Effect of Feeding Supplemental Fibrolytic Enzymes or Soluble Sugars with Malic Acid on Milk Production

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ABSTRACT

Two trials were conducted to evaluate effects of feeding supplemental fibrolytic enzymes or soluble sugars and malic acid on milk production. In trial 1, 257 cows at four sites were fed a basal diet consisting of no more than 60% of forage DM as corn silage and less than 40% as alfalfa hay. Cows were assigned randomly within site, parity, and two stages of lactation to: 1) control; 2) enzyme A; 3) enzyme B; and 4) soluble sugars and malic acid. There was a 14-d pretreatment and an 84-d treatment period. Enzyme solutions were sprayed on either the forage component or the TMR each day while mixing feed. Trial 2 was similar, except 122 cows at one site in the United Kingdom were fed diets containing forage that was 75% corn silage and 25% grass silage, and all cows began the study between 25 to 31 DIM. Mean milk productions for 233 cows that completed trial 1 were 32.9, 32.5, 32.4, and 32.9 kg/d for control, enzyme A, enzyme B, and soluble sugars and malic acid, respectively. Mean milk productions for 116 cows that completed trial 2 were 28.2, 27.9, 28.8, and

28.4 kg/d, respectively. In vitro analyses of the activities of enzyme solutions indicated that all major cellulose and hemicellulose degrading activities were present; however, the pH optima (approximate pH = 4 to 5) were more acidic, and the temperature optimum (approximately 50°C) was greater than normal pH and temperature in the rumen. If fibrolytic activity in the rumen is a major mechanism of action of supplemental fibrolytic enzymes, it appears that considerable activity of these preparations was lost due to conditions in the rumen. In conclusion, feeding supplemental fibrolytic enzymes or malic acid with soluble sugars had no effect on milk production under the conditions used in this study.

(**Key words:** fibrolytic enzyme, malic acid, feed additive, milk production)

Abbreviation key: CM = carboxymethyl; CNY = Cornell, NY; CPM = Cornell-Penn-Miner; DMSO = dimethyl sulphoxide; DNS = dinitrosalicylic acid; IL = Illinois; MD = Maryland; MP = metabolizable protein; SSMA = soluble sugars/malic acid; SNY = Spruce Haven, NY.

INTRODUCTION

Feed costs represent the highest expense to dairy producers and, as a consequence, maximizing utilization of nutrients is essential to the profitability and sustainability of dairy farms. The largest fraction of feed energy that is not available to the animal is the

Received March 6, 2002.

Accepted May 24, 2002.

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undigested fibrous fraction. Methods that reduce this fraction could provide considerable benefit to dairy producers. Ruminant microorganisms, including bacteria and fungi, secrete specific enzymes to digest the fibrous fraction of feedstuffs. The host animal derives energy from the microbial cells and from various end products of fermentation. One potential method of improving utilization of fibrous feedstuffs is to increase their digestibility by increasing the quantity of fibrolytic enzymes that are available in the rumen.

Several approaches have been attempted for incorporating enzymes into diets for dairy cattle. Experiments have been conducted to study effects of adding fibrolytic enzymes to dry forages (Yang et al., 1999) or concentrates (Yang et al., 2000) before feeding and adding amylolytic and proteolytic enzymes targeted towards the starch-protein matrix of sorghum (Chen et al., 1995). In other studies, the effects of direct-fed fibrolytic enzymes applied either to the forage component of the diet (Schingoethe et al., 1999; Kung et al., 2000) or to a TMR (Beauchemin et al., 1999) have been examined. Phipps et al. (2002) reviewed recent literature on the effect of direct-fed enzymes on feed intake and milk production. They noted that with few exceptions (Lewis et al., 1999), the direct-fed enzymes produced no significant effect on feed intake. While significant increases in milk production following treatment with direct-fed enzymes have been reported by Lewis et al. (1999), Schingoethe et al. (1999), and Zheng et al. (2000), other workers, including Beauchemin et al. (1999), Rode et al. (1999), and Kung et al. (2000), reported only numerical, nonsignificant increases. Also, Yang et al. (1999, 2000) reported significant changes in milk yield when enzymes were mixed at the time of manufacturing with alfalfa hay cubes or the concentrate portion, respectively. In the studies reviewed, the range in milk production response varied from 0 to 6.3 kg/d. Changes in milk composition due to the application of direct-fed enzymes were variable.

