CHAPTER 3

ISOLATION AND IDENTIFICATION OF CELLULOLYTIC RUMEN BACTERIA

3.0. INTRODUCTION

The rumen harbours diverse groups of bacterial population and most species of the rumen bacteria such as the cellulolytic bacteria ferment more than one kind of substrate. However, the presence of active cellulolytic bacteria population is still the key to the successful utilisation of fibrous feed materials by the ruminants.

It was shown in Chapter 2 that usage of the *Vigna* bean sprout wastes caused increase in the rumen bacterial population whereas the usage of POME caused the decreased in the population. Feeding the goats with high concentration of soluble carbohydrate diet may inevitably eliminate some of the cellulolytic bacteria population and as such the representative bacteria may not be present in the *Vigna* bean wastes or the POME-based concentrate (Abdul Manan, 1987). Apart from the latter and due to feasibility reason, only the cellulolytic rumen bacteria from the normal grass (Napier grass) fed goat was further characterised.

3.1. ISOLATION OF CELLULOLYTIC RUMEN BACTERIA

3.1.1. Materials and methods

A total of 220 colonies of bacteria from the roll tubes (section 2.1.1 f) inoculated with rumen samples from goat fed with Napier grass were selected. All the colonies from roll tubes with 30-60 colonies and half the total colonies from roll tubes with 60-100 colonies were selected and subcultured into pre-reduced slant of CM medium (section 2.1.1 d). The slants were incubated for 3 days at 37°C.

Viable culture from each of the slants was suspended in anaerobic dilution solutions, vigorously vortex mixed for 3 minutes and the number of cells per ml was estimated using phase contrast microscope. The suspension was serially diluted and 0.1 ml samples from each dilution tubes which contained 10-100 cell per ml were rolled into triplicates molten cellulose agar (Latham and Wolin, 1978).

The composition of the cellulose agar is as follows: K_2HPO_4, 0.45g; NaCl, 0.9g; $(NH_4)_2SO_4$, 0.9g; $MgSO_47H_2O$, 0.19g; cystein-HCl, 0.5g; Na_2CO_3, 4g; resazurin, 0.0001g; Trypticase, 5g; yeast extract, 1g; Agar, 10g; cellulose, 2g; 1 ml. of volatile fatty acid mixture (section 2.1.1 d), and 1000 ml distilled water.

The cellulose roll tubes were then incubated for 14 days at 37°C. Single colony or duplicate colonies from the roll tubes with more than one viable cultures were each re-inoculated into medium 10 broth (Caldwell *et al*, 1966). The isolates were checked for purity by comparing the colony morphology of cultures from the same tube (Dehority, 1969).

Medium 10 broth has the following composition: Trypticase, 2g; yeast extract, 0.5g; glucose, 0.5g; cellobiose, 0.5g; starch, 0.5g; hemin, 0.0001g; resazurin, 0.0001g; K_2HPO_4, 0.45g; KH_2PO_4, 0.45g; $(NH_4)_2SO_4$, 0.9g; NaCl, 0.9g; $MgSO_47H_2O$, 0.19g; $CaCl_22H_2O$, 0.09g, 3.1 ml of VFA mixture (section 2.1.1 d), and 1000 ml distillled water.

The inoculated tubes were incubated for 3 days at 37°C. The cultures were again serially diluted in triplicates, rolled in cellulose agar medium and single colony from each tubes was reisolated and incubated at 37°C in Medium 10 broth. The culture purification steps were repeated and the pure cultures were picked and stocked in CM medium slant for further use.

3.2. DETERMINATION OF CELLULOLYTIC ACTIVITY

3.2.1. Materials and methods

The pure cultures were inoculated into cellulose broth (Latham *et al*, 1978) and incubated for 5 days. The incubated cellulose broth was centrifuged at 4000 *g* for 20 minutes. The supernatant was used as crude cellulase preparation in cellulase assay as described in section 2.2.3a.2 using two types of cellulose as substrate:

1. Acid swollen-cellulose (Section 2.2.3.a1).
2. Microcrystalline cellulose (Sigma).

3.2.2. Results

Only 26 isolates showed positive cellulolytic activity using acid swollen cellulose or/and microcrystalline cellulose. Cellulolytic activity of the isolates is shown in table 3.1

Table 3.1.

