale factor (*Y*-axis intercept), the gas pool size, potential gas production and the rate constant (the fractional rate of gas production per hour), respectively.

The model also allowed for the estimation of a lag phase before rapid gas production began and gave the closest agreement between gas production, digestion rates, and cell growth. Gas production and FP cellulose disappearance rates were fitted to a model by the nonlinear (NLIN) procedure of SAS (1996) using Marquardt's compromise. The constants (*a*, *b*, *k* and *lag*) were evaluated.

### Computation of data and statistical analysis

A spread-sheet program was used for data handling and processing of gas accumulation data, and to determine relationships between gas accumulation and FP cellulose digestion or enzyme activities. Cumulative gas production data were corrected to 0.1 g dry matter (DM). The NLIN procedure of SAS (1996) was used to fit curves to experimentally derived gas accumulation profiles using the NLIN model of Merry et al. (1991) and Herrero et al. (1996) as described above. Statistical differences were also determined by an analysis of variance with mean separations performed by Duncan's new multiple range test using GLM procedures of SAS (1996), and *p* value of <0.05 were considered significant.

## RESULTS

### Gas production

The cumulative *in vitro* gas production at different times for treatments is given in table 1. There was some

**Table 1.** Effects of long chain fatty acids (C<sub>18:1</sub>, C<sub>18:2</sub>, C<sub>18:1</sub> and C<sub>18:3</sub>) on cumulative *in vitro* gas production and its parameters by rumen anaerobic fungus, *Neocallimastix frontalis* RE1

Incubation time (h)	Treatments				
	Control	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Gas production (mL/0.1 g DM of substrates)					
12	9.69±0.56 <sup>A</sup>	8.83±0.38	10.48±0.89	9.63±0.53	11.13±0.22
24	12.83±0.68	12.04±0.52	13.60±0.88	13.24±0.53	14.52±0.25
48	13.64±0.63 <sup>b</sup>	15.87±0.27 <sup>ab</sup>	17.42±0.30 <sup>a</sup>	17.08±1.40 <sup>a</sup>	18.34±1.05 <sup>a</sup>
72	31.29±1.00 <sup>b</sup>	35.91±1.19 <sup>a</sup>	34.95±1.07 <sup>ab</sup>	34.52±1.99 <sup>ab</sup>	23.66±0.94 <sup>c</sup>
120	46.26±0.21 <sup>a</sup>	48.11±1.15 <sup>a</sup>	47.15±1.45 <sup>a</sup>	47.59±1.57 <sup>a</sup>	37.73±2.97 <sup>b</sup>
168	50.47±0.35 <sup>a</sup>	51.15±1.17 <sup>a</sup>	49.51±1.29 <sup>a</sup>	50.87±1.69 <sup>a</sup>	45.42±1.52 <sup>b</sup>
216	50.81±0.35	50.47±1.17	49.96±1.46	50.52±1.82	46.41±1.28
Gas production parameters*					
<i>a</i> + <i>b</i> (mL·0.1g <sup>-1</sup> )	52.29	48.12	50.11	49.45	47.47
<i>k</i> (Gp·h <sup>-1</sup> )	0.0289	0.0675	0.0331	0.0561	0.0202
<i>lag</i> (h)	47.66	47.67	40.57	47.75	59.56

<sup>A</sup> Mean ± SEM.

<sup>a,b</sup> Means with different superscripts in the same rows are different (p<0.05).

\* The parameters *a*, *b*, *k* and *lag* for the negative exponential equation,  $G_p = a + b[1 - \exp^{-k \cdot (time - lag)}]$ ; *G<sub>p</sub>* is a gas production (mL/0.1g DM of substrate) of time *t*; *a*+*b*, potential gas production; *k*, the fractional rate of gas production per hour; *lag*, time required before rapid gas production began.

differences in the cumulative gas production as a result of the addition of LCFAs at some incubation times which could be estimated as exponential phase (48-120 h) based on the figure 1. The gas production was significantly increased by the addition of stearic, oleic and linoleic acids, but that was significantly reduced by the addition of linolenic acid. Linolenic acid reduced the cumulative gas production to about 32.3 and 20.0% compared to a control treatment when measured after 72 and 120 h of incubation, respectively. Total gas produced when fermentation was almost completed (after 168 h) was similar for all five treatments. In all of the treatments, the cumulative gas production by the fungus was quickly increased according to the incubation time, especially from after 48 h incubation. The control and the stearic, oleic, linoleic and linolenic acids treatments reached maximum gas production after 120 h of incubation with amounts generally in the range of 45~50 mL·0.1 g DM<sup>-1</sup>. Some of these results indicate that gas production by this ruminal fungus was affected by the addition of LCFAs, even if maximum gas production was not decreased.