The objective of this study was to examine the effects of feeding one of two enzyme treatments on animal performance. In addition, a treatment group was also included to supply nutrients designed to support microbial growth. The nutrients included nonstructural carbohydrates, which were intended to supply energy to microbes and increase ruminal ammonia utilization and dicarboxylic acids to increase lactate utilization by *Selenomonas ruminantium* (Martin et al., 2000).

MATERIALS AND METHODS

Trial #1

This trial was conducted at four sites. All sites followed the same experimental design, and the data from

all four sites were pooled. Sites were: Central Maryland Research and Education Center, Ellicott City (MD); Spruce Haven Research Farm, Auburn, New York (SNY); University of Illinois research farm, Urbana (IL); and Cornell University Teaching and Research Center, Dryden, New York (CNY).

A total of 257 primiparous and multiparous Holstein cows were assigned to the study. All cows were between 8 and 166 DIM at the start of the pretreatment period and were in good overall health, including good foot, leg, and udder conformation. The numbers of animals assigned within each site and parity group are presented in Table 1. Cows were assigned to the study at the beginning of a 14-d pretreatment period within site, parity, and stage of lactation. Stages of lactation were early or mid-lactation. Early-lactation cows were <81 DIM at the initiation of the treatment period at all sites except IL where they were <73 DIM.

Following the 14-d pretreatment period, cows were assigned randomly within blocks to one of four treatment groups. Treatments were: 1) control; 2) fibrolytic enzyme solution A (Finnfeeds, Helsinki, Finland); 3) fibrolytic enzyme solution B (Biovance Technologies, Inc., Omaha, NE); and 4) additive-containing soluble sugars and malic acid (SSMA; Milk Specialties, Dundee, IL). Both enzyme solutions contained activities derived from *Trichoderma longibrachiatum*. Enzyme solution A was added at 1.25 L/1000 kg of forage DM and was diluted with water and sprayed on forage in the TMR wagon at 10 L/1000 kg of forage (as fed). Enzyme solution B was added at 2 L/1000 kg of total ration DM and was diluted and sprayed on the mixed ration at 10 L/1000 kg (as fed). Enzymes were sprayed on feed each morning during the mixing process. Treatment #4 was added daily to the diet as a dry supplement to provide approximately 0.227 kg/cow per day. Levels of enzyme addition used and the type of feed (forage only or TMR) to apply the enzyme mixture were based on recommendations from the enzyme manufacturers. The treatment phase lasted for 84 d. Feeds were mixed at each site for a specified time in a mixer wagon. Times used across sites ranged from 5 to 10 min, and the time used at each site resulted in a homogeneous mixture and additives were well dispersed.

Cows were fed a TMR (Table 2) formulated to meet or exceed NRC (1989) requirements. At each site, the feed for 1 d was mixed once daily, and cows were fed once daily (except Illinois where the daily ration was weighed out every morning but fed in a.m. and p.m. offerings). Also, diets were evaluated using the Cornell-Penn-Miner (CPM) model (Barry et al., 1994) to assure that metabolizable protein (MP) allowable milk was at least 2 kg/d greater than ME allowable milk. Across sites, forages were restricted to no more than 60% of

Table 1. Numbers of cows starting the trials at each site and numbers of cows within parity and start DIM blocks.

Site	#Primiparous		#Multiparous		Total
	Early block	Late block	Early block	Late block	
Trial 1					
MD	14	5	14	27	60
SNY	15	22	6	34	77
IL	8	8	16	28	60
CNY	17	13	18	12	60
Total	54	48	54	101	257
Trial 2					
UK	37		85		122

forage DM as corn silage and no less than 40% of forage as alfalfa hay. Cows were fed ad libitum.

Cows were milked twice (CNY, MD, IL) or three (SNY) times daily. Samples of each milking were collected 1 d/wk, and they were analyzed using an automated infrared analyzer for fat and protein (AOAC, 1990; Dairy One, Ithaca, NY). Amounts of feed offered and refused were weighed daily for each cow. At IL, weighback for each cow was scored every morning for

moisture content of the refused feed. Scores were from 1 (driest) to 4 (wettest). Individual cow refusal samples were measured for DM at 2- to 4-wk intervals. Using these samples, an average DM was calculated for scores 1 through 4. The average for a specific score was then used to estimate the DM content of all refusal amounts with that wetness score.

