CHAPTER 3

MATERIALS AND METHODS

3.1 Sources of Microorganisms

Fifty samples of various bacterial sources were collected during September 1993 to April 1994. Those samples included solar heated soils, wastewater, mud, sediments, peat soil, compost, manure, feces, sludge, municipal wastes, thermal springs, etc.

3.2 Raw Materials and Sources

3.2.1 Pure cellulose

The 99.5% α-cellulose fiber (Sigma, St. Louis, Missouri, U.S.A.) and carboxymethylcellulose (CMC) were used in the isolation of thermophilic cellulolytic bacteria. The α-cellulose was also used as the standard in 3.8.3 and in the cellulose fermentation (See **3.5**).

3.2.2 Paper waste

At least 5 brands of brand-new toilet paper and facial tissues sold in convenient stores were chosen as paper waste. Each sample was ground into small pieces using an electronic warring blender. The cellulose content was then determined individually as described in 3.8.3. The amounts of cellulose in all samples were ranging from 73.5 to 82.3%. All toilet paper and facial tissue samples were consequently mixed into one sample as a substrate and called 'paper waste' throughout the study for the biogas production.

3.3 Chemicals and Reagents, Gases, and Special Instruments

3.3.1 Chemicals and Reagents

Chemicals and reagents used in this study were all analytical grade.

3.3.2 Gases

All anaerobic gases throughout the thesis work were specially made by the Thai Industrial Gases (TIG) Limited which were:

(i) 10% H_2 + 5% CO_2 + 85% N_2 (Oxygen free)
(ii) 99.99% N_2
(iii) 99.99% CH_4

3.3.3 Special Instruments

The special instruments used in the thesis work were as follows :

(i) Anaerobic chamber - Anaerobic System Model 1024, dual type, Forma Scientific, Marietta, Ohio, U.S.A.
(ii) Electron microscope - JSM-35CF, JEOL, Japan
(iii) Gas chromatography - Shimadzu GC 7AG, Shimadzu, Kyoto, Japan
(iv) Spectrophotometer - Spectronic 20, Bausch & Lomb, U.S.A.

3.4 Culture Media*

3.4.1 Media for Thermophilic Cellulolytic Bacteria

Cellulose agar (CA) was the solid medium. Fluid growth medium was cellulose broth (CB) that is CA without agar. The components of this modified medium were as same as those of medium ZAP (Hudson *et al.*, 1990) in which the quantity of cellulose (in g l^{-1}) was differed. Stock cultures were maintained on cellulose broth (CB).

3.4.2 Media for Thermophilic Methanogens

Balch's medium II agar (BMA ; Balch *et al.*, 1979) was the solid medium. Stock cultures were maintained on Balch's medium II broth (BMB).

3.5 Samples and Cultivation Procedures

Samples of bacterial sources were transferred into the rubber-stoppered glass bottles containing some toilet paper and filled with an inorganic solution composed of 0.1 percent $(NH_4)_2SO_4$, 0.05 per cent K_2HPO_4, 0.03 per cent KH_2PO_4, 0.01 per cent $CaCl_2$, 0.01 per cent $MgSO_4$ and 1.0 per cent $CaCO_3$. All samples were immediately transferred to anaerobic chamber filled with 10% H_2 + 5% CO_2 + 85% N_2. Active cellulose digestion was evident after a week of incubation at 55°C. When fermentation became apparent (by gas production) the enrichment was subjected to a dilution series and kept there until proceeded.

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

3.5.1 Isolation and Screening of Thermophilic Cellulolytic Bacteria

First of all, all steps here were done in anaerobic chamber. A loopful amount of diluted fluids, after incubation at 55°C for 24, 48 and 72 hours, was streaked onto CA. All plates were placed in the plastic bags to preserve moisture, and incubated for one to two weeks. The cellulolytic thermophiles could be easily identified by the occurrence of clear zones around colonies in the cellulose medium. If the lytic zone was not typically clear, the further detection for cellulolytic activity could be implemented by screening with Congo red. Likewise modified by Bragger *et al.* (1989), the plates of media containing little amount of carboxymethylcellulose were flooded with aqueous Congo red (0.1% w/v, BDH, U.K.) for 30 min, then destained for 15 min with 1 M NaCl. Cellulolytic colonies were surrounded by a yellow halo on a red or purplish background. Thermophilic bacteria directly showed their higher cellulolytic activity with larger lytic zones. They were individually picked and re-streaked on CA (about other 2-3 times) until the pure cultures were obtained.

