# EFFECT OF PHOSPHORUS SUPPLEMENTATION ON GAS PRODUCTION, RUMEN FERMENTATION AND PRODUCED AMYLASE AND CARBOXYMETHYLE CELLULASE ACTIVITY, *IN VITRO*

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**Abstract**

*An in vitro gas production technique was used to study the effect of different levels of dicalcium phosphate (0, 0.037, 0.074, and 0.111 of 1 g DM) on gas production, rumen fermentation, dry and organic matter digestibility, and the activity of amylase and carboxymethyl cellulase (CMCase). A gas production technique was performed using rumen fluid collected from three fistulated Santa Ines sheep. Cumulative gas production was recorded at 3, 6, 9, 12, 16, 24, 30, 36, 48 and 72 h of incubation. Kinetics of gas production was fitted to an exponential model. Volatile fatty acids (VFA), Ammonia-N ($NH_3$-N) concentrations, dry and organic matter digestibility and enzyme activity (CMCase and amylase) were determined at 24 h of incubation. The cumulative volume of gas production increased with increasing the level of phosphorus. Total gas produced at 72 h of incubation was higher for concentrate and hay (H) +concentrate (C). The highest value of individual VFA for H+C was at level 2 of treatment and for concentrate at level 4, while the addition of different levels of P was decreased the value of individual VFA for hay. No significant effects were observed on pH, $NH_3$-N levels or truly dry and organic matter digestibilities with P addition. The specific activity of amylase and CMCase of sonicated the bottles contents after incubation time were significantly increased at level 2 for H, C or C+H.*

**Key words**: Phosphorus, gas production, rumen fermentation and *in vitro*

## INTRODUCTION

Phosphorus is very important for normal rumen metabolism, reproduction, skeletal growth, and production. Low phosphorus intake frequently occurs in young stock and dry cows from lack of supplementation or concentrates feeding. Sometimes the problem can be due to poor availability of phosphorus sources. Most of the normal forages consumed by ruminants are little more than adequate with respect to their P content. Furthermore, increasing use of poor-quality roughages and by-products, generally deficient in P, so animals need to be supplemented with this mineral to meet their nutritional requirements (McDowell and Conrad, 1990). In herds reared at pasture, phosphorus can be considered, worldwide, the major mineral deficiency causing economic impact (Underwood and Suttle, 1999). Phosphorus (P) is generally the most costly mineral to supplement in animal diets. Chandler (1996) indicated that P accounts for more than 50% of the cost of typical vitamin-mineral mixes used on dairy farms. There are a strong interaction between the host animal and the rumen microorganisms with respect to P supply and utilization. Rumen microbes have specific phosphorus requirements to degrade the cell walls of feedstuffs. Also, rumen microbes need P to maintain metabolism and growth (Komisarczuk-Bony and Durand, 1991); and total P content of rumen microorganisms ranges from 2 to 6% of the dry matter (Valk *et al*., 2000). The suggested lower concentration of P to maintain normal microbial growth in the rumen is 100 mg/L of ruminal fluid (Durand and Kawashima, 1980). For optimum

plant cell degradation and microbial protein synthesis within the rumen, the available P should be at least 5 g/kg fermented organic matter, supplied via the diet and saliva (Komisarczuk-Bony and Durand, 1991). Microorganisms are largely dependent on dietary P for their P requirement and the host animal is affected first under a marginal P deficiency (Durand and Kawashima, 1980). There are many studies that report data on the P requirements of ruminal microbes. These data have mainly been obtained using batch-culture systems (Milton and Ternouth, 1984) and using continuous culture techniques (Komisarczuk *et al*., 1987b). In the present work, in vitro gas production technique was used to investigate the effects of different levels of inorganic P on gas production, rumen fermentation and the activity of amylase and carboxymethyle cellulose.

## MATERIAL AND METHODS

The present study was carried out at the Laboratory of Animal Nutrition (LANA), Center of Nuclear Energy in Agriculture (CENA), University of Sao Paulo (USP), Brazil.