Gas accumulation profiles for each of the five treatments are shown in figure 1. In all the treatments, the gas production rate by fungus showed a marked lag time, with lag times of 47.66, 47.67, 40.57, 47.75 and 59.56 h for the control, stearic, oleic, linoleic and linolenic acids, respectively. And there were some notable differences between gas accumulation profiles with the additions of stearic and linoleic acid producing the most gas, and linolenic acid producing the least gas during the exponential

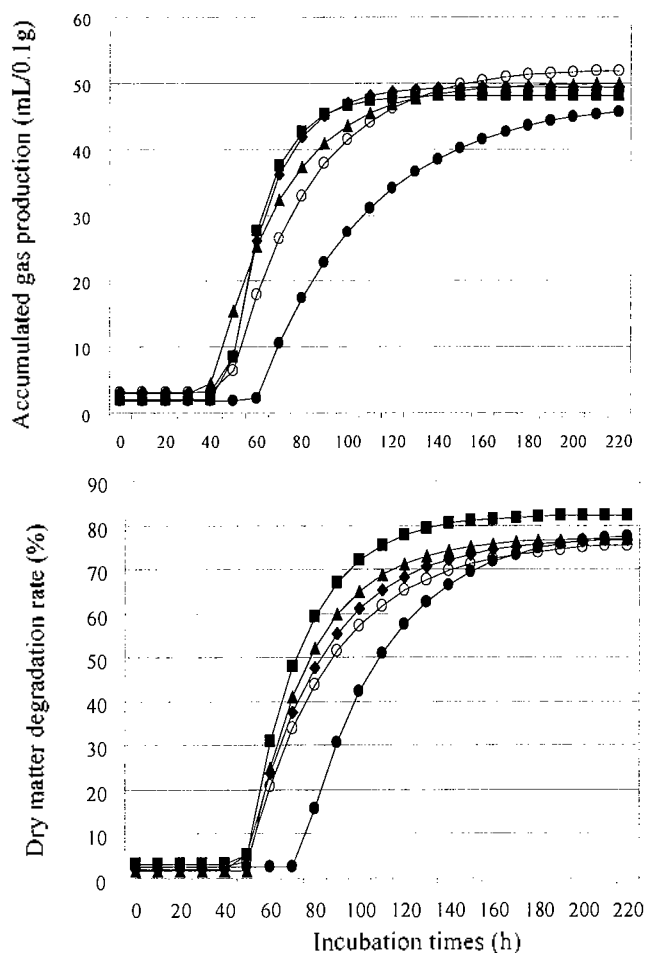
phase. In general, the addition of stearic, oleic and linoleic acids resulted in higher gas accumulation rates and greater gas pools than those of control treatment. However, the linolenic acid treatment resulted in the lowest gas accumulation and the smallest gas pools. Our data show that the gas accumulation rate (%·h<sup>-1</sup>) of the control, stearic, oleic, linoleic and linolenic acids treatments were 2.89, 6.75, 3.31, 5.61 and 2.02%, respectively. These results indicate that the addition of LCFAs have significant effects on the gas production rate by rumen anaerobic fungi.

#### FP cellulose degradation

The effects of LCFAs on FP cellulose degradation by the fungus, *N. frontalis* RE1 are shown in table 2. There were some significant differences in degradation rates due to the addition of stearic, oleic and linoleic acids. These acids did not have any significant inhibitory effects at any of the incubation times. The degradation rate at exponential phase (48~120 h) of incubation tended to be slightly increased by the addition of these acids, yet there were no statistical differences (p>0.05) in 120 h and before 48 h of incubation. However, the rate of FP cellulose degradation was significantly (p<0.05) reduced by the addition of linolenic acid and degradation rates were reduced by about 3, 4 and 2 times compared to the control treatment when measured after 48, 72 and 120 h of incubation, respectively. In all of the treatments, the degradation rate by the fungus showed a marked lag up to 48 h of incubation; after 72 h of incubation, the degradation rates quickly increased. The control, stearic and oleic acids treatments reached

maximum degradation after 120 h of incubation, while in the other treatments (linoleic and linolenic acids treatments) maxima were reached after 168 h of incubation. There were no differences in maximum degradation rates amongst the treatments with all treatments exhibiting degradation in the range of 75–80%. These results indicate that either the growth, or the length of the lag phase or both were affected by the addition of LCFAs, even when maximum degradation was not decreased. The lag periods required before rapid gas production or degradation began by the fungus seem very long even for the control. This results may be caused by the difficulty to degrade substrate (FP, 100% of cellulose) by this kind of fungus.