230 bacterial isolates were grown in cellulose broth. After incubation, the culture broth was tested for cellulolytic activity for swollen cellulose and crystalline cellulose. Only 26 isolates showed positive cellulolytic activity as shown in this table.

	Cellulolytic activity (mg glucose/ 10 ml/ 24 hours)			
	swollen cellulose		crystalline cellulose	
Isolate	Mean	Std. error	Mean	Std. error
14	143.83	39.54	47.17	15.25
15	162.00	31.37	74.33	15.00
19	83.00	9.02	65.00	8.17
110	33.88	4.51	66.83	9.99
112	73.58	9.16	72.50	4.34
113	128.33	29.34	63.50	2.45
114	113.17	12.30	78.50	9.47
115	124.00	14.20	156.00	21.19
119	57.33	17.83	48.50	15.86
24	51.00	9.69	43.17	4.68
25	40.17	10.75	78.67	5.97
29	67.50	8.30	45.67	10.02
211	76.00	12.19	66.83	14.17
212	34.50	15.10	50.67	12.98
216	135.33	38.40	59.50	10.74
32	94.66	33.50	78.00	11.31
33	87.00	2.96	76.17	2.86
34	138.83	25.96	61.33	25.08
35	47.66	5.36	56.33	9.48
36	229.83	36.11	48.17	4.12
37	126.83	20.85	62.00	17.21
311	45.67	16.70	72.33	5.12
314	105.33	27.20	80.33	12.25
315	58.33	8.98	72.17	1.77
316	127.00	16.52	16.35	3.31
317	32.00	10.01	19.17	5.64

3.3. IDENTIFICATION OF CELLULOLYTIC ISOLATES

The key for the identification of cellulolytic rumen bacteria is based on the list in the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), the 4th edition of Anaerobes Laboratory Manual (Holdeman, Cato, and Moore, 1977) and the Atlas of Rumen Microbiology (Ogimoto and Imai, 1981).

The first step for identification of the rumen bacteria (Cowan and Lister, 1974) is to have a pure culture and this was established in section 3.1.1. The next step is to determine the Gram reaction, cellular morphology, cell size, motility, spore formation, and catalase production followed by analysing the volatile fatty acid and alcohol production. At this stage, a cluster of gene a can be listed. The final step is to check for ability of ' nera to ferment various sugars.

3.3.1. Celllar morphology, Gram Reactio , Spore formation, motility and catalase production

3.3.1.1. Materials and methods

Cellular morphology, cell arrangement, si , motility, and Gram reaction were determined at 1 day, 2 day , 4 days, and 7 days of incubation. Four replicates of the pu e cultures were

inoculated into medium 10 broth (Caldwell *et al*, 1966) and incubated at 37°C. Gram reaction was done using Hucker's method (Gerhardt, 1981), cell shape, arrangement, and size was determined from a wet mount (Carpenter, 1977), the presence of endospore was determined by Schaeffer-Fulton method (Gerhardt, 1981), and motility was observed by the hanging drop method (Carpenter, 1977). Catalase was determined by adding 3% hydrogen peroxide solution into the culture broth and observed for gas release (Bailey and Scott, 1974).

3.3.1.2. Results

The results for Gram reaction, cell shape, the presence of endospore, motility and catalase of the isolates is shown in table 3.2. All cultures were catalase negative. 12 isolates were either coccus or ovoid/elongated coccus with positive Gram reaction for 24 hours culture with the tendency to show weak negative reaction., nonmotile and did not have endospore; 10 isolates were Gram negative rods or short rods, nonmotile and without endospore; 3 Gram negative motile curve rod; 2 Gram positive rods without endospore and a Gram negative rod with endospore.

Table 3.2.

26 isolates that showed positive cellulase production were further characterised. Standard procedures for bacterial characterization and identification was carried out including Gram stain, spore stain, motility and catalase production.