### 1. Feedstuffs:

Coast cross hay (H) and concentrate mixture (C) (cassava meal, soybean, urea, molasses and mineral mixture), were ground in mills to pass a 1 mm sieve prior to chemical analysis and *in vitro* gas production measurements. The chemical composition of hay and concentrate mixture are presented in Table (1). Four levels of dicalcium phosphate (0 (L1), 0.037 (L2), 0.074 (L3), and 0.111 (L4) of 1 g DM) were added on the substrates.

Table 1. Chemical composition of concentrate mixture and hay

| Items | DM* | Ash* | OM* | CP* | NDF* | ADF* | EE* | P* |
|---|---|---|---|---|---|---|---|---|
| Concentrate | 892.53 | 25.03 | 974.97 | 149.21 | 90.38 | 034.55 | 20.70 | 1.42 |
| Hay | 905.75 | 52.92 | 947.08 | 113.76 | 804.07 | 442.52 | 19.10 | 2.59 |

DM express as g/kg sample.
* express as g/kg DM.

### 2. Chemical Analyses:

Feedstuffs were analyzed according to AOAC, (1990) for dry matter (DM), organic matter (OM), crude protein (CP), ether extract (EE), crude fiber (CF), ash, Neutral detergent fiber (NDF), acid detergent fiber (ADF) according to Van Soest *et al*. (1991). Enzyme assays were measured according to Nasser *et al*. (2005). Reducing sugar was determined as glucose equivalents by the Somogi method (1960). Protein concentration of the crude enzymes (amylase and CMCase) was determined by Bradford method (1976).

### 3. In vitro gas production and rumen fermentation

*An in vitro* gas production technique was carried out using a pressure transducer and data logger for measuring the produced gas in 160 ml serum bottle incubated at 39$^{o}$C (Mauricio *et al*., 1998). Rumen fluid was collected before the morning feeding from three fistulated sheep fed on Coast cross hay and concentrate mixture, into a pre-warmed thermos flask. The rumen fluid was strained through four layers of surgical gauze and flushed with $CO_2$, then was added to the buffered mineral solution (1:2 v/v), which was maintained in water bath at 39$^{o}$C, and mixed. Ground samples (1 g DM) of H, C and mixture of both (70% H: 30% C, w/w) were incubated with 75 ml of diluted rumen fluid into 160 ml serum bottle. The bottles were closed by rubber stoppers, shaken and placed in the incubator at 39$^{o}$C. Four bottles with only buffered rumen fluid were incubated and considered as the blank. The gas headspace pressure was recorded before incubation (0) and 3, 6, 9, 12, 18, 24, 30, 36, 48, and 72 h after incubation using a pressure transducer (Theodorou *et al*., 1994). Total gas values were corrected for blank incubation and expressed as milliliter of gas produced per 200 mg of dry matter. Cumulative gas production data were fitted to the model of Ørskov and McDonald (1979). Four bottles containing 1 g samples and 75 ml buffered rumen fluid were incubated for determination pH, ammonia nitrogen ($NH_3$-N), volatile fatty acids (VFA) concentrations, protozoa count, digested dry and organic matter and enzyme activity at 24 h of incubation.

**4. Enzyme assay**

At 24 h of incubation time, the whole content of two bottles was transferred to a 100 ml beaker. The contents were carefully mixed then sonicated at 4°C using a sonicator (Labsonic U model; B. Braun Biotech International). The sonicated samples were centrifuged at 24 000×g for 20 min at 4°C and clear supernatant was used for the estimation of enzyme activities. The reaction mixture contained 0.5 ml phosphate buffer (0.1 M, pH 6.8), 0.250 ml carboxymethylcellulose (1.0 g/100 ml phosphate buffer) and 0.250 ml extracted supernatant for the estimation of carboxymethylcellulase (CMCase). For amylase activity, the reaction mixture contained 0.5 ml phosphate buffer, 0.250 ml corn starch (2 g/100 ml phosphate buffer) and 0.250 ml extracted supernatant. The reaction mixtures were incubated for 45 min (amylase) and 60 min (CMCase) at 39°C. The reducing sugars thus released estimated according to Somogyi method (1960) using glucose as standard.

