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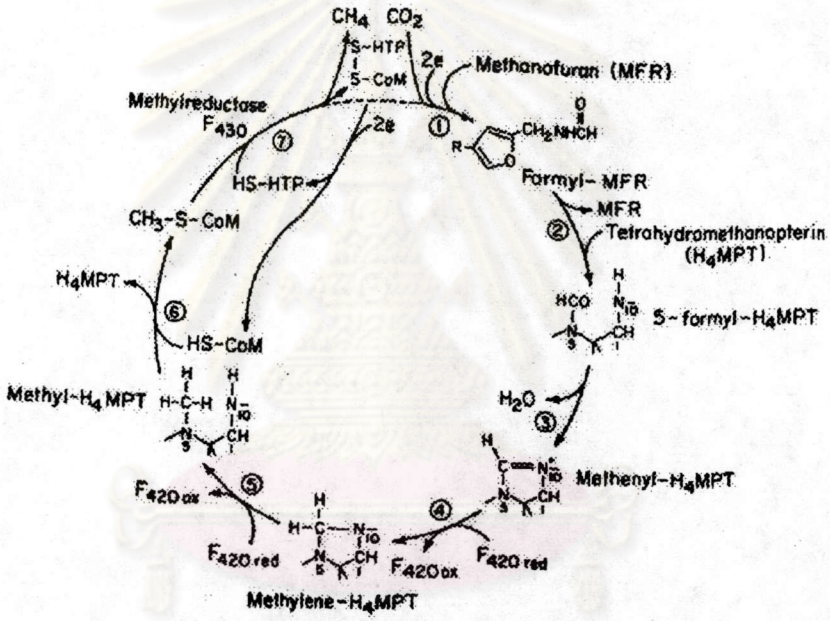
APPENDICES

APPENDIX A—METHANOGENESIS PATHWAY AND STRUCTURE OF COENZYMES

Source: Wolfe, 1990.

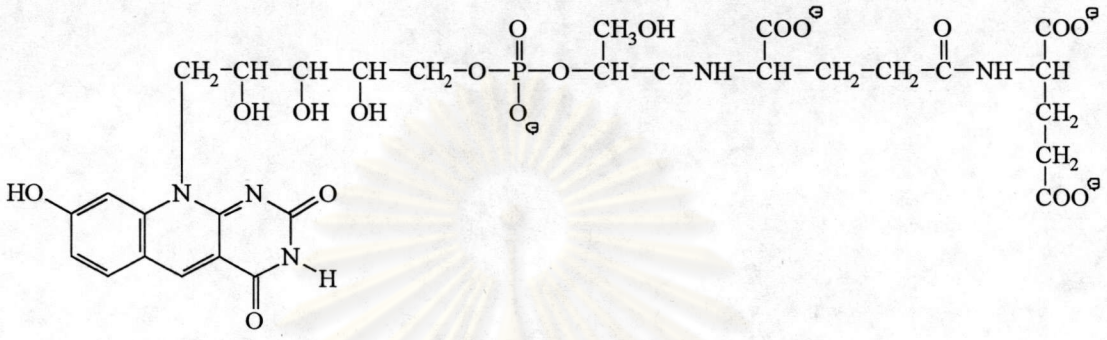
1. Propose Pathway of Methanogenesis



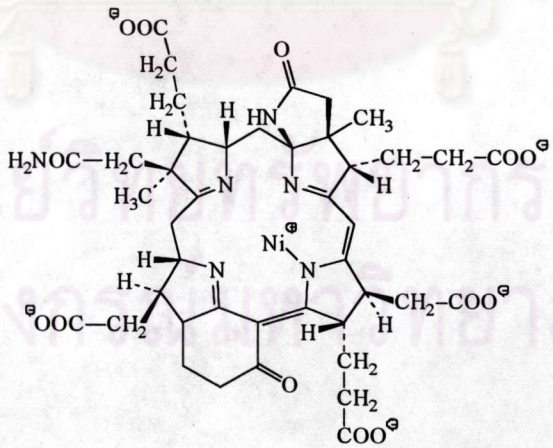
2. Structure of Coenzyme M

$HS\text{-}CH_2\text{-}CH_2\text{-}SO_3^-$

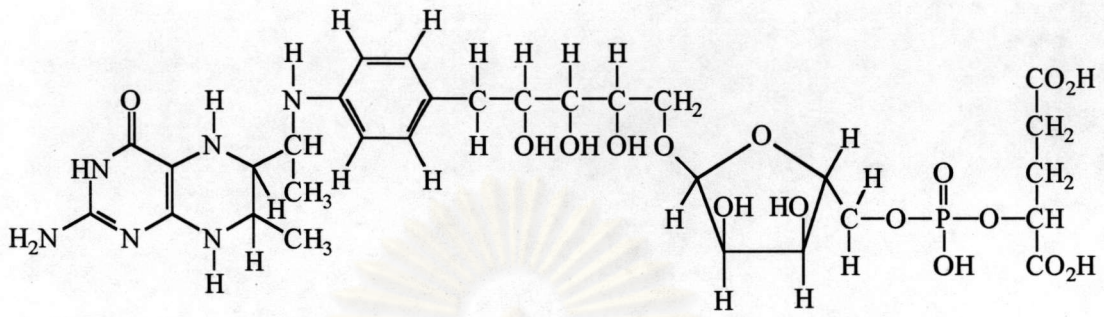
3. Structure of Factor F_{420}



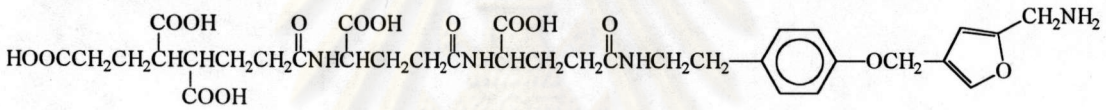
4. Structure of Factor F_{430}



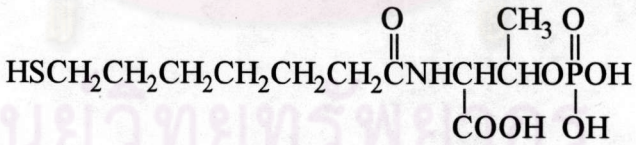
5. Structure of Methanopterin



6. Structure of Methanofuran



7. Structure of Component B



APPENDIX B -- MEDIA

Formula

1. Cellulose Agar (CA)

Formula in milliliter and gram per 1 liter

KH_2PO_4	0.75
K_2HPO_4	1.5
NH_4Cl	0.9
$MgCl_2.6H_2O$	0.4
NaCl	0.9
Yeast Extract (Difco; Detroit, Michigan, U.S.A.)	0.6
Peptone (Difco; Detroit, Michigan, U.S.A.)	2.0
Cysteine HCl	1.0
$FeSO_4$ (10% solution)	30 µl
Trace Minerals	9.0
Trace Vitamins	1.0
Resazurin (0.1% solution)	1.0
Cellulose[a]	10
Bacto Agar	40
Distilled Water	989

Final pH 7.2

Preparation of this medium would be discussed later.

[a] Only when isolating cellulolytic thermophiles, 7 g α-cellulose plus 3 g carboxymethylcellulose (CMC) were used as a substrate.

2. Cellulose Broth (CB)

Formula in milliliter and gram per 1 liter

KH_2PO_4	0.75
K_2HPO_4	1.5
NH_4Cl	0.9
$MgCl_2.6H_2O$	0.4
NaCl	0.9
Yeast Extract (Difco; Detroit, Michigan, U.S.A.)	0.6
Peptone (Difco; Detroit, Michigan, U.S.A.)	2.0
Cysteine HCl	1.0
$FeSO_4$ (10% solution)	30 µl
Trace Minerals	9.0
Trace Vitamins	1.0
Resazurin (0.1% solution)	1.0
Cellulose	0.1
Distilled Water	989

Final pH 7.2

Preparation of this medium would be discussed later.

