CHAPTER 4

GROWTH AND ACTIVITIES OF PURE CULTURES OF *F. SUCCINOGENES*, *R. ALBUS*, *R. FLAVEFACIENS*, *B. FIBRISOLVENS*, AND *C. CELLOBIOPARUM* IN POME, POME EXTRACTS PLUS CELLULOSES AND POME EXTRACTS

4.0. INTRODUCTION

Rumen of the goats that have been mainly sustained on grass alone contained normal bacterial cellulolytic population (Chapter 3) but introduction of POME into rumen changed this population (Abdul Manan, 1987). In Chapter 2, it was shown that the goats which have been supplemented with POME-based concentrates have of the same order of total bacterial population as the goats that have mainly been sustained on grass alone but the cellulolytic bacterial population of the former was an order lower than the latter. Hence, in this chapter the growth of pure cultures of cellulolytic rumen bacteria will be investigated in order to elucidate whether POME has any selective effect on the bacteria.

4.1. NUTRIENT ANALYSIS OF POME

4.1.1. Materials and Methods

Freshly collected POME samples were dried at 90°C, grounded and sieved. The samples were dried before carrying

out the nutrient analysis. All analyses were done in six replicates.

4.1.1a. Ash

One gram of dried samples (W_1) were placed in porcelain crucibles and weighed (W_2). The crucibles were heated at 600°C for 3 hours in Muffle furnace and then left to cool to room temperature. The crucibles were placed in a desiccator and weighed (W_3) again. Ash content was calculated from the formula:

$$\% \text{ Ash} = [W_3-(W_2-W_1)]/W_1 \text{ X } 100$$

4.1.1b. Fat

Soxhlet system M 6 (Tecator) was used for the determination of fat content. Dried samples were weighed (W_1) and placed into cellulose thimbles of size 26 X 100 mm (Whatman). The timbles were dried in oven at 95°C for 3 hours before attaching to the extraction unit. The extraction cups were weighed (W_2). The thimbles were then placed into the extraction cups containing diethylether and refluxed at 75°C for 15 minutes. Diethylether were evaporated and the extraction cup was dried in an oven at

95°C for 1 hour. The cups were cooled in a desiccator and weighed (W_3). Percentage of fat was calculated according to the formula:

$$\% \text{ Fat} = (W_3-W_2)/W_1 \times 100$$

4.1.1c. Fibre

Fibretec System (Tecator) was used for the determination of fibre content. 1 gram of sample was weighed (W_1) and transferred into filter crucibles of porosity size 40-90 μm (Tecator). 150 ml of preheated 0.128 M H_2SO_4 was added to each sample and boiled for 30 minutes. The samples were then filtered and washed three times with 30ml hot distilled water. 150 ml preheated 0.233 M KOH solution was added to each sample and boiled for 30 minutes. The samples were filtered and washed three times with hot water followed by washing with 25ml acetone three times. The crucibles were dried at 100°C overnight, cooled, and weighed (W_2). The samples were ashed at 600°C for 3 hours, cooled, placed in a desiccator and weighed (W_3). Fibre content was calculated from the formula:

$$\% \text{ Fibre} = (W_2-W_3)/W_1 \times 100$$

4.1.1d. Protein

Kjeltec Digestion System, and Kjeltec distiller (Both of Tecator) for Kjeldahl nitrogen were used for the determination of protein content. 0.2 g samples were placed in the digestion flasks and 20 ml distilled water was added followed by 15 ml concentrated H_2SO_4. Three (Na_2SO_4 + $CuSO_4$) catalyst digestion tablets (Ajax Chemical, Australia) was added and the digestion flask was placed into the heating block. The tubes were heated at a medium heating for 45 minutes until frothing stopped and the samples started to fumes. This was followed by a maximum heating until the mixture turned clear and finally, heating was set back to medium heating for 15 minutes. The heating sequence was found to be important for complete digestion of the samples. Approximately 100 ml of water was added to the cooled sample and the tube was fixed to the distillation unit. 50 ml of 10 N of sodium hydroxide solution was automatically added and the steam distillation was started. The distillate was collected in conical flask containing 0.05 M H_2SO_4.

