CHAPTER 4

RESULTS

4.1 Isolation and Selection of Pure Cultures

4.1.1 Thermophilic Cellulolytic Bacteria

From 123 pure cultures of thermophilic cellulolytic bacteria, two strains were selected, namely C23 and C73. Their characteristics were as follows :

C23 : Colonial characteristics grown on cellulose agar -- 5 mm in diameter, yellow, convex, rugged edge, surrounded with 1-1.2 cm lytic zone (See **Figure 4.1**)

Morphological characteristics -- rod-shape, gram-negative bacilli, 0.5 by 4-6 μ (See **Figure 4.2**)

C73 : Colonial characteristics grown on cellulose agar -- 3 mm in diameter, yellow, convex, rugged edge, surrounded with 6-9 mm lytic zone (See **Figure 4.3**)

Morphological chacteristics -- rod-shape, gram-negative bacilli, 0.4 by 6-8 μ (See **Figure 4.4**)

The two thermophilic cellulolytic bacteria were pre-tested for their cellulolytic activities by cultivating in the media which contained 0.1% α-cellulose fibre. **Figure 4.5** showed the rates of cellulose utilization whereas C23 is better than C73. The thermophilic cellulolytic bacteria strain C23 and C73 were able to utilize 10

mg cellulose within 12 days and 15 days, respectively. The pH reduction in each monoculture was gradually decreased and there was similar in the rate of both cellulolytic strains. The CO_2 production rates were shown in **Figure 4.6** in which the rates were constant for about 7 and 6 days in C23 and C73, respectively. The results of their acid formation were shown in **Table 4.1**. Acetic acid was the only acid product. However, the amount of acetic acid produced by strain C23 was lower than that of strain C73. In **Table 4.2**, the production of CO_2 after cellulose fermentation was presented. The CO_2 production by strain C23 was higher than that of strain C73. In an agreement with a larger lytic zone observed, the cellulolytic activity of C23 was greater than C73.

4.1.2 Thermophilic Methanogens

Three isolates from 147 pure cultures of thermophilic methanogens, namely M38, M47, and M48, were selected. They were characterized as :

M38 : Colonial characteristics grown on Balch's medium II agar -- 1 mm in diameter, smooth, round, convex, shining, translucent, yellow-brown (See **Figure 4.7**).

Morphological characteristics -- rod-shape, filamentous, gram-negative bacilli, 0.4 by 1.2-1.5 μ (See **Figure 4.8**)

Methanogenic activity -- 21.35 μmol of CH_4 were produced within 5 days of incubation at 55°C.

M47: Colonial characteristics grown on Balch's medium II agar -- smaller than 1 mm in diameter, smooth, round, convex, opaque, grey-white.(See **Figure 4.9**).

Morphological characteristics -- rod-shape, filamentous, gram-negative bacilli, smaller than 0.1 by 1 μ (See **Figure 4.10**)

Methanogenic activity -- 19.25 μmol of CH_4 were produced within 5 days of incubation at 55°C.

M48: Colonial characteristics grown on Balch's medium II agar -- 1.5-2 mm in diameter, smooth, round, convex, translucent, yellow-brown (See **Figure 4.11**).

Morphological characteristics -- oval rod-shape, gram-negative bacilli, 0.3 by 1.1-1.2 μ (See **Figure 4.12**)

Methanogenic activity -- 23.52 μmol of CH_4 were produced within 5 days of incubation at 55°C.

Under the UV light, all three strains of selected thermophilic methanogens were autofluorescent indicating that they contained coenzyme F_{420}.

In all methanogens, H_2-CO_2 utilization is a common character. In **Table 4.3** the capacity of CO_2 utilization by the three selected thermophilic methanogens were shown. It was found that strain M48 showed the highest ability followed by M38 and M47, respectively.

4.2 Fermentation of Cellulose by Thermophilic Coculture

Among six coculture sets, the amounts of remained cellulose were shown in **Table 4.4** and the detailed results of remained cellulose contents and the pH reduction of each set are presented in **Figure F-1 to F-7** in **Appendix F**. The coculture sets of strain C23 were able to utilize all cellulose in day 12, and those of strain C73 required 15 days to degrade cellulose. On contrary, the mixed culture were not able to utilize cellulose within 15 day-fermentation. The pH of the medium gradually decreased from 7.2, at the beginning of fermentation period, to 6.7-6.8, on the last day.

During the fermentation peroid, the amounts of acetic acid produced were presented in **Table 4.5**. All sets of coculture were able to utilize acetate as their substrate. The percentages of acid loss were ranged from 20.7-30.6%. The coculture set of C73 and M48 utilized acetates higher than others.

The gas production in all cocultures of thermophilic cellulolytic bacteria and thermophilic methanogen were summarized in **Table 4.6** and their detailed results were shown in **Figure F-8 to F-14** in **Appendix F**. Carbon dioxide content was decreased in all sets. After 10-day fermentation, the CH_4 production of cocultures were widely ranged from approximately 4.5-6.4 mmol per gram of cellulose.

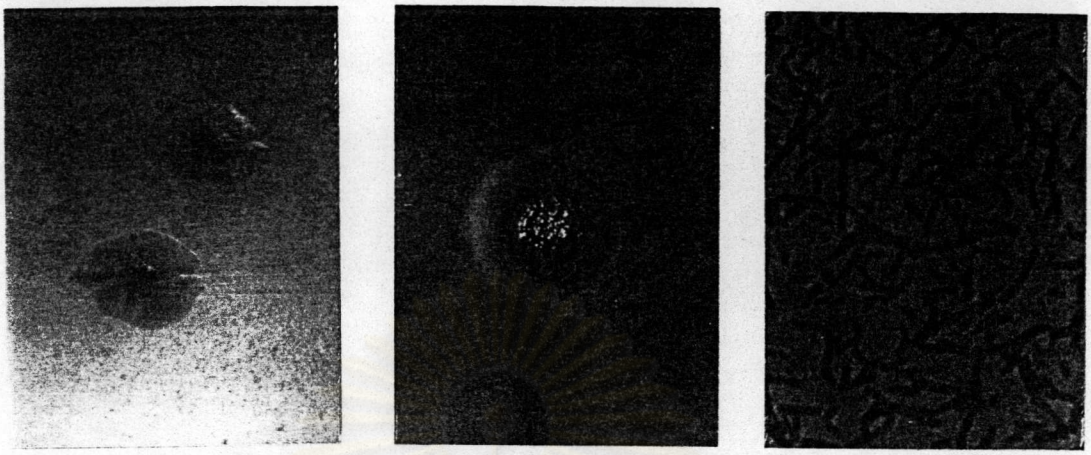
The comparison of cellulose degradation and CH_4 production of all sets were in **Table 4.7**. It was shown that the coculture set of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M48 showed the highest degree in cellulose fermentation and biogas production.

4.3 Fermentation of Paper Waste by Thermophilic Coculture

The amounts of remained cellulose were summarized in **Table 4.8**. and the detailed results of remained cellulose contents and the pH reduction of each set could be found in **Figure G-1** to **G-7** in **Appendix G**. Depended on the paper waste digestion capacity of selected thermophiles, C23 and C73, paper waste was not detectable on day 26 and day 30, respectively. Similar to the experiment in **4.2**, the pH of the medium decreased from 7.2, at the beginning of fermentation period, to 6.7-6.8, on the last day. On contrary, the pH of a mixed culture differed from those coculture sets. Its pH on the last day was about 6.6, lower than others.