The correlation equation between gas produced (in mL



**Figure 1.** Cumulative gas production and filter paper cellulose degradation profiles by rumen anaerobic fungus, *Neocallimastix frontalis* RE1 grown in a medium with added long chain fatty acids (○-○, Control; ■-■, Stearic acid; ▲-▲, Oleic acid; ◆-◆, Linoleic acid; and ●-●, Linolenic acid); representing the rate of gas production expressed in mL gas·0.1g<sup>-1</sup>DM·h<sup>-1</sup>. The gas production and degradation curves were derived from a negative exponential equation with lag time:  $Gp = a + b[1 - \exp^{-kx(\text{time} - \text{lag})}]$ .

per 0.1 g DM) and the proportion of dry matter disappearing during fermentation (*in vitro* digestibility) was:

$$\text{In vitro digestibility} = (\text{Gas produced} \times 1.916) - 20.52$$

(correlation coefficient 0.963,  $r^2$  92.82%,  $n=105$ )

The FP cellulose degradation curve fitted to a negative exponential equation is also given in figure 1. The effective degradability and potential degradation rates of each treatment were quiet similar. However, the degradation rate per hour and the lag time were quite different among treatments with the value of  $k$  (%·h<sup>-1</sup>) of the control, stearic, oleic, linoleic and linolenic acids treatments being 2.67, 4.03, 3.67, 2.99 and 2.69, respectively. These results indicated that the addition of LCFAs have significant effects on the degradation rate, but not the extent of degradation by rumen fungi.

The initial pH of the culture media ( $6.65 \pm 0.02$ ) declined during the incubation time up to 72 or 127 h post-inoculation (data not shown). When measured during lag phase (12 and 24 h of incubation), the pH value was slightly decreased ( $p > 0.05$ ) by the addition of all of the fatty acids tested. However, after 48 h of incubation, there were significant decreasing of pH values as a result of the addition of stearic, oleic and linoleic acids. The pH was significantly ( $p < 0.05$ ) increased in the linolenic acid treatment after 72 and 120 h of incubation, however. This trend is corroborated by the cumulative *in vitro* gas production and the FP cellulose degradation rate.

#### Enzyme activity

Enzymatic activities in the different cultures are shown graphically in figures 2 and 3 for CMCase and xylanase, respectively. CMCase activity in the culture supernatants in the of stearic, oleic and linoleic acids treatments was higher than that for the control treatment from after 72 h of incubation with statistical differences at some observed times. The CMCase activity of culture supernatant for linoleic acid was significantly ( $p < 0.05$ ) lower after 48 and 72 h of incubation, and slightly lower ( $p > 0.05$ ) after 120 and 168h of incubation. The CMCase activity in all of the treatments increased relative slowly and reached maximal activity after 120 and 168 h of incubation. The correlation equations between gas production and CMCase activity were:

$$\text{CMCase activity} = (\text{Gas produced} \times 0.389) + 0.112$$

(correlation coefficient 0.909,  $r^2$  82.57%,  $n=105$ ).

The xylanase activity of culture supernatant for the treatments with oleic and linoleic acids was significantly ( $p < 0.05$ ) higher, but that for the treatments of stearic and linolenic acids was quiet similar compared to the control treatment when measured up to 12 h post inoculation.

**Table 2.** Effects of long chain fatty acids (C<sub>18:1</sub>, C<sub>18:2</sub>, C<sub>18:1</sub> and C<sub>18:3</sub>) on the degradation rate of filter paper cellulose and degradation parameters by rumen anaerobic fungus, *Neocallimastix frontalis* RE1