In addition, each pure colony of selected thermophilic bacteria with the highest cellulolytic activity was inoculated to 10 ml of CB in 25 ml serum vial and incubated at 55°C for 15 days. Gaseous products were drawn back and re-checked for cellulolytic activity (CO_2 yield) by using a gas chromatography. The analysis of gaseous products was discussed in 3.8.1. Volatile fatty acids were analyzed on day 15 using a gas chromatography. The analysis of volatile fatty acids was discussed in 3.8.2.

All isolates were examined microscopically for morphology and gram reaction aspects. Electron-microscopic was also recommended. Every two weeks, the pure cultures of selected strains were subcultured into fresh CB.

3.5.2 Isolation and Screening of Thermophilic Methanogens

Again, all cultivation steps here were done in anaerobic chamber. A loopful of diluted fluids, after incubation at 55°C for 24, 48 and 72 hours, was streaked onto BMA, the selective media. Next, all plates were placed in plastic bags to preserve moisture. After a week incubation in anaerobic chamber at 55°C, the colonies was present. Moreover, methanogens could be readily identified on agar plates by exposing them to ultraviolet (UV) light. Individual cells autofluorescenced a blue-green color under these conditions, owing to their content of $Factor_{420}$, a low-potential electron carrier found only in methanogens. Certain colonies were individually picked and re-streaked on BMA. The same procedure was repeated for other 2-3 times to obtain the thermophilic methanogenic pure cultures. Consequently, the pure cultures were examined under a microscope for morphology and by gram reaction.

All isolates were tested for the methanogenic activity by cultivating in BMB, in 25 ml serum vials stoppered with black butyl rubber septum and crimped with aluminum seal. After 5-day incubation at 55°C, CH_4 content produced in the head space was drawn to check by using the gas chromatography. The analysis of gaseous products was discussed in 3.8.1. The selected strains with high methanogenic activity (high CH_4 formation) were subcultured into BMB every two weeks and kept as stock cultures. The detailed morphology in high resolution of selected strains was scanned by the electron-microscope.

3.5.3 Mixture of All Samples

Diluted fluids from all bacterial sources were inoculated into CB and incubated at 55°C. These mixed cultures were transferred every two weeks into fresh CB for keeping as a stock culture.

3.6 Fermentation of Cellulose

3.6.1 Fermentation of Cellulose by Coculture

The procedure was performed by inoculating 0.1 ml of 3-day culture of the selected thermophilic methanogens (grown in BMB at 55°C) and 0.1 ml of 3-day culture of the select thermophilic cellulolytic bacteria (grown in CB at 55°C) together into 25 ml serum vials contained 10 ml CB plus 0.1% α-cellulose. The inoculum size of each bacteria was approximately 2.1 - 2.3 $\times$ 10^6 cells per ml. The coculture combinations were shown in **Table 3.1**.

All tests, comprising all six combinations of coculture, control cultures of the coculture, and the control, were performed in 5 replicates. Also, all vials were stoppered with black butyl rubber septums and sealed with aluminum caps in the same anaerobic atmosphere (10% H_2 5% CO_2 and 85% N_2).

During the 15-day fermentation period, the gaseous products, volatile fatty acids, and remained cellulose contents were determined periodically. Gaseous products were analyzed on day 0, 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 14, and 15. Volatile fatty acids were analyzed twice, on day 0 and 15. Finally, the remained cellulose contents were determined on day 2, 4, 6, 8, 10, 12, and 15; pH of fermented slurry was also determined at the same period.

3.6.2 Fermentation of Cellulose by Mixed Culture

In order to compare with pure culture technique, 0.1 ml of 3-day culture of mixed strains (grown in CB at 55°C) was cultured in 25 ml serum vials contained 10 ml CB plus 0.1 % α-cellulose. The inoculum size was about 3.8 $\times$ 10^6 cells per ml.

All tests, comprising mixed culture and the control, were performed in 5 replicates. Also, all vials were stoppered with black butyl rubber septums and sealed with aluminum caps in the same anaerobic atmosphere (10% H_2 5% CO_2 and 85% N_2).

During the 15-day fermentation period, the gaseous products, volatile fatty acids, and remained cellulose contents were determined periodically. Gaseous products were analyzed on day 0, 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 14, and 15. Volatile fatty acids were analyzed twice, on day 0 and 15. Finally, the remained cellulose contents were determined on day 2, 4, 6, 8, 10, 12, and 15; pH of fermented slurry was also determined at the same period.

Table 3.1 Six coculture combinations of two selected thermophilic cellulolytic bacteria and three thermophilic methanogens.

No. of Coculture	Thermophilic Cellulolytic Bacteria + Thermophilic Methanogens
1	C23 + M38
2	C23 + M47
3	C23 + M48
4	C73 + M38
5	C73 + M47
6	C73 + M48

3.7 Fermentation of Paper Waste

3.7.1 Fermentation of Paper Waste by Coculture

The experiment was conducted in the same manner as in 3.6.1, except paper waste (described in 3.2.2) was used in lieu of α-cellulose. In addition, all cultivated cultures were incubated for 30 days at 55°C instead of the 15-day incubation.