Feed ingredients and control TMR were sampled weekly. An aliquot of each sample was frozen, and for-

Table 2. Ingredient and nutrient composition of basal diet at each site.

	MD	SNY	IL	CNY	UK
Ingredient (%) DM					
Corn silage	29.1	29.2	27.0	25.7	42.7
Alfalfa hay	19.4	19.5	25.0	16.7	
Grass silage					14.3
Cottonseed	10.2	7.1	10.0	9.9	
Corn, ground shelled	26.1		22.9		
Corn, high moisture		19.7		29.4	
Wheat					20.1
Soybean meal, 48% CP	5.4	9.5	11.0	5.2	
Bypass SBM ¹	7.7	3.9		8.0	5.9
Canola meal					10.1
Fishmeal		0.6	1.0		1.4
Distillers grain		3.2			
Bakery waste		3.8			
Urea		0.3			
Protein supplement ²				2.1	
Molasses					4.0
Fat ³					0.6
Minerals	2.2	3.3	3.4	2.0	0.9
Nutrient analysis, % DM					
DM ⁴	49.15	53.80	67.90	53.04	44.60
CP	16.00	17.77	17.24	18.49	19.10
NE _L ⁵	1.59	1.61	1.57	1.68	1.61
ADF	23.38	27.40	25.42	19.74	22.32
NDF	39.98	37.60	38.06	30.14	37.60
NFC ⁶	32.73	33.27	33.40	40.30	33.32
Ether extract	5.95	5.53	4.96	4.81	3.62

¹Soyplus (West Central Soy, Ralston, IA).

²Hi-Lys (Agway Inc., Syracuse, NY).

³Megalac (Volac International, Royston, UK).

⁴Percentage of wet weight.

⁵Mcal/kg.

⁶Nonfiber carbohydrate [100 - (NDF, % + CP, % + ether extract, % + ash, %)].

age samples were tested weekly for DM to adjust the as-fed amounts of forage and concentrate in the TMR on a weekly basis. At the completion of the trial, samples were composited for the pretreatment period and for every 4-wk interval during the treatment period. Composite samples were analyzed by wet chemistry methods (Dairy One, Ithaca, NY).

Body weights were measured at the beginning of the treatment period and every 4 wk thereafter. Cows were observed daily and health findings were recorded.

Trial #2

A second trial using the same four treatment groups was conducted at the University of Reading using 122 British Holstein-Friesian cows (Table 1). The experimental design for trial 2 was similar to trial 1, except there was no blocking by stage of lactation. Instead, all cows began the pretreatment between 11 and 17 DIM, and treatments were initiated 14 d later between 25 and 31 DIM. Also, the diet contained approximately 75% of the forage DM as corn silage and 25% as grass silage (Table 2).

Enzyme Assays

Enzyme preparations A and B were assayed for xylanase, endoglucanase, exoglucanase, β -glucosidase, β -xylosidase, α -L-arabinofuranosidase, α -L-arabinopyranosidase, acetyl esterase, and α -glucuronidase as described below.

Xylanase activity. Xylanase was assayed as described by Bailey et al. (1992). A 15-ml test tube containing 1.8 ml of 1.0% (wt/vol) birchwood xylan (Roth 7500) prepared in 50 mM sodium citrate buffer pH 5.3, and 0.196 ml of distilled water were preincubated at 50°C for 10 min. The enzyme reaction was initiated by adding 4 μ l of 1:200 diluted enzyme sample, and the reaction continued for 5 min at 50°C. The reaction was terminated by adding 3 ml of dinitrosalicylic acid (DNS) reagent, and the reducing sugars released were determined by the DNS method (Miller, 1959). The unit of enzyme activity was expressed as the amount of enzyme required to release 1 μ mol of reducing sugars as xylose equivalent $\text{min}^{-1} \cdot \text{g}^{-1}$ of the enzyme sample.

