CHAPTER 3

MATERIALS AND METHODS

3.1 Sources of Microorganisms

3.1.1 Sources of Cellulolytic Bacteria

Bacterial sources in these studies were :

(i) digestive fluids. Samples were collected from an anaerobic digester, semi-continous type, seeded with live stock manure and used pineapple peel as digesting substrate, at the Department of General Science, Faculty of Science, Chulalongkorn University. Samples were obtained during December 1982 through February 1983.

(ii) rumen contents. Samples were collected immediately from ruminant carcasses that had recently been killed at the Bangkok Livestock Trading Limited (The assistance of the staff members of this company in obtaining these samples is greatfully acknowledged). Samples were obtained during December 1982 through March 1983.

3.1.2 Sources of Methanogens

Methanogenic sources in these studies were :

(i) digestive fluids. Samples were collected in the same manner as above and obtained during December 1982 through February 1983.

(ii) animal dungs. samples of hen dung and pig dung were collected from the Chicken Co-operative and Pig Sty, respectively, at the Faculty of Agricultural Science, Kasetsart University, Bangkok Campus (The assistance of the staff members of this University in obtaining these samples is kindly acknowledged). Samples were obtained in February 1983.

3.2 Raw Materials and Sources

Pure cellulose or 99.5% α - cellulose fiber (Sigma, St.Louis, Missouri, U.S.A.) and some cellulosic wastes were used as the substrates. Five from seven kinds of cellulosic wastes were chosen. Those were :

(i) straw (Oryza sativa). Samples were collected from paddy field in Chacheongsao Province.

(ii) pineapple peel (Ananas comosus). Samples were collected from a cannery in Cholburi Province.

(iii) bermuda grass (Cynodon doctylon). Samples were collected from lawn in front of Main Hall in Chulalongkorn University.

(iv) water hyacinth (Eichharnia crassipes). Samples were collected from small canal along Wipavadee Road at Din Daeng area.

(v) waste paper. Samples were collected from the document digester machine of the Bank of America, Suriwong Branch.

And other two raw materials were bagasses (Saccharum officinarum) and corn cob (Zea mays). Samples of each were collected from Samyan Market. All samples of each were collected periodically, the pool sample were proceeded. Ground or small pieces of the cellected materials were dried at 70°C. After overnight drying, they were allowed to cool and weighed immediately. All dried materials were determined for cellulose content. Those are 25.03, 70.79, 44.69, 24.39, 19.36, 38.34 and 28.45% in pineapple peel, paper, straw, Bermuda grass, water hyacinth, bagass and corn cob respectively.

The amounts of straw, pineapple peel, bermuda grass, water hyacinth and waste paper were 30, 50, 50, 50 and 20 mg., respectively, and used as substates for the biogas production.

3.3 Media*

3.3.1 Media for General Bacteria

Eosin-methylene blue agar (EMB; Difco, Detroit,

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*Formula and preparations of these media are shown in Appendix B

Michigan, U.S.A.), nutrient agar (NA ; Difco), salmonella-shigella agar (SS ; Difco), triple sugar iron agar (TSI ; Difco), mannitol salt agar (MSA ; Difco) and streptococci fecalis agar (SFA ; Difco), were the solid media.

3.3.2 Media for Cellulolytic Bacteria

Rumen fluid cellulose agar (RFCA ; 46) was the solid medium. Fluid growth medium was rumen fluid cellulose broth RFCB ; 46). Stock cultures were maintained on cellulose broth (CB).

3.3.3 Media for Methanogens

Balch's medium I agar (BMA ; 15) was the solid medium. Stock cultures were maintained on Balch's medium I broth (BMB ; 15).

3.3.4 Media for Multiple-strain or Mixed Cultures

VSL medium agar and broth (VSLA, VSLB), versatile media for bacteria in digestive tank presented by Ueki (127) were the solid and fluid growth media, respectively.