3. Trace Minerals

Formula in gram per liter of distilled water

Nitrilotriacetic Acid	1.5
$MgSO_4.7H_2O$	3.0
$MnSO_4.2H_2O$	0.5
NaCl	1.0
$FeSO_4.7H_2O$	0.1
$CoCl_2$	0.1
$CaCl_2.2H_2O$	0.1
$ZnSO_4$	0.1
$CuSO_4.5H_2O$	0.01
$AlK(SO_4)_2$	0.01
H_3BO_3	0.01
$Na_2MoO_4.2H_2O$	0.01

Final pH 7.0

Preparation of trace mineral solution would be discussed later.

4. Trace Vitamins

Formula in milligram per liter of distilled water

Biotin	2
Folic Acid	2
Pyridoxine Hydrochloride	10
Thiamine Hydrochloride	5
Riboflavin	5
Nicotinic Acid	5
DL-Calcium Pantothenate	5
Vitamin B_{12}	0.1
p-Aminobenzoic Acid	5
Lipoic Acid	5

Sterilized by 0.45 μ millipore filter, freezed and stored in a refrigerator in the absence of light util used.

5. Balch's Medium II Agar (BMA)

Formula in milliliter and gram per liter

Mineral	25
NH_4Cl	1.25
Trace Minerals	10
Trace Vitamins	10
$Fe(NH_4)_2(SO_4)_2.7H_2O$	0.02
$NaHCO_3$	7.5
Cysteine HCl	0.6
$Na_2S.9H_2O$	0.6
Agar	40
Distilled Water	955

Final pH 7.2

Preparation of this medium would be discussed later.

6. Balch's Medium II Broth (BMB)

Formula as same as Balch's Medium II Agar but agar was omitted.

7. Mineral

Formula in grams per liter of distilled water.

KH_2PO_4	6
$(NH_4)_2SO_4$	6
NaCl	12
$MgSO_4.7H_2O$	2.6
$CaCl_2.2H_2O$	0.16

Preparation

1. Preparation of Medium number 1, 2, 5, and 6

The 500 ml solution of ingredients was added to a 1-liter ground joint round bottom flask[b] and heated. When the solution was boiled, cysteine hydrochloride and/or sodium sulfide were added which time the color turned from blue to reddish-pink. During boiling the solution for several minutes, the pink color disappeared, indicating reduction. The flask was suddenly stoppered with a rubber stopper, covered with cloth and tied tightly, and autoclaved for 15 minutes. After transferring to the anaerobic chamber, trace vitamin solution was added. Before used, any plate or vial contained certain medium was incubated over night.

2. Preparation of Trace Minerals

The nitrilotriacetic acid was suspended in 500 ml distilled water and dissolved by titrating with 2-3 N KOH until the pH was stabilized at 6.5. The rest of the ingredients were added and dissolved in the order they were listed. Finally, the volume was adjusted to 1 liter with distilled water.

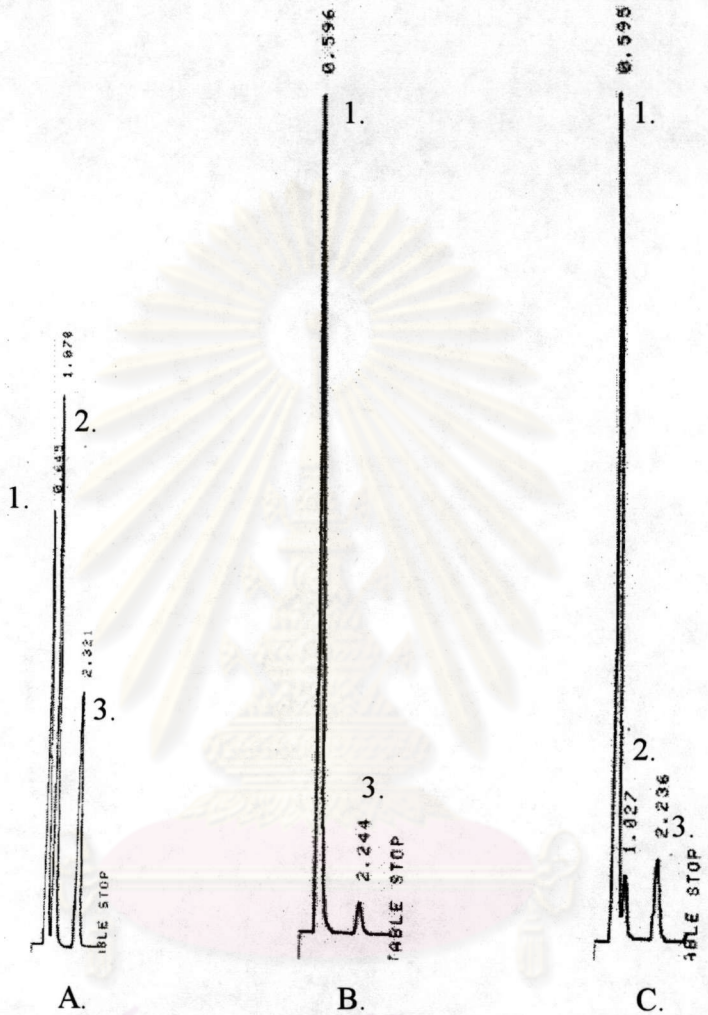
[b] The round shape of the flask prevents breakage due to the sterilization pressure.

APPENDIX C -- CONDITION OF CHROMATOPAC, STANDARD CHROMATOGRAM AND CALCULATION METHOD FOR GASEOUS PRODUCTS ANALYSIS

1. Condition of Chromatopac

Column :	Porapak QS, 80-100 mesh, 2 m long, 3 mm inside diameter, 4 mm outside diameter.
Detector :	Thermal Conductivity Detector
Temperature :	Column temp 25°C Injector temp 90°C
Current :	100 mA
Flowrate :	20 ml per min
Carrier Gas :	He

2. Standard Chromatogram



1. N_2 2. CH_4 3. CO_2

A. Standard gas

B. Gas drawn from a fermentaion vial inoculated with a thermophilic cellulolytic bacteria

C. Gas drawn from a fermentation vial inoculated with a thermophilic cellulolytic bacteria and a thermophilic methanogen or coculture

3. Calculation Method of Gas Analyzed from Gas Chromatographic Method (Mah, Smith, and Baresi, 1978)

$$\text{Micromoles of gas formed} = [\{\%Gas \times (V_f + V_r)\} /2.24] + M_p$$

while ;

%Gas = the volume (ml) per 100 ml of gas phase

V_f = the volume (ml) of gas phase in culture vial

V_r = the volume (ml) of gas phase removed by syringe following equilibration of atmospheric pressure

M_p = the sum of micromoles removed after equilibration

= $[\Sigma (\%Gas \times V_r) /2.24]$

APPENDIX D -- PREPARATION OF STANDARD SOLUTION, CONDITION OF CHROMATOPAC, AND CHROMATOGRAM OF STANDARD VOLATILE FATTY ACIDS

1. Preparation of Volatile Fatty Acid Standard Solutions

   1.1 Prepared standard solutions of each volatile acid by diluting the following volumes to 100 ml with distilled water :

   - Acetic acid 5.7 ml
   - Propionic acid 7.5 ml
   - Butyric acid 9.1 ml

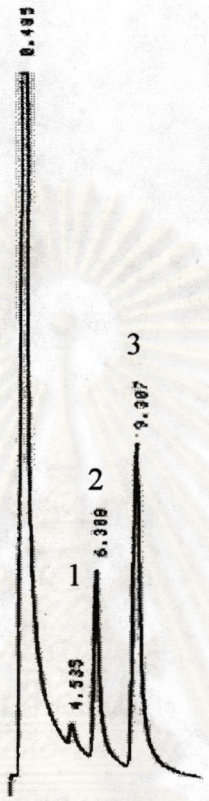
   1.2 Kept each standard solution in glass bottles with ground glass stoppers.

