CHAPTER 5

DIGESTION OF *P. PURPUREUM* AND POME IN SEMI-BATCH FERMENTATION SYSTEM

5.0. Introduction

In chapter 2, it was shown that rumen ingesta of POME fed goat was lower in cellulolytic activity against swollen cellulose, POME fibre, crystalline cellulose, and grass fibre than the rumen digesta of grass fed goats. In Chapter 4, although POME was shown to support growth of all important cellulolytic bacterial species, but rate of growth and maximum count attained by more active cellulolytic bacteria such as *F. succinogenes* and *R. flavefaciens* were low in POME as compared to less efficient cellulolytic but highly saccharolytic bacteria such as *Butyrivibrio* spp and *Clostridium* spp. Thus, the rumen population might be altered if goats are fed with POME as there is high tendency that the rumen will become acidic. To investigate the ability of mixed ruminal bacterial population to digest cellulosic materials in POME, a semi-batch laboratory fermentation system was set-up. Using this system, pH was maintained at 6.8 (for optimum cellulase enzyme activity) and cellulosic components would be retained for microbial digestion. Effect of soluble sugar digestion and cellulolytic activity against POME cellulose could be studied under controlled pH condition and this system could approximately simulate the natural rumen system.

5.1. NUTRIENT ANALYSES OF *PENNISETUM PURPUREUM*.

5.1.1. Materials and method.

Napier grass (*Pennisetum purpureum*) was collected from the Genetic Garden, University of Malaya. Leaves of freshly collected *P. purpureum* were cut into 2-4 cm and dried at 90°C overnight. Dried leaves were grounded and passed through 1 mm-pore size sieve. Determination of ash, fat, fibre, protein and nitrogen free extract contents of the leaves were carried out as described in Section 4.1 (Chapter 4). Soluble sugar content of the leaves was determined as follows: 0.5g sample was added to 50 ml distilled water and boiled for 15 minutes. The boiled sample was filtered through Whatman (Fast) filter paper. The filtrate was spun at 10,000 g for 20 minutes and the supernatant was analysed for soluble sugar content as described in Section 2.2.1a.

5.1.2. Results

The nutrient content for the grass is shown in table 5.1. Nitrogen-free extract is the major component of *P. purpureum* (nearly half of the total dry weight) followed by fibre, protein, ash, and fat. *P. purpureum* also contains 9.45 % (% of dry weight) of soluble sugar.

Table 5.1: Chemical constituents of *Pennisetum purpureum*

Samples	Chemical contents (% dry wt.)					
	Ash	Fat	Fibre	Protein	NFE	Sol.Sugar
1	10.66	4.30	29.52	11.86	43.66	10.92
2	10.63	4.54	28.58	11.16	45.09	8.40
3	10.20	4.11	29.76	15.59	40.34	9.12
4	10.41	4.39	29.18	13.89	42.13	8.94
5	9.92	4.62	30.17	15.14	40.15	10.56
6	11.11	4.22	29.19	15.33	39.65	8.78
Mean ± Std. deviation	10.47± 0.37	4.36± 0.18	29.40± 0.50	13.83± 1.73	41.84± 1.20	9.45± 0.94

5.2. SEMI-BATCH FERMENTATION

5.2.1. Materials and Methods

5.2.1.1 Culture apparatus

The semi batch fermentation system was set up using Biostat M bench top fermentor (B. Braun, Germany). It was made up of a culture vessel, a parameter measurement and regulation assembly. The 2-liter culture vessel was double-walled with a top cover plate with a number of opening for pH and Eh electrodes, temperature sensor, fitting tubes for influent, effluent, sampling port, gas inlet, gas outlet and acid-alkali buffer input. The openings were sealed with O-

rings to prevent contamination as well as maintaining anaerobiosis within the vessel. Mounted at the center of the cover plate was a strong stirrer. Thermostat controlled circulating water passed through the double layer of the culture vessel to maintain the temperature of the fluid in the culture vessel at 38 ± 0.1°C. The details of the apparatus is shown in figure 4.1.

The mineral solution reservoir was fitted with two glass tubings, one for attachment of a gas inlet, and the other was used as for mineral solution. The gas inlet was connected to an oxygen-free CO_2 cylinder via a chromous acid oxygen scrubber so as to maintain a slight positive pressure of oxygen-free CO_2 in the mineral reservoir. In addition, it served to fill back the reservoir headspace during the outflow of the mineral solution. This was considered essential since the reducing condition of the mineral solution failed to be maintained in the absence of the oxygen-free CO2.

The mineral solution was delivered to the fermentor culture vessel through silicon tubing (2-mm internal diameter) by a variable-speed peristaltic pump (Prista, Atto Corp., Japan). During fermentation, the culture fluid was continuously removed at pre-determined rate through the effluent tube. The opening to the effluent tube inside the culture vessel was covered with a single layer of muslin cloth (0.25 mm X 0.25 mm pore size) to prevent the outflow of large particulate solid substrate.

The effluent was removed through a silicon tubing (4 mm inner diameter) by a variable speed peristaltic pump (Setric Genie Ind., France) into effluent collection bottle to which 10 ml of saturated $HgCl_2$ was added twice daily. The speed of effluent discharged was maintained equal to the speed of intake of influent into the culture vessel. The medium in the culture vessel was maintained under anaerobic condition by continually purging of $89\%N_2$-$5.5\%CO_2$-$5.5\%H_2$ that was passed through an oxygen scrubber. The outlet of the scrubber was equipped with a gas filter housing a 0.22 µm membrane filter to ensure sterility of the gas. The gas released from the culture vessel through the gas outlet was passed into a conical flask containing mineral oil. The pH of the culture media was automatically regulated. The output of the pH regulator controlled the discontinuous acting pumps that provide either 0.5 M HCl or 0.5 M NaOH to the culture fluid in the culture vessel to maintain the pre-set pH.

Sample from the culture vessel was collected through a sampling port to which a 4.0mm-internal diameter-silicone tubing was attached to the withdrawal pipe of the sampling port. A T-junction was attached to the free end of the silicone tubing to which one end was connected to a sterile 5.0 ml-syringe which act as the sampler and the other end attached to an O_2-free CO_2 gas source. Between samplings the tube was clamped with laboratory clamp and one end of the tubing was left attached to a sterile 50 ml-syringe.