3.4 Samples and Cultivation Procedures

Samples of rumen contents were kept in RFCB flasks, sealed with paraffin oil and stopped with a rubber. Samples of rumen fluids were collected by collecting device (Figure 3.1) suggested by Hungate

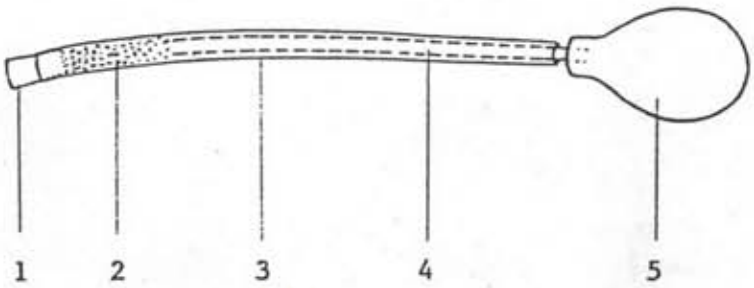


Figure 3.1 Collecting Device.

1. Rubber Stopper
2. Hole (2mm in diameter)
3. Outer Plastic tube
4. Inner Plastic tube
5. Suction Bulb

Figure 3.2 Anaerobic cylinder.

(46), and also samples of digestive fluids drawn from the bottom of digestive tank were kept in culture tubes sealed with oil. Fresh animal dungs were collected. All samples were immediately transferred to anaerobic chamber (Anaerobic System, Model 1024, dual type; Forma Scientific,Marietta, Ohio, U.S.A.), and kept there until proceeded.

3.4.1 Procedures for General Bacteria in Digestive Fluids

Serial dilutions of digestive fluids were spreaded on NA and different selective media for certain bacteria,e.g., EMB for gram-negative, MSA for Bacillus species and staphylococci. NA plates were incubated separately in aerobic and anaerobic conditions but only in aerobic condition for selective plates. The results were read after 24 hour incubation at 37°C.

3.4.2 Procedures for Cellulolytic Bacteria

A loopful amount of rumen contents or digestive fluids was inoculated into RFCB. After incubation at 37°-39°C for 24, 48 and 72 hours, each culture was streaked on RFCA. To preserve moisture, all plates were packed in plastic bags and incubated for three to four weeks. Cellulose-positive colonies with an oppearance suggestive of cellulolytic bacteria were individually picked and identified as cellulolytic bacteria by occurence of typical clear zone in the cellulose medium. Again, other two times on RFCA, pure cultures of cellulolytic bacteria were obtained. Due to strong recommendations, all steps here were done in anaerobic chamber. In addition,all individual isolates were examined

under microscope for morphology and gram reaction aspects. Moreover, all isolates were selected for higher cullulolytic activity by using multipoint inoculator devices (128). The selected strains of cellulolytic bacteria were subcultured every two weeks into fresh CB and kept as stock cultures.

3.4.3 Procedures for Methanogens

Similar to cellulolytic bacteria, all cultivation steps of methanogens were done in anaerobic chamber. A loopful amounts of digestive fluid and two types of animal dungs were inoculated into BMB and incubated at 37°–39°C. After 48 hours, each culture was streaked on BMA. All plates were packed in an anaerobic cylinder (See Figure 3.2). Outside the anaerobic chamber, the cylinders were pressurized to be two atmospheres with H_2 and CO_2 gas mixture in the ratio of 80 to 20, and incubated at 37°C for two weeks. Under ultraviolet (UV) long wave length the colonies of methanogens could be detected in ambient atmosphere. After UV identification, the methanogen plates were immediatly transferred to the chamber again. Certain colonies were individually picked and streaked on BMA. Again, other two times on BMA, pure cultures of methanogens were obtained. Morphology and gram reaction of all isolates were performed microscopically. Isolates with higher activity of methanogenicity were selected by inoculating into BMB. Those tubes were filled with 80% H_2-20% CO_2 gas mixture into the head-spaces (Hungate technique, 46), and incubated at 37°C for 5 days. CH_4 content in the produced gas in the head-spaces were drawn to check

(procedures of gas analysis would be discussed). The selected strains of methanogens subcultured every week into fresh BMB and kept as stock cultures.