Incubation Time (h)	Treatments				
	Control	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
Degradation rate (%)					
12	1.66±1.80 <sup>A</sup>	4.14±0.25	2.25±0.48	1.42±0.88	0.24±1.53
24	4.26±1.14	6.27±0.75	4.70±1.29	2.58±1.09	8.72±1.13
48	9.20±1.29 <sup>b</sup>	13.86±1.63 <sup>a</sup>	15.07±1.22 <sup>a</sup>	10.05±1.67 <sup>b</sup>	5.51±0.48 <sup>c</sup>
72	30.98±2.38 <sup>b</sup>	49.15±7.60 <sup>a</sup>	36.34±4.14 <sup>ab</sup>	36.26±2.17 <sup>ab</sup>	9.32±3.34 <sup>c</sup>
120	73.44±3.02 <sup>ab</sup>	73.96±0.20 <sup>ab</sup>	76.30±1.48 <sup>a</sup>	69.66±1.24 <sup>b</sup>	35.12±16.15 <sup>c</sup>
168	75.77±2.00	77.06±1.82	73.36±1.38	75.53±0.79	76.04±2.52
216	75.77±0.23	81.37±3.46	76.80±0.38	80.25±1.43	77.97±0.34
Degradation constants*					
<i>a</i> + <i>b</i> (%)	76.27	82.39	77.08	77.07	79.04
<i>k</i> (h <sup>-1</sup> )	0.0267	0.0403	0.0367	0.0299	0.0269
<i>lag</i> (h)	49.26	49.32	49.95	48.55	72.96
ED** (%)	33.85	41.19	37.24	34.84	32.85

<sup>A</sup> Mean ± SEM.

<sup>a,b</sup> Means with different superscripts in the same rows are different (p<0.05).

\* The parameters *a*, *b*, *k* and *lag* for the negative exponential equation,  $P=a+b[1-\exp^{-k(t-lag)}]$ ; *P* is a disappearance rate at time *t*; *a*+*b*, potential degradation rate; *k*, the fractional rate of degradation per hour; *lag*, time required before rapid degradation began by fungus.

\*\* ED: Effective degradability (substrate availability) of filter paper cellulose was calculated as  $ED=a+b[k_d/(k_d+k_p)]$ , *k<sub>d</sub>* (*k*) is a digestion rate constant, and *k<sub>p</sub>* is a passage rate constant assumed to be 0.05 h<sup>-1</sup>.

However, after 72 h of incubation, xylanase activity was significantly increased by the addition of all LCFAs except linoleic acid. The control and oleic acid treatments reached maximum xylanase production after 120 h of incubation, stearic and linolenic acid treatments reached maxima after 168h, and the linoleic acid treatment reached a maximum after 216h of incubation. There were significant differences in maximum activity of xylanase between treatments and these varied in the range of 73–108 μmol xylose/min/mL. These results indicate that the rate and extent of production of xylanase by the ruminal fungus were affected by the addition of LCFA.

The correlation equations between gas production and xylanase activity were:

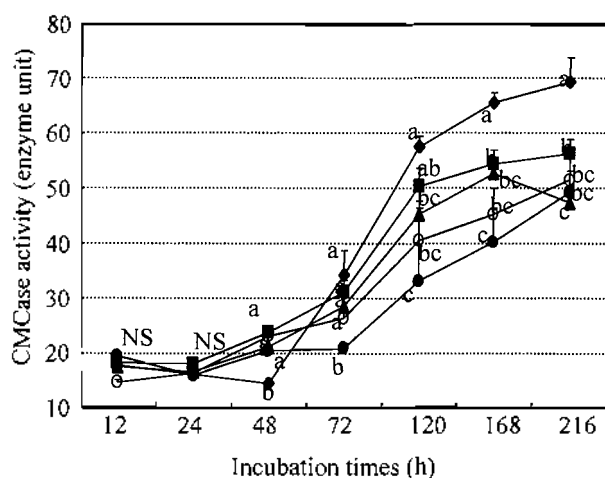
Xylanase activity={Gas produced×(-7.481)+1.788 (correlation coefficient 0.8766,  $r^2$  76.85%, *n*=105)}. The correlation coefficient ( $r^2$ =76.85) obtained between gas production and xylanase activity was the lowest among the various comparisons such as gas production rate versus degradation rate, CMCase activity, or xylanase activity.

## DISCUSSION

There have been many studies that show that growth and fermentation in anaerobic fungi are closely linked. For example, Lowe et al. (1987) showed that the fermentation end-products, formic acid and acetic acid, could be used as a convenient indicator of fungal growth and Mountfort and

Asher (1983) demonstrated that H<sub>2</sub> production could be used as a convenient estimate of fungal growth. Theodorou et al. (1995) also demonstrated that monitoring the accumulation of fermentation gasses during fungal growth is a useful method for the rapid and precise determination of fungal growth on soluble and particulate substrates. Similar trends were also observed in our experiments and there were high correlations between cumulative *in vitro* gas production and fungal growth (94.78%, data not shown), FP cellulose degradation (96.34%), CMCase activity (90.86%) or xylanase activity (87.67%). Thus, measuring of cumulative gas production could be a useful tool for evaluating fungal growth and/or digestion rates. There is a need to make more extensive comparisons between gas production and other parameters using different classes of feeds and species of ruminal fungi.