In **Table 4.9**, the production of acetic acid during the 30-day fermentation of paper waste was presented. The percentages of acetate loss were ranging from 20.2-28.7%, where the set of C73 + M48 showed the highest acetate utilization compared to other sets.

The gas production in the 30-day fermentation of paper waste were summarized in **Table 4.10**. The results in detail were presented in **Figure G-8** to **G-14** in **Appendix G**. On day 26, the CH_4 formation of thermophilic cocultures was around 4.9-6.7 mmol per gram of paper waste. The degree of paper waste degradation and the CH_4 production were shown in **Table 4.11**. The coculture set of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M48 showed the highest degree in paper waste fermentation and biogas production.



(a) (b) (c)

Figure 4.1 Colonial characteristic (a), lytic zone (b), and gram staining (c) of thermophilic cellulolytic bacteria strain C23 on cellulose agar, incubated 5 days at 55°C in 10% H_2 + 5% CO_2 + 85% N_2 atmosphere.

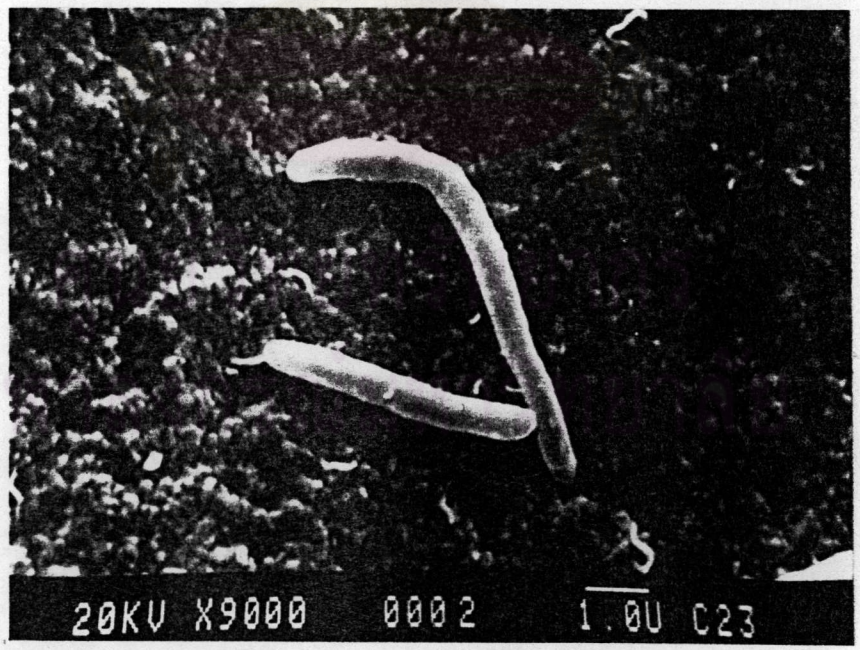
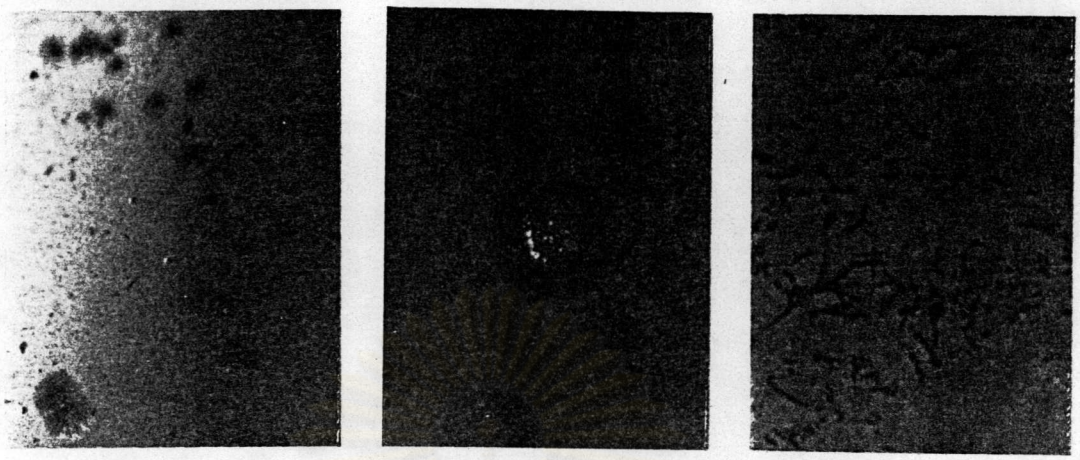


Figure 4.2 High resolution scanning electron micrograph of thermophilic cellulolytic bacteria strain C23 (x 9000).



(a) (b) (c)

Figure 4.3 Colonial characteristic (a), lytic zone (b), and gram staining (c) of thermophilic cellulolytic bacteria strain C73 on cellulose agar, incubated 5 days at 55°C in 10% H_2 + 5% CO_2 + 85% N_2 atmosphere

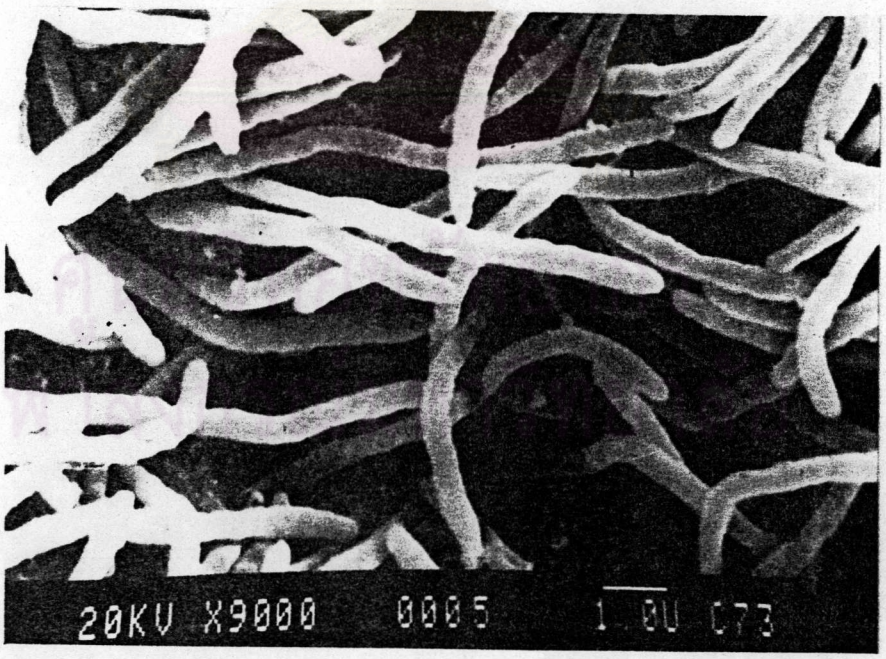
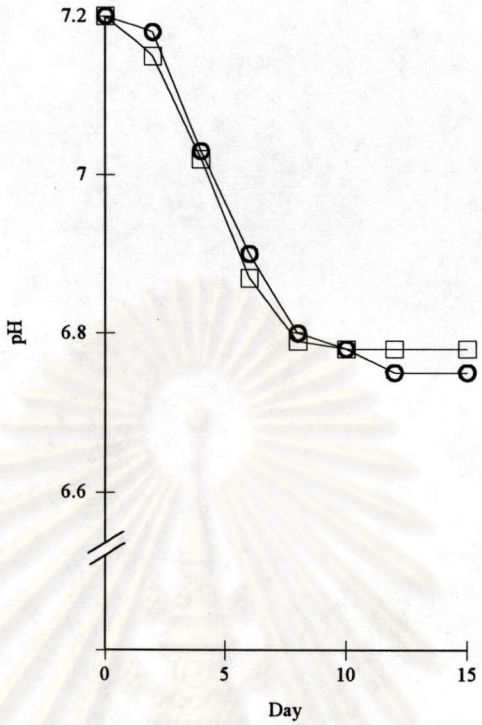


Figure 4.4 High resolution scanning electron micrograph of thermophilic cellulolytic bacteria strain C73 (x 9000).