3.4.4 Procedures for Mixed Culture

Few dropful amounts of digestive fluids were inoculated into VSLB and incubated at 37°C for two weeks. These mixed culture were transferred every week into fresh VSLB and kept as stock cultures.

3.5 Fermentation of Cellulose by Co-culture and Mixed Culture

In co-culture studies, one-tenth ml of 4-day culture (grown in CB at 37°C) of the tested cellulolytic organisms and 0.1 ml of 4-day culture (grown in BMB at 37°C) of the tested methanogens were inoculated together into tubes contained 10-ml CB plus either 0.1 or 0.2 % cellulose. The inoculum of each organism was about 2-3 x 10^7 cells or about 0.2 - 0.3 x 10^7 cells per ml. All tested organisms were individually inoculated into the control tubes. Comparison with pure culture technique, 0.1 ml of 4-day culture (grown in VSLB at 37°C) of mixed strains was also inoculated into the CB. The inoculum of mixed strains was about 2.3 x 10^8 cells or 2.3 x 10^7 cells per ml. All combinations of co-cultures (Table 3.1), control cultures of the co-culture, mixed culture and control tube were done in three replicates. During the 17-day incubation of those in the actually anaerobic chamber at 37°C, the amounts of fermentatives products, e.g., gases and acids,

Table 3.1 Fifteen co-culture sets of binary combination between three selected cellulolytic bacteria and five selected methanogens.

No. of Co-culture	Cellulolytic + Methangens bacteria
1	CU 1 + Sc 1
2	CU 1 + Sc 2
3	CU 1 + Sc 3
4	CU 1 + Sc 4
5	CU 1 + Sc 5
6	CU 3 + Sc 1
7	CU 3 + Sc 2
8	CU 3 + Sc 3
9	CU 3 + Sc 4
10	CU 3 + Sc 5
11	CU 4 + Sc 1
12	CU 4 + Sc 2
13	CU 4 + Sc 3
14	CU 4 + Sc 4
15	CU 4 + Sc 5

and remained cellulose content, were determined periodically. Samples of gasous products were drawn from gas space of the culture tubes on day 0, 1, 2, 3, 4, 5, 7, 8, 10, 13, 15 and 17. Samples of acid products of each tube were collected 2 times, i.e., on day 0 and at the end of incubation period. The amounts of remained cellulose were determined on day 2, 4, 6, 8, 10, 12, 15 and 20, and pH of fermented slurry was also recorded at the same period.

3.6 Fermentation of Some Cellulosic Wastes by Co-culture and Mixed Culture

The experiment was divided into 2 parts, i.e., actual substrate and pre-treated substrate by 1.0% NaOH solution at 15 pound (lb) per square inch, at 140°C for 45 minutes, and neutralized with 1.0 % HCl solution.

3.6.1 Actual Wastes as Substrates

Four milliliters of Hungate buffer (See Appendix B) were added into each tube contained certain amount of actual substrate, made final volume to 10ml with distilled water, stopped with screw caps and sterilized by autoclaving. After sterilization the substrate tubes were transferred into anaerobic chamber and kept overnight before used. Fermentation of the actual wastes--straw, pineapple peal, bermuda grass, water hyacinth and waste paper, were conducted in the same manner as in 3.5. Instead of the 17-day incubation, all cultivated cultures were incubated for 30 days at 37°C. During the certain

period of incubation in the chamber, the amounts of digestive products, e.g. gases and acids, and remained cellulose contents were determined periodically. Samples of gaseous products were drawn on day 0, 5, 9, 10, 13, 17, 25 and 30. Samples of acid products of each tube were cellected 2 times, i.e., day 0 and 30. The amounts of remained cellulose were determined on day 0, 11, 16, 21 and 30,and pH of digestive slurry was recorded at the same period.