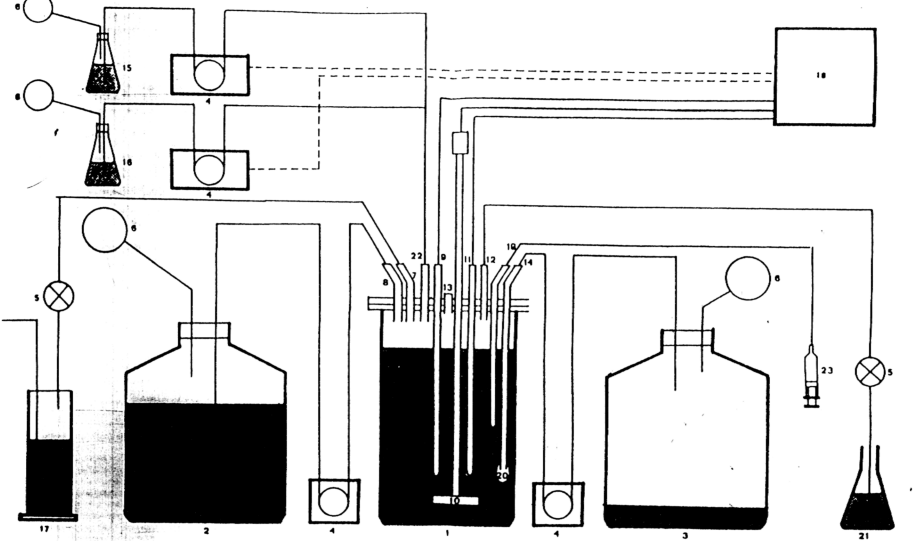


FIGURE 5.1. SEMI-BATCH FERMENTATION SYSTEM SET-UP:
(1) Culture vessel, (2) Influent reservoir, (3) Effluent flask, (4) Peristaltic pump, (5) Gas filter, (6) Balloon, (7) Gas inlet, (8) Influent inlet, (9) pH electrode, (10) Stirrer, (11) Redox measurement electrode, (12) Gas outlet, (13) Inoculating port, (14) Effluent port, (15) Acid solution, (16) Alkali solution, (17) Chromous acid oxygen scrubber, (18) Control Panel, (19) Sampling port, (20) Muslin cloth, (21) Mineral oil, (22) Acid-alkali inlet, (23) Sterile syringe.

5.2.1.2. Media preparation

The mineral reservoir contained pre-reduced artificial saliva solution of McDougall (1949) of the following composition: $NaHCO_3$, 9.8g; KCl, 0.57g; $CaCl_2$, 0.04g; $Na_2HPO_412H_2O$, 9.3g; NaCl, 0.47g; $MgSO_47H_2O$, 0.12g, resazurin, 0.0001g, cysteine-HCl, 0.25g, Na_2S, 0.25g, and 1000 ml distilled water. It was prepared according to the method for dilution solution as described in section 2.1.1c.

In experiment using grass as cellulosic substrate, 1 litre medium was prepared in the culture vessel with the following constituents: Grass, 70 g; $NaHCO_3$, 8 g; Modified Pfennig mineral solution, 5 ml; Pfennig metal solution, 5 ml; B vitamins solution, 1 ml, cysteine-HCl, 0.25 g; Na_2S, 0.25 g; volatile fatty acid solution (section 2.1.1d), 3.1 ml; 50 ml clarified rumen fluid (Holdeman, Cato, and Moore, 1974) and distilled water, 950 ml.

Pfennig mineral solution (McInerney, Bryant, and Pfennig, 1979) as modified by Pavlostathis, Miller, and Wolin (1988) had the following composition: KH_2PO_4, 10g; $MgCl_2.6H_2O$, 6.6g; NaCl, 8g; $CaCl_2.2H_2O$, 1g, and 1000 ml distilled water. Pfennig metal solution (McInerney *et al*, 1979) had the following composition: $ZnSO_47H_2O$, 0.1g; $CoCl_26H_2O$, 0.2g; $CaCl_22H_2O$, 0.01g; $NiCl_26H_2O$, 0.02g; $Na_2MoO_42H_2O$, 0.03g; $FeCl_24H_2O$, 1.5g, and distillled water,

1000 ml. B-vitamins solution (McInerny *et al*, 1979) had the following composition: Nicotinic acid, 20mg; cyanocobalamin, 20mg; thiamine, 10mg; p-aminobenzoic acid, 10mg; pyridoxine, 50mg; and pantothenic acid, 5mg, and distilled water, 1000 ml.

In experiment using POME, growth medium of 1 liter was prepared with the following constituents: Palm oil mill sludge, 1000 ml; $NaHCO_2$, 8 g; cysteine-HCl, 0.25 g; Na_2S, 0.25 g; and clarified rumen fluid, 50 ml.

Both grass and POME media was prepared as followed: All ingredients were mixed in the culture vessel except B-vitamins solution, bicarbonate, clarified rumen fluid, and the reductants. The culture vessel was completely fitted with cover plates with attached tubing and electrodes and was sterilized in an autoclave for 30 minutes. After sterilization, the culture vessel was mounted to its holder at the fermentor unit and left to cool to about 55°C. The medium was bubbled with 100% CO_2. Sterile bicarbonate solution, vitamin Bs solution, and filter sterilized rumen fluid were added to the culture vessel medium by injecting the respective solution through viton diaphragm at the inoculation port on the cover plate by using hypodermic syringe. Finally the filter sterilized cystein-HCl and sodium sulfide solutions were added the same way as the addition of bicarbonate, rumen fluid, and B-vitamins solutions were added. The pH of the media was adjusted and maintained at 6.8. Stream of 100% CO_2 was changed to 89%N_2-

5.5%H_2-5.5% CO_2 until inoculation with rumen ingesta was done.

5.2.1.3. Preparation of inoculum

Rumen ingesta from Napier grass fed goat were shown to contain all the important cellulolytic bacteria species and high in cellulolytic activity. Hence it was used as inoculum for the present study. 100 ml of the rumen ingesta (section 2.1.1e) in a 500 ml conical flask was diluted with 300 ml anaerobic mineral solution (section 2.1.1c) in an anaerobic cabinet. The mixture was magnetically stirred for 30 minutes and then it was left to stand for half an hour to allow the course solid to settle down.