Isolate	Cell shape	Gram	Spore	motility	catalase
110	eC	V	-	-	-
24	eC	+	-	-	-
311	eC	V	-	-	-
15	C	+	-	-	-
115	eC	V	-	-	-
211	eC	V	-	-	-
314	eC	+	-	-	-
315	eC	+	-	-	-
19	C	+	-	-	-
29	eC	+	-	-	-
35	eC	+	-	-	-
316	eC	+	-	-	-
112	sR	-	-	-	-
113	sR	-	-	-	-
114	R/sR	-	-	-	-
216	sR	V	-	-	-
32	sR	-	-	-	-
34	sR	-	-	-	-
37	sR	-	-	-	-
317	R/sR	-	-	-	-
119	cR	-	-	-	-
28	cR	-	-	-	-
36	cR	-	-	-	-
212	R	+	-	-	-
33	R	+	-	-	-
14	R	-	+	-	-

(+) denotes Gram-positive, spore former, motile or catalase producer;

(-) denotes Gram-negative, nonsporing, nonmotile and or non catalase producer

(v) denotes Gram variable reaction

(R) denotes rod shape of > 1 µm long

(sR) denotes rod shape of <1 µm long

(C) denotes coccus shape

(eC) denotes elongated coccus shape

(cR) denotes curve-rod shape

3.3.2. Volatile Fatty acids and alcohol productions

3.3.2.1. Materials and Methods

In order to determine the volatile fatty acids pattern and alcohol production, cultures were inoculated into cellulose broth and incubated for 5 days at 37°C. After the incubation period, the cultures were centrifuged at 2500 *g* and the supernatant was used in the determination of VFA as described in section 2.2.2a. Alcohol content was also determined by Gas-chromatography using column 5% Carbowax on 80/120 Carbopack B AW (Supelco Inc.) with column temperature 170°C and nitrogen flow rate of 20 ml/min. Peaks resolved were compared with peak produced by standard methanol and ethanol at concentration of 79 µg/µl and 78 µg/µl respectively.

3.3.2.2. Results

VFA production and alcohol production are shown in table 3.3. Twenty of the isolates produced acetate as major fermentation product, 3 isolates have formic and butyrate as the major end products of fermentation. One of the isolates has formic alone as the major product whereas one isolate did not have any major fermentation products.

Table 3.3: **26 cellulolytic isolates were grown in cellulose broth. 5 days after incubation, the culture broth was analysed for fermentation products such as volatile fatty acid (VFA) (acetic, formic, propionic, butyric, and valeric acid) and alcohol (ethanol and methanol) content using gas chromatography. The concentration of each acid or alcohol produced by the isolate was expressed in millimole per litre. Acid that is more than one millimole per litre is taken as major fermentation product.**

	Fermentation products	
Isolates	Major (> 1 mmolar)	Minor (< 1 mmolar)
110	Acetic	Ethanol
24	Acetic	Ethanol
311	Acetic	Ethanol
15	Acetic	ND
115	Acetic	Formic
211	Acetic	Formic
314	Acetic	ND
315	Acetic	ND
19	Acetic	ND
29	Acetic	ND
35	Acetic	Formic
316	Acetic	ND
112	Acetic	ND
113	Acetic	Formic, Propionic
114	Acetic	Formic
216	Acetic	ND
32	Acetic	ND
34	Acetic	ND
37	Acetic	ND
317	Acetic	ND
119	Formic, Butyric	ND
28	Formic, Butyric	Ethanol
36	ND	Formic, Butyric, Ethanol
212	Formic, Butyric	ND
33	Formic	Butyric
14	Acetic	ND