   1.3 To prepare a working standard solution, mixed together 1 ml of each of the standards prepared in step 1.1; then diluted to 100 ml with distilled water. Stored this standard in a stoppered glass bottle.

2. Condition of Chromatopac

Column :	10% FFAP, 100-120 mesh, 1.5 m long, 4 mm inside diameter
Detector :	Flame Ionization Detector
Temperature :	Column temp 140°C Injector temp 240°C
Current :	100 mA
Flowrate :	50 ml per min
Carrier Gas :	N_2

3. Chromatogram of Standard Volatile Fatty Acids



1. Acetic Acid	2. Propionic Acid	3. Butyric Acid

APPENDIX E -- PREPARATION OF REAGENTS, STANDARD CURVE, AND CALCULATION METHOD OF CELLULOSE DETERMINATION

1. Preparation of Reagents

Acetic/Nitric Reagent

Mixed 150 ml 80% acetic acid and 15 ml concentrated nitric acid

Phenol Reagent

Added 5 g reagent-graded phenol to 100 ml distilled water

2. Preparation of Standard Curve

To prepare the stock standard, dissolved 100 mg pure cellulose (α-cellulose fiber, Sigma catalog No. C-8002, dried for 6 hours at 105°C and cooled in desicator) in 10.0 ml 67% sulfuric acid with gentle heated. Diluted the solution to 100 ml with 0.15% (wt/vol) benzoic acid, which was added as a preservative. Stored at 5°C. This solution was stable for serveral months. Diluted 1:10 in distilled water just before use to give a solution containing 100 μg cellulose per ml. Prepared standards (10 to 100 μg/ml) from the diluted solution, then proceeded as in step xii of the procedure.

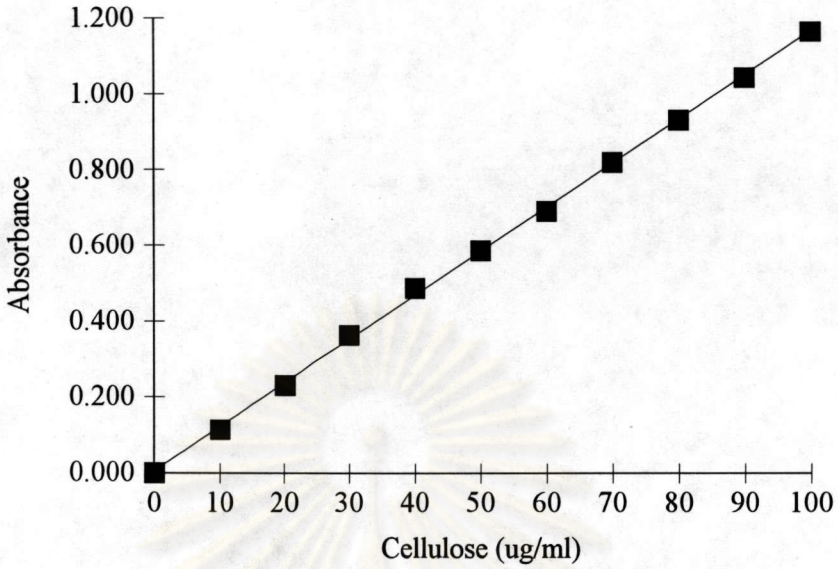


Figure E.1 A linear standard curve of cellulose detected by this method.

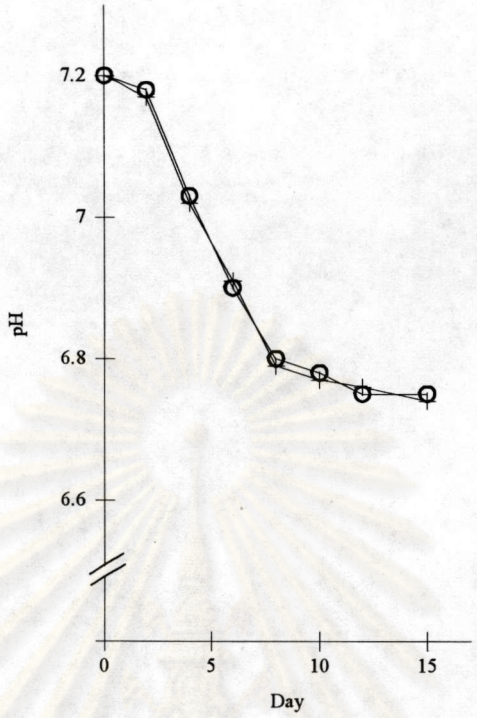
3. Calculation Method

$$\text{Cellulose } (\mu g) = \frac{\text{Absorbance read}}{\text{Slope constant}}$$

where slope constant equals to 0.0116.

APPENDIX F -- FERMENTATION OF CELLULOSE BY THERMOPHILIC COCULTURE

(a)



(b)

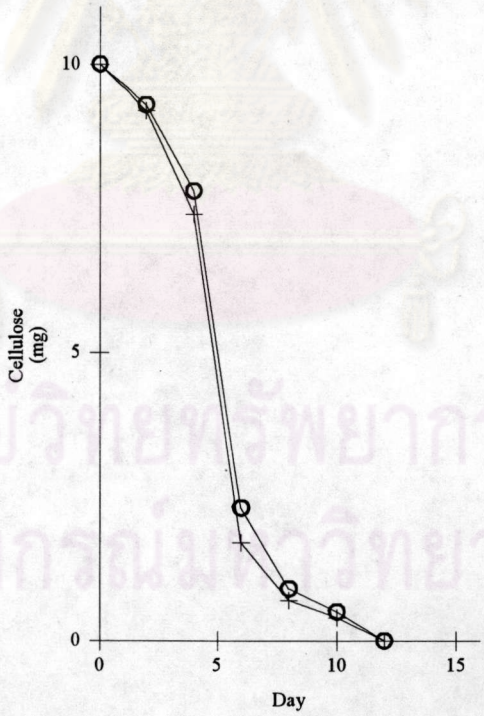
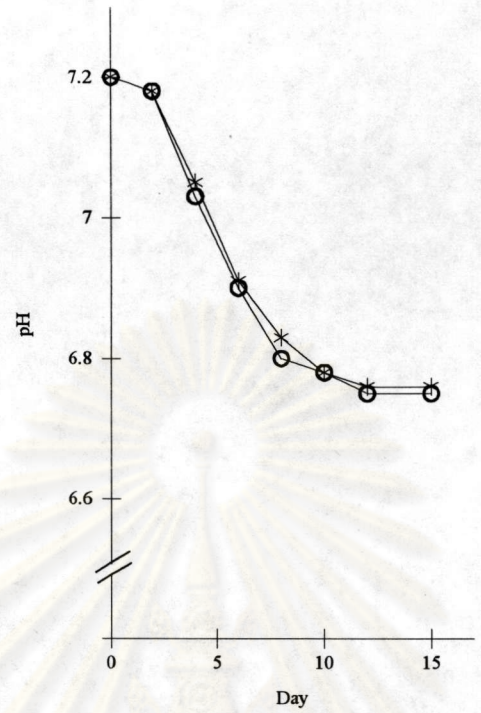