**5. Volatile fatty acid estimation**

At the end of incubation (24 h) 1ml of the supernatant was collected in a microfuge tube containing 0.20 ml metaphosphoric acid (25 ml/100 ml). The mixture was allowed to stand for 2 h at room temperature and centrifuged at 5000×g for 10 min. The clear supernatant was collected and stored at −20 °C until analyzed. The VFA's were measured by gas chromatography (ThermoQuest mod. 8000top, FUSED SILICA capillary column 30m×0.25mm×0.25mm film thickness) as described by Cottyn and Boucque (1968).

**6. *In vitro* dry matter degradability**

At the end of the incubation period (24 h), contents of each serum bottle were filtered through pre-weighed Gooch crucibles and residual dry matter was estimated. The per cent loss in weight was determined and presented as IVDMD. The dried feed sample and residue left above was ashed at 550 °C for determination of IVOMD.

**7. Statistical analyses**

Data were subjected to analysis of variance (ANOVA) using the General Linear Model. Significant differences between individual means were identified using least significance difference (LSD) multiple range test (SAS, 2000).

## RESULTS AND DISCUSSION

**1. Gas production**

The results of gas production as affected by different levels of phosphorus are presented in Figure 1 and Table 2. The cumulative volume of gas production increased with increasing time of incubation and the level of phosphorus. Total gas produced at 72 h of incubation was higher for concentrate and hay +concentrate than hay. The high values of produced gas at 24 and 72 were observed for concentrate and hay with level 3 of phosphorus, while hay + concentrate with level 4. The low values were observed for all substrates with level 1. Estimated gas production rate (C) varied from o.oo6 ml/h in hay to 0.088 ml/h in concentrate. There was no significant ($P>0.05$) differences between the same type of substrate of gas production and parameters while, there are significant ($P<0.05$) differences among the substrates in terms of total gas production and parameters (Table 2). Cell wall (as NDF), lignin (as ADL) and acid detergent insoluble ash (ASHI) contents seem to be important factors for in vitro kinetic parameters obtained with cow and dromedary rumen micro-flora Hadi *et al*., (2003). Ndlovu and Nherera (1997) found that gas production rate (c) was negatively correlated with NDF and ADF. On the other hand, gas production and estimated parameters (c, a, b and a + b) were positively correlated with CP protein which is one of the limiting factors for microbial growth (Larbi *et al*., 1998 and Kamalak, 2006). When hay was mixed with concentrate, the total gas production and the rate of gas production increased which are in agreement with that reported by Sampath *et al*., 1995. Significant ($P< 0.05$) positive associative effects on in vitro gas production were observed with untreated finger millet straw at different levels of peanut cake supplementation after 12, 52 and 166 h of incubation (Sampath *et al*., 1995).

Table 2. Cumulative gas production (ml/200 mg) at different times of incubation for different levels of phosphorus and parameters of gas production

| Items | 12 h | 24 h | 48 h | 72 h | a + b | C ml/h |
|---|---|---|---|---|---|---|
| Concentrate(C) | | | | | | |
| L1 | 31.00$^{a}$ | 41.70$^{a}$ | 51.90$^{a}$ | 54.70$^{a}$ | 52.9$^{a}$ | 0.084$^{a}$ |
| L2 | 31.70$^{a}$ | 41.80$^{a}$ | 51.80$^{a}$ | 54.60$^{a}$ | 52.6$^{a}$ | 0.086$^{a}$ |
| L3 | 32.80$^{a}$ | 43.00$^{a}$ | 52.60$^{a}$ | 55.30$^{a}$ | 53.3$^{a}$ | 0.088$^{a}$ |
| L4 | 35.18$^{a}$ | 43.10$^{a}$ | 52.40$^{a}$ | 55.00$^{a}$ | 53.9$^{a}$ | 0.088$^{a}$ |
| Hay (H) | | | | | | |
| L1 | 7.00$^{c}$ | 13.00$^{c}$ | 25.00$^{c}$ | 32.60$^{c}$ | 66.90$^{b}$ | 0.006$^{c}$ |
| L2 | 7.50$^{c}$ | 14.20$^{c}$ | 26.30$^{c}$ | 33.80$^{c}$ | 67.20$^{b}$ | 0.007$^{c}$ |
| L3 | 8.40$^{c}$ | 14.90$^{c}$ | 27.30$^{c}$ | 34.80$^{c}$ | 68.60$^{b}$ | 0.007$^{c}$ |
| L4 | 8.18$^{c}$ | 14.70$^{c}$ | 27.00$^{c}$ | 34.40$^{c}$ | 71.10$^{b}$ | 0.006$^{c}$ |
| H + C | | | | | | |
| L1 | 17.10$^{b}$ | 25.50$^{b}$ | 37.70$^{b}$ | 43.50$^{b}$ | 50.50$^{a}$ | 0.027$^{b}$ |
| L2 | 17.50$^{b}$ | 26.00$^{b}$ | 38.00$^{b}$ | 43.90$^{b}$ | 51.38$^{a}$ | 0.026$^{b}$ |
| L3 | 18.10$^{b}$ | 26.80$^{b}$ | 38.60$^{b}$ | 44.50$^{b}$ | 51.95$^{a}$ | 0.026$^{b}$ |
| L4 | 19.30$^{b}$ | 27.80$^{b}$ | 40.50$^{b}$ | 46.40$^{b}$ | 54.17$^{a}$ | 0.026$^{b}$ |