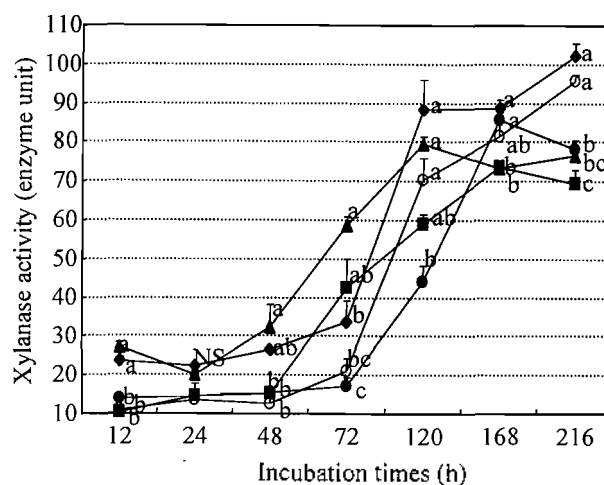
Unsaturated FAs with C<sub>18</sub> chain lengths seem to have the most widespread inhibitory effect on pure cultures of rumen anaerobic bacteria and protozoa. The most consistent effect of lipid and/or FAs supplementation *in situ* is the considerable decrease in rumen protozoal numbers in sheep and cows (Henderson et al., 1977; Ikwuegbu and Sutton, 1982). With 40 mL of linseed oil given per day, the rumen of a sheep was almost completely defaunated (Ikwuegbu and Sutton, 1982). Higher FAs are toxic to eukaryotic cell such as rumen protozoa and to Gram-positive methanogenic bacteria (Nieman, 1954; Galbraith and Miller, 1973; Henderson, 1973). The mechanism of toxicity has not yet



**Figure 2.** Influence of long chain fatty acids (O-O, Control; ■-■, Stearic acid; ▲-▲, Oleic acid; ◆-◆, Linoleic acid; and ●-●, Linolenic acid) on CMCase activity (enzyme unit;  $\mu\text{mol}\cdot\text{glucose}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) in supernatant of growing rumen anaerobic fungus, *Neocallimastix frontalis* RE1. The letters at the tops of the spots indicate statistical significance; means with different letters are significantly different ( $p < 0.05$ ).

been elucidated (Girard and Hawke, 1978). Under aerobic condition, polyunsaturated FAs have been reported to inhibit microorganisms via autoxidation and by the formation of peroxides and radicals (Ismail et al., 1977). Knapp and Melly (1986) also reported that the bactericidal effects of polyunsaturated FAs were mediated by a peroxidative process involving hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Wang and Johnson (Wang and Johnson, 1992) also reported that linolenic acid ( $\text{C}_{18:3}$ ) was more inhibitory than linoleic acid ( $\text{C}_{18:2}$ ) and oleic acid ( $\text{C}_{18:1}$ ) against *Listeria monocytogenes* which could be consistent with a peroxidative mechanism. However, these mechanisms are less favorable in an anaerobic environment such as the rumen. Therefore, there may be other explanations for FAs toxicity such as coating effects of FAs on the fungal cells.

Interestingly, in this study we demonstrated that the addition of emulsified LCFAs including stearic acid ( $\text{C}_{18:0}$ ), oleic acid ( $\text{C}_{18:1}$ ) and linoleic acid ( $\text{C}_{18:2}$ ) did not have any significant inhibitory effects on cellulolysis by *N. frontalis* RE1, based on the results of gas production, FP cellulose degradation rate and cellulase activities. In contrast, the addition of linolenic acid ( $\text{C}_{18:3}$ ) caused a significant inhibitory effect on fungal cellulolysis. The marked growth inhibition effect of linolenic acid is very interesting because of Orpin and Letcher (1979) showed that  $\alpha$ -linolenic acid stimulated the *in vitro* growth of *N. frontalis*. Our present work has shown, for the first time, that cellulolysis by a ruminal fungus is slightly stimulated when the organism is