Figure F-1 pH reduction (a) and remained cellulose (b) in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M38: C23 (O) and C23 + M38 (+)

(a)



(b)

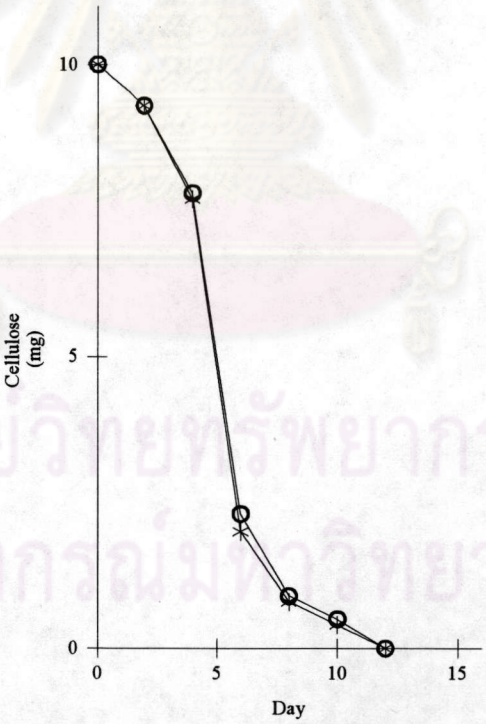
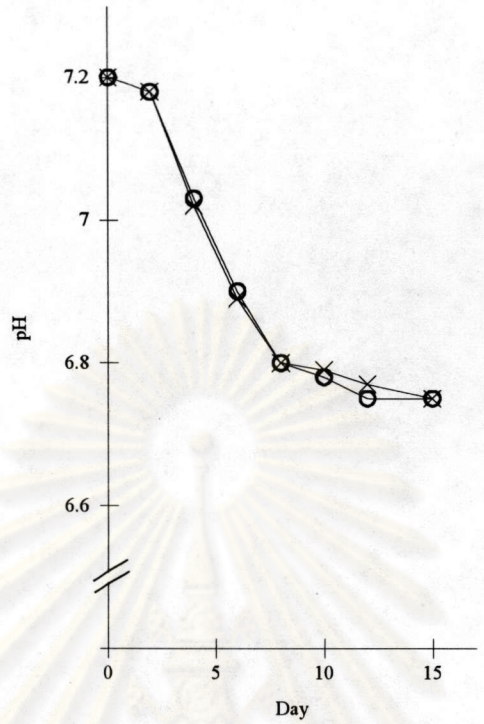


Figure F-2 pH reduction (a) and remained cellulose (b) in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M47: C23 (O) and C23 + M47 (*)

(a)



(b)

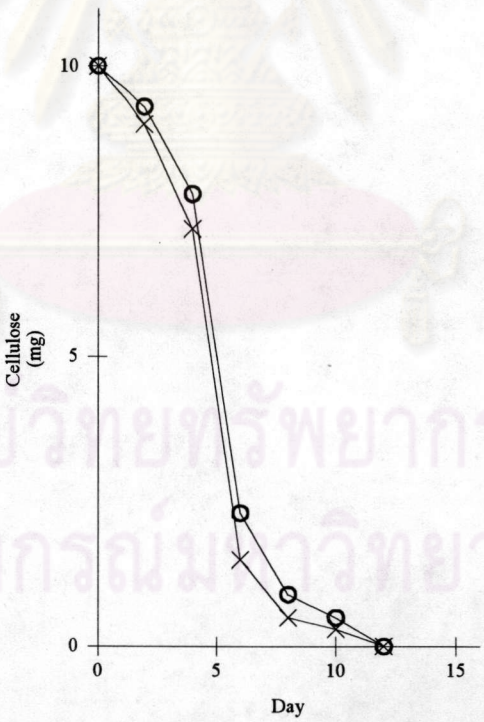
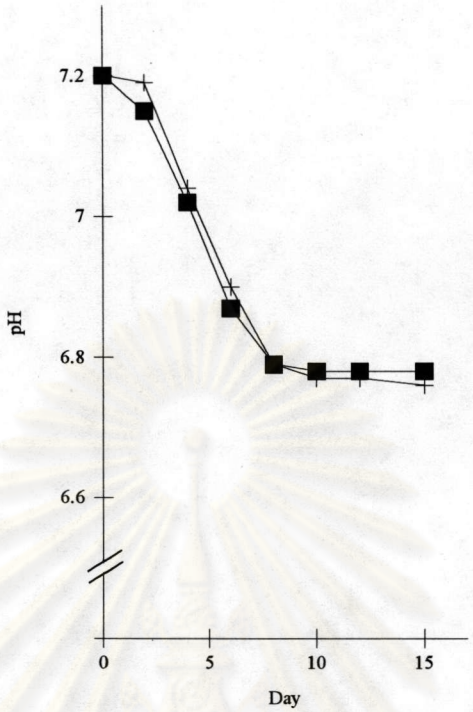


Figure F-3 pH reduction (a) and remained cellulose (b) in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M48: C23 (O) and C23 + M48 (×)

(a)



(b)

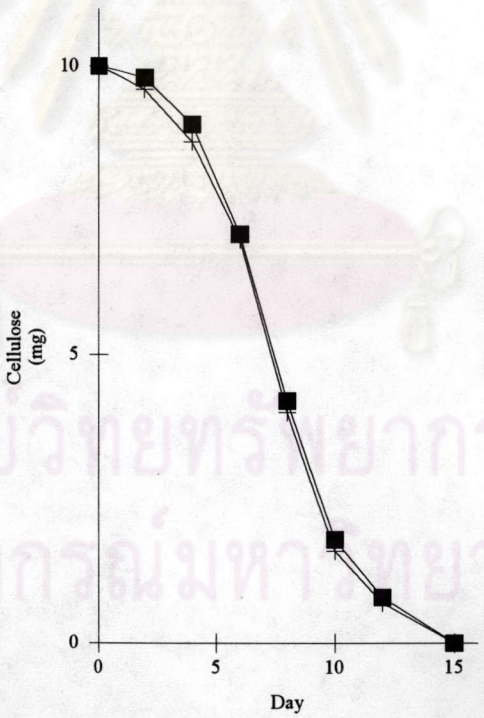
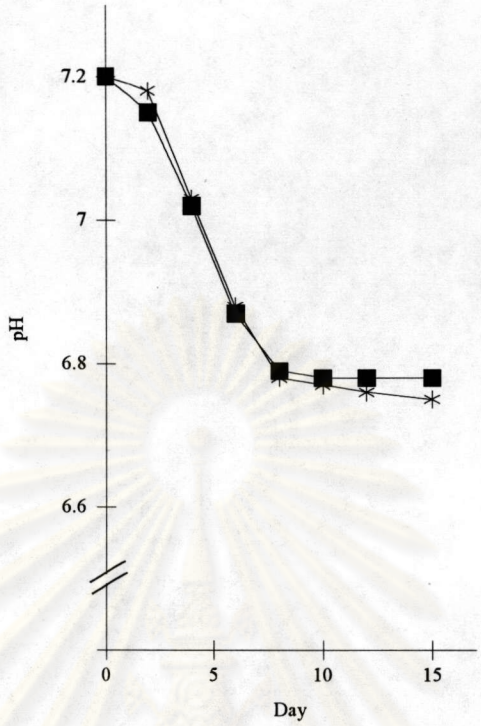


Figure F-4 pH reduction (a) and remained cellulose (b) in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M38: C73 (■) and C73 + M38 (+)

(a)



(b)