Endoglucanase activity. Endoglucanase was assayed as described by Wood and Bhat (1988). A 15-ml test tube containing 1 ml of 1.0% (wt/vol) of carboxymethyl cellulose (CM-cellulose, medium viscosity), 0.5 ml of 100 mM sodium acetate buffer pH 5.0, and 0.492 ml of distilled water was preincubated at 50°C for 10 min. At the end of 10 min, 8 μ l of 1:200 diluted enzyme sample was added and incubated for a further 15 min at 50°C. The reaction was terminated by adding 3 ml

of DNS reagent, and the amount of reducing sugars released was determined as described above. The unit of enzyme activity was expressed as the amount of enzyme required to release 1 μ mol of reducing sugars as glucose equivalent $\text{min}^{-1} \cdot \text{g}^{-1}$ of the enzyme sample.

Exoglucanase activity. Exoglucanase was assayed as described by Wood and Bhat (1988). A 15-ml test tube containing 1 ml of 1.0% (wt/vol) of Avicel (Fluka, Sigma-Aldrich, Dorset, UK), 0.5 ml of 100 mM sodium acetate buffer pH 5.0, and 0.492 ml of distilled water was preincubated at 50°C for 10 min. At the end of 10 min, 8 μ l of 1:250 diluted enzyme sample was added, and the tubes were incubated for a further 2 h at 50°C. The reaction was terminated by placing the tubes in a boiling water bath for 10 min. The tubes were centrifuged at $1350 \times g$ for 10 min, and the reducing sugars present in the supernatant were determined by the DNS method (Miller, 1959). The unit of enzyme activity was expressed as the amount of enzyme required to release 1 μ mol of reducing sugars as glucose equivalent $\text{min}^{-1} \cdot \text{g}^{-1}$ of the enzyme sample.

β -Glucosidase, β -xylosidase, α -L-arabinofuranosidase, α -L-arabinopyranosidase, and α -D-glucuronidase activities. β -Glucosidase, β -xylosidase, α -L-arabinofuranosidase, α -L-arabinopyranosidase, and α -D-glucuronidase were assayed using microtiter plates in duplicate and using two different enzyme concentrations. A 150- μ l reaction volume containing 100 μ l of 3 mM substrate, 37.5 μ l of 100 mM sodium acetate buffer, pH 5.0, and 12.5 μ l of enzyme (1:20 to 1:100 diluted) was incubated at 40°C for 15 min. The substrate for each activity was the respective *p*-nitrophenyl-glycoside (i.e., *p*-nitrophenyl- β -D-glucoside for β -glucosidase). The reaction was terminated by adding 150 μ l of 0.4 M glycine buffer, pH 10.8. The absorbance was read using an Anthos Microplate reader at 405 nm. The unit of enzyme activity was expressed as μ mol of *p*-nitrophenol released $\text{min}^{-1} \cdot \text{g}^{-1}$ of enzyme sample.

Acetyl esterase activity. Acetyl esterase was measured using *p*-nitrophenyl acetate by microtiter plate method. *p*-Nitrophenyl acetate (10 mM) was dissolved in 20% (vol/vol) of dimethyl sulphoxide (DMSO), since it was partially soluble in water. However, the above concentration of DMSO had little effect on enzyme activity. Also, the *p*-nitrophenyl acetate was unstable at temperatures >50°C and pH 7.0. Therefore, the initial rate of the reaction was measured using potassium phosphate buffer at pH 6.8 and at 37°C for 10 min at 405 nm. The reaction was linear up to 10 min and the enzyme activity was determined based on the absorbance at the end of 5 min of incubation. Thus, a typical 200- μ l reaction mixture contained 100 μ l of 10 mM *p*-nitrophenyl acetate in 20% DMSO, 50 μ l of 50 mM potassium phosphate buffer, pH 6.8 and 50 μ l of

Table 3. Numbers of cows that started trial, removed from study, and in final analyses for each trial.

	Control	Enzyme A	Enzyme B	SSMA	Total
Trial 1					
Started trial	62	66	63	66	257
Removed during study	3	3	2	3	11
Did not follow protocol ¹	1	3	1	0	5
Health drop ²	2	2	2	2	8
Total in analysis	56	58	58	61	233
Trial 2					
Started trial	28	32	30	32	122
Removed during study	2	1	1	2	6
Did not follow protocol	0	0	0	0	0
Health drop	0	0	0	0	0
Total in analysis	26	31	29	30	116

¹Dropped because not within 7 to 166 DIM as required by study protocol.