**Figure 3.** Influence of long chain fatty acids (O-O, Control; ■-■, Stearic acid; ▲-▲, Oleic acid; ◆-◆, Linoleic acid; and ●-●, Linolenic acid) on xylanase activity (enzyme unit;  $\mu\text{mol}\cdot\text{xylose}\cdot\text{min}^{-1}\cdot\text{mL}^{-1}$ ) in supernatant of growing rumen anaerobic fungus, *Neocallimastix frontalis* RE1. The letters at the tops of the spots indicate statistical significance; means with different letters are significantly different ( $p < 0.05$ ).

grown in a medium with added stearic, oleic and linoleic acids. Our results are in disagreement with those of numerous researchers (Demeyer and Henderickx, 1967; Henderson, 1973; Maczulak et al., 1981) who reported that  $\text{C}_{18}$  USFAs cause a strong inhibition of microbial growth.

The significant stimulatory effects after the addition of stearic, oleic and linoleic acids could be explained by the hydrogenation of UFA, compounds which can stimulate the growth and activity of anaerobic fungi by capturing the  $\text{H}_2$  that is produced as a main fermentation end-product by fungus. Hydrogen is an intermediate produced during plant cell wall breakdown by anaerobic fungi (Fonty and Joblin, 1991). Various workers (Bauchop and Mountfort, 1981; Mountfort et al., 1982; Fonty et al., 1988; Joblin et al., 1990; Marvin-Sikkema et al., 1990; Joblin and Williams, 1991) also have shown that the anaerobic fungi symbiotically interact with some hydrogen-utilizing bacteria, especially methanogens, and provide  $\text{H}_2$  which can be utilized by the bacteria (Wolin and Miller, 1988). Unsaturated FA in LCFA also has the ability to capture  $\text{H}_2$  in the rumen by the process of biohydrogenation (Harfoot, 1978), which reduces the number of double bonds (Ward et al., 1964).

If our presumption that rumen fungi could have the ability to hydrate UFAs to SFAs, is right, the strongest stimulatory effects should have been observed in the linolenic treatment. We failed to observe this phenomenon, however. Further research is needed to determine whether rumen

anaerobic fungi have the ability to hydrate UFAs to SFAs.

Another possible explanation is that exogenous LCFA have little, if any, energy-sparing effects on the growth of rumen anaerobic fungi under the limiting substrate concentrations used in this growth study. Some previous reports have evidenced the role of energy sparing effects and nutrient up take provided by LCFA in the stimulation of rumen microorganisms. Furthermore, yeast extract has been shown to be a growth factor for an acetogenic strain isolated from the rumen of a deer. Rates of biohydrogenation *in vitro* also vary with concentration of substrate in the media, the age and type of microbial inoculum, and the presence of required cofactors in rumen fluids (Kellens et al., 1986). If the cellulase enzyme is cell surface bound, adsorption of the LCFA either on the fungal cell or substrate would probably interfere with the digestion of insoluble cellulose. Fatty acids have been demonstrated to affect cell permeability and transport of nutrients (Greenway et al., 1979). Recent studies have indicated that micromolar concentrations of fatty acids can affect the activity of enzymes in the cell membrane (Viegas and Sa-Correia, 1991).

In conclusion, we have found that certain LCFAs (particularly stearic, oleic and linoleic acids) are not inhibitory to anaerobic fungi. The activity we observed suggests that these FAs could stimulate growth and cellulolysis by anaerobic gut fungi. However, linolenic acid strongly inhibits the process of fungal cellulolysis. These preliminary results are the first report of the effect of LCFAs on the anaerobic fungi. Further research is needed to identify the mode of action of LCFAs on fungal cells and to verify whether rumen anaerobic fungi have the ability to hydrate UFAs to SFAs.

#### ACKNOWLEDGEMENTS

This research was partially supported by High-Technology Development Project of Ministry of Agriculture and Forestry in Korea, the Brain Korea 21 Project and KOSEF (Korea Science and Engineering Foundation, Taejon, Korea). Appreciation is expressed to Lindsey J. Yanke, Research Centre, Agriculture and Agri-Food Canada, Lethbridge, AB, for kindly providing the microorganisms used in this experiment.