The ammonium content of the distillate was determined according to a modified method of Harwood and Kuhn (1970). In this method, distillates were diluted to give a nitrogen concentration within a range of 0.1-2.0 µg N

per ml. Phenol-nitroprusside solutions (reagent A) followed by NaOH-hypochlorite solutions (reagent B) were added in the ratio of 15ml sample: 5ml reagent A: 2.5ml reagent B. The concentration of nitrogen in the samples were determined from the intensity of the color using spectrophotometer (Spectronic 20, Bausch and Lomb) against reagent blank at 630 nm. Ammonium chloride solution at 2 µg N per ml was used as standard in the analysis. The concentration of nitrogen per gram of the dried samples were multiplied by 6.25 to give the protein content of the samples.

4.1.1e. Nitrogen-free extract

Nitrogen-free extract (NFE) was calculated by the formula of Crampton and Harris (1969):

$$\% \text{ NFE} = 100 - \%(\text{Protein} + \text{Fat} + \text{Fibre} + \text{Ash})$$

4.1.1f. Soluble sugar

0.5 g 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 centrifuged at 10000 *g* for 20 minutes and the supernatant

was analyzed for soluble sugar content using the Anthrone method as described in section 2.2.1a.

4.1.2. Results

POME contains nitrogen-free extract, 40.15%; ash, 18.67%; fibre, 16.81%; fat, 13.60%; and protein, 10.77%. 100 g of POME contains 12.19g soluble sugars. Table 4.1 gives a comparison of the results of the present study with those previously reported by other authors for POME. There appears to be general agreement and slight differences are due to different in mill-to-mill operating system.

Table 4.1: Chemical constituents of Palm oil mill effluents (% of dry weight)

Protein	Fat	Ash	NFE	Fibre	Sol. sugar	Reference
9.60	21.30	11.10	46.50	11.50	-	Devendra (1975)
12.20	21.10	11.90	43.70	11.10	-	Muthurajah and Devendra (1976)
10.90	25.60	11.70	42.40	9.70	-	Huang, Ong, Seow, and Tan (1978)
10.77 ± 0.44	13.60 ± 0.90	18.67 ± 0.25	40.15 ± 2.30	16.81 ± 1.65	12.19± 0.30	**Current Results** (Mean ± std. deviation)

4.2. GROWTH AND CELLULOLYTIC ACTIVITY OF BACTERIA IN POME

4.2.1. Materials and Methods

4.2.1.1. Media Preparation

POME was collected from local Palm oil mill and stored at -12°C until further used. POME medium was prepared by diluting POME with equal volume of distilled water to give 50% POME with solid content of 25 g/l. pH was adjusted to 7. It was boiled and dispensed into pregassed 50 ml-roll streak tube (Bellco Glass Inc.). The tubes were again gassed with oxygen free CO_2 before they were tightly capped with butyl rubber stoppers.

The 50 ml-roll streak tubes were used as the culture vessel since 180 tubes were required for this batch fermentation study. They were more convenient to handle when compared to 100 ml or larger conical flasks especially in maintaining the required anaerobic growth conditions of the cultures. The use of fermenters was rather impractical since a total of 30 fermentations would be required for the 3 different medium and 5 test cultures carried out in duplicates.

A POME extract based medium was also used for this study. In order to obtain the POME extract, the POME was centrifuged at 4000 *g* for 15 minutes. The supernatant was dried in a stainless steel tray at 90°C. The dried POME extract was powdered using laboratory mill (Thomas Wiley, Model 4, Philadelphia) and passed through 0.1 mm pore size sieve and kept in air tight bottle until further use.

The POME extract medium was prepared by dissolving 12g of POME extract powder in distilled water followed by adjusting pH to 7. The solution was boiled before dispensing into the roll streak tube, gassed with 100% CO_2 and stoppered. POME extract with cellulose was similarly prepared but with the addition of 0.5% swollen cellulose (section 2.2.3a) and 0.5% crystalline cellulose (Sigma). All tubes were autoclaved for 30 minutes and cooled to about 55°C in water bath. The tubes were gassed with 100% CO_2 and required amount of sodium carbonate solution was added using a sterile hypodermic syringe. The solution was further bubbled with 100% CO_2 before solution of volatile fatty acid mixture (section 2.1.1 d), cystein-HCl, and sodium sulphide were added to the concentration of 0.1%(v/v), 0.25%(w/v) and 0.25%(w/v) respectively. The tubes were gassed out with 100% CO_2 and cooled to room temperature before inoculation.

4.2.1.2 Determination of soluble sugar concentration in POME extract and POME media

As concentration of soluble sugar may influence cellulase production or activity, soluble sugar in POME extract media and POME media was analysed as described in Section 4.1.1f.