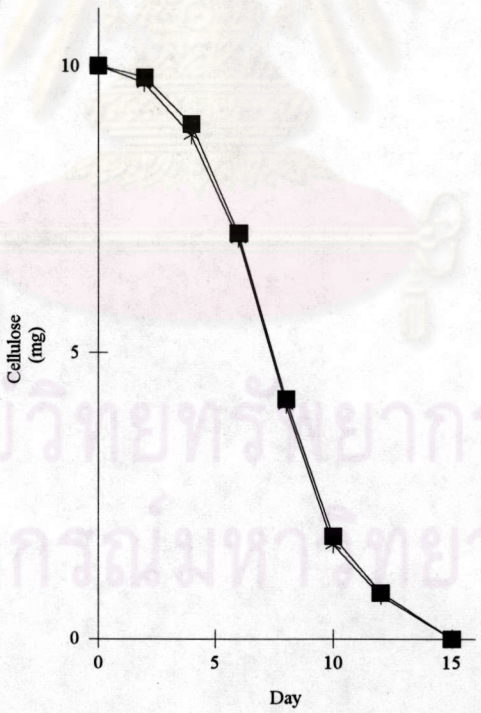
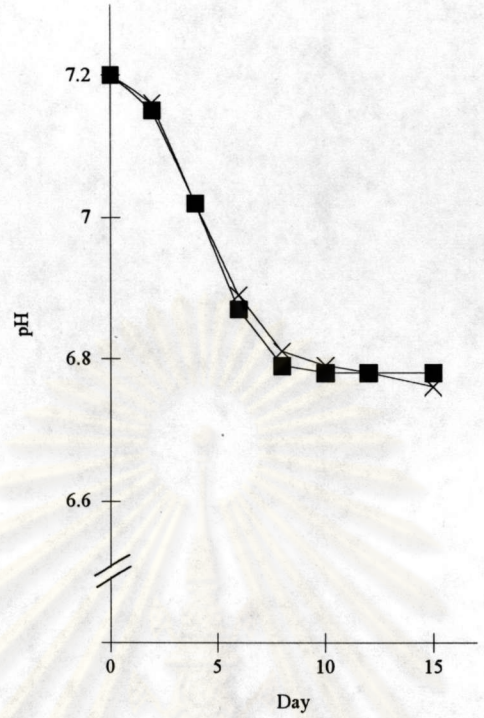


Figure F-5 pH reduction (a) and remained cellulose (b) in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M47: C73 (■) and C73 + M47 (*)

(a)



(b)

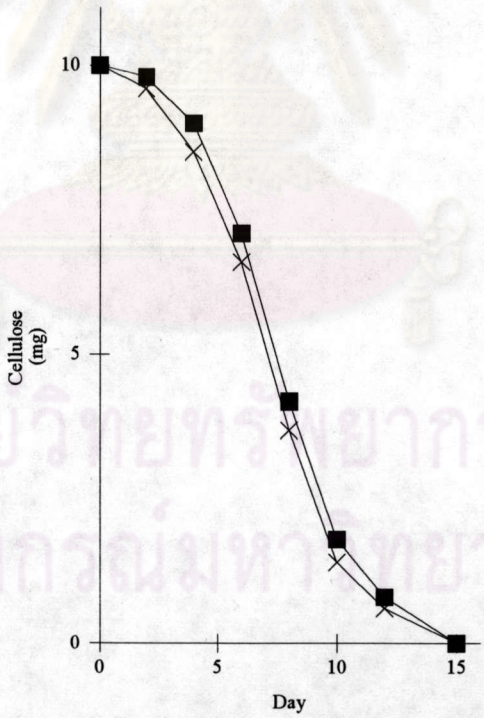
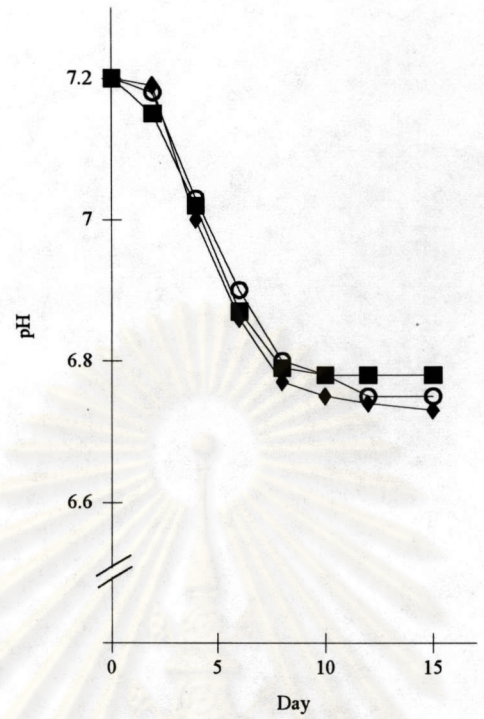


Figure F-6 pH reduction (a) and remained cellulose (b) in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M48: C73 (■) and C73 + M48 (×)

(a)



(b)

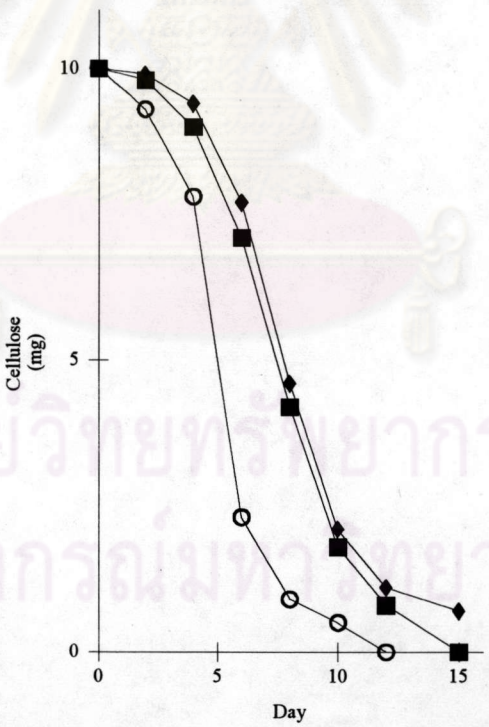


Figure F-7 pH reduction (a) and remained cellulose (b) in cellulose fermentation by a thermophilic mixed culture: C23 (○), C73 (■) and mixed culture (♦)

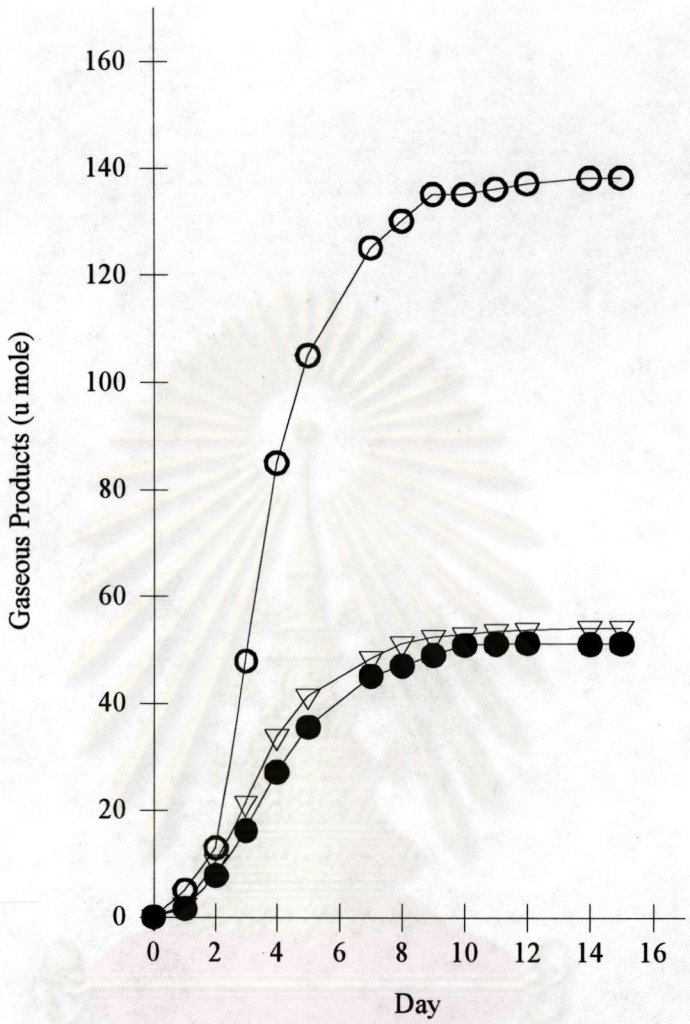


Figure F-8 Gas production in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M38: C23 (O = CO_2) and C23 + M38 (● = CO_2 , ∇ = CH_4)