5.2.1.4. Inoculation and Fermentation Procedures

100 ml of the liquid portion from section 5.2.1.3 was inoculated into the culture vessel through the inoculation port by using two 50 ml-hypodermic syringe. The fermentor stirrer was started and the medium was allowed to stabilized for 3 hours. After the stabilization period, 50 ml of the culture fluid was sampled out and the continuous delivery of artificial saliva (influent line) and the

outflow of the effluent were started. Speeds of both pumps were adjusted to get the liquid retention time in the culture vessel at 1.5 turnover per day (16 hours retention time). At this turnover rate, soluble sugar is removed faster and the time is long enough for the cellulose dependent-bacteria to proliferate. Sampling was done at every 12 hours for a period of 3 days. Duplicate experiments were carried out for each fermentation and results obtained were averaged.

5.2.1.5. Analyses of samples

Samples were analyzed for total microbial count, total cellulolytic count, sugar concentration, cellulolytic activity, and volatile fatty acids concentration. Total bacterial count and total cellulolytic bacterial count was carried out using the roll tube technique as described in section 2.1.1d and 2.1.1f. The concentration of soluble sugar was determined by the Anthrone method as described in section 2.2.1a. Cellulolytic activity was determined according to the method described in section 2.2.3a.2. Volatile fatty acids was analyzed as described in section 2.2.2 a.

5.2.2. RESULTS

5.2.2.1. Total viable and cellulolytic count of bacteria

The introduction of 100 ml of inoculum into the fermentor would give an initial count of 4.5 $x10^6$ cell per ml of the broth. However, stabilizing the system for 3 hours before commencing the continuous flow has caused the population to increase to 1.33 x 10^8 and 1.12 x 10^8 using the grass and POME as substrate respectively as shown in figure 5.2. The number of bacteria increased during first 12 hours to 9.17 x 10^8 in the grass system whereas in the POME system it increased to 6.58 x 10^9, hence indicating a higher growth rate in the latter system. In both systems the bacterial population decreased at 24 hours indicating a population washout. In the system using grass as substrate, the population was rather stable from 24 to 48 hours, thereafter increased in size from 48 to 60 hours of fermentation indicating the population growth rate exceeding the set dilution rate before a second population washed out at 72 hours. In the system using POME as substrate, the number of bacteria decreased rapidly from 12 to 48 hours of fermentation followed by a more gradual decreased until day 3. Although the counts of bacteria were always higher in POME compared to those of the grass but the continual washed-out from 12 to 72 hours of fermentation indicated that the initial burst in population could have been followed by growth under substrate limited condition.

The total viable count of cellulolytic bacteria is shown in figure 5.3. The same pattern was observed as for the total viable bacterial count although the count of cellulolytic bacteria was always lower than the total bacteria count. The population of cellulolytic bacteria during the first 3 hours of sampling was 2.53×10^7 and 2.94×10^7 for the grass and POME systems respectively and in all cases the count in POME was higher than in the grass system.

5.2.2.2. Total soluble sugar

Figure 5.4 showed the total soluble sugar presence in the culture fluid. Using 70 g of grass and 1000 ml of POME would give an initial concentration of soluble sugar at 6.62 g/l and 8.53 g/l in grass and POME systems respectively. However, after 3 hours of stabilization, the concentration of sugar in grass was only 1.18 g/l whereas in POME it was 8.43 g/l. In the grass system, it decreased to 0.27g/l in 12 hours but in the POME system it decreased rapidly to 0.93 g/l. However, the sugar concentration in the system with POME as substrate was higher than in grass until 60 hours. The sugar concentration decreased with time in both systems and in the POME system, it reached its lowest value at 72 hours.

Figure 5.2: Rumen ingesta of goat fed with *P.purpureum* was inoculated into a semi-bath fermentation system containing the following substrate: (i) *P.purpureum, or* (ii) POME. The growth medium was analysed for total bacterial count using roll tube method.

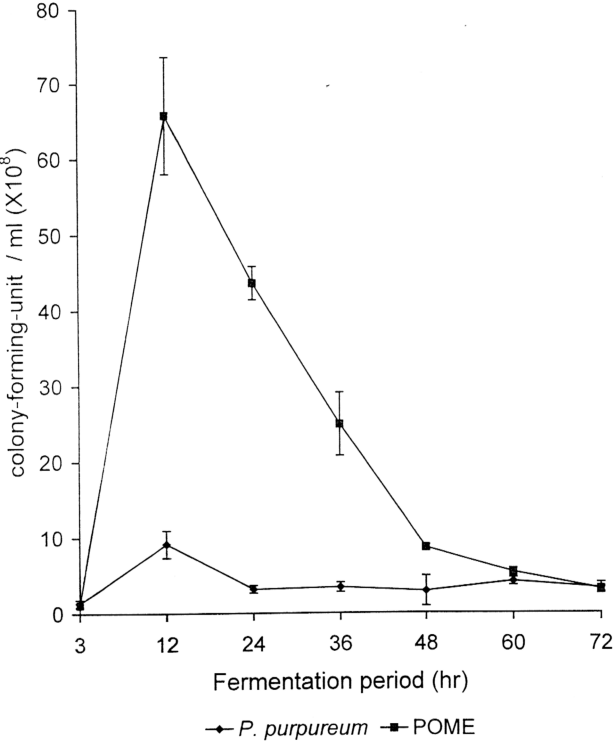


Figure 5.3:Rumen ingesta of goat fed on *P.purpureum* was inoculated into a semi-batch fermentation system containing the following substrate:(i) *P.purpureum* , or (ii)POME.The fermentation medium was analysed for cellulolytic bacteria number using roll tube.