4.2.1.3 Preparation of inoculum

Five cellulolytic bacteria isolated in chapter 3 were chosen to study their growth and activities in POME (medium 'P'), POME extract (medium PE) and POME extract plus celluloses (medium PE+C). *Fibrobacter succinogenes*, *Ruminococcus albus*, *R.flavefaciens*, *Butyrivibrio fibrisolvens*, and *Clostridium cellobioparum*, which have been maintained in complete medium (section 2.1.1 d), were used in this study.

Inoculum was prepared as described in section 3.3.3.1. The number of bacteria cells in suspension was counted using Helber counter (0.2 mm depth heamocytometer) and serially diluted if necessary in dilution solution to

obtaine a bacterial suspension of about 1.0 X 10^5 cells per ml.

4.2.1.4 Inoculation and incubation procedures

0.5 ml of bacterial suspension of each isolates from the dilution solution containing about 1.0 X 10^5 cell per ml was each inoculated into 20 ml of the respective POME, POME extract and POME extract plus cellulose media in a 50 ml-roll streak tubes. Duplicate tubes per incubation period per medium were prepared for each bacterial isolates and incubated at 37°C for 0, 2, 4, 8, 16, and 24 hours in an orbital shaker (Gallenkamp, England). The termination of incubation was done by storing the respective tubes at 4°C.

4.2.1.5 Analyses of samples

Samples were analyzed for count of bacterial cells and cellulolytic activity. Microbial count was first estimated with the aid of Helber counter and subsequently the number of viable cells were confirmed as described in

section 2.1.1e and 2.1.1f. and cellulolytic activity was done as described in section 2.2.3a.2.

4.2.2. Results

4.2.2.1 Soluble sugar concentration in POME extract media and POME media

Concentration of soluble sugar in various POME media is shown in Table 4.1. POME has 1.8 times higher soluble sugar concentration than POME extract. This result indicates that the extraction method used in this study did not extract all the soluble carbohydrates in POME.

Table 4.2: Total soluble sugar in POME extract broth medium and POME medium (g glucose/10 ml medium)

	POME extract media	POME media
Replicate 1	1487	2600
Replicate 2	1375	2125
Replicate 3	1250	2637
Average ± Std. deviation	1371 ± 119	2454 ± 285

4.2.2.2. **Bacterial count**

Figure 4.1a, 4.1b, and 4.1c showed the growth of cellulolytic bacteria in different POME medium. In all media, highest cell count was obtained with *C. cellobioparum* followed by *B. fibrisolvens, R. albus, R. flavefaciens,* and *F. succinogenes. C. cellobioparum, B. fibrisolvens and R. albus* grew more rapidly than *R. flavefaciens* and *F. succinogenes.*

B. fibrisolvens showed comparable growth rate in POME extract and POME extract with celluloses but grew better in POME medium. Growth of *C.cellobioparum, R.albus,* and *R.flavefaciens* were highest in POME medium followed by POME extract with celluloses and POME extract medium. *F. succinogenes* grew slightly better in POME than POME extract or POME extract plus celluloses.

Figure 4.1a: Growth of cellulolytic bacteria species in POME extract broth medium. Cell number was counted from colony forming unit in roll tube.

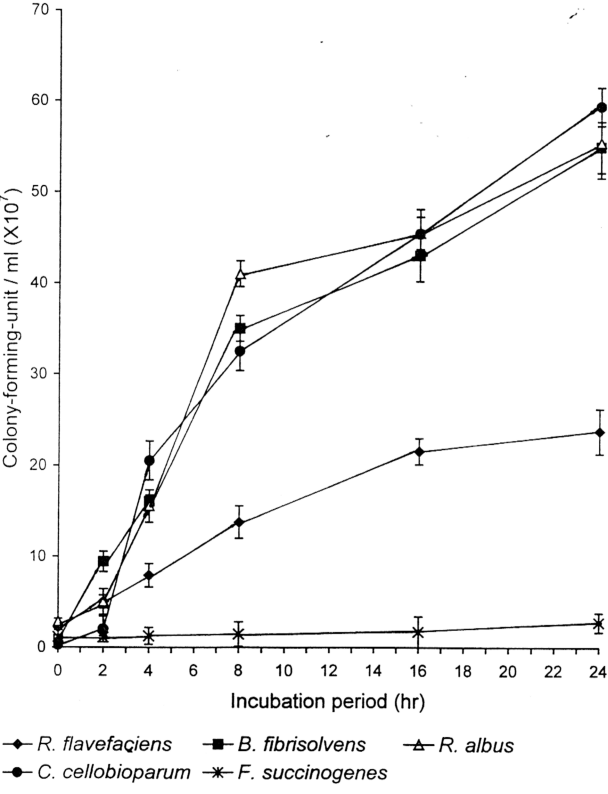


Figure 4.1b: Growth of cellulolytic bacteria species in POME extract broth medium with addition of acid-swollen cellulose and crystalline cellulose. Cell number was counted from colony forming unit in roll tube.