(a)



(b)

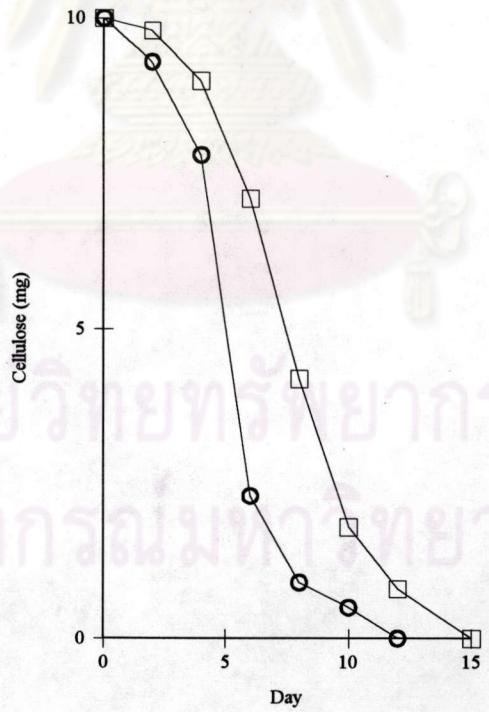


Figure 4.5 pH reduction (a) and remained cellulose contents (b) in cellulose fermentation by two selected strains of thermophilic cellulolytic bacteria : C23 (○) and C73 (□).

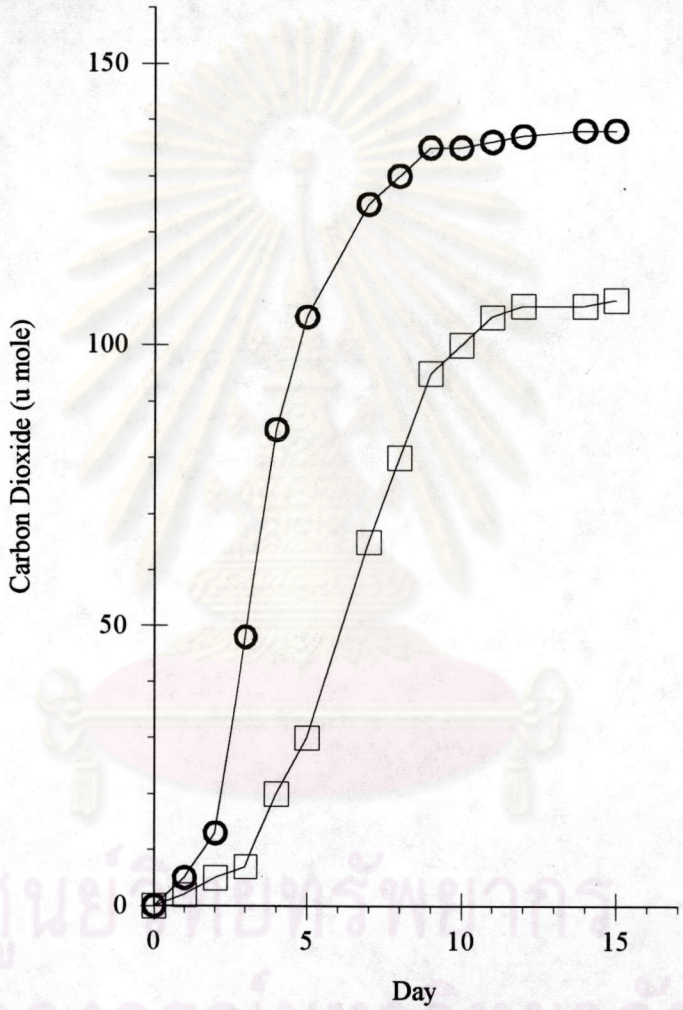


Figure 4.6 CO_2 production in cellulose fermentation by two selected strains of thermophilic cellulolytic bacteria : C23 (O) and C73 (□).

Table 4.1 Acid production after 15-day fermentation of α-cellulose by two selected thermophilic cellulolytic bacteria

Organism	Initial no. of organisms (cells × 10^6)	α-cellulose (mg)	Production (μmol) of		
			Acetic acid	Propionic acid	Butyric acid
C23	2.3	10	5.23 ± 0.11	ND	ND
C73	2.1	10	6.05 ± 0.17	ND	ND
Mixed Culture[a]	3.8	10	10.24 ± 0.45	3.27 ± 0.20	6.25 ± 0.36

Alls were means of 5 replicates.

ND = not be detectable

Alls were cultivated in 10 ml cellulose broth under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources

Table 4.2 CO_2 production after 15-day fermentation of α-cellulose by two selected thermophilic cellulolytic bacteria

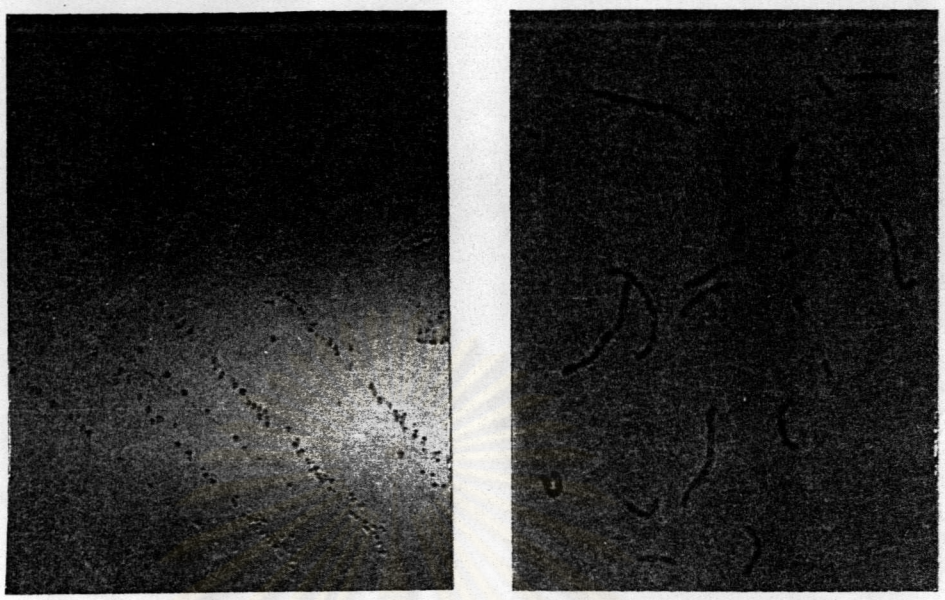
Organism	Production (μmol) of	
	CO_2	CH_4
C23	138.2 ± 10.21	ND
C73	108.6 ± 9.21	ND
Mixed Culture[a]	60.5 ± 5.94	43.2 ± 4.01

Inoculum size = 2.1-3.8 × 10^6 cells/ml

ND = not detectable

Alls were cultivated in 10 ml cellulose broth under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources



(a) (b)

Figure 4.7 Colonial characteristic (a) and gram staining (b) of thermophilic methanogenic bacteria strain M38 on Balch's medium II agar, incubated 5 days at 55°C in 10% H_2 + 5% CO_2 + 85% N_2 atmosphere.