ND = Not detected

3.3.3. Carbohydrates utilisation

3.3.3.1. Materials and methods

Cellulolytic isolates were tested for their ability to utilise the following carbohydrates: Glucose, lactose, sucrose, maltose, arabinose, starch, cellobiose, mannose, melezitose, rhamnose, sorbitol and trehalose. The isolates were inoculated into medium 10 broth and incubated for 3 days. The cultures were serially diluted and rolled in molten cellulose agar (Latham *et al*, 1978) and incubated for 5 days. Several colonies were picked with the aid of 1-ml syringe attached with needle filled with 0.25 ml anaerobic dilution solution. A drop of the dilution solution was layered over the colony, mixed by touching the tip of the needle and sucked into the syringe. This was repeated with other colonies until satisfactory turbidity of the inoculum was obtained. The inoculum was transferred to 5 ml prereduced dilution solution contained in 14 ml-bottle and gassed with oxygen-free carbon dioxide. 0.1 ml of the diluted inoculum was inoculated into 4 ml of prereduced media containing test sugar contained in 7 ml-bottle, gassed and capped. The media had the following constituents: Trypticase, 5g; yeast extract, 5g; NaCl 2.5g; L-tryptophan, 0.2g; L-cystein, 0.4g; hemin, 0.01g, bromocressol purple, 0.02g (API 20A Instruction Manual, API System) and 1000 ml distilled water. The inoculated bottles were incubated at 37^{o}C and measured at 24 hours and 48 hours for acid formation.

3.3.3.2. Results

The results for the fermentation of various carbohydrates are shown in table 3.4. All the tested isolates were capable of fermenting cellobiose and glucose to acid. All the Gram positive cocci and elongated cocci and the Gram positive rods were not able to ferment starch to acid. Seven of the nine Gram negative rods and short rods, all the curve rods and the Gram negative endospore forming rods fermented starch to acid.

3.3.4. Characterisation of isolates

The ability of the 26 isolates to produce cellulase, the absence of catalase and their inability to grow in the presence of oxygen indicated that they were obligate anaerobic cellulolytic bacteria. Using the results of the above tests, the isolates were characterised as shown in table 3.5. Isolates 110, 24, 311, 15, 115, 211, 314, 315, 19, 29, 35 and 316 were Gram positive coccus or slightly elongated coccus, nonmotile and did not possess endospore indicating that they were *Ruminococcus*. Isolate 110, 24 and 311 produced acetate and ethanol and as such were *R. albus*. Nine of the isolates 15, 115, 211, 314, 315, 19, 29, 35, and 316 produced only acetate and formate and as such were *R. flavefaciens*.

Table 3.4:

Cellulolytic isolates were tested for its ability to ferment various carbohydrates including Arabinose (Ara), cellobiose (Cel), lactose (Lac), maltose (Mal), mannose (Man), mellibiose (Mel), rhamnose (Rha), sorbitol (Sor), sucrose (Suc), trehalose (Tre), glucose (Glu), and starch (Sta). Test was carried out in medium containing bromocressol purple (pH indicator). Fermentation of carbohydrate produces acid/s and this will turn the pH indicator to yellow (+). If the carbohydrate is not fermented, the fermentation medium remain purplish (-).

	Ara	Cel	Lac	Mal	Man	Mel	Rha	Sor	Suc	Tre	glu	Sta
14	+	+	+	+	+	-	+	+	+	+	+	+
15	-	+	-	-	-	-	-	-	-	-	+	-
19	-	+	-	-	+	-	-	-	-	+	+	-
110	-	+	-	+	+	-	-	-	-	-	+	-
112	-	+	-	-	-	-	-	-	-	+	+	+
113	-	+	-	-	+	-	-	-	-	-	+	+
114	-	+	-	-	-	-	-	-	-	-	+	+
115	-	+	-	-	-	-	-	-	-	-	+	-
119	-	+	+	-	-	-	-	-	-	+	+	+
24	-	+	+	-	-	+	-	-	-	-	+	-
28	+	+	+	-	-	-	+	+	+	-	+	+
29	-	+	-	-	+	-	-	-	+	-	+	-
211	-	+	-	-	+	-	-	-	-	+	+	-
212	+	+	-	-	+	+	-	+	-	-	+	-
216	-	+	-	+	+	-	-	-	-	-	+	+
32	-	+	-	-	-	-	-	-	-	-	+	+
33	+	+	-	+	+	+	-	-	-	+	+	-
34	-	+	-	+	+	-	-	-	-	-	+	+
35	-	+	-	-	+	-	-	-	+	-	+	-
36	-	+	+	+	+	-	-	-	-	-	+	+
37	-	+	-	+	-	-	-	-	-	-	+	-
311	-	+	-	-	+	-	-	-	+	+	+	-
314	-	+	-	-	+	-	-	-	-	-	+	-
315	-	+	+	+	-	-	-	-	+	-	+	-
316	-	+	+	+	+	-	-	-	-	+	+	-
317	-	+	-	+	+	-	-	-	-	-	+	+