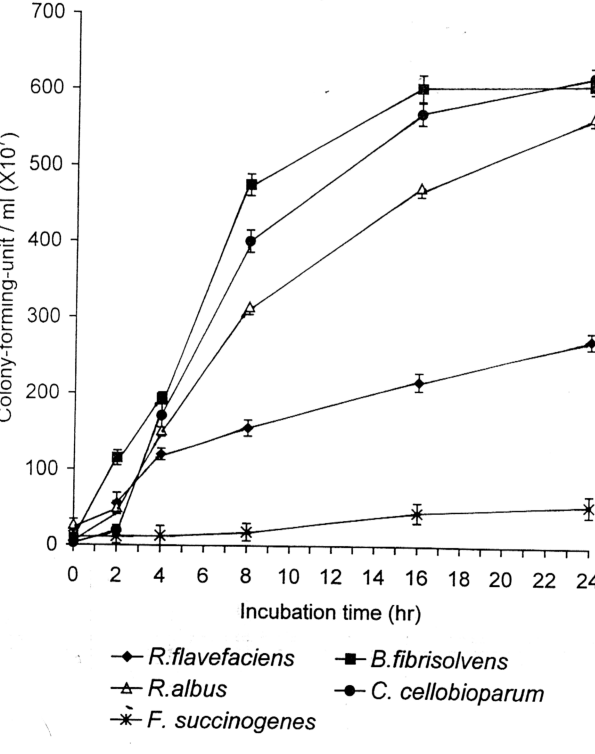
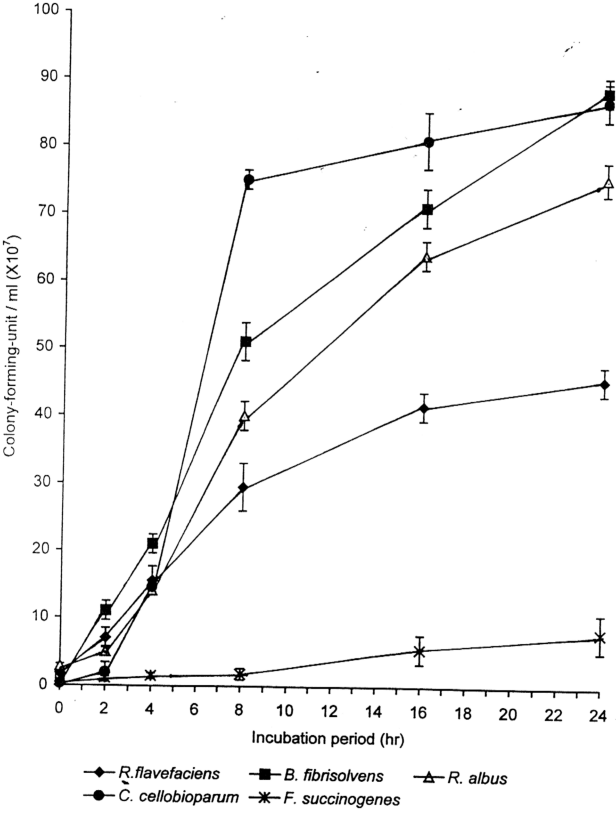


Figure 4.1c: Growth of cellulolytic bacteria species in POME broth medium. Cell number was counted from colony forming unit in roll tube.



4.2.2.3. Cellulolytic activity

In POME extract (Figure 4.2a) cellulolytic activity was detected only for *R. flavefaciens*. Sharp increase was observed from 0 to 8 hour incubation. Further increase occurred until 16 hours whereafter the value was constant until 24 hours.