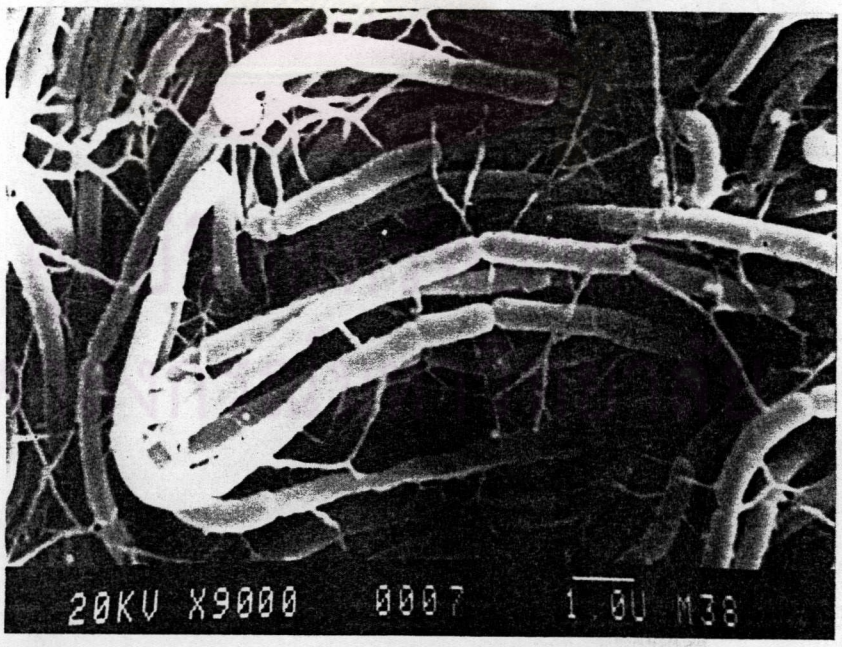
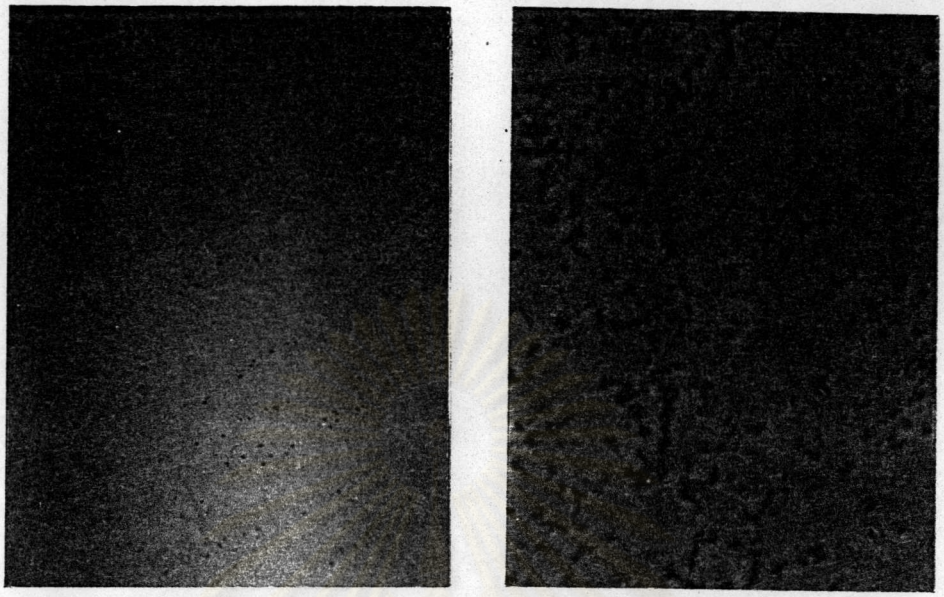


Figure 4.8 High resolution scanning electron micrograph of thermophilic methanogenic bacteria strain M38 (x 9000).



(a) (b)

Figure 4.9 Colonial characteristic (a) and gram staining (b) of thermophilic methanogenic bacteria strain M47 on Balch's medium II agar, incubated 5 days at 55°C in 10% H_2 + 5% CO_2 + 85% N_2 atmosphere

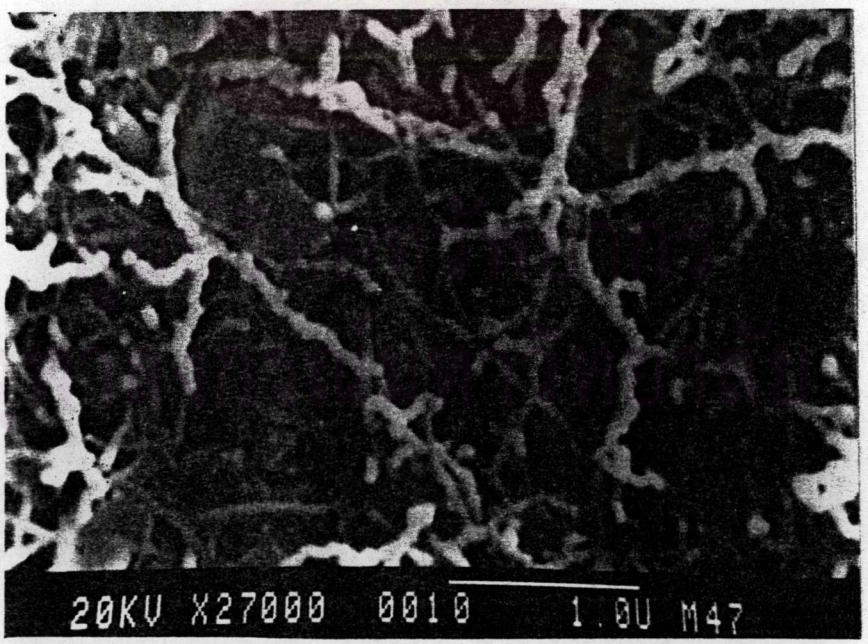
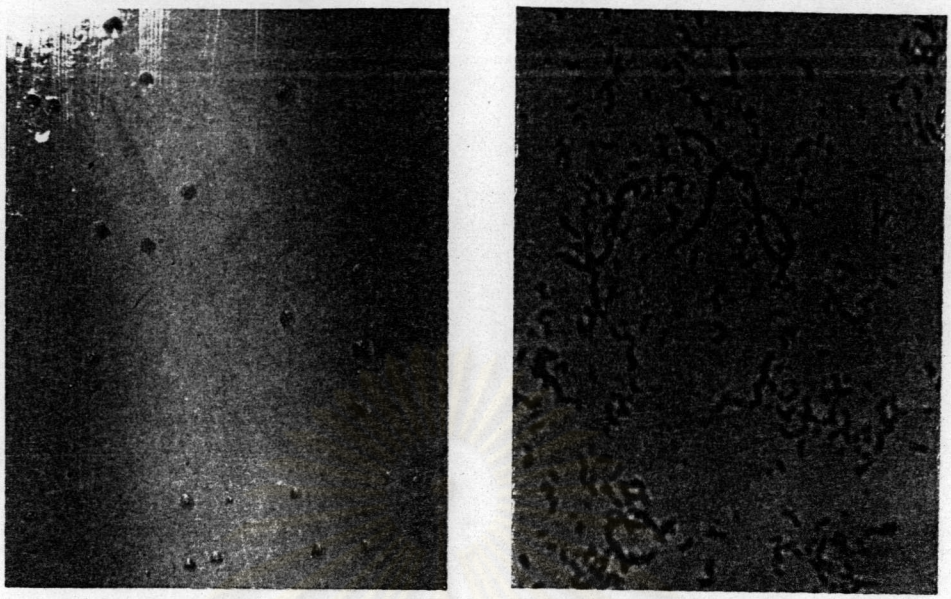


Figure 4.10 High resolution scanning electron micrograph of thermophilic methanogenic bacteria strain M47 (x 27000).