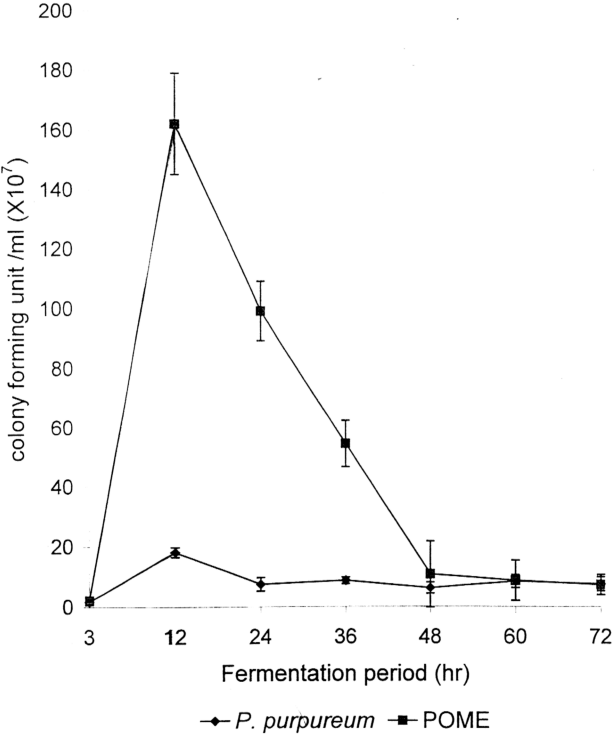
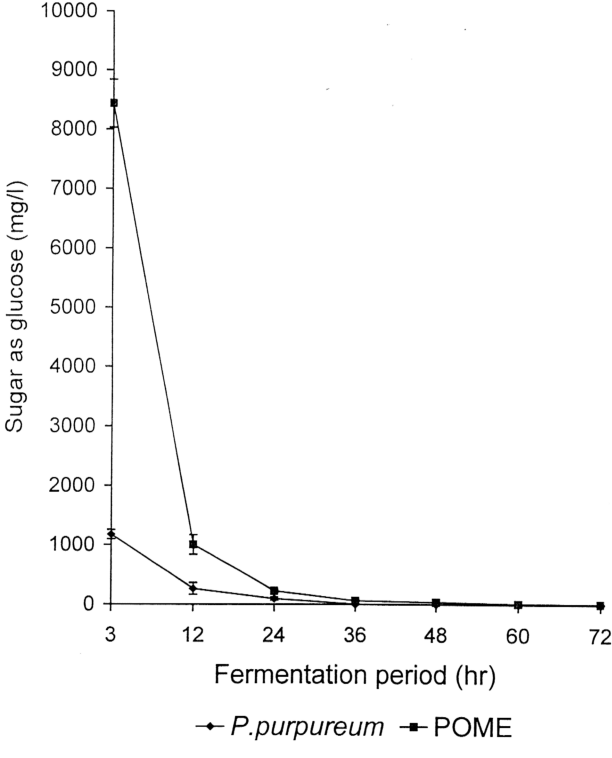


Figure 5.4:Rumen ingesta of goat fed on *P.purpureum* was inoculated into a semi-batch fermentation system containing the following substrate: (i) *P. purpureum* , or (ii) POME. The fermentation broth was analysed for total soluble sugar expressed as glucose.



5.2.2.3. Volatile fatty acid concentration and pattern

Total fatty acids were shown in figure 5.5. The concentration of VFA in the 3 hour-sample was higher in the system using POME as substrate (6.69 g/l) compared to the usage of grass as substrate (1.83 g/l). In the POME fermentation, the volatile fatty acid concentration decreased with time with the highest rate of decrease for the system using POME during first 24 hours of fermentation. However, in the grass fermentation, VFA concentration increased at 12 hour of incubation period but decreased sharply from 12 to 36 hour of incubation period. From 36 hours of incubation period, VFA concentration was approximately constant although slight increased was observed at 72 hours for the system using grass as substrate. Table 5.3 showed individual carbon 2 to carbon 5 VFA concentration. Acetic acid was the major VFA produced from 12 hours onwards for both systems. Propionic acid was higher than butyric in the grass system but the reverse was observed in the POME system. Iso-acids decreased until 36 hours where its presence was no longer detected until 72 hours in the grass system. However, in POME system, the iso-acids were absent from 12 hours onwards. The valeric acid was found to be higher in POME system but it was found to decrease with time in both systems.

Figure 5.6 showed the ratio of acetic to propionic acid. The ratio increased until 24 hours in the POME system but decreased at 36 hours where the value remained approximately constant at 36 until 72 hours. In the grass system, the ratio increased to 9.5 at 12 hours but decreased

to 4.1 and 5.31 at 24 hours and 36 hours respectively. The ratio dropped to 2.5 at 48 hours and remained approximately constant (2.5 -3.0) until 72 hours.

5.2.2.4. Cellulolytic activity

The results for cellulolytic activities are shown in figure 5.7a, 5.7b, 5.7c, and 5.7d. The cellulase activity for swollen cellulose, crystalline cellulose, and POME fibre generally decreased from 12 hours to 36 hours and then increased until 72 hours for swollen cellulose.

The action of the crude cellulase extracts against the grass fiber as substrate was rather different. Extracts from the grass system, showed decreased in cellulolytic activities for grass fibre for the first 12 hours and then remained constant for the next 12 hours but the extracts from the POME system gradually decreased in cellulolytic activities until 24 hours. Both the extracts showed increased in activity at 36 hours of fermentation. The extracts generally decreased in activity after 36 hours of fermentation although from the grass system it remained constant from 36 to 48 hours of fermentation.

Figure 5.5: Rumen ingesta of goat fed on *P.purpureum* was inoculated into a semi-batch fermentation system containing the following substrate: (i) *P.purpureum*, or (ii) POME. Fermentation medium was analysed for total VFA using gas chromatography.

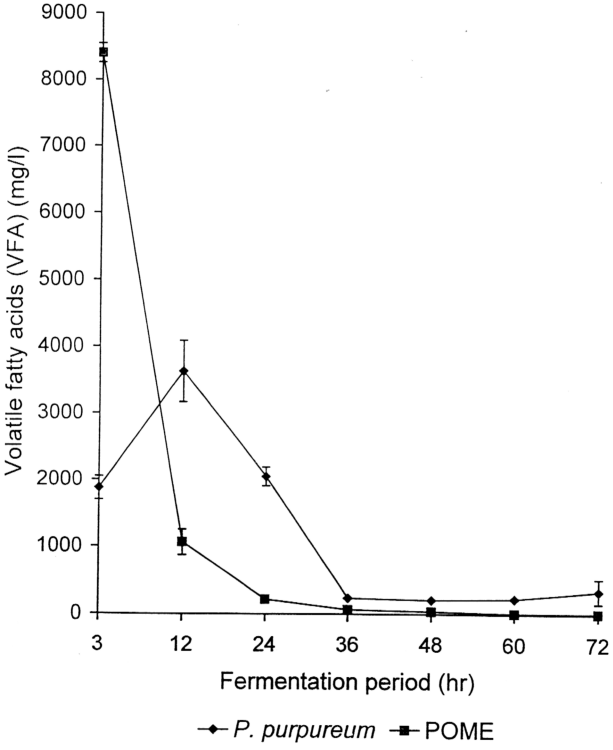


Figure 5.6: Semi-batch fermentation of *P. purpureum* and POME by mixed ruminal bacterial population from rumen ingesta of goat fed on *P. purpureum*. Concentration of acetic and propionic acid produced during fermentation was analysed by GLC and expressed in molar. Graph below is the molar ratio of acetic to propionic acid during fermentation of POME or *P. purpureum*.