3.6.2 Pre-treated wastes as substrates

Same amounts of each substrate were treated as mentioned before. The pre-treated or treated substrates were used as substrates. Fermentation of treated wastes--straw, pineapple peel, bermuda grass, water hyacinth and waste paper, were performed in the same manner as in 3.6.1.

3.7 Chemical Analysis Procedures

3.7.1 Analysis of Gaseous Products

Determinations of hydrogen, carbon dioxide and methane in biogas samples were monitored by gas-solid chromotographic methods (Using Shimadzu GC 7AG and Chromatography Proportional Counter, Shimadzu C-RIA, Shimadzu, Kyoto, Japan). The condition of chromatopac (129, 130, 131) standard curve, and calculation methods were described (132) in Appendix C.

The Porapak QS column was conditioned overnight before used. After flushing with O_2-free nitrogen gas, 100-µl Harmilton gastight syringe was used to draw gas samples (100 µl). Standard curves of H_2, CO_2 and CH_4 were prepared by using standand gases (Japanese Standard). Under this condition at 25°C, retention times of H_2, N_2, CH_2 and CO_2 were 0.75, 0.87, 1.36 and 2.45 minutes, respectively. The volume of produced gas was determined daily at room temperature, 25°C. Micromoles of methane, carbon dioxide and hydrogen were computed. It is noted that the amounts of gas volumes were not corrected to standard temperature (273°K) computations, so that the quantity of gases might be over estimated by about 8% (32).

3.7.2 Analysis of Acid Products

The quantities of produced acids were conducted by the technique of continuous thin layer chromatography. Thin-layer chromatographic set (Desaga ; Heidelberg,West Germany) was used. The preparation of carrier plates, solvents and spray reagent were performed (See Appendix D).

The analysis procedure of acids in digestive fluids were proceeded (133). Serial steps are :

(i) 6 ml of culture supernatants were acidified to below pH 2.0 by addition of 0.1-ml 10N sulfuric acid.

(ii) 1 ml of diethyl ether was added to the acidified supernant, and the tube was closed tightly.

(iii) shaken thoroughly for 10 min.

(iv) centrifuged at 1,000 G for 5 min.

(v) the ether parts were spotted on the carrier plates and run continuously (134) for 12 hours (See Figure 3.3).

(vi) after 10-minute drying in room temperature, the plates were sprayed with pH indicator and heated at 100°C for 30 min. The volatile acids were detected as pink or yellow spots on a green background (See Figure 3.4).

(vii) the spots were recorded. For differentiation of formic and propionic acid, the plates were sprayed again with 1.0% ammonical silver oxide solution and heated at 100°C for 15 min (See Figure 3.4).

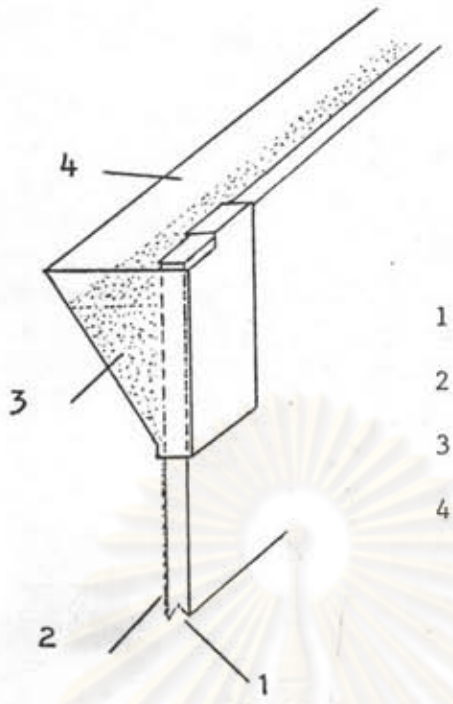
Standard acids (0.2% W/V in diethylether) and the unknowns were run together. For calculation the concentration of unknown acids, various volumes of unknowns were initiated (See Figure 3.5).