L1, the first level of phosphorus; L2, the second level of phosphorus; L3, the third level of phosphorus, L4, the fourth level of phosphorus

$^{abc}$ Means within the same columns with different superscript are significantly different (P<0.05)

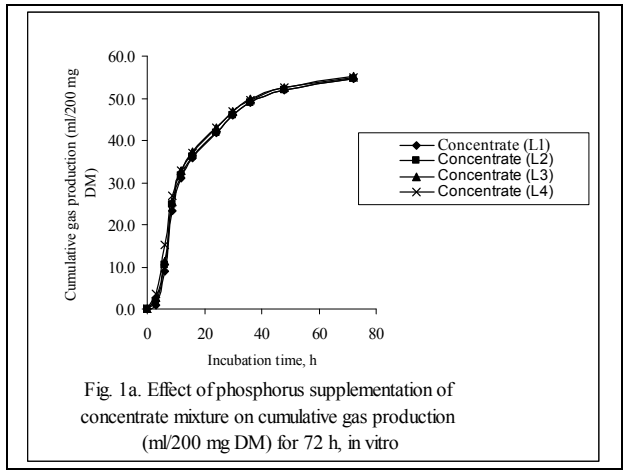


Fig. 1a. Effect of phosphorus supplementation of concentrate mixture on cumulative gas production (ml/200 mg DM) for 72 h, in vitro

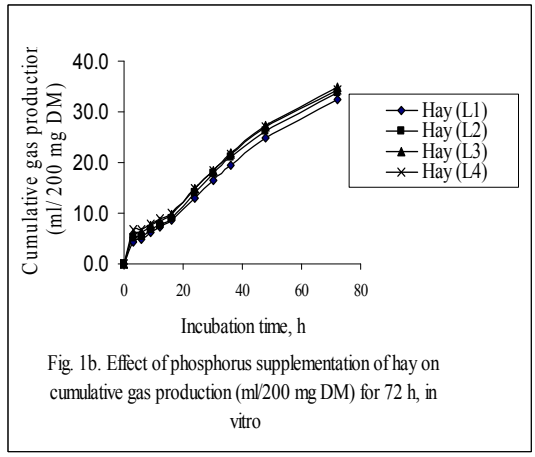


Fig. 1b. Effect of phosphorus supplementation of hay on cumulative gas production (ml/200 mg DM) for 72 h, in vitro

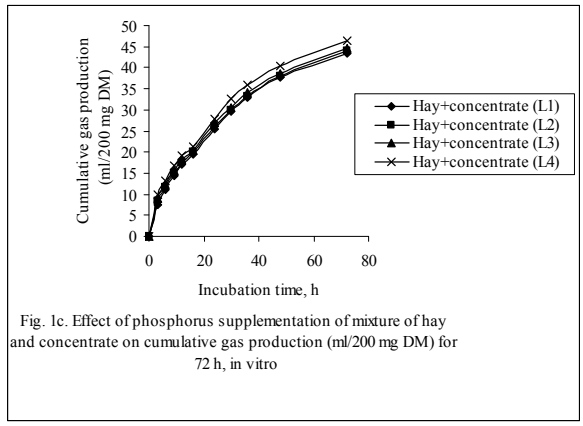


Fig. 1c. Effect of phosphorus supplementation of mixture of hay and concentrate on cumulative gas production (ml/200 mg DM) for 72 h, in vitro