(a) (b)

Figure 4.11 Colonial characteristic (a) and gram staining (b) of thermophilic methanogenic bacteria strain M48 on Balch's medium II agar, incubated 5 days at 55°C in 10% H_2 + 5% CO_2 + 85% N_2 atmosphere

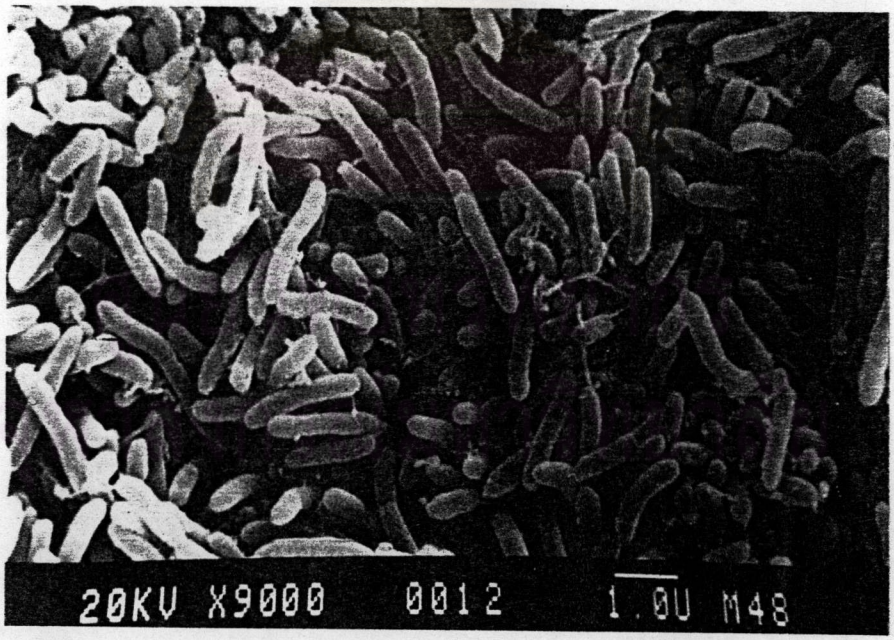


Figure 4.12 High resolution scanning electron micrograph of thermophilic methanogenic bacteria strain M48 (x 9000).

Table 4.3 CO_2 utilizing capacity of the three selected thermophilic methanogen after 5-day incubation.

Methanogen	CO_2 loss (μmol)	CH_4 Production (μmol)	Ratio CH_4/CO_2 cons
M38	18.57 ± 1.27	21.35 ± 1.95	1.15
M47	18.87 ± 1.32	19.25 ± 1.84	1.02
M48	19.44 ± 1.41	23.52 ± 2.11	1.21

Alls were cultivated in 10 ml Balch's medium II broth under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere at 55°C.

Initial stage CO_2 = 58.26 μmol

CH_4 = 0 μmol

Table 4.4 Remained cellulose in the fermentation of α-cellulose by monoculture of thermophilic cellulolytic bacteria C23 and C73, by coculture with thermophilic methanogen M38, M47, and M48, and by a mixed culture.

Organism	Remained cellulose (mg)		
	day 0	day 6	day 12
C23 system	10	2.3 ± 0.12	0
C23 + M38	10	1.7 ± 0.09	0
+ M47	10	2.0 ± 0.07	0
+ M48	10	1.5 ± 0.09	0
C73 system	10	7.1 ± 0.51	0.8 ± 0.02
C73 + M38	10	7.0 ± 0.43	0.7 ± 0.03
+ M47	10	7.0 ± 0.47	0.75 ± 0.03
+ M48	10	6.6 ± 0.38	0.6 ± 0.02
Mixed culture[a]	10	7.7 ± 0.74	1.1 ± 0.08

Alls were cultivated in 10 ml cellulose broth under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources

Table 4.5 Production of acetic acid in the 15-day fermentation of α-cellulose by monoculture of thermophilic cellulolytic bacteria, and by coculture of thermophilic cellulolytic bacteria and thermophilic methanogen

Organism	Initial no. of organisms (cells) × 10^6	Production of acetic acid (μmol)	% Acetic acid loss
C23 system	2.3	5.23 ± 0.30	0.00
C23 + M38	2.3 + 2.1	3.96 ± 0.43	24.3
+ M47	2.3 + 2.1	4.15 ± 0.13	20.7
+ M48	2.3 + 2.2	3.74 ± 0.08	28.4
C73 system	2.1	6.05 ± 0.12	0.00
C73 + M38	2.1 + 2.1	4.65 ± 0.32	23.1
+ M47	2.1 + 2.1	4.76 ± 0.20	21.3
+ M48	2.1 + 2.2	4.20 ± 0.24	30.6

Alls were cultivated in 10 ml cellulose broth under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

Table 4.6 Gas production in the 15-day fermentation of α-cellulose by monoculture of thermophilic cellulolytic bacteria, by coculture of thermophilic cellulolytic bacteria and thermophilic methanogen, and by a mixed culture

Organism	CO_2 production (μmol)		CH_4 production (μmol)		Ratio $CH_4 : CO_2$	
	day 5	day 10	day 5	day 10	day 5	day 10
C23 system	105.0 ± 9.25	135.0 ± 12.4	ND	ND	-	-
C23 + M38	35.65 ± 29.5	51.01 ± 5.21	41.51 ± 3.64	53.02 ± 4.92	1.16	1.04
+ M47	36.21 ± 3.24	50.64 ± 4.51	38.52 ± 3.14	49.68 ± 4.36	1.06	0.98
+ M48	37.67 ± 3.52	48.62 ± 4.13	46.87 ± 3.98	64.78 ± 5.27	1.24	1.33
C73 system	30.0 ± 2.53	100.1 ± 8.79	ND	ND	-	-
C73 + M38	13.20 ± 1.12	45.36 ± 3.97	16.80 ± 1.53	49.87 ± 4.38	1.27	1.10
+ M47	15.00 ± 1.04	47.60 ± 4.03	14.20 ± 1.17	45.32 ± 3.96	0.95	0.95
+ M48	13.10 ± 1.20	43.10 ± 3.89	17.32 ± 1.13	50.23 ± 4.59	1.32	1.17
Mixed culture[a]	17.8 ± 1.37	53.12 ± 4.92	7.89 ± 0.64	35.21 ± 2.57	0.44	0.66
Control	ND	ND	ND	ND	-	-

ND = not detectable

Alls were cultivated in 10 ml cellulose broth under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources.