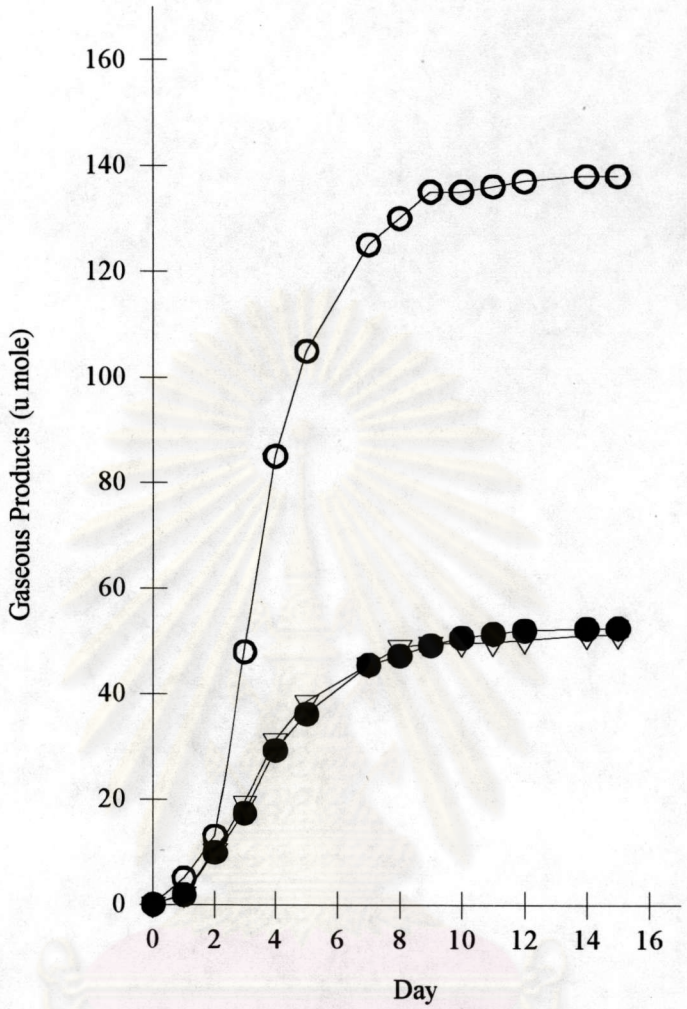


Figure F-9 Gas production in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M47: C23 (O = CO_2) and C23 + M47 (● = CO_2 , ∇ = CH_4)

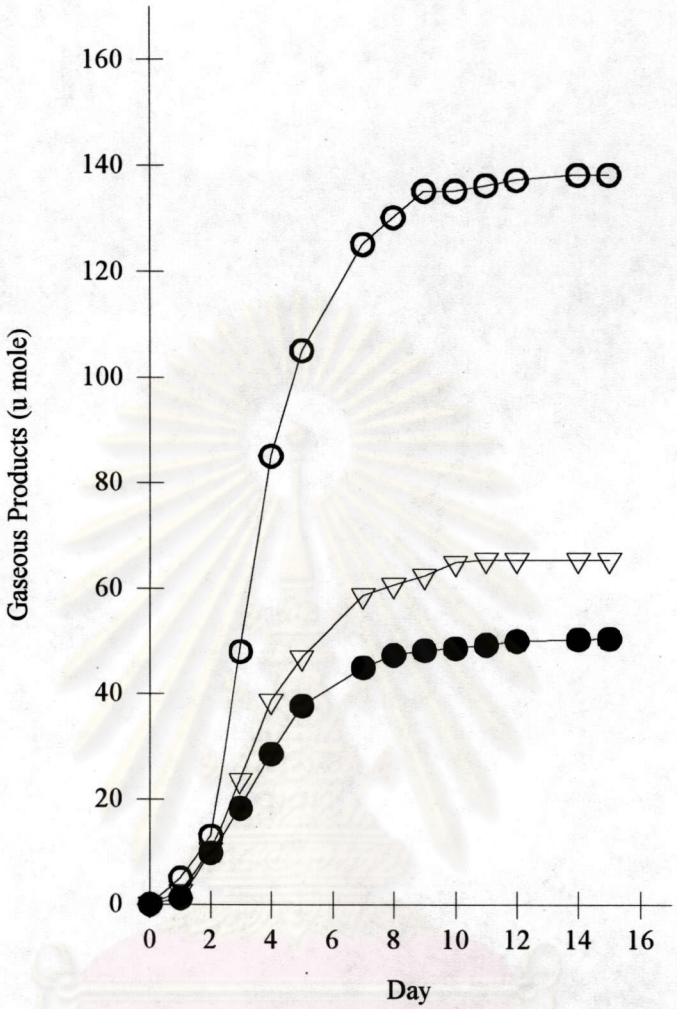


Figure F-10 Gas production in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M48:
C23 (O = CO_2) and C23 + M48 (● = CO_2 , ∇ = CH_4)

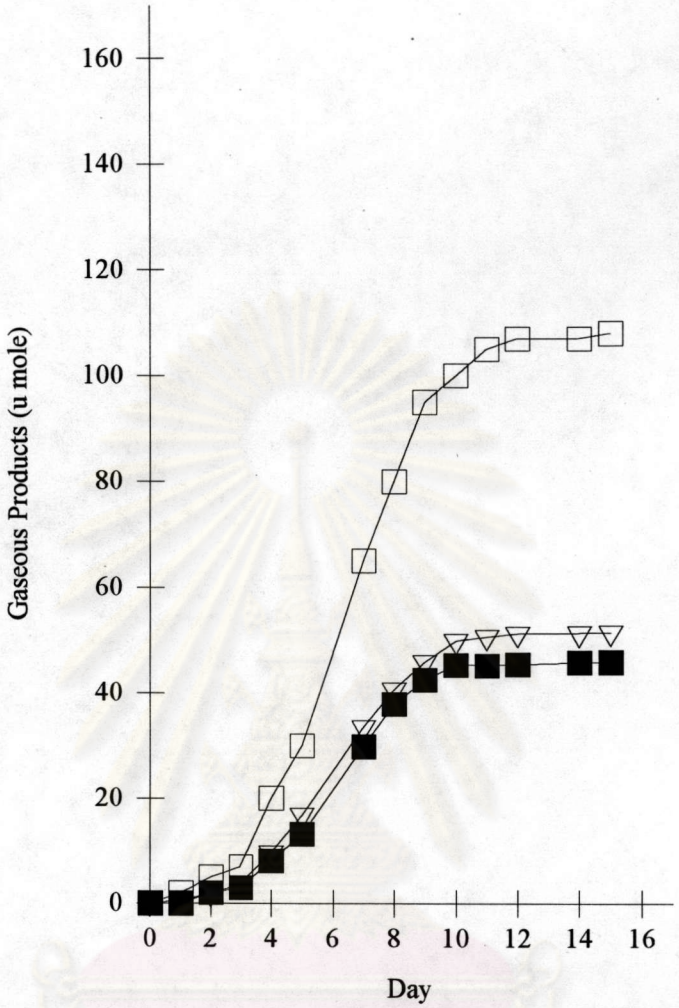


Figure F-11 Gas production in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M38:
C73 (□ = CO_2) and C73 + M38 (■ = CO_2 , ∇ = CH_4)