²Completed study but dropped from analysis due to health malady that may have affected response.

1:50 diluted enzyme. The unit of enzyme activity was expressed as μmol of *p*-nitrophenol released $\text{min}^{-1}\cdot\text{g}^{-1}$ of enzyme sample.

Determination of pH optimum of cellulase and hemicellulase activities. For the determination of pH optimum of xylanase, endoglucanase, and exoglucanase, 1 ml of 1% substrate (birchwood xylan, CM-cellulose, medium viscosity, or Avicel) prepared in distilled water was mixed with 0.9 ml of buffer (50 mM) having varying pH values (citrate-phosphate, pH 2.6 to 7.0; potassium-phosphate, pH 6.6 to 8.0, and boric acid-sodium hydroxide, 8.0 to 9.0) and incubated at 50°C for 10 min. At the end of 10 min, 0.1 ml of suitably diluted enzyme sample was added to the reaction mixture, and the assay was carried out as described above. The reducing sugars released were determined by the DNS method (Miller, 1959), and the pH optimum was determined by plotting the unit of enzyme activity versus pH.

The pH optimum of β -glucosidase, β -xylosidase, α -L-arabinofuranosidase, and α -L-arabinopyranosidase was determined by using microtiter plates as described above and using the above mentioned three buffer systems. In all cases, the pH optimum was determined by plotting the unit of enzyme activity versus pH.

Determination of temperature optimum of xylanase, endoglucanase, and exoglucanase activities. The optimum temperatures were determined by measuring the xylanase, endoglucanase, and exoglucanase activities present in samples A and B at temperatures between 10 and 70°C at the optimum pH of each enzyme activity. The assay conditions for each activity were as described above. The optimum temperature of each enzyme activity was determined by plotting the activity versus temperature.

Statistical Analyses

Due to differences in the experimental design of the two production trials, each trial was analyzed separately.

Data were analyzed by SAS (1997) using the following model:

$$Y_{ijkl} = U + T_i + S_j + TS_{ij} + B_k + TB_{ik} + M_l + TM_{il} + bPRE_{ijkl} + e_{ijkl},$$

where U = overall mean, T_i = treatment effect, S_j = site effect, TS_{ij} = treatment by site interaction, B_k = stage of lactation block, TB_{ik} = treatment by stage of lactation interaction, M_l = parity group, TM_{il} = treatment by parity interaction, PRE_{ijkl} = pretreatment covariate, b = regression coefficient for PRE_{ijkl} , and e_{ijkl} = residual error. When $P < 0.25$, the treatment by site interaction was used as the error term to test significance of the treatment main effect. Treatment effects were deemed significant at $P < 0.05$. Data for trial 2 were analyzed separately using a similar model excluding the site and stage of lactation terms.

RESULTS

Production Trials

Health of cows was generally good throughout both trials 1 and 2. Eleven cows were removed from trial 1, and six cows were removed from trial 2 (Table 3). The most common reasons for removal were displaced abomasum, mastitis, foot problems, and not adapting to tiestalls. Additionally, data from animals that completed the study but were identified as having health problems that could impact production results were removed. Both the study investigator and the investigator for each site evaluated these cows individually, and if both investigators agreed, data for these animals were excluded from analysis. Data from eight animals in trial 1 were removed due to health problems such as early dry-off and foot health. Data from five additional animals were not included due to not meeting protocol defined DIM at the initiation of treatments. There were

Table 4. Feed consumption, milk production and composition, and change in BW of cows fed basal diets or diets supplemented with enzymes or soluble nutrients at four US sites (trial 1).

	Control	Enzyme A	Enzyme B	SSMA	SEM ¹
No. cows	56	58	58	61	
DMI, kg/d	21.2	21.8	21.4	21.4	0.24
Milk, kg/d	32.9	32.5	32.4	32.9	0.45
3.5% FCM, kg/d	33.9	33.5	33.5	34.2	0.49
Milk fat, %	3.73	3.70	3.71	3.74	0.041
Milk CP, %	3.15	3.17	3.17	3.18	0.017
Log ₁₀ SCC	4.8	4.8	4.9	4.9	0.04
BW change ² , kg	28.0	37.2	28.7	31.9	4.92

¹There were no differences ($P > 0.10$) between treatments.