**2. Effect on VFA**

The TVFA concentration (mM) was significantly ($P<0.05$) increased for H, C and H+C at L1, L4 and L2, respectively (Table 3). Amounts of total VFA produced from H were markedly reduced, contributing in part to increased pH values and there were indications of changes in the pattern of fermentation with reduced acetic acid production. These results are in agreement with Leedle and Hespell, (1984), Durand *et al*. (1987) and Komisarczuk, *et al*. (1987a). The highest value of acetate and propionate for H+C was at level 2 and for C at level 4, while the addition of different levels of P was decreased the value of both acids for H. Propionate and butyrate were significantly decreased for H, while significantly increased for C or mixture of H and C (Table 3). Acetate and propionate ratio was significantly decreased for H by adding P, whereas no significant effect for C or H+C. Isobutyrate, isovalerate and valerate concentrations were significantly increased for H + C at L2 . No significant effects were observed on isovalerate and valerate for H or C. Komisarczuk, *et al*. (1987b) suggested that total volatile fatty acids (VFA) produced averaged about 6.83 mmol/h with Pi levels of 48 and 28 mg/l. Reduction in inorganic phosphorus (Pi) concentrations to 4 and < 1 mg/l resulted in significant reductions in total VFA to approximately 6.25 and 3.75 mmol/h respectively, accompanied by a rise in pH from 6.5 to 7.3. Reduction of P conceration to < 1 mg/l resulted in further reductions in the amounts of total VFA produced. There were also changes in the molar proportions of the individual VFA and propionate increased significantly. Increased butyrate and decreased acetate proportions were also observed, both of which approached significance at the 5% level. Amounts of total VFA produced were markedly reduced, contributing in part to increased pH values and there were indications of changes in the pattern of fermentation with reduced acetic acid and increased propionic acid production (Komisarczuk, *et al*. 1987b).

**3. Effect on pH, ammonia nitrogen and degradability of feed**

The values of pH, ammonia-N concentrations ($NH_3$-N) and dry matter and organic matter degradability at 24 h of incubation time are presented in Table 4. The average values of pH ranged from 6.50 to 6.54, 6.92 to 6.98 and 6.77 to 6.82 for concentrate, hay and hay + concentrate, respectively. pH value was increased significantly ($P<0.05$) for H by adding phosphorus. No significant effects were observed on pH for C or H + C. The present results showed that there were no significant ($P > 0.05$) differences among all substrates in $NH_3$-N levels or degradability of dry (DMD) and organic matter (OMD) at 24 h of incubation time (Table 4). Komisarczuk, *et al*. (1987a) showed that the microbial efficiency of protein synthesis was significantly decreased (by 5 g of N/kg

OMF) in P-deficient vessels. The $NH_3$-N production in P-deficient vessels was increased by about 30 mg/day compared to control vessels. However, it can be calculated that P deficiency had little effect on proteolysis in the system. P deficiency induced a marked decrease in microbial protein synthesis, ammonia utilization and cellulose digestion (Komisarczuk, *et al*., 1987b). The beneficial effects of additional P with respect to increased N utilization (Durand *et al*., 1983).

Table 3. Effect of inorganic phosphorus levels on molar proportions of total and individual VFA (mmol/l) and acetate to propionate ratio