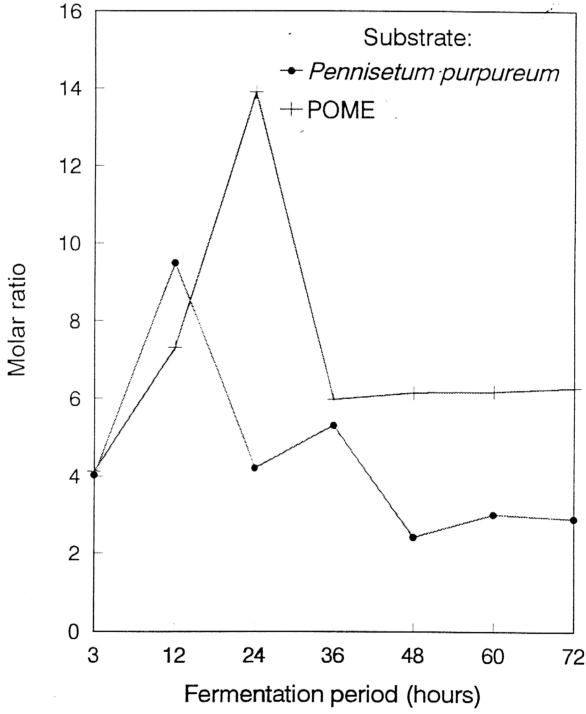


Table 5.2a: Volatile fatty acids production during semi-batch fermentation of *P.purpureum* by mixed ruminal bacteria population in rumen ingesta of goat fed on *P.purpureum*.

Fermentation period (Hrs)	Volatile fatty acids (mmole/litre)					
	Acetic	Propio-nic	Iso-butyric	n-butyric	Iso-valeric	n-valeric
3	7.31 ± 0.82	1.81 ± 0.30	3.54 ± 0.47	2.24 ± 0.15	2.36 ± 0.64	3.33 ± 0.55
12	5.98 ± 0.33	0.63 ± 0.08	0.51 ± 0.10	0.38 ± 0.04	2.01 ± 0.04	0.38 ± 0.09
24	3.24 ± 0.08	0.77 ± 0.06	0.27 ± 0.01	0.50 ± 0.10	0.47 ± 0.07	0.13 ± 0.02
36	2.92 ± 0.91	0.55 ± 0.09	0.05 ± 0.02	0.30 ± 0.01	0.03 ± 0.02	0.05 ± 0.03
48	2.78± 0.06	1.15 ± 0.04	ND	0.29 ± 0.07	ND	0.06 ± 0.01
60	2.53 ± 0.03	0.84 ± 0.09	ND	0.49 ± 0.05	ND	0.08 ± 0.04
72	2.34 ± 0.08	0.81 ± 0.10	ND	0.85 ± 0.06	ND	0.04 ± 0.02

ND= Not detected

Table 5.2b: Volatile fatty acids production during semi-batch fermentation of POME by mixed ruminal bacteria population in rumen ingesta of goat fed on *P.purpureum*.

Fermentation period (hrs)	Volatile fatty acids (mmole/litre)					
	Acetic	Propio-nic	Iso-butyric	n-butyric	Iso-valeric	n-valeric
0	12.86 ± 0.15	3.12 ± 0.88	0.56 ± 0.30	2.51 ± 0.33	17.60 ± 1.50	38.06 ± 2.66
12	15.65 ± 0.42	2.14 ± 0.05	ND	4.17 ± 1.05	ND	10.82 ± 0.33
24	10.98 ± 0.08	0.79 ± 0.09	ND	4.19 ± 0.89	ND	0.30 ± 0.15
36	6.39 ± 0.27	1.07 ± 0.15	ND	2.95 ± 0.30	ND	0.34 ± 0.09
48	4.61 ± 0.55	0.75 ± 0.18	ND	1.72 ± 0.10	ND	0.29 ± 0.03
60	3.70 ± 0.36	0.60 ± 0.05	ND	3.31 ± 0.52	ND	0.08 ± 0.03
72	3.64 ± 0.10	0.58 ± 0.10	ND	0.72 ± 0.06	ND	0.09 ± 0.02

Table 5.3 showed the relative rates of digestion of different form of cellulose. At 3 hours of fermentation the digestion rate for the extracts from both systems was highest for the swollen cellulose (S) and as such was the most readily digestible form of fibre. This was followed by POME fibre (P), grass fibre (G) and crystalline cellulose (Cr). This pattern was the same as the pattern observed for the extracts described in chapter 2. After 12 hours of fermentation the extracts from the grass system, showed highest digestion of POME fibre followed by the swollen cellulose, grass fibre and crystalline cellulose (P>S>G>Cr.). The order changed to P>G>S>Cr from 24 to hours of fermentation. In POME system, the relative rates of digestion of different form of cellulose changed for every 24 hours of incubation. The rates were of the order S>P>Cr>G, P>G>S>Cr, and P>S>G>Cr during first, second, and third 24 hours of incubation respectively. Digestion of the POME fibre, grass fibre and crystalline cellulose was higher using the samples from the grass system. However for the samples obtained from the POME system, higher cellulolytic activity was observed using POME fibre rather than the grass fibre as substrate.

Figure 5.7a: Rumen ingesta of goat fed on *P.purpureum* was inoculated into a semi-batch fermentation system containing the following substrate:(I) *P.purpureum* ,or (ii)POME. The fermentation medium was analysed for cellulolytic activity for swollen celulose.