In situation where POME extract was added with celluloses, cellulolytic activity was detected from 4 hours incubation in *R. flavefaciens*, from 8 hours in *R. albus*, and from 16 hours in both *C. cellobioparum* and *B. fibrisolvens* (Figure 4.2b). At 8 hours of incubation *R. flavefaciens* had higher activity than *R. albus*. After a sharp increase in cellulolytic activity of *R. albus* from 8 to 16 hours of incubation, its activity exceeded that of *R. flavefaciens* at 16 to 24 hours. Cellulolytic activity for both *C. cellobioparum* and *B. fibrisolvens* were much lower compared to *R. albus and R. flavefaciens*. However, *F. succinogenes* showed no cellulolytic activity up to 24 hours incubation.

When POME was used, cellulolytic activity was highest for *R. flavefaciens* at 8 hours incubation period followed by *R. albus* (Figure 4.2c). However, no cellulolytic activity was detected for *C. cellobioparum, B. fibrisolvens*

or *F. succinogenes*. From 8 to 16 hours, *R. flavefaciens* and *R. albus* showed further increase in cellulolytic activity with the former being higher. Cellulolytic activity for *C. cellobioparum* and *B. fibrisolvens* were detected at 16 hour but was very low when compared to the *R.flavefaciens* and *R. albus*. From 16 hour to 24 hours, only *R. flavefaciens* exhibited increase in cellulolytic activity. The cellulolytic activity in *B. fibrisolvens*, *R. albus*, and *C. cellobioparum* was the same as the value at 16 hours incubation. Cellulolytic activity for *F. succinogenes* was observed only from 24 hours of incubation.

4.3. DISCUSSION

POME harbours cellulolytic, amylolytic, proteolytic and lipolytic bacteria (Shaiful *et al*, 1987). Proximate analysis of POME in this study indicates that the POME could support growth of bacteria since it contains relatively high nitrogenous matter and soluble sugar. In the present study, POME supports growth of all selected rumen cellulolytic bacterial species. Except *F. succinogenes*, rapid increase in count was observed during first eight hours of incubation.

Figure 4.2a: Cellulolytic activity in culture broth of cellulolytic bacteria species grown in POME extract broth. Cellulolytic activity was tested against acid-swollen cellulose.

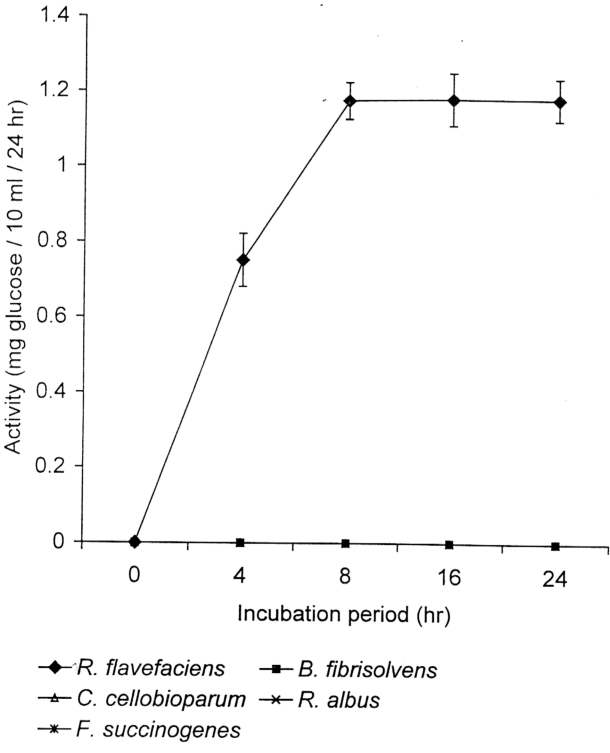


Figure 4.2b: Cellulolytic activity in culture broth of cellulolytic bacteria species grown in POME extract broth with addition of acid-swollen cellulose and crystalline cellulose. Cellulolytic activity was tested against acid - swollen cellulose.