| Items | TVFA | A | P | A/P ratio | IB | B | IV | V |
|---|---|---|---|---|---|---|---|---|
| C | | | | | | | | |
| L1 | 49.70$^{c}$ | 23.58$^{bc}$ | 13.69$^{c}$ | 1.73$^{d}$ | 0.21$^{ab}$ | 9.40$^{c}$ | 0.44$^{b}$ | 0.66$^{a}$ |
| L 2 | 55.06$^{ab}$ | 25.83$^{ab}$ | 15.51$^{ab}$ | 1.67$^{d}$ | 0.21$^{ab}$ | 10.73$^{b}$ | 0.44$^{b}$ | 0.68$^{a}$ |
| L 3 | 53.01$^{ab}$ | 24.62$^{ab}$ | 15.04$^{b}$ | 1.64$^{d}$ | 0.09$^{c}$ | 10.51$^{b}$ | 0.42$^{b}$ | 0.68$^{a}$ |
| L 4 | 56.53$^{a}$ | 26.18$^{a}$ | 16.16$^{a}$ | 1.62$^{d}$ | 0.00$^{e}$ | 11.44$^{a}$ | 0.43$^{b}$ | 0.70$^{a}$ |
| H | | | | | | | | |
| L1 | 33.44$^{f}$ | 18.71$^{efg}$ | 6.46$^{e}$ | 2.92$^{b}$ | 0.10$^{c}$ | 4.77$^{f}$ | 0.20$^{de}$ | 0.28$^{de}$ |
| L2 | 29.92$^{fg}$ | 17.27$^{fg}$ | 5.11$^{f}$ | 3.38$^{a}$ | 0.09$^{cd}$ | 3.70$^{g}$ | 0.17$^{e}$ | 0.21$^{de}$ |
| L3 | 29.37$^{g}$ | 17.12$^{g}$ | 4.81$^{f}$ | 3.56$^{a}$ | 0.08$^{cd}$ | 3.45$^{g}$ | 0.17$^{e}$ | 0.19$^{e}$ |
| L4 | 29.02$^{g}$ | 16.95$^{g}$ | 4.70$^{f}$ | 3.60$^{a}$ | 0.08$^{cd}$ | 3.36$^{g}$ | 0.16$^{e}$ | 0.17$^{e}$ |
| H+C | | | | | | | | |
| L1 | 36.07$^{e}$ | 19.68$^{def}$ | 7.30$^{e}$ | 2.70$^{bc}$ | 0.13$^{bc}$ | 5.66$^{e}$ | 0.28$^{cd}$ | 0.33$^{cd}$ |
| L2 | 41.76$^{d}$ | 21.89$^{cd}$ | 8.93$^{d}$ | 2.45$^{c}$ | 0.27$^{a}$ | 6.70$^{d}$ | 0.58$^{a}$ | 0.65$^{a}$ |
| L3 | 39.77$^{d}$ | 20.94$^{de}$ | 8.59$^{d}$ | 2.44$^{c}$ | 0.16$^{bc}$ | 6.79$^{d}$ | 0.38$^{b}$ | 0.47$^{b}$ |
| L 4 | 39.91$^{d}$ | 20.98$^{de}$ | 8.53$^{d}$ | 2.46$^{c}$ | 0.16$^{bc}$ | 6.97$^{d}$ | 0.35$^{bc}$ | 0.46$^{bc}$ |

A, acetate; P, propionate; IB, isobutyrate; B, butyrate; IV, isovalerate; V, valerate
$^{abcdefg}$ Means within the same columns with different superscript are significantly different (P<0.05)

Table 4. Effect of different levels of phosphorus on pH, ammonia N and degradability of dry matter and organic matter

| Items | pH, 24h | NH3-N mg/l | DMD,24h % | OMD, 24h % |
|---|---|---|---|---|
| Concentrate (C) | | | | |
| L1 | 6.50$^{d}$ | 36.7$^{a}$ | 84.01$^{a}$ | 82.85$^{a}$ |
| L2 | 6.54$^{d}$ | 33.5$^{abc}$ | 86.13$^{a}$ | 85.79$^{a}$ |
| L3 | 6.53$^{d}$ | 34.5$^{ab}$ | 85.09$^{a}$ | 84.00$^{a}$ |
| L4 | 6.54$^{d}$ | 35.3$^{a}$ | 84.23$^{a}$ | 81.95$^{a}$ |
| Hay | | | | |
| L1 | 6.92$^{b}$ | 20.4c$^{d}$ | 26.99$^{c}$ | 24.33$^{c}$ |
| L2 | 6.98$^{a}$ | 19.8$^{d}$ | 25.56$^{c}$ | 22.26$^{c}$ |
| L3 | 6.97$^{a}$ | 20.4$^{cd}$ | 27.14$^{c}$ | 23.64$^{c}$ |
| L4 | 6.94$^{ab}$ | 21.0$^{cd}$ | 24.40$^{c}$ | 19.80$^{c}$ |
| H+C | | | | |
| L1 | 6.82$^{c}$ | 24.2$^{abcd}$ | 46.81$^{b}$ | 45.24$^{b}$ |
| L2 | 6.79$^{c}$ | 21.2$^{bcd}$ | 46.32$^{b}$ | 44.57$^{b}$ |
| L3 | 6.77$^{c}$ | 25.8$^{abcd}$ | 50.03$^{b}$ | 48.08$^{b}$ |
| L4 | 6.80$^{c}$ | 23.3$^{abcd}$ | 47.03$^{b}$ | 43.95$^{b}$ |