Table 4.7 Degree of α-cellulose degradation and CH_4 production from 15-day fermentation by monoculture of thermophilic cellulolytic bacteria, by coculture of thermophilic cellulolytic bacteria and thermophilic methanogen, and by a mixed culture

Organism	Remained cellulose (mg)	Gas production (μmol)		Ratio CH_4:CO_2	CH_4 production per cellulose (mmol/g)
		CO_2	CH_4		
C23	0	138.0 ± 10.2	ND	-	-
C23 + M38	0	51.16 ± 4.32	54.15 ± 4.96	1.06	5.41
C23 + M47	0	52.34 ± 4.65	51.1 ± 4.63	0.98	5.11
C23 + M48	0	50.28 ± 4.41	65.36 ± 5.36	1.30	6.54
C73	0	108.0 ± 10.1	ND	-	-
C73 + M38	0	45.86 ± 3.92	51.50 ± 3.67	1.12	5.15
C73 + M47	0	51.40 ± 4.02	47.32 ± 3.24	0.92	4.73
C73 + M48	0	45.17 ± 3.87	55.50 ± 5.02	1.23	5.55
Mixed culture[a]	0.7 ± 0.04	60.54 ± 5.94	43.20 ± 35.7	0.71	4.32
Control	10.0	ND	ND	-	-

ND = not detectable

Alls were cultivated in 10 ml cellulose broth under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources

Table 4.8 Remained cellulose in the fermentation of paper waste by monoculture of thermophilic cellulolytic bacteria C23 and C73, by coculture with thermophilic methanogen M38, M47, and M48, and by a mixed culture.

Organism	Remained cellulose (mg)		
	day 0	day 16	day 26
C23 system	8.2 ± 0.34	2.57 ± 0.25	0
C23 + M38	8.2 ± 0.34	2.32 ± 0.17	0
+ M47	8.2 ± 0.34	2.45 ± 0.21	0
+ M48	8.2 ± 0.34	2.32 ± 0.23	0
C73 system	8.2 ± 0.34	3.67 ± 0.33	0.20 ± 0.07
C73 + M38	8.2 ± 0.34	3.54 ± 0.28	0.25 ± 0.09
+ M47	8.2 ± 0.34	3.62 ± 0.24	0.19 ± 0.09
+ M48	8.2 ± 0.34	3.42 ± 0.21	0.15 ± 0.08
Mixed culture[a]	8.2 ± 0.34	4.20 ± 0.32	0.92 ± 0.02

Alls were cultivated in 10 ml cellulose broth (paper waste was in place of α-cellulose) under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources

Table 4.9 Production of acetic acid in the 30-day fermentation of paper waste by monoculture of thermophilic cellulolytic bacteria, and by coculture of thermophilic cellulolytic bacteria and thermophilic methanogen

Organism	Initial no. of organisms (cells) $\times 10^6$	Production of acetic acid (μmol)	% Acetic acid loss
C23 system	2.1	10.36 ± 0.32	0
C23 + M38	2.3 + 2.1	7.96 ± 0.19	23.2
+ M47	2.3 + 2.1	8.12 ± 0.17	21.6
+ M48	2.3 + 2.2	7.53 ± 0.26	27.3
C73 system	2.1	11.64 ± 0.22	0
C73 + M38	2.1 + 2.1	8.79 ± 0.11	24.5
+ M47	2.1 + 2.1	9.29 ± 0.24	20.2
+ M48	2.1 + 2.2	8.30 ± 0.25	28.7

Alls were cultivated in 10 ml cellulose broth (paper waste was in place of α-cellulose) under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

Table 4.10 Gas production in the 30-day fermentation of paper waste by monoculture of thermophilic cellulolytic bacteria, by coculture of thermophilic cellulolytic bacteria and thermophilic methanogen, and by a mixed culture

Organism	CO_2 production (μmol)		CH_4 production (μmol)		Ratio $CH_4 : CO_2$	
	day 16	day 26	day 16	day 26	day 16	day 26
C23 system	78.64 ± 6.11	136.0 ± 12.6	ND	ND	-	-
C23 + M38	45.67 ± 3.95	54.92 ± 4.88	45.62 ± 3.82	53.65 ± 4.86	1.00	0.98
+ M47	48.26 ± 4.03	57.35 ± 5.06	46.21 ± 3.94	51.98 ± 4.33	0.96	0.91
+ M48	40.27 ± 3.77	53.04 ± 4.99	50.97 ± 4.38	67.04 ± 5.67	1.27	1.26
C73 system	51.36 ± 4.26	107.6 ± 9.89	ND	ND	-	-
C73 + M38	29.80 ± 2.16	52.08 ± 4.36	52.08 ± 4.20	52.98 ± 4.86	1.75	0.98
+ M47	35.74 ± 2.97	55.97 ± 4.77	55.97 ± 4.76	49.25 ± 4.31	1.57	0.88
+ M48	29.84 ± 2.04	48.12 ± 4.16	48.12 ± 3.97	55.98 ± 4.78	1.61	1.16
Mixed culture[a]	51.36 ± 46.7	63.25 ± 4.96	27.64 ± 1.94	41.98 ± 3.25	0.54	0.66
Control	ND	ND	ND	ND	-	-

ND = not detectable

Alls were cultivated in 10 ml cellulose broth (paper waste was in place of α-cellulose) under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources

Table 4.11 Degree of paper waste degradation and CH_4 production from 30-day fermentation by monoculture of thermophilic cellulolytic bacteria, by coculture of thermophilic cellulolytic bacteria and thermophilic methanogen, and by a mixed culture

Organism	Remained cellulose (mg)	Gas production (μmol)		Ratio CH_4:CO_2	CH_4 production per paper waste (mmol/g)
		CO_2	CH_4		
C23	0	134.0 ± 11.2	ND	-	-
C23 + M38	0	55.43 ± 4.98	56.31 ± 5.01	1.02	5.63
C23 + M47	0	57.62 ± 5.12	51.64 ± 4.46	0.9	5.16
C23 + M48	0	53.24 ± 4.58	67.32 ± 5.87	1.26	6.73
C73	0	109.5 ± 9.42	ND	-	-
C73 + M38	0	52.36 ± 4.21	54.89 ± 3.97	1.05	5.49
C73 + M47	0	56.21 ± 5.03	49.65 ± 4.04	0.88	4.97
C73 + M48	0	48.56 ± 4.12	57.92 ± 4.76	1.19	5.79
Mixed culture[a]	0.5 ± 0.03	64.25 ± 5.69	42.35 ± 3.26	0.66	4.24
Control	10.0	ND	ND	-	-

ND = not detectable

Alls were cultivated in 10 ml cellulose broth (paper waste was in place of α-cellulose) under 10% H_2 + 5% CO_2 + 85% N_2 atmosphere incubated at 55°C.

[a] A mixed culture of all thermophilic bacteria from 50 sources

CHAPTER 5

DISCUSSION AND CONCLUSION

In the study of biogas production from cellulose and paper waste at 55°C, the two important groups of anaerobic bacteria involved were thermophilic cellulolytic bacteria and thermophilic methanogens. By using a coculture technique at high temperature, this thesis was demonstrated and originated in Thailand.

Two strains of thermophilic cellulolytic bacteria, C23 and C73, were isolated, characterized, and chosen as the tested organisms. They both were gram-negative, small rod-shaped, and 0.4-0.5 × 4-8 μ size. These two cellulolytic thermophiles were able to utilize cellulose and paper waste as their substrates. Based on the size of lytic zone, the cellulolytic activity of strain C23 was higher than that of strain C73. Their major fermentation products included CO_2 and acetic acid. Acetate was the only volatile fatty acid found. The high resolution scanning electron micrographs and a key for the determination of generic position of organisms (Buchanan and Gibbons, 1974) showed that they both might resemble any of bacteroid or clostridium groups.

The difference between bacteroid and clostridium groups is that the clostridia are sporeforming rods. To create a starvation condition for the two selected thermophilic cellulolytic bacteria may cause the spore formation. In the present study, it might be possible that the nutrients in the both culture media, CA and CB, were adequate, so the bacteria did not form spores. If, however, these two bacteria are not bacteroid nor clostridium, they may be new species. For further determination, further investigations are recommended.