²Weight at end of treatment period minus weight at beginning of treatment period.

no indications that removals were affected by treatments.

Results from trials 1 and 2 are presented in Tables 4 and 5, respectively. For both trials, milk production was not affected ($P > 0.10$) by treatments. Milk fat and milk protein percentages also were not affected by feeding these additives. Additionally, there were no effects of treatment on SCC, feed intake, or BW change during the 84-d treatment period. In trial 1, there were no treatment $\times$ site interactions, indicating that responses were not obtained within any one site. There were no treatment interactions with stage of lactation from trial 1, indicating that responses were not dependent on stage of lactation. Additionally, for both trials 1 and 2, there were no treatment $\times$ parity interactions, suggesting that treatments were not effective for either parity group.

In Vitro Enzyme Activities

Enzyme preparations A and B contained all the major hemicellulase (xylanase, β -xylosidase, α -L-arabinofuranosidase, α -L-arabinopyranosidase, acetyl esterase, and α -D-glucuronidase) and cellulase (endoglucanase, exoglucanase, and β -glucosidase) activities (Table 6). In general, the levels of all cellulase and hemicellulase activities in sample A were higher than in sample B, except for α -L-arabinofuranosidase (Table 6). Also,

both A and B enzyme samples had greater xylanase and endoglucanase activities compared with the other cellulase and hemicellulase activities (Table 6).

The pH activity profiles and the pH optimum of each activity in enzyme samples A and B when measured at 50°C are presented in Figure 1 and Table 6. The xylanase from enzyme samples A and B was active between pH 3.0 to 9.0 (Figure 1) and had broad pH optima between 5.0 to 6.0 and 5.5 to 5.8, respectively (Table 6). The highest activity of xylanase from both enzyme samples at optimal pH was similar (Figure 1).

The endoglucanase from enzyme samples A and B was active between pH 2.6 to 8.0, but the endoglucanase from sample A had slightly broader pH optimum (4.0 to 5.5) and higher activity than that from sample B (4.0 to 4.5; Figure 1). The exoglucanase from samples A and B was active between pH 2.6 to 7.0 (Figure 1), but this enzyme had the optimal activity between pH 4.2 to 4.6 and at pH 5.0, respectively. The β -glucosidase from both enzyme samples was active between pH 2.6 to 8.0, but optimally at pH 4.5 (Table 6). Nevertheless, the β -glucosidase from sample A had a broader pH optimum and greater activity than that from sample B.

The β -xylosidase from samples A and B was active between pH 2.6 to 8.0 (Figure 1), but optimally active between pH 2.6 to 4.0. The β -xylosidase activity in both enzyme samples decreased with the increase in pH from 4.0 to 8.0. Thus, the β -xylosidase from enzyme samples

Table 5. Feed consumption, milk production and composition, and change in BW of cows fed basal diets or diets supplemented with enzymes or soluble nutrients at one UK site (trial 2).

	Control	Enzyme A	Enzyme B	SSMA	SEM ¹
No. cows	26	31	29	30	
DMI, kg/d	18.5	18.3	18.3	18.2	0.24
Milk, kg/d	28.2	27.9	28.8	28.4	0.46
3.5% FCM, kg/d	30.2	29.4	29.6	29.9	0.52
Milk fat, %	3.95	3.83	3.69	3.88	0.097
Milk CP, %	3.34	3.35	3.36	3.34	0.029
Log ₁₀ SCC	4.5	4.5	4.6	4.5	0.06
BW change ² , kg	23.9	25.1	27.8	34.1	6.72

¹There were no differences ($P > 0.10$) between treatments.

²Weight at end of treatment period minus weight at beginning of treatment period.

Table 6. Enzyme activities and pH optima of *Trichoderma longibrachiatum*-derived enzyme preparations A and B.