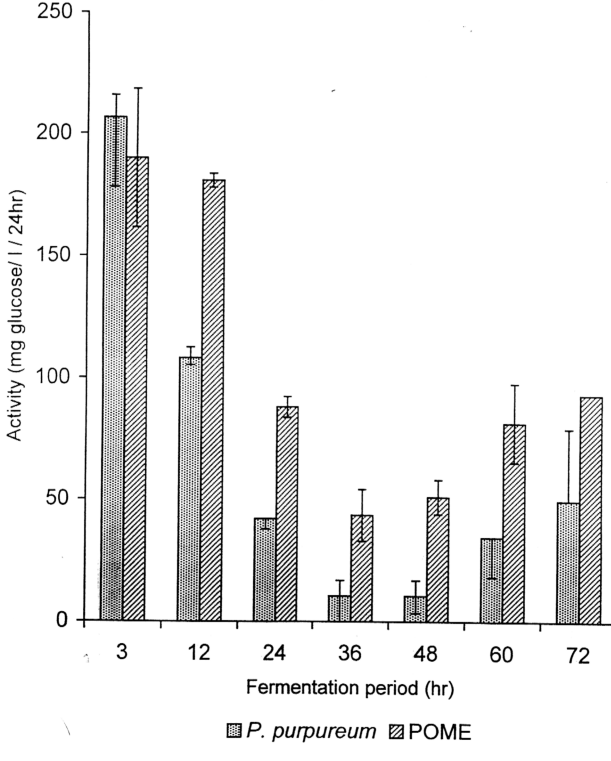


Figure 5.7b: Rumen ingesta of goat fed on *P.purpureum* was inoculated into a semi-batch fermentation system containing the following substrate: (I) *P.purpureum* ,or (ii) POME. The fermentation medium was analysed for cellulolytic activity for POME fibre.

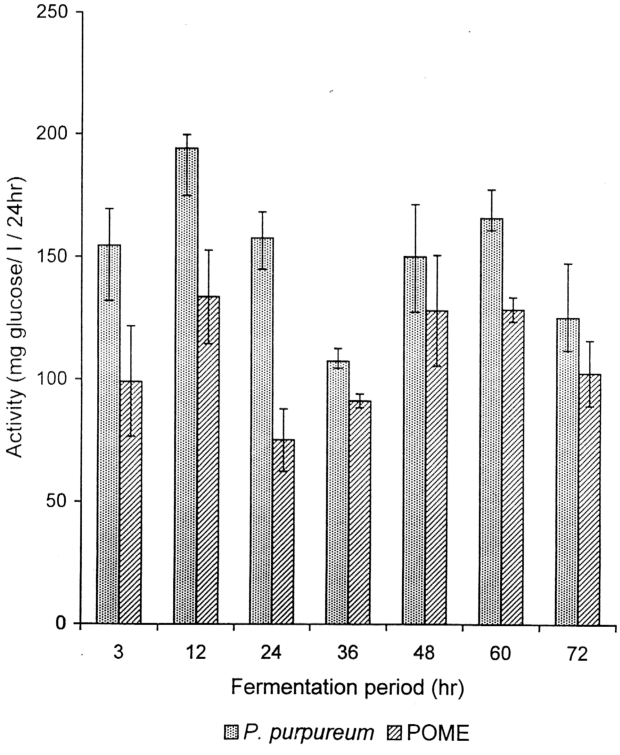


Figure 5.7c: Rumen ingesta of goat fed on *P.purpureum* was inoculated into a semi-batch fermentation system containing the following substrate:(I) *P.purpureum*, or (ii)POME. The growth medium was analysed for cellulolytic activity for crystalline cellulose.

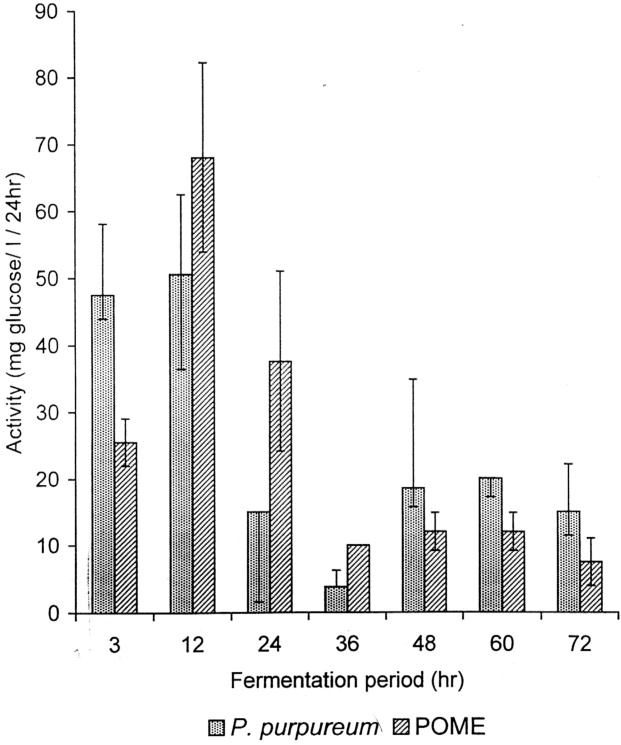


Figure 5.7d: Rumen ingesta of goat fed on *P.purpureum* was inoculated into a semi-batch fermentation system containing the following substrate: (I) *P.purpureum*, or (ii) POME. The fermentation medium was analysed for cellulolytic activity for grass fibre.

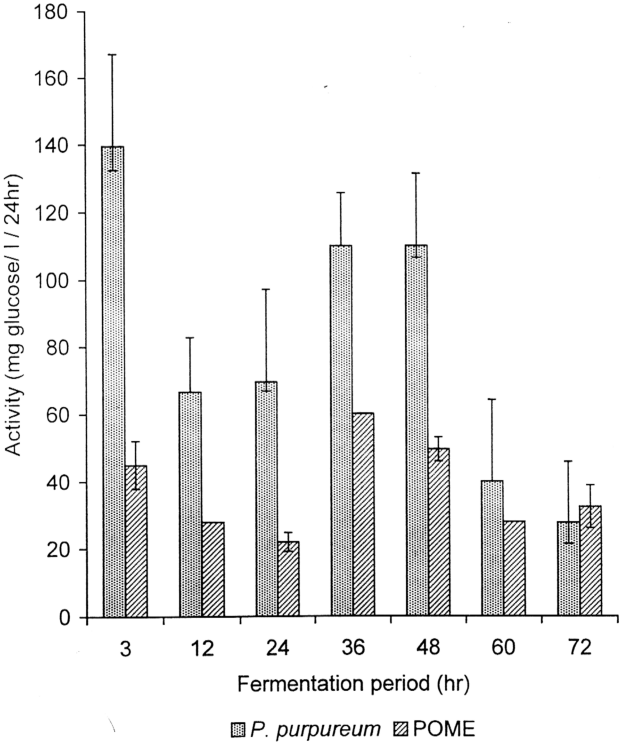


Table 5.3: Fermentation medium in the semi-batch fermentation of *P.purpureum* or POME by mixed ruminal bacteria population in rumen ingesta of goat fed on *P.purpureum* was analysed for cellulolytic activity for swollen cellulose (s), crystalline cellulose (Cr), grass fibre (G) and POME fibre (P). Table below shows the relative cellulolytic activity of the different form of cellulose by the cellulase enzyme produced in the semi-bath fermentation of grass or POME at 3, 12, 24, 36, 48, 60, and 72 hours of fermentation period.