When isolating the thermophilic cellulolytic bacteria, the clear zones were observed within 3-5 days, then developed areas of incomplete clearing. One reason

was due to the nature of agar-based media that usually lose gel strength and often exhibit syneresis, with the presence of significant amounts of surface water escaping from the gel (Lin and Casida, 1984). Another reason proposed was that the bacteria migrated through the medium and were found in a thin layer at the junction between digested and undigested cellulose. Also, it was possible that an extracellular cellulase was less formed as Johnson *et al.* (1982) indicated that most anaerobic cellulolytic rods, such as *Bacteroides succinogenes*, *Clostridium thermocellum*, and *Acetivibrio cellulolyticus*, had so far failed to show extracellular cellulolytic activities on derived or native forms of cellulose, though grew more rapidly, comparable to that from fungi, like *Trichoderma* sp.

Without ethanol determination in the present study, acetate became the main liquid product of the cellulose fermentation. Jones (1989) indicated that the moderate thermophiles (at 50-55°C) were all ethanologenic with either a mixed acid (formic, lactic, and/or acetic) or an acetogenic fermentation pattern. This study might follow an acetogenic fermentation pattern. In a future work, ethanol determination would be recommended.

The present study indicated that sites, where organic wastes decay, are suitable thermophilic cellulolytic bacterial source because cellulose is a major component of the wastes. Upon emplacement, the initial breakdown of refuse is aerobic but oxygen is soon depleted and the environment becomes anaerobic. High temperature (higher than 50°C) have been recorded within the anaerobic regions (Archer and Peck, 1989). Fermentation of the cellulose yields a range of volatile fatty acids, which have been shown to make up a major part of the leachate organic carbon, and will, when present, cause acidic condition. That is to say there is a possibility to further this present study forward the thermoacidophiles, one of the archaebacterial mricroorganisms, besides thermophilic methanogens.

In the isolation of methanogens, three strains of thermophilic methanogenic bacteria, M38, M47, and M48, were selected, characterized, and chosen as the test

organisms, based on the H_2-CO_2 utilization which is a common character of methanogens. They were gram-negative, very slim rod-shaped, and filamentous. Their sizes were widely ranged from smaller than 0.1 by 1 μ to 0.3-0.4 by 1.1-1.5 μ Strain M47 was very small in size compared to the other two. They all utilized CO_2 and acetate as substrates for CH_4 production. The methanogenic activity of M38 were higher than that of M47. Strain 48 showed the highest methanogenic activity among the three selected strains. All three resembled any of methanobacterium group (methane-producing rods). The high resolution electron micrographs revealed that these thermophilic methanogens existed in clumps or filaments. It could be seen that there were fibrillae, like a net, adhere to the cells. Among three selected thermophilic methanogens, the fibrous net of strain M47 was higher than others, followed by those of M38 and M48, respectively.

By comparing the colonial size, morphological size, CH_4 production, and fibrillae formation among the three thermophilic methanogens, it might be a relation among these aspects. It was shown that strain M47 possessed the smallest size and lowest methanogenic activity. On contrary, strain M47 had more fibrillae than strains M38 and M48. There are some possibilities of why there was fibrillae formation among the methanogens, which can be proposed in this thesis as follows:

1) Due to the very small sizes of thermophilic methanogens compared to normal flora, it might be a necessity for the bacteria to form a net where they could link or gather in a clumped form.
2) A methanogen with a lower methanogenic activity, like strain M47, would have more fibrillae because most of its energy were required to form fibrillae and there were lower energy available to form methane.
3) The bacterial fibrillae might be a means of adherence, including (i) self-adherence to protect the cells and to self-secure, (ii) substrate adherence when there were substrates available, and (iii) substratum adherence, and;

4) The fibrillae could be a communication network of methanogenic thermophiles.

Similarly, the bacterial fibrillae formation is also found among microaerophilic bacteria, like lactobacilli, to adhere to the intestinal mucosa (Tannock, 1995), though the mechanism has not yet been determined.

The isolation of both anaerobic cellulolytic and methanogenic bacteria under thermophilic anaerobic conditions encountered problems with the dehydration of selective media. In this study, when isolating the bacteria 4% agar was added to solidify the culture media, CA and BMA, to help preserve moisture, although all plates were kept in plastic bags. Previously, the 2% agar in the media was used during the preliminary test, but all plates were dehydrated within 2 weeks due to the high temperature. Despite requiring to reactivate it many times during the work, silica gel was preferably used to help absorb the moisture content in an anaerobic chamber.

Besides the dehydration problem during the work, agar became cloudy when incubated at temperature higher than 50°C. This brought about difficulty in isolating cellulolytic bacteria because the clear zones were hardly observed. Chi and Casida (1984) recommended that gelrite might be an agar substitute for the cultivation of thermophilic bacteria. It is composed of glucose, glucoronic acid, and rhamnose moieties. Its outstanding features are as follows : (i) consistent batch-to-batch quality due to a stringent control of the fermentation process; (ii) economic use: as little as one half treatment of agar is required for the same purpose; (iii) fast hydration and easy control of gel setting in which gel strength is determined by choice and concentration of divalent cations (Mg^{2+}, Ca^{2+}); and (iv) the resulting gels are stable even at high temperature resisting a normal autoclaving cycle without significant loss of gel strength, and with its uniform clarity facilitating excellent visual screening of test plate.

Besides gelrite, Hermann, Noil, and Wolfe (1986) recommended an improved agar bottle plate for isolation of methanogens or other anaerobes in a defined gas atmosphere. The bottle plate effectively prevented moisture leakage.

Thus, gelrite and bottle plates could be used in the future work due to their efficiency, even if the costs were fairly high.

In cellulose fermentation by six thermophilic coculture sets, cellulose was utilized by thermophilic cellulolytic bacteria and the digestion products, CO_2 and acetate, were then consumed by thermophilic methanogens to produce methane. In the monocultures, it suggested that the thermophilic cellulolytic bacteria with higher cellulolytic activity, like strain C23, produced higher CO_2 yield but the lower amounts of acetic acid were formed. The coculture set of thermophilic cellulolytic bacteria C23 and thermophilic methanogen strain M48 showed the highest biogas production. In this coculture, CO_2 and acetic acid (acetates) were utilized the highest. This could be explained that there were less accumulation of acetate and CO_2 in cocultures because methanogens utilized them to terminal products, CH_4 and CO_2. The accumulation of acid and CO_2 may create extreme environments in culture vials where it might be formed and led to inhibition of other biological conversions (Koster and Cramer, 1987; Wilkie and Smith, 1989). Thus, cellulose digestion and cellulase production were not inhibited while methanogenesis continued. When further applied to a thermophilic digester, the acetic acid concentration should be controlled, otherwise the gas production would decrease (Zinder, Anguish, and Cardwell, 1984).

In the study, the difference of pH reduction in all coculture sets was not a major interest. On contrary, if applied the coculture technique to a large thermophilic digestor, the difference of pH reduction would bring about the study of costs and benefits due to the addition of bases to neutralize the system. When the system is more acidic, the more bases are surely needed.