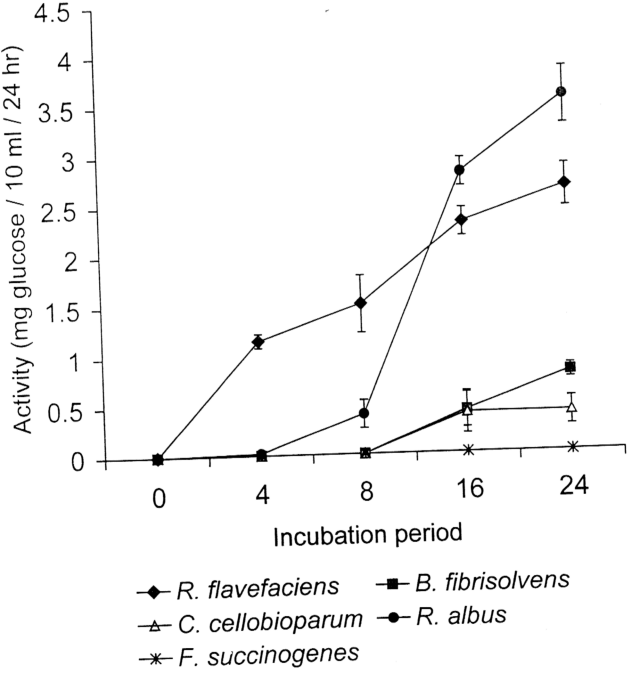
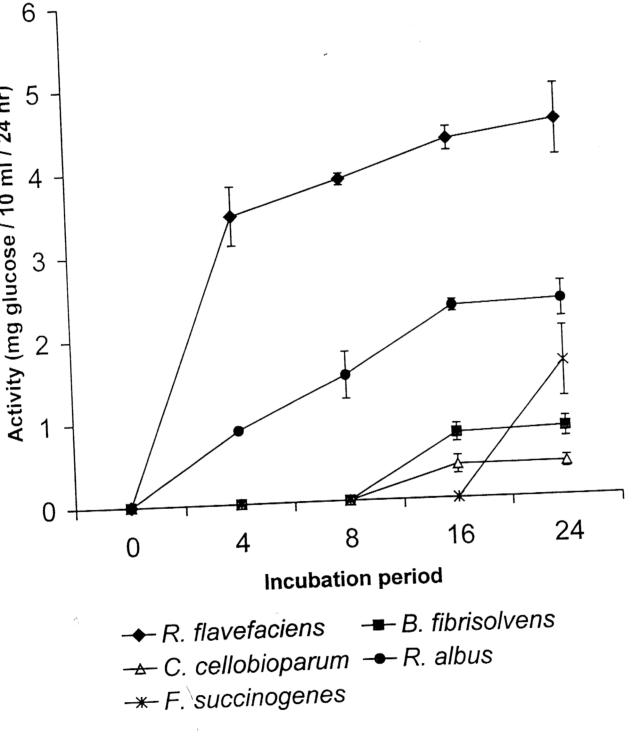


Figure 4.2c: Cellulolytic activity in culture broth of cellulolytic bacteria species grown in POME broth. Cellulolytic activity was tested against acid-swollen cellulose



Carbohydrate was the major form (70%) of organic matter in POME with extractable carbohydrate amounting to 70.5% of the total carbohydrate (Shaiful *et al*, 1987). Analysis of POME content by Abdul Manan (1987) shows that POME contains 13% neutral carbohydrates. Glucose, utilizable by most rumen cellulolytic bacteria, is the major form of neutral carbohydrates (65.6%). Other sugars in POME are xylose (19.2%), Mannose (6.5%), Arabinose (4.7%) and galactose (4.0%).

In the present study, cellulolytic activity of *B. fibrisolvens*, *C. cellobioparum* and *F. succinogenes* were detected only after 16 hours of incubation when the growth rate decreased due to depletion of fermentable carbohydrate (Russell *et al*, 1978). Rapid increase of cellulolytic activity of *R. albus* after 8 hours of incubation was also corresponding to decrease in growth rate.

Higher count attained by *B. fibrisolvens* and *C. cellobioparum* might be due to the variety of sugars that these bacteria could ferment (Stewert & Bryant, 1988). *C. cellobioparum* isolate used in this study (isolate no. 14) ferment 11 out of 12 sugars tested including the three major sugars in POME (glucose, arabinose, and mannose) *and B. fibrisolvens* isolate (isolate no. 28) ferment 9 out of 12 sugars tested. They also have been found to be amongst the

most numerous cellulolytic bacteria in rumens under certain dietery conditions (Prins *et al*, 1972; Gouws and Kistner, 1965; Latham *et al*, 1971).