#### REFERENCES

- Bauchop, T. and D. O. Mountfort. 1981. Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. *Appl. Environ. Microbiol.* 42:1103-1110.
- Bernalier, A., G. Fonty and P. Gouet. 1990. Fermentation properties of four strictly anaerobic rumen fungal species: H<sub>2</sub>-producing microorganisms. In: *Microbiology and Biochemistry of Strict Anaerobes Involved in Interspecies Hydrogen Transfer* (FEMS Symposium Sept. 1989). (Ed. J. P. Belaich). Plenum, NY. pp. 301-364.
- Bryant, M. P. and L. A. Burkey. 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. *J. Dairy Sci.* 36:205-217.
- Demeyer, D. I. and H. K. Henderickx. 1967. The effect of C<sub>18</sub> unsaturated fatty acids on methane production *in vitro* by mixed rumen bacteria. *Biochem. Biophys. Acta.* 137: 484-497.
- Fedorak, P. M. and S. E. Hrwdey. 1983. A simple apparatus for measuring gas production by methanogenic cultures in serum bottles. *Environ. Technol. Lett.* 4:425-432.
- Fonty, G. and K. N. Joblin. 1991. The rumen anaerobic fungi, p. 625-654. In: *Physiological Aspects of Digestion and Metabolism in Ruminants*. Academic Press, (Ed. T. Tsuda, Y. Sasaki, and R. Kawashima). San Diego, California.
- Fonty, G., E. Grenet, M. Fevre, A. Breton and P. Gouet. 1988. *Ecologie et fonction des champignons anaerobies du rumen*. *Reprod. Nutr. Dev.* 28(Suppl.1): 1-18.
- Galbraith, H. and T. B. Miller. 1973. Effect of long chain fatty acids on bacterial respiration and amino acid uptake. *J. Appl. Bacteriol.* 73:659-675.
- Girard, V. and J. C. Hawke. 1978. The role of holotrichs in the metabolism of dietary linoleic acid in the rumen. *Biochim. Biophys. Acta.* 528:17-27.
- Gordon, G. L. R. and M. W. Phillips. 1989. Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine rumen. *Appl. Environ. Microbiol.* 55:1703-1710.
- Greenway, D. L. A. and K. G. H. Dyke. 1979. Mechanism of the inhibitory action of linoleic acid on the growth of *Staphylococcus aureus*. *J. Gen. Microbiol.* 115:233-245.
- Harfoot, C.G. 1978. Lipid metabolism in the rumen. *Prog. Lipid Res.* 17:21-54.
- Henderson, C. 1973. The effects of fatty acids on pure cultures of rumen bacteria. *J. Agric. Sci.* 81:107-112.
- Henderson, C., C. S. Stewart and R. S. Hins. 1977. The effect of added tallow on the rumen digestion rate and microbial populations of sheep fed dried grass. *Proc. Nutr. Soc.* 36:48A.
- Herrero, M., I. Murray, R. H. Fawcett and J. B. Dent. 1996. Prediction of the *in vitro* gas production and chemical composition of kikuyu grass by near-infrared reflectance spectroscopy. *Anim. Feed Sci. Technol.* 60:51-67.
- Hungate, R. E. 1950. The anaerobic mesophyllic cellulolytic bacteria. *Bacteriol. Rev.* 14:1-49.
- Ikwuegbu, O. A. and J. D. Sutton. 1982. The effect of varying the amount of linseed oil supplementation on rumen metabolism in sheep. *Br. J. Nutr.* 48:365-375.
- Ismail, G., W. D. Sawyer and W. S. Wegener. 1977. Effect of hydrogen peroxide and superoxide radical on viability of *Neisseria gonorrhoeae* and related bacteria. *Proc. Soc. Exp. Biol. Med.* 155:264-269.
- Joblin, K. N., G. E. Naylor and A. G. Williams. 1990. The effect of *Methanobrevibacter smithii* on the xylanolytic activity of rumen fungi. *Appl. Environ. Microbiol.* 56: 2287-2295.
- Joblin, K. N. and A. G. Williams. 1991. Effects of cocultivation of ruminal chytrid with *Methanobrevibacter smithii* on lucerne stem degradation and extracellular fungal enzyme activities. *Lett. Appl. Microbiol.* 12:121-124.
- Kellens, M. J., H. L. Goderis and P. P. Tobback. 1986.