Isolates 112, 113, 114, 216, 32, 34, 37 and 317 were Gram negative rods or short rods, nonmotile and did not possess endospore. They were *Fibrobacter*. Isolates 112, 216, 32, 34, 37 and 317 produced only acetate, and isolate 114 acetate with formate, 113 acetate with formate and propionate and hence were *F. succinogenes*.

Isolate 119, 28 and 36 were Gram negative curve rods, no endospore and motile, indicating that they were either *Butyrivibrio* or *Acetivibrio*. However, the latter is characteristic of sewage sludge and hence the former could be more appropriate since the isolates were obtained from rumen. Isolate 119 produced formate and butyrate as major products, isolate 28 produced formate, butyrate with ethanol as minor product and isolate 36 produced formate, butyrate and ethanol as non major product and as such differed from the fermentation products of *Acetivibrio* which is mainly acetate and ethanol (Coughlan and Meyer, 1991). Hence the isolates were *B. fibrisolvens*.

Isolates 212 and 33 were motile Gram positive rods without endospore, produced formate with butyrate as major or minor product and as such were *E. cellulosolvens*. Isolates 212 and 33 Isolate 14 although gave Gram negative reaction but possessed endospore and as such it was *Clostridium*. It was C. cellobioparum since it produced acetic acid as the major fermentation product.

Table 3.5:

Based on various characterisation tests done including Gram stain, cellular morphology, spore stain, motility, catalase production, volatile fatty acids and alcohol production in cellulose broth, and fermentation of various carbohydrates, the 26 cellulolytic isolates were identified.

Cellulolytic bacteria	Isolates
Ruminococcus albus	110, 24, 311
Ruminococcus flavefaciens	15, 115, 211, 314, 315, 19, 35, 316, 29
Fibrobacter succinogenes	112, 113, 114, 216, 32, 34, 37, 317
Butyrivibrio fibrisolvens	119, 28, 36
Eubacterium cellulosolvens	212, 33
Clostridium cellobioparum	14

However, some of the isolates in this study differed in their ability to ferment some of the listed carbohydrates in Bergey's Manual, Anaerobes Laboratory Manual and Atlas of Rumen Microbiology. This could probably be attributed to differences at strain level. Some of the *Ruminococcus* could ferment lactose, maltose, mannose and sucrose but not starch. Some of the *Bacteroides* could ferment maltose, mannose and most could ferment starch but not

lactose or sucrose. The *Butyrivibrio* could ferment starch but not mannose, the *Eubacterium* could ferment lactose, sucrose and starch. However, the *Clostridium* could ferment all the test carbohydrates except melezitose.

3.4. DISCUSSION

Although 220 isolates were screened in this study only 26 of the isolates showed cellulolytic activity on crystalline cellulose and swollen cellulose. All common ruminal cellulolytic bacteria species were found in the ingesta of rumen fed with Napier grass but differ in proportion of each individual species. Five cellulolytic genera were identified. Ruminococci was the most numerous cellulolytic bacteria isolated (46.15% of the total isolates) which comprised of 11.54% *R. albus* and 34.62% *R. flavefaciens*. *F. succinogenes* was the second largest number of species isolated which constitute 30.77% of the total isolates. *Butyrivibrio, Eubacterium* and *Clostridium* occurred in low number under present dietary condition, constitute 11.54%, 7.69% and 3.85% of the total cellulolytic bacteria respectively.

For the ***Ruminococcus***, 2 out of 3 of the *R. albus* showed higher activity against crystalline cellulose compared to the swollen cellulose with average activity on the former

being 39% higher than the latter. However for *R. flavefaciens*, 3 out of 9 isolates showed higher activity on the crystalline cellulose but the average activity on swollen cellulose was 18.1% higher than the crystalline cellulose. In *Eubacterium* and *Butyrivibrio*, one isolate from each genera had higher activity for crystalline cellulose but all the *Fibrobacter* and *Clostridium* isolates showed higher activity for swollen cellulose.