When subjected to the paper waste fermentation by thermophilic cocultures, paper waste was degraded and consumed as a substrate for biogas production. During the first week, the CH_4 production were very slow. Thereafter, the gaseous products dramatically increased whereas the paper waste decreased. Paper waste contained some of lignified plant tissues which were partly recalcitrant to anaerobic digestion. Op den Camp *et al.* (1988) pointed out that materials with lignin contents higher than 25 % were not degraded within 72 hours. Acetate and CO_2 contents decreased in all six thermophilic cocultures. As in the cellulose fermentation, the coculture set of thermophilic cellulolytic bacteria strain C23 and thermophilic methanogen strain M48 produced the highest amount of biogas. A proposed diagram presenting the biogas production from paper waste by thermophilic cocultures was depicted and shown in **Figure 5.1**. In comparison to the coculture technique, thermophilic bacteria from all 50 bacterial sources were added to ferment cellulose in the same vial. The mixed culture set showed the lowest gas production. This could be explained that a mixed thermophilic population, as in natural environments, acted antagonistically and synergistically to the others bringing about the accumulation of acids and gaseous products which inhibited the biogas production. Also, in a mixed culture of fermentation, there could be sulfate-reducing bacteria outcompeting methanogenic organisms for H_2 and acetate (Sowers, Baron, and Ferry, 1984; Westermann and Ahring, 1987).

Compared to the biogas production from cellulose, the CH_4 production in the paper waste fermentation by coculture was higher. It could be due to the favorable nutrients from paper waste for both types of bacteria. From the present knowledge, calcium is used as a binding agent in toilet paper and facial tissue and is required for cell maintenance of both cellulolytic and methanogenic bacteria. In an agreement with Grohmann and Himmel (1991) that calcium is important in cellulases where calcium binds with an amino acid sequence, but it is unclear whether calcium ion is required for stability or not.

From the present study, it was demonstrated that paper waste was effectively digested and converted to biogas within 30 days. Compared to the former investigations, the amount of CH_4 production from paper waste fermentation was presented in **Table 5.1**. The CH_4 formation (as mol/mol hexose) from cellulose and paper waste of a thermophilic coculture in this study was higher than those of many researches done so far, but less than a triculture system. To enhance this present thermophilic coculture, the effective thermophilic acetate-degrading bacteria, such as *Syntrophomonas* sp. and *Desulfovibrio* sp., and/or thermophilic aceticlastic methanogens, such as *Methanosarcina thermophila* or *Methanosarcina* strain CHTI 55 (Lowe *et al.*, 1993), should be isolated and added to the system to help utilize acetate during the fermentation. In other words, a triculture or quadculture is recommended. Thus, the CH_4 yield would be higher. This could provide an attractive means of paper waste reduction with the recovery of methane as an energy source. The potential for converting cellulosic wastes into industrial substrates surely stimulated the interest in thermophilic fermentations.

In addition to the above mentioned, if coculture technique were still preferable, finding the optimum ratios of thermophilic cellulolytic bacteria and thermophilic methanogen would help increase the biogas production because the acid and methane production could be controlled properly. Hence, unfavorable conditions in biogas production, such as high acid contents which is toxic to all methanogens, could be eliminated, effectively.

In conclusion, research on bacteria and the enzymes they produce has made a significant contribution to the understanding of the biological conversion of organic matter, like cellulose and paper waste, to methane. This has been due in part to recent scientific interest in the anaerobic thermophilic bacteria as biological material useful in developing an understanding of basic life process. Thus, the isolation of selected new thermophilic bacterial species could provide valuable material for future genetic and other biological investigations at the molecular level, in addition to providing insights into the microbial interactions occurring during anaerobic thermophilic digestion.

In pursuit of unique thermophilic anaerobes provided by selection, this study showed a variety of unknown bacteria. The isolates included bacteria which hydrolyze cellulose and bacteria which produce methane under conditions of specific interest to this experiment. A general conclusion which can be drawn from the coculture data reported here is that methanogenesis from specific intermediates is enhanced by the interactions of a multiplicity of substrate linked microbial species. Thus, the selected thermophilic bacteria could be used and applied to other thermophilic digesters, effectively.

Further investigation of not only individual microbial species but, equally important, their interactions and recycling processes, should provide new insight into the control and regulation of anaerobic digestion and paper waste treatment.

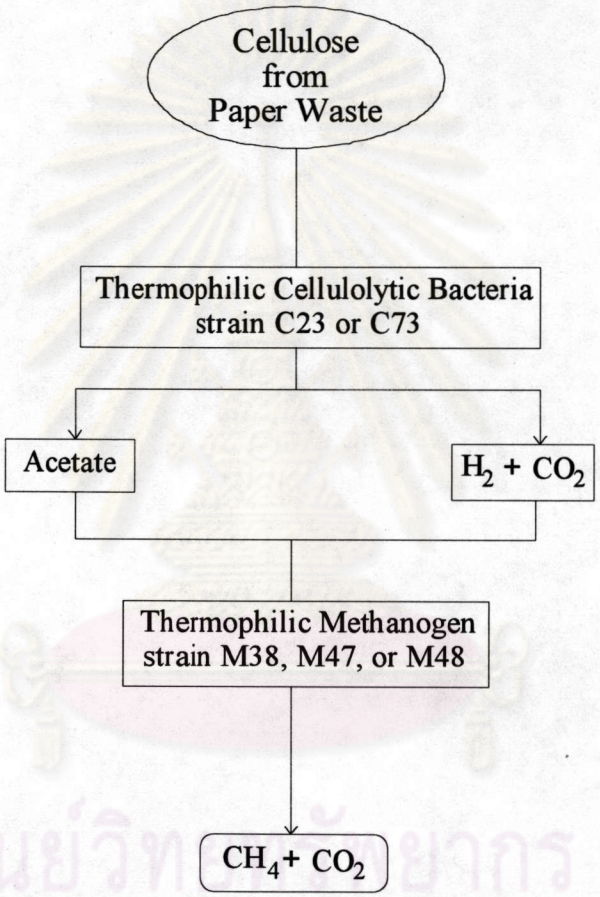


Figure 5.1 Proposed diagram presenting the biogas production from paper waste by thermophilic cocultures

Table 5.1 The amount of methane production compared to the former investigations

CH_4 (mol/mol hexose)	Substrate	Organisms	References
0.45	cellulose	Cellulolytic bacteria CU1 + Methanogen Sc4	Sribenjalux and Vejjanukroh, 1984
0.50 (mol/mol butyrate)	butyrate	a butyrate-degrading bacteria + *M. thermoautotrophicum*	Ahring and Westermann, 1987
0.52	cellulose	Cellulolytic bacteria CU3 + Methanogen Sc5	Sribenjalux and Vejjanukroh, 1984
0.56	cellulose	*C. thermocellum* + *M. thermoautotrophicum*	Weimer and Zeikus, 1977
0.64	cellulose	*A. cellulolyticus* + *Methanosarcina barkeri*	Laube, 1981
0.75	cellulose	*Ruminococcus albus* + *Methanobrevibacter smithii*	Pavlostathis *et al.*, 1990
1.18	cellulose	Thermophilic cellulolytic bacteria C23 + Thermophilic methanogen M48	This study
1.21	paper waste	Thermophilic cellulolytic bacteria C23 + Thermophilic methanogen M48	This study
1.45 (mol/mol lactate)	lactate	*C. formicoaceticum* + *Methanosarcina mazei*	Yang and Tang, 1991
2.08	cellulose	*A. cellulolyticus* + *Methanosarcina barkeri* + *Desulfovibrio* sp.	Laube, 1981
2.33 (mol/mol butyrate)	butyrate	a butyrate-degrading bacteria + *M. thermoautotrophicum* + TAM	Ahring and Westermann, 1987