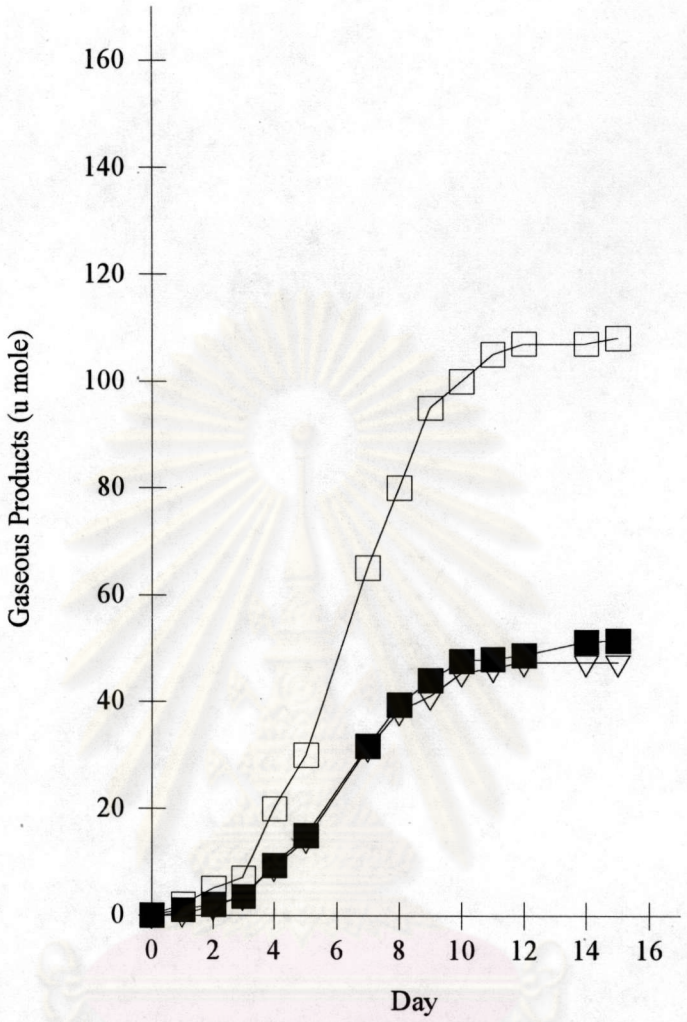


Figure F-12 Gas production in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M47: C73 (□ = CO_2) and C73 + M47 (■ = CO_2 , ∇ = CH_4)

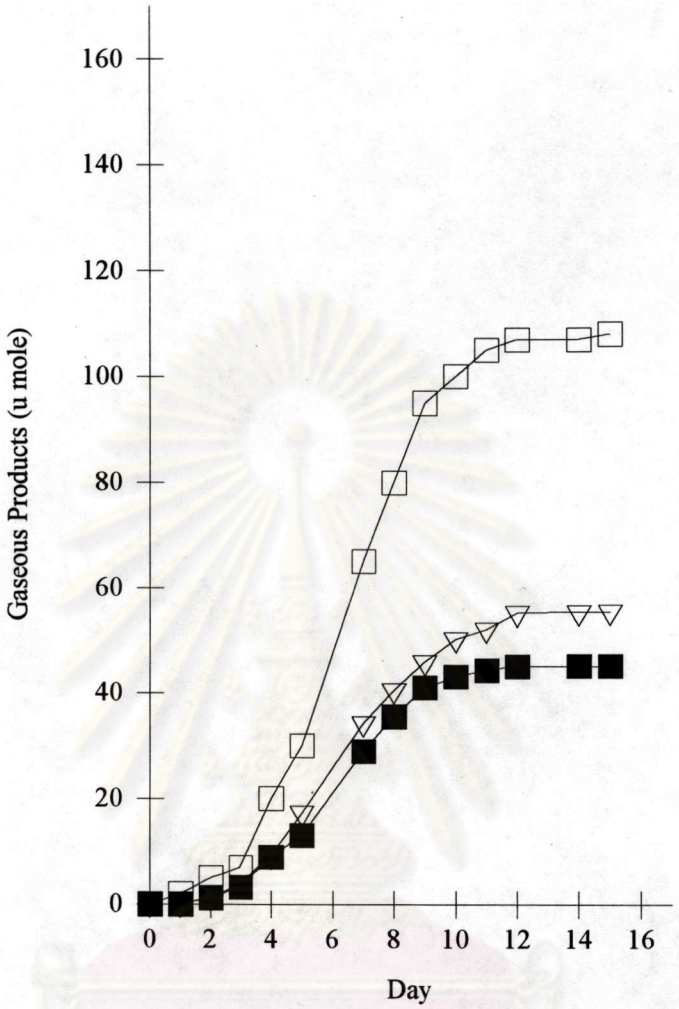


Figure F-13 Gas production in cellulose fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M48: C73 (□ = CO_2) and C73 + M48 (■ = CO_2 , ∇ = CH_4)

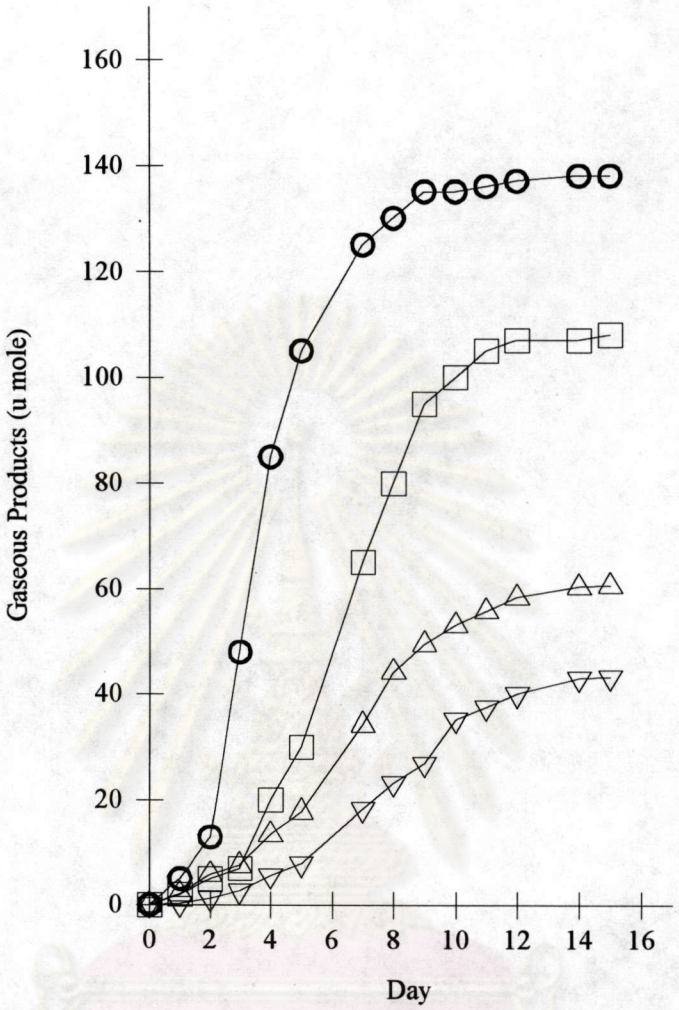


Figure F-14 Gas production in cellulose fermentation by a thermophilic mixed culture: C23 (O = CO_2), C73 (□ = CO_2) and mixed culture (Δ = CO_2 , ∇ = CH_4)