This difference could probably be attributed to the difference in cellulase enzymes components produced by different isolates. Endoglucanase is produced by all cellulolytic organisms, which is responsible for the digestion of carboxymethylcellulose and swollen cellulose. However, digestion of crystalline cellulose requires exoglucanase, which combine its action with endoglucanase. In the digestion of cellulose, the rumen bacteria adhered tightly to the cellulose and released of cellulase into the surrounding medium may differ between the bacteria species. Leatherwood (1965) reported that as much as 58% of the cellulase was released from the cells of *R. albus*. However, Hungate (1947); Gong and Forsberg (1989) and Coughland and Meyer (1991) described that the cellulase of *F. succinogenes* was mainly cell bound. As such, determination of cellulase activity from cell-free supernatant as was carried out in this work could have excluded the cell bound cellulase.

The composition of cellulolytic rumen bacteria reflected that of a normal rumen. *Ruminococcus* sp. are

usually the most abundant cellulose digesting bacteria in ruminant on good practical diets, with or without concentrate (Kistner and Gouws, 1964; Bryant and Burkey, 1953) and was more dominant than the Gram negative rod (Warner, 1962; Giesecke, 1970). Both *R. albus* and *R. flavefaciens* may occur together in approximately equal number or as in the present studies the latter predominate (Hungate, 1950; Kistner *et al*, 1965; Varel and Dehority, 1989).

In animal on high roughage diet of low protein content, *F. succinogenes* or *Butyrivibrio* sp. would be more numerous [Geisecke, 1970 (table 3.6); Bryant *et al*, 1953; Bryant and Doetsch, 1954; Latham, Sharpe and Sutton, 1973; Varel *et al*, 1989]. It was generally recognised that Ruminococci digested the more readily degradable form of cellulose, whereas *F. succinogenes* could also digest the more resistant cellulose (Dinsdale, Morris and Bacon, 1978). The composition of the ration was recognised as being the most important single factor influencing both the total number and the proportion of different species of rumen bacteria since no significant difference was observed between bison and cattle fed the same type of diet (Towne, Nagaraja, Cochran, Harmon, Owensby and Kaufman, 1988).

Cellulolytic bacteria in rumen of goat fed with PBC was not enumerated and characterised. It was done by Abdul Manan (1987). He found that feeding PBC to goat had caused lower number of total bacteria and fewer cellulolytic species in the rumen. *Ruminococcus* sp., *F. succinogenes*, *E.*

cellulosolvens, *B. fibrisolvens* and *C. cellobioparum* were isolated from rumen of Napier grass fed goat but *B. succinogenes* and *F. succinogenes* were absent in 10^{-8} dilution of rumen ingesta of goat fed with PBC. *Butyrivibrio*, *Eubacterium* and *Clostridium* occurred in the same proportion in the rumen of goat fed with the latter diet.

Table 3.6: Occurrence of cellulolytic bacteria in the rumen of sheep with different diets (Giesecke, 1970)

Diet	Occurrence of bacteria
Teff hay	68% Gram-negative rod, 17% Gram-positive cocci
Lucerne Hay	16% gram-negative rod, 84% gram-positive cocci
Wheat straw	93% gram-negative rod, 7% *Butyrivibrio* sp.
Teff hay	36% *R. flavefaciens*, 39% *R. albus*, 24% *Butyrivibrio* sp.
Wheat straw - urea + molasses	69% Ruminococci, 18% *F. succinogenes* 13% *Butyrivibrio* sp.

In conclusion, the rumen of goat fed with *Pennisetum purpureum* contained all important cellulolytic bacteria species. More active cellulolytic species such as the ruminococci and *Fibrobacter succinogenes* are more dominant than the less active cellulolytic species such as *Butyrivibrio* spp, *Eubacterium* spp. and *Clostridium* spp. This composition of cellulolytic bacterial population reflects that of a normal rumen.