During the fermentation period, the amounts of digestive products, e.g. gases and volatile fatty acids, and the remained cellulose contents were analyzed periodically. The gaseous products were drawn to check on day 0, 4, 8, 12, 16, 19, 22, 26, and 30. The volatile fatty acids were collected twice, on day 0 and 30. The amounts of remained cellulose and pH of digestive slurry were determined on day 0, 5, 10, 15, 20, 25, and 30.

3.7.2 Fermentation of Paper Waste by Mixed Culture

The experiment was conducted in the same manner as in 3.6.2, except paper waste (described in 3.2.2) was used in lieu of α-cellulose. In addition, all cultivated cultures were incubated for 30 days at 55°C instead of the 15-day incubation.

During the fermentation period, the amounts of digestive products, e.g. gases and volatile fatty acids, and the remained cellulose contents were analyzed periodically. The gaseous products were drawn to check on day 0, 4, 8, 12, 16, 19, 22, 26, and 30. The volatile fatty acids were collected twice, on day 0 and 30. The amounts of remained cellulose and pH of digestive slurry were determined on day 0, 5, 10, 15, 20, 25, and 30.

3.8 Procedures of Chemical Analysis

3.8.1 Analysis of Gaseous Products

Methane and carbon dioxide in biogas samples were analyzed by using gas chromatography. The chromatopac condition, standard gas chromatogram, and calculation methods (Mah, Smith, and Baresi, 1978) were described in **Appendix C**.

The 1.0 ml gas-tight syringe (Pressure Lok, Precision Sampling Corp., Baton Rouge, Louisiana, U.S.A.), after flushing with O_2-free 99.99% nitrogen gas, was used to draw the gas samples (0.5 ml). The 99.99% CH_4 was used as the standard gas. The retention times of N_2, CH_4, and CO_2 were approximately 0.595, 1.027, and 2.236 minutes, respectively. The volume of gaseous product was determined daily at room temperature (25°C). Micromoles of methane and carbon dioxide were finally computed.

3.8.2 Analysis of Volatile Fatty Acids

The quantities of volatile fatty acids, acetic acid, propionic acid, and butyric acid were analyzed by gas chromatography technique. The preparation of volatile fatty acid standard solutions, gas chromatograph conditions, and column packing (Levett, 1991) were described in **Appendix D**. The retention times of acetic acid, propionic acid, and butyric acid, were approximately 4.535, 6.388, and 9.307, respectively.

The analysis procedures in serial steps were as follows :

(i) pipetted 1.0 ml of spent culture medium (or pus) into a centrifuge tube.

(ii) added 0.2 ml of 50% aqueous H_2SO_4.

(iii) added 1.0 ml diethyl ether (special for chromatography).

(iv) sealed and mixed the tube for 10-15 sec on a vortex mixer.

(v) centrifuged briefly to break the ether/water emulsion.

(vi) allowed the aqueous and ether layers to separate.

(vii) injected 1 µl of upper (ether) layer into the gas chromatography.

(viii) compared retention times of peaks obtained with those of standard volatile fatty acid mixture run on the same day.

3.8.3 Determination of Cellulose Contents

Total or remained contents of cellulose were determined by the phenol-sulfuric reaction (Dubois *et al.*, 1956; Wood and Kellogg, 1988). Preparation of reagents and the standard curve were described in **Appendix E**. The process would be performed in serial steps as follows :

(i) placed 10.0 ml sample in 15 centrifuge tube.

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

(iii) decanted and discarded supernatant.

(iv) added 3.0 ml acetic nitric reagent.

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

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

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

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

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

(x) let stand 1 hour

(xi) diluted into 100 ml volumetric flask with distilled water.

(xii) placed 1.0 ml of this solution in a 6-inch by 1-inch test tube.

(xiii) added 1.0 ml of the phenol reagent; mixed rapidly and thoroughly.

(xiv) added 5.0 ml of concentrated sulfuric acid, mixed rapidly, and let stand for 10 min.

(xv) placed the tubes in a water bath at 25°C for 15 min.

(xvi) read the absorbance of each tube at 490 nm against the blank without cellulose using the spectrophotometer.

(xvii) determined the concentration of cellulose in the samples from a standard curve prepared by plotting the absorbances of the standards versus the concentration of cellulose.

Note: Step i to viii were to delignify the paper waste samples which contained other plant components, namely lignin and hemicellulose. The white residue, after performed step viii, was cellulose. When preparing the standard cellulose curve by using α-cellulose as the standard, the process began at step xii.