- Biohydrogenation of unsaturated fatty acids by a mixed culture of rumen microorganisms. *Biotechnol. Bioeng.* 28:1268-1276.
- Knapp, H. R. and M. A. Melly. 1986. Bactericidal effect of polyunsaturated fatty acids. *J. Inf. Dis.* 154:84-94.
- Lowe, S. E., M. K. Theodorou, A. P. J. Trinci and R. B. Hespell. 1985. Growth of anaerobic rumen fungi on defined and semidefined media lacking rumen fluid. *J. Gen. Microbiol.* 131:2225-2229.
- Lowe, S. E., G. G. Griffith, A. Milne, M. K. Theodorou and A. P. J. Trinci. 1987. The life cycle and growth kinetics of an anaerobic rumen fungus. *J. Gen. Microbiol.* 133:1815-1827.
- Maczulak, A. E., B. A. Dehority and D. L. Palmquist. 1981. Effects of long-chain fatty acids on growth of rumen bacteria. *Appl. Environ. Microbiol.* 42:856-862.
- Marvin-Sikkema, F. D., A. J. Richardson, C. S. Stewart, J. C. Gottschal and R. A. Prins. 1990. Influence of hydrogen consuming bacteria on cellulose degradation by anaerobic fungi. *Appl. Environ. Microbiol.* 56:3793-3797.
- Menke, K.H., L. Raab, A. Salewski, H. Steingass, D. Fritz and W. Schneider. 1979. The estimation of the digestibility and metabolisable energy content of ruminant feeding stuffs from the gas production when they are incubated with rumen liquor *in vitro*. *J. Agric. Sci. Camb.* 93:217-222.
- Merry, R. J., M. K. Theodorou, M. G. Raurich and M. S. Dhanoa. 1991. Use of head-space gas pressure in batch cultures to assist in determination of nutritive value of silage to rumen bacteria. Presented at a Conference on Forage Conservation Towards 2000. Institute of Grassland and Forage Research, Federal Research Centre of Agriculture, Braunschweig-Volkenrode, Germany.
- Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.
- Miller, T. L. and M. J. Wolin. 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. *Appl. Microbiol.* 27: 985-987.
- Mountfort, D. O., R. A. Asher and T. Bauchop. 1982. Fermentation of cellulose to methane and carbon dioxide by a rumen anaerobic fungus in a triculture with *Methanobrevibacter* sp. strain RA1 and *Methanosarcina barkeri*. *Appl. Environ. Microbiol.* 44:128-134.
- Mountfort, D. O. and R. A. Asher. 1983. Role of catabolite regulatory mechanisms in control of carbohydrate utilization by the rumen anaerobic fungus *Neocallimastix frontalis*. *Appl. Environ. Microbiol.* 46:1331-1338.
- Nieman, C. 1954. Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriol. Rev.* 18:147-163.
- Orpin, C. G. 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.
- SAS. 1996. SAS user's guide: Statistics, SAS Institute. Inc., Cary, NC, USA.
- Theodorou, M. K., B. A. Williams, M. S. Dhanoa and A. B. McAllan. 1991. A new laboratory procedure for estimating kinetic parameters associated with the digestibility of forages. International Symposium on Forage Cell Wall Structure and Digestibility. US Dairy Forage Research Center, USDA, Agricultural Research Service, Wisconsin, USA.
- Theodorou, M. K., R. J. Merry, B. B. Nielsen, D. Isac, D. D. Davies, M. Lawrence and A. P. J. Trinci. 1992. A new method for studying growth and activity of surface colonizing micro-organisms. In: Sixth International Symposium on Microbial Ecology (ISEM-6), September, 1992. C2-5 Microbial Surface Colonization. Barcelona. p. 39.
- Theodorou, M. K., D. R. David, B. B. Nielsen, M. I. G. Lawrence and A. P. J. Trinci. 1995. Determination of growth of anaerobic fungi on soluble and cellulosic substrates using a pressure transducer. *Microbiology.* 141:671-678.
- Viegas, C. A. and I. Sa-Correia. 1991. Activation of plasma membrane ATPase of *Saccharomyces cerevisiae* by octanoic acid. *J. Gen. Microbiol.* 137:645-651.
- Wang, L. L. and E. A. Johnson. 1992. Inhibition of *Listeria monocytogenes* by fatty acids and monoglycerides. *Appl. Environ. Microbiol.* 58:624-629.
- Ward, P. F. V., T. W. Scott and R. M. C. Dawson. 1964. The hydrogenation of unsaturated fatty acids in the ovine digestive tract. *Biochem. J.* 92:60-68.
- Wolin, M. J. and T. L. Miller. 1988. Microbe-microbe interactions, In: *The Rumen Microbial Ecosystem* (Ed. P. N. Hobson). Elsevier, Amsterdam. pp. 361-386.