$NH_3$-N, ammonia nitrogen; DMD, dry matter degradability; OMD, organic matter degradability
$^{abcd}$ Means within the same columns with different superscript are significantly different (P<0.05)

**4. Effect on enzyme profile**

The effects of different levels of phosphorus on specific activity (μg/mg) of amylase and CMCase are presented in Table 5. The specific activities of amylase were significantly increased for all substrates at the second level of phosphorus, whereas specific activity of CMCase was reduced significantly (P<0.05) at L4 for C and H+C. No significant effects were observed on the specific activity of CMCase for H. Witt and Owens (1983) showed that increasing ruminal P concentration from 208 to 398 mg/liter did not increase microbial cellulose digestion,

but the low level was inadequate for maintaining the adult ruminant animal's P stores. Durand *et al*., (1987) suggested that phosphorus is specifically required for the degradation of cell wall constituents and particularly for cellulolysis which seems to have a higher P requirement than hemicellulolysis and amylolysis. Marked changes in metabolic activities were observed with concentrations of about 4 mg/l or less. This change in fermentative activity was confirmed by significant reductions in cellulose and hemicellulose digestion with no apparent depression of starch digestion, indicating perhaps a change in the balance of the bacterial population (Leedle and Hespell, 1984). A minimum requirement of 20 mg Pi/l for the maximum cellulolytic activity of pure cultures of cellulolytic bacteria, and extreme sensitivity of cellulolytic bacteria to P deficiency has been reported by Durand ***et al.*** (1983). It has also been shown that cellulases isolated from mixed rumen bacteria associated with fibre have specific P requirements and that the enzymes show different affinities for different fibre fractions (Francis ***et al***., 1978**).** The present results showed that cellulose digestion was affected not only by decreasing phosphorus level but also by increasing the level of phosphorus. It has been shown that CMCase have specific P requirements.

Table 5. Effect of different levels of phosphorus on specific activity (µg/mg) of amylase and CMCase

| Items | Amylase | CMCase |
|---|---|---|
| Concentrate(C) | | |
| L1 | $58.7^{bc}$ | $13.4^{de}$ |
| L2 | $79.5^{a}$ | $17.4^{d}$ |
| L3 | $59.5^{bc}$ | $11.1^{e}$ |
| L4 | $52.9^{bc}$ | $8.6^{f}$ |
| Hay (H) | | |
| L1 | $48.9^{c}$ | $56.8^{c}$ |
| L2 | $61.8^{bc}$ | $63.0^{c}$ |
| L3 | $49.2^{c}$ | $59.3^{c}$ |
| L4 | $49.8^{c}$ | $58.5^{c}$ |
| H + C | | |
| L1 | $53.6^{c}$ | $104.0^{a}$ |
| L2 | $66.9^{b}$ | $119.0^{a}$ |
| L3 | $57.2^{bc}$ | $102.0^{a}$ |
| L4 | $56.0^{bc}$ | $74.4^{b}$ |

$^{abcdef}$ Means within the same columns with different superscript are significantly different (P<0.05)

## CONCLUSION

The current study suggested that the addition of phosphorus improves rumen metabolism and microbial rumen activity and the percentage of addition depends on the quality of feed materials and their content of phosphorus. Phosphorus requirements of rumen microbes are important because decrease or increase the phosphorus concentration rapidly decrease microbial activity and animal production and increase the pollution.

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