Incubation period (hrs)	Grass fermentation	POME fermentation
3	S > P > G > Cr	S > P > G > Cr
12	P > S > G > Cr	S > P > Cr > G
24	P > G > S > Cr	S > P > Cr > G
36	P > G > S > Cr	P > G > S > Cr
48	P > G > S > Cr	P > G > S > Cr
60	P > G > S > Cr	P > S > G > Cr
72	P > G > S > Cr	P > G > G > Cr

5.3. DISCUSSION

The usage of the semi-batch system allowed the continuous removal of fine particulate organic matter, soluble carbohydrates and the end products of fermentation in the culture vessel while retaining the larger fibrous organic portion for microbial enzymatic action. Together with the continual input of artificial saliva and the maintenance of a constant optimum pH, the semi-batch system could approximately simulate the natural rumen system. By eliminating the effect of pH, the fibre digestion would be a function of fibre source (Grant and Weidner, 1991), bacterial species (Akin, 1986), and the environment in which digestion occurred. This would also include the soluble carbohydrate concentration (Mould, Orskov, and Mann, 1984) but may have little effect since the content of the N-free extract in grass and POME was comparable.

However, based on the nutrient analysis of *P. purpureum* (this chapter) and POME (Chapter 4) it was found that the organic matter content was 89.51% and 81.33% for *P.purpureum* and POME respectively and the protein content for the former was 13.88% and the latter was 10.77%. This indicates that the grass would be better nutritionally. The fibre content for the grass was 29.4% and cellulose content was 81.63% of the fibres and for the POME the fibres was only 16.81% and the cellulose was 14.6 % of the fibres

(Abdul Manan, 1987). In POME 76.5% of the fibres was lignin. A 70 g grass per litre used in this study would contain 16.8 g of cellulose whereas 1000 ml. of POME would only contain 1.78 g of cellulose. With the N-free extract at 29.3 g per 70 g of grass and 28.4 g per litre of POME, the semi-batch system using POME as substrate would have a much lower ratio of cellulose to non-fibrous carbohydrate compared to the semi-batch system using grass as substrate. The soluble sugar in the grass sample was 9.45% and this would give an initial value of 6.6 g per litre in the fermentor whereas for the POME system where the soluble sugar was 12.2% in the POME, an initial value of 8.5 g per litre would be present in the fermentor.

Samples obtained at the end of the 3 hours stabilization period showed that the bacterial population were comparable in both the grass or POME systems and was 2 order higher than the initial inoculum. However, the soluble sugar concentration using POME as substrate was 8.4 g/l which was 7.0 times higher than using grass as substrate (1.2 g/l) and the total VFA with POME as substrate (6.69 g/l) was 3.7 times higher than that of grass as substrate (1.83 g/l). Hence, in the absence of the continuous flow of influent and effluent during the first 3 hours of fermentation, it would appear that the system with grass as substrate was rather limited in carbohydrate that could readily be digested to soluble sugar and consequently the production of volatile fatty acids compared to the system using POME as substrate.

The artificial saliva introduced into the system was free from energy substrate. The net changed in fermentor after the first 3 hours of fermentation would be due to the production of sugar and VFA by the microbial population less the amount that was washed out with the effluent and the amount utilized by the microbial population. A steady state population size after the first 3 hours of fermentation could only be achieved if population growth rate was equal to 0.065/hr, for a flow rate of 1 litre per 16 hours and a constant culture volume of 1.0 litre. An increase in population size from 1.33×10^8 and 1.12×10^8 at 3 hours of fermentation to 9.17×10^8 and 6.58×10^9 at 12 hours of fermentation in grass and POME as substrate respectively indicated that the population growth rate in both systems exceeded 0.065/hr, and POME as substrate to be better in supporting the initial growth of the population. At the end of the first three hours of fermentation the system using POME as substrate contained 8.43 g/l sugar and 6.69 g/l VFA whereas with grass as substrate the concentration of sugar and VFA were only 1.18 g/l and 1.83 g/l respectively. This indicated that in the system using grass as substrate the sugar and VFA could rapidly be assimilated by the bacterial population (Pavlostathis *et al*, 1988), but using POME as substrate accumulation apart from assimilation of sugar and VFA have occurred. The higher concentration of sugar and VFA in the system using POME as substrate allowed high increase in the population size from 3 to 12 hours of fermentation. However, the continual decreased in the population size for the total bacteria and cellulolytic bacteria populations

indicated that the populations growth rate were lower than the dilution rate set up for the semi-batch systems. The higher washed out rate for the POME system compared to grass system indicated that the readily digestible substrate initially present in the former was exhausted. Hence, the growth of both the cellulolytic and non-cellulolytic populations from the rumen was dependent on the availability of soluble carbohydrate (Mould, Orskov, and Mann, 1984) and volatile fatty acids (Bryant and Robinson, 1962).

The cellulolytic activities of crude extracts from initial inoculum showed better digestion for the swollen cellulose, followed by the POME fibres, grass fibres and the crystalline cellulose. However, the digestion of the cellulosic substrates differed between the extracts obtained from the two fermentation systems. For the first 3 hours of fermentation the digestion of the swollen cellulose was approximately of the same rate for the cellulase extracts from the two systems but for the extracts from the grass system digested the POME fibres, grass fibres and crystalline cellulose 1.6, 3.5 and 1.9 times better respectively than the extracts from the POME systems. The release of cell free cellulases into the surrounding medium was the function of population and the growth stage of the populations. (Gilkes, Kilburn, Miller and Warren, 1991).

In the present work only the cell free cellulase was determined and as such would be subjected to the variation in the growth stages of the bacterial populations.