Unlike *C. cellobioparum* and *B. fibrisolvens*, *R. albus*, *R.flavefaciens* and *F. succinogenes* have limited ability to utilize various sugars (Stewert & Bryant, 1988). They do not ferment arabinose and xylose found in POME. Limited versatility in using the sugars caused their lower count and led them to be more dependent on cellulose digestion than other rumen cellulolytic species (Hungate, 1966). However, *R. albus* grew better than *R.flavefaciens* in all media. This difference in growth rate has also constantly been observed by Gylswyk *et al* (1971) and they thought it would be due to difference in nutrient requirements or else due to difference in genetic make-up.

Cellulolytic activity showed by *B. fibrisolvens in vitro* was consistently low as similarly observed by Van Gylswyck *et al* (1971). Their extent of solubilization of cellulose was less efficient than the *Ruminococci* (Kock and Kistner, 1969) and the majority of *Butyrivibrio* strains described were not cellulolytic (Bryant and Small, 1959). Low cellulolytic activity but high population number attained in POME suggested that other carbohydrate sources than cellulose were utilized.

Although *R.flavefaciens* and *R. albus* grew less rapidly than *B.fibrisolvens* and *C. cellobioparum,* both showed very much higher cellulolytic activity. Unlike *R. albus* which cellulolytic enzyme was not detected when grown in POME extract, *R. flavefaciens* showed cellulolytic activity in all POME media. This bacterium was reported to secrete both exoglucanase and endoglucanase into culture fluid even in glucose or cellobiose-containing media (Gardner *et al,* 1987). When the cellulolytic activity was expressed as per million of cells, same rate of cellulolytic activity was found during first 16 hours in all media before it slightly dropped at 24 hour. This situation was similarly observed by Hiltner and Dehority (1983) who found that the rate of cellulose digestion by *R. flavefaciens in vitro* was similar between 10 and 21 hours after which the rate of cellulose digestion slowed down. Lowest cellulolytic activity value achieved in POME was found to be comparable to the highest cellulolytic activity achieved in PE+C. High cellulolytic activity in POME could be related to the presence of higher soluble sugar in POME that resulted in rapid increase in bacterial number and hence higher cellulolytic activity. Ability of *R. flavefaciens* to produce cellulase in all media supports the suggestion by Pettipher *et al* (1979) that cellulase production in this bacterium is constitutive.

Cellulase of *R. albus* was not detectable in POME extract medium but detectable when grown in media containing cellulose (PE+C and POME). Cellulolytic activity calculated per million of cells showed that the cellulolytic activity was about constant in POME but increase markedly in PE+C. It was calculated that PE+C has higher cellulose concentration (1%) as compared to POME (0.02%). This situation suggest that presence of higher concentration of cellulose may trigger more cellulase secretion as similarly been found by Ohmiya, Nokuza and Shimidzu (1983). Higher cellulase activity in the media containing cellulose was in agreement with the suggestion by McGavin *et al* (1990) that in the presence of cellulose the distribution of the endoglucanase activity shifts from cell-associated to more approximately equal to the amount of cell-associated and extracellular activities. The results support the suggestion that cellulase enzymes *in R. albus* are subjected to either induction or repression (Greeve *et al*, 1984).

Cellulolytic activity of *F. succinogenes* was not detectable in cell-free culture fluid of POME extract and POME extract with cellulose and a very low cellulolytic activity was detected at the later growth stage in POME medium. Maglione, Russell and Wilson (1997) reported that cell-free culture broth of this bacterium was unable to produce reducing sugar from ball-milled cellulose or

crystalline cellulose and extracellular exoglucanase has never been identified from this bacterium. Although cellulolytic activities were found in cells grown on glucose, cellobiose or cellulose medium (Huang & Forsberg, 1990), endoglucanase activity was primarily cell-associated in cultures grown on soluble sugars (McGavin *et al*, 1990) and there was a lag in the digestion of cellulose when either glucose or cellobiose was present in conjunction with cellulose (Huang *et al*, 1990). The released endoglucanase was found bound tightly to cellulose particle (McGavin *et al*, 1990). Thus, very low cellulolytic activity of this bacterium as assayed in this study does not reflect the inability of this bacterium to utilize cellulose in POME.

In conclusion, important cellulolytic bacteria such as *R. albus, R. flavefaciens*, and *F. succinogenes* could grow and express their cellulase in POME. However, their growth rate is lower as compared to less important cellulolytic bacteria such as *C. cellobioparum and B. fibrisolvens*. Cellulose concentration in POME is too low for maintenance of several species of important cellulolytic bacteria and high concentration of readily utilizable soluble carbohydrates in POME may promote rapid growth of less important cellulolytic bacteria and hence lowering the pH.