Enzyme	Enzyme A	Enzyme B
	Activity ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$)	
Cellulase		
Endoglucanase ¹	6,913.7	2,644.7
Exoglucanase ²	2.90	1.86
β -Glucosidase	31.03	17.37
Hemicellulase		
Xylanase ²	38,883.8	26,483.1
β -Xylosidase	3.69	1.36
α -L-Arabinofuranosidase	3.36	13.05
α -L-Arabinopyranosidase	4.16	1.27
Acetyl esterase	4.69	1.19
α -D-Glucuronidase	0.015	0.032
	pH optima	
Xylanase	5.0 to 6.0	5.5 to 5.8
Endoglucanase	4.0 to 5.5	4.0 to 4.5
Exoglucanase	4.2 to 4.6	4.6 to 5.0
β -Glucosidase	4.5	4.5
β -Xylosidase	2.6 to 4.0	2.6 to 4.0
α -L-Arabinofuranosidase	2.6 to 4.0	2.6 to 4.0
α -L-Arabinopyranosidase	4.3	4.0 to 4.2

¹DNS method.²Birchwood xylan at pH 5.3.

A and B showed an identical pH versus activity profile except the total activity in sample A was twofold higher than that from sample B.

Both α -L-arabinofuranosidase and α -L-arabinopyranosidase from enzyme samples A and B were active between pH 2.6 to 7.0 (Figure 1). The α -L-arabinofuranosidase from enzyme samples A and B was optimally active between pH 2.6 to 4.0, whereas the α -L-arabinopyranosidase from enzyme samples A and B was optimally active at pH 4.3 and at pH between 4.0 to 4.2, respectively. At optimum pH, the activity of α -L-arabinofuranosidase from enzyme sample B was five times higher than that from enzyme sample A, whereas the activity of α -L-arabinopyranosidase from enzyme sample A was four times higher than that from enzyme sample B.

The xylanase from enzyme sample B was optimally active at 50°C, whereas that from enzyme sample A was optimally active at 60°C (Figure 2). Xylanase from both enzyme samples showed only 10% of the optimal activity up to 20°C (Figure 2). Nevertheless, the activity of xylanase from both samples increased sharply with an increase in temperature up to 50 to 60°C and decreased sharply above 60°C. The endoglucanase from both enzyme samples was optimally active at 60°C (Figure 2). Above 60°C, the endoglucanase activity from sample B decreased rapidly, whereas the endoglucanase activity of sample A decreased more slowly. However, up to 40°C, the endoglucanase from both enzyme samples showed only 20% of the optimal activity. The exoglucanase from enzyme samples A and B was opti-

mally active at 50°C (Figure 2). The pH and temperature activity profiles clearly suggested that the cellulase and hemicellulase activities from these enzyme samples would be less efficient in the rumen because pH and temperature would not be optimum.

DISCUSSION

The current trials consisted of a relatively large number of cows, were conducted at multiple sites, used both early and mid-lactation dairy cows, and were of a relatively long duration. Under these circumstances, there were no differences in milk production between control cows and any of the additive-fed cows. These results contrast with those of Lewis et al. (1999), Schingoethe et al. (1999) and Zheng et al. (2000), that reported significant differences, and Beauchemin et al. (1999), Rode et al. (1999), and Kung et al. (2000), that only reported numerical but nonsignificant increases in milk production due to addition of direct-fed enzymes.

Several mechanisms have been proposed whereby supplemental enzymes could improve animal performance (Beauchemin and Rode, 1996). The most obvious mechanism would be improved digestibility of cellulose or hemicellulose. Improved digestibility has been measured in several studies with dairy cattle (Beauchemin et al., 1999; 2000; Rode et al., 1999; Yang et al., 1999; 2000) and has been associated with increased rate of particle outflow from the rumen, possibly caused by increased rate of digestion and/or lower rumen fluid viscosity, but has not been associated with increased

Figure 1. Effect of pH on cellulase and hemicellulase activities of enzyme samples A (closed symbols) and B (open symbols). Buffers used were: sodium acetate, pH 3.0 to 7.0 (squares); potassium phosphate, pH 6.6 to 8.0 (triangles); and boric acid/sodium hydroxide, pH 8.0 to 9.0 (circles).

DMI. Under these conditions, if retention time is reduced, the benefits of enhanced fibrolytic activity may be small.

The lack of production responses and the pH optima curves and optimum temperatures suggest that the amount of supplemental enzymatic activity for degradation of cellulose and hemicellulose may be limited in the environment of the rumen of the cows offered the diet used in the current study. Typical pH of the rumen is higher than the pH optima that were obtained, and ruminal temperature is lower than the optimal temperature for enzymatic activity. In general, at pH = 6 most of the enzymatic activities of either enzyme A or B will be reduced by about two-thirds of their optimal activity, and at 38°C, these activities will be further reduced by two-thirds. Therefore, the combined effects of pH and temperature that exist in the rumen would suggest that

Figure 2. Effect of temperature on xylanase, endoglucanase, and exoglucanase activities of enzyme samples A (closed symbols) and B (open symbols).

moderate fibrolytic activity, compared with the amount added, could occur within the rumen with these exogenous enzymes.