The relative distance moves used butyric acids as the arbitary standard were shown in Table 3.2.

Spot areas of unknowns and standards were recorded in square millimeter. The quantities of unknown and standard acids--butyric, formic, propionic, acetic, lactic and succinic acids, were calculated from the spot areas. The method of calculation would be discussed in Appendix D.

3.7.3 Determination of Cellulose Contents

Total or remained contents of cellulose were



1. Glass plate
2. Layer
3. Absorbant
4. Angled trough

Figure 3.3 Continuous thin layer chromatography technique.

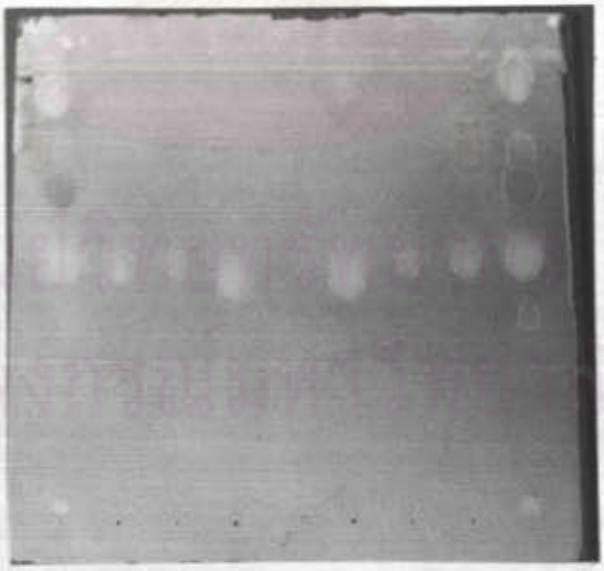


Figure 3.4 Twelve hours continuous thin layer chromatography plate sprayed with indicators.