Although the cellulolytic bacterial population increased from 3 to 12 hours of fermentation, the production of cell-free cellulase only increased for POME and crystalline cellulose as substrate. Digestion of the other two substrates were lower for the 12 hours of fermentation. If the activity against swollen cellulose could be taken as endoglucanase (Cx-cellulase) activity it would implied that the released of cell-free endoglucanase was low during active growth whereas the activity against crystalline cellulose indicated that release of exoglucanase (C1-cellulase) was higher during active growth or otherwise the production of exoglucanase was proportionately higher than endoglucanase. This could probably be true since at the 12 hours of fermentation, free sugar and VFA were already on the decline and as such the bacterial population has to be dependent on the fibrous substrate as carbon and energy sources. This could also explained the decrease in bacterial population from both systems after the 12 hours of fermentation. However, it also suggested that cellulase production was not totally depressed by the presence of soluble sugar but the utilization of available sugar was more important than deriving it from cellulose digestion.

Cellulolytic activity for all the substrates increased from 36 hours might be due to the exhaustion of the soluble carbohydrates as seen from the low soluble sugar concentration at 36 hours. The presence of soluble carbohydrates may shorten the lag phase of cellulose digestion and this is presumably due to greater numbers of

bacteria (Hiltner and Dehority, 1983). To maintain the population size at the steady state in the system where dilution rate and volume are constant, the rate of utilization of growth limiting substrate has to be constant (Slater, 1979). With no further addition of grass or POME, the population has to depend on the fibrous portion of grass or POME for their carbon requirement and this could be achieved by populations that are active in producing cellulase enzymes. This could be seen by further increased in cellulolytic activity when the soluble sugar has decreased (from 36 hours for swollen cellulose, POME fibre, and crystalline cellulose and from 24 hours in the case for grass fibre).

Decrease in cellulolytic activity for crystalline cellulose, grass fibre, and POME fibre at 72 hours was probably due to decreased in digestible fraction of the fibres presence in the culture fluid. In the case of grass, the following trend exist for the rate of digestion of grass tissues: mesophyl phloem > epidermis parenchyma sheath > lignified vascular tissue (Akin and Burdick, 1975; Hanna, Monson, and Burton, 1973). The rate of utilization of cellulose was found to be dependent on the steady state concentration of cellulose (Pavlostathis *et al*, 1988) and followed the first order kinetics of cellulose disappearance (Fan and Lee, 1983; Stack and Cotta, 1986) when the dilution rate is less than 30 hours (Shi and Weimer, 1992). A given proportion of the remaining potentially digestible material would disappear each hour and consequently would caused

lower microbial yield, lower cellulolytic activity and bacterial count at 72 hours.

The volatile fatty acids pattern for the 3-hour sample represents the VFA produced during 3 hours batch fermentation. Availability of high concentration of readily fermentable carbohydrates in POME caused a rapid increase in microbial activities resulting in higher production of volatile fatty acids (Hungate, 1966; Leedle, Bryant, and Hespell, 1982) as well as the total number of viable bacteria. However, the molar ratio of individual VFA was not typical of that of abnormal rumen (Church, 1969). Valeric acid was high in both system especially in POME system and this could be due to the high initial amount of soluble sugar in POME than in grass. In rumen, volatile fatty acids are absorbed through the rumen wall with the order of butyric > propionic > acetic (Church, 1969) and absorbtion increased as the chain length of the acids increased (Sutton, McGilliard, and Jacobson, 1963). However, the accumulation of acids in the fermentor system resulted in high proportion of C_6 and C_5 acids.

Acetate to propionate ratio obtained for both systems indicated that cellulolytic population was active (Murphy, Baldwin, and Koong, 1982). The increased in acetate to propionate ratio was probably due to higher production of acetate rather than decrease in propionate concentration which implies that cellulolytic population could have dominated during the digestion of both substrates (Hoover,

Kincaid, Varga, Thyn, and Junkins, 1984). The decreased in the iso-acids proportion was associated with the greater utilization by cellulolytic bacteria (Rumsey, Putnam, Bond, and Oltjen, 1970) and was in accordance with the increased in cellulolytic activities and total viable cellulolytic bacterial count. In the POME system, iso-acids were utilized at a higher rate. This could be probably due to greater rate of utilization by the higher number of cellulolytic bacteria in the POME system and also higher degradation rate of protein and amino acids (Erfle, Boila, Teather, Mahadevan, and Sauer, 1981) present in POME. However, elevated butyric acid concentration in the culture fluid of POME system was an indication of a less efficient butyric fermentation compared to fermentation of grass (Orskov, Flatt, and Moe, 1968). The latter situation could probably be due to different population of bacteria in the POME and the grass system.

Cellulolytic activity was generally better for the grass system although higher concentration of cellulolytic bacteria was present in the POME system. POME fibre comprised of fruits constituent in dissolved or suspended state and was predominantly colloidal (Wood, 1977). More solid cellulosic materials in liquid slurries in grass system permits more colonization (Cheng, Fay, Howarth, and Costerton, 1980) and cellulase production (Ohmiya *et al*, 1983).

The difference in the pattern of the relative rate of digestion of different form of cellulose in grass system compared to POME system indicates that cellulolytic population was more stable on the grass as substrate. In POME, the synthesis of a different set of cellulase enzymes could have occurred (Rutella and King, 1968). The cellulolytic population stabilized on the grass as substrate produced more active enzymes for the grass and POME fibres whereas the population stabilized on POME as substrate was more active on swollen and crystalline cellulose.

The low digestibility of swollen cellulose relative to grass fibre in grass system showed that ruminal microflora adapted to specific cellulose structure did not necessarily be able to utilize the more digestible form of cellulose and ruminal microflora could not rapidly adapt toward the utilization of cellulose with altered unit cell structure (Weimer *et al*, 1990). Hence this could probably explain the changes in the relative rate of digestion of different cellulosic material in cellulolytic enzyme assay from the original inoculum (from rumen of grass fed goat) introduced into the POME system compared to the inoculum introduced into the grass system. Adaptation of cellulolytic population to certain type of cellulose may involve the synthesis of cellulase enzymes for the particular substrate or selection of species that could utilize the substrate which might result in different pattern of cellulolytic activity as well as the rate of cellulose digestion.

In conclusion, POME contains higher readily assimilable sugars as compared to grass. Rumen bacteria population assimilated these sugars rapidly resulting in an increase of number of bacteria and VFA concentration. However, the resulting cellulolytic bacteria population adapted on POME was generally poorer in cellulolytic activity and they were less efficient in saccharification of fibers as compared to the population adapted on grass. This could be due to different in composition of cellulolytic bacteria in population adapted on POME as compared to grass.