Wallace (1997) indicated that two roles supplemental enzymes could fill are either to amplify or complement ruminal activities. If their role was to amplify an existing activity, then the amount of activity added to the rumen would need to significantly add to that already present. Loss of activity at temperatures or pH values that significantly deviate from the optima would make this less likely. However, if complementing an existing activity, the combination of feeding high-energy diets and supplementing an enzyme with a pH optimum in the acidic range might be desirable (Wallace, 1994) and effective. Recent work by Morgavi et al. (2000) has shown the existence of a synergistic effect between ruminal and exogenous enzymes and that this can increase the hydrolytic potential within the rumen environment and could be an important mechanism by which enzyme additives improve fiber digestion. But with any diets and under any conditions, it would be necessary that the enzymes be effective near 39°C in order to have ruminal activity.

Although no effects of the direct-fed enzymes were recorded for milk production in the current study, reports using the same enzymes exist in the literature, which have shown significant milk yield responses (Zheng et al., 2000). Among a range of factors that could affect enzyme efficacy, diet composition may be extremely important as in studies with high concentrate-to-forage ratios rumen pH may be considerably closer to the optima for the enzymes, which could greatly improve fiber digestibility. In addition, a number of other factors such as method of application, specific enzyme complexes, time, and duration of enzyme application before feeding, dryness of the feed, dose rate, and stage of lactation in which the diet is fed can all influence milk production response noted with direct-fed enzyme additives. Wallace (2001) suggested that effects of fungal enzyme preparations are most likely at prefeeding. In the present study, enzymes were added each morning when mixing the TMR. Also, compounds that may or may not have fibrolytic activity in the enzyme mixtures may have acted as adjuvants that stimulated ruminal digestibility. If there were changes in the substrates that were used in producing the enzyme mixtures, these other compounds and/or activities may have changed as well.

Grant and Mertens (1992) have demonstrated that fiber digestion in the rumen is inhibited by low pH, and a more favorable ruminal environment may stimulate growth rate of ruminal fiber-digesting bacteria; however, milk or other production variables were not improved by the addition of SSMA. Few published studies

are available where SSMA or malic acid alone were fed to dairy cows (Kung et al., 1982). Most research with malic acid examined effects on ruminal organisms and are reviewed by Martin (1998). Results of previous studies indicate two potential mechanisms of production responses to supplemental SSMA. One potential mechanism is that malic acid may stimulate lactic acid uptake by *Selenomonas ruminantium*, resulting in a greater ruminal pH. The lack of a production effect may indicate that ruminal acidosis was not a factor that affected milk production for the cows at these sites. Additionally, there was no indication of acidosis based on mean milk fat content (Table 4 and 5). Ruminal pH is affected by the fermentable carbohydrate in the diet. Nocek (1997) recommended that NSC in diets of dairy cows range from 30 to 40% to avoid acidosis. The average NFC at four sites was approximately 33% and at Cornell was 40%. Fiber (ADF and NDF) percentages of the diets were well above the minimum amounts recommended by the current NRC (2001).

The second mechanism would be increased incorporation of ruminal ammonia by the addition of the soluble or nonstructural sugars. The diet may not have been limiting in soluble sugars because NSC was within the recommended range (Nocek, 1997), and diets were formulated at all sites using the CPM model with the restriction that MP allowable milk was 2 kg/d greater than ME allowable milk.

CONCLUSION

Data from in vitro testing of the enzymes used in these studies indicate that most of the cellulase and hemicellulase activities are present; however, activity is lowered at pH and temperature conditions that exist in the rumen. No changes in milk production, milk composition, or BW gain were obtained in either trial in which supplemental enzymes or SSMA were added to the diet.

ACKNOWLEDGMENTS

The authors thank Dr. Jim Nocek, Mr. Doug Young, and the staff at the Spruce Haven Farm for their contributions to this study.

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