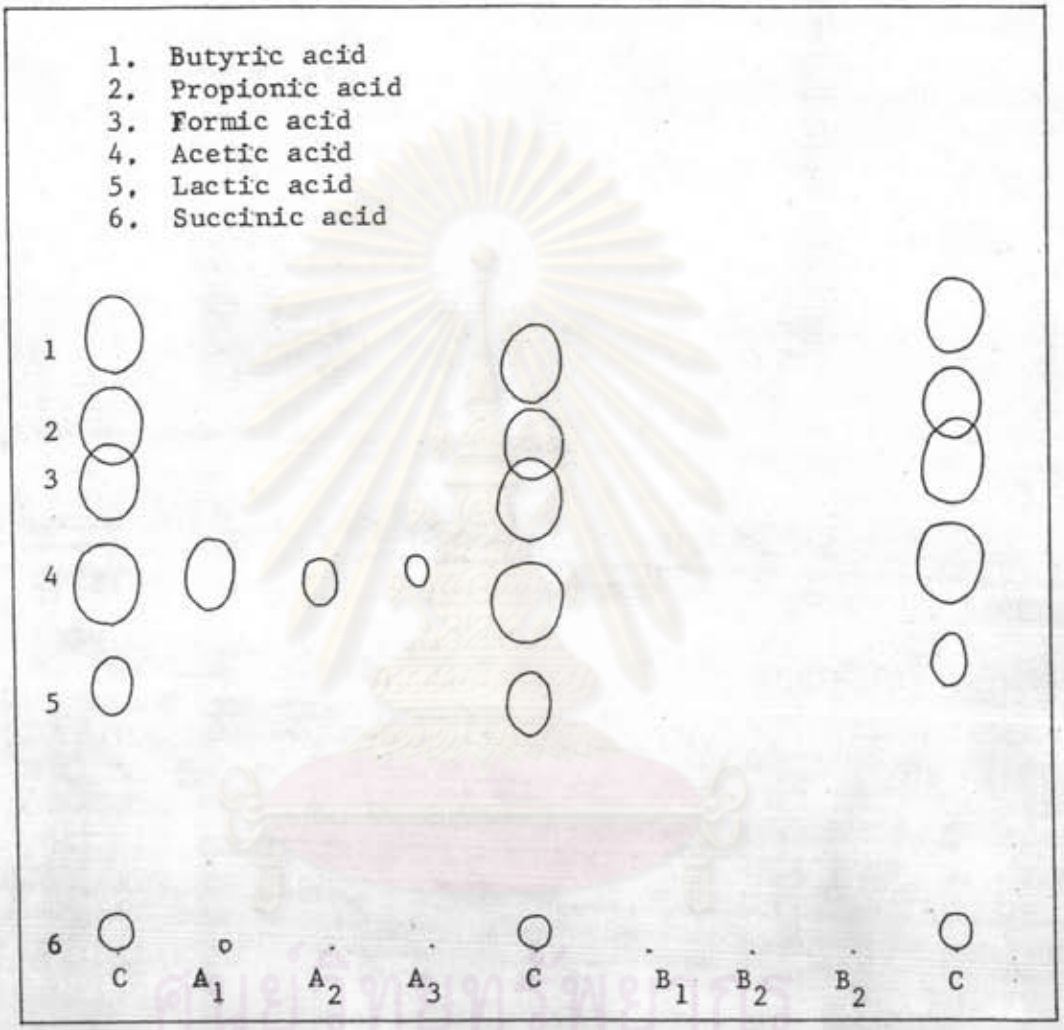


Figure 3.5 Separation of bacterial fermentation acids by continuous thin layer chromatography, c = Control standard acid 0.2 % ($^{W}/_{V}$) 10 ul, A_1, A_2, A_3 = unknow, B_1, B_2,B_3 = Broth control.

Table 3.2 The Relative Distance Move of the Acids.

Succinic acid	3.2 (± 0.48)
Lactic acid	40.2 (± 3.5)
Acetic acid	59.0 (± 2.3)
Formic acid	73.8 (± 2.2)
Propionic acid	83.7 (± 2.2)
Butyric acid	100.0*

*Butyric acid was taken as the arbitrary standard.

Determination of Cellulose Contents

Total or remained contents of cellulose were determined by Updegraff (135). Preparation of reagents, standard curve and method of calculation were described in Appendix E. The process would be performed in serial steps, those were :

(i) homogenized sample of microbial culture in a waring blender.

(ii) placed 10.0 ml sample in 15 ml centrifuge tube

(iii) centrifuged 5 min at 2000 to 3000 rpm in a dinical-type centrifuge.

(iv) decanted and discarded supernatant

(v) added 3.0 ml acetic nitric reagent (it is convenient to do this by adding 1.0 ml, mixing well on vortex mixer, then adding the remaining 2.0 ml and mixing.)

(vi) with a marble on top to reduce evaporation and created a refluxing action, placed tubes in a boiling water bath for 30 min. Maintained bath level at same level as the liquid in the tubes.

(vii) centrifuged 5 min at high speed. Decanted and discarded supernatant.

(viii) added 10 ml distilled water and washed in the manner similar to step v.

(ix) centrifuged 5 min at high speed and discarded the supernatant

(x) added 2 ml of 67% H_2SO_4 (v/v) in a manner similar to step 5.

(xi) let stand 1 hour

(xii) diluted into 100 ml volumetric flask with distilled water, centrifuged if any precipitate or turbidity was present

(xiii) placed 1.0 ml of this dilution in a 150 x 18 mm. tube, added 4.0 ml distilled water, place tubes in an ice bath to cool.

(ixv) added 10 ml cold anthrone reagent by layering with a pipet

(xv) mixed well, placed a marble on top of each and placed tubes in a boiling water bath for 16 min.

(xvi) cooled in ice bath 2-3 min, let stand at room temperature 5-10 min

(xvii) read absorbance of each at a wave length of 620 mμ agant a reagent blank.

The amounts of cellulose were read from the standard curve and calculated further.