APPENDIX G -- FERMENTATION OF PAPER WASTE BY THERMOPHILIC COCULTURE

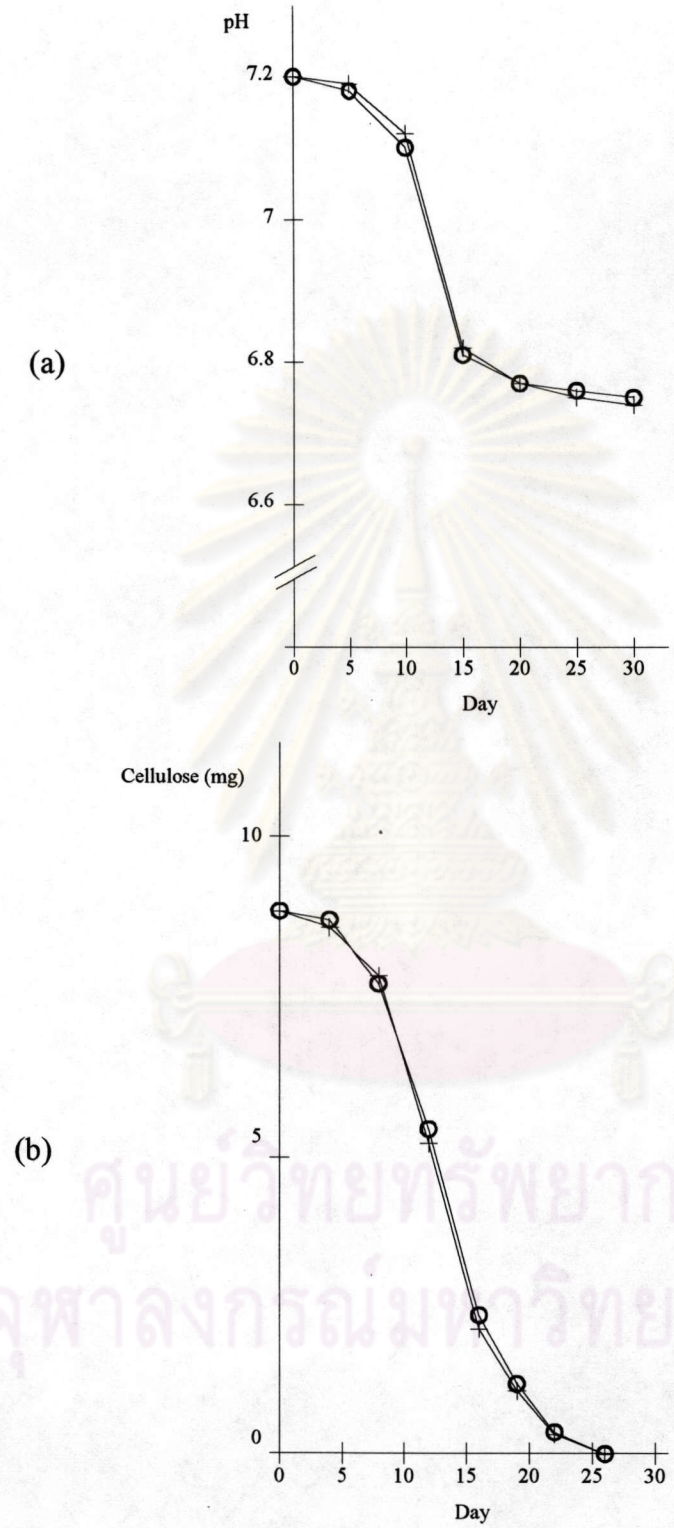


Figure G-1 pH reduction (a) and remained cellulose (b) in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M38: C23 (O) and C23 + M38 (+)

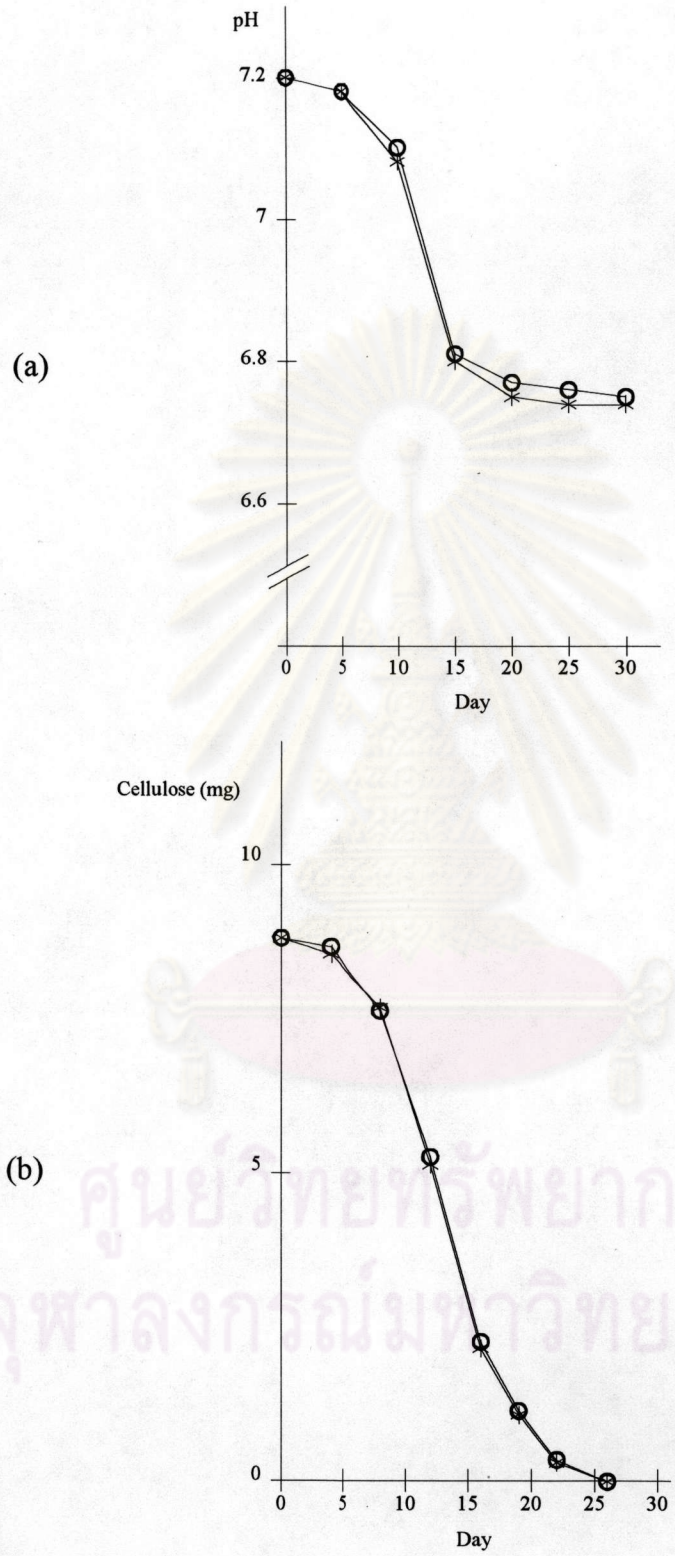


Figure G-2 pH reduction (a) and remained cellulose (b) in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M47: C23 (O) and C23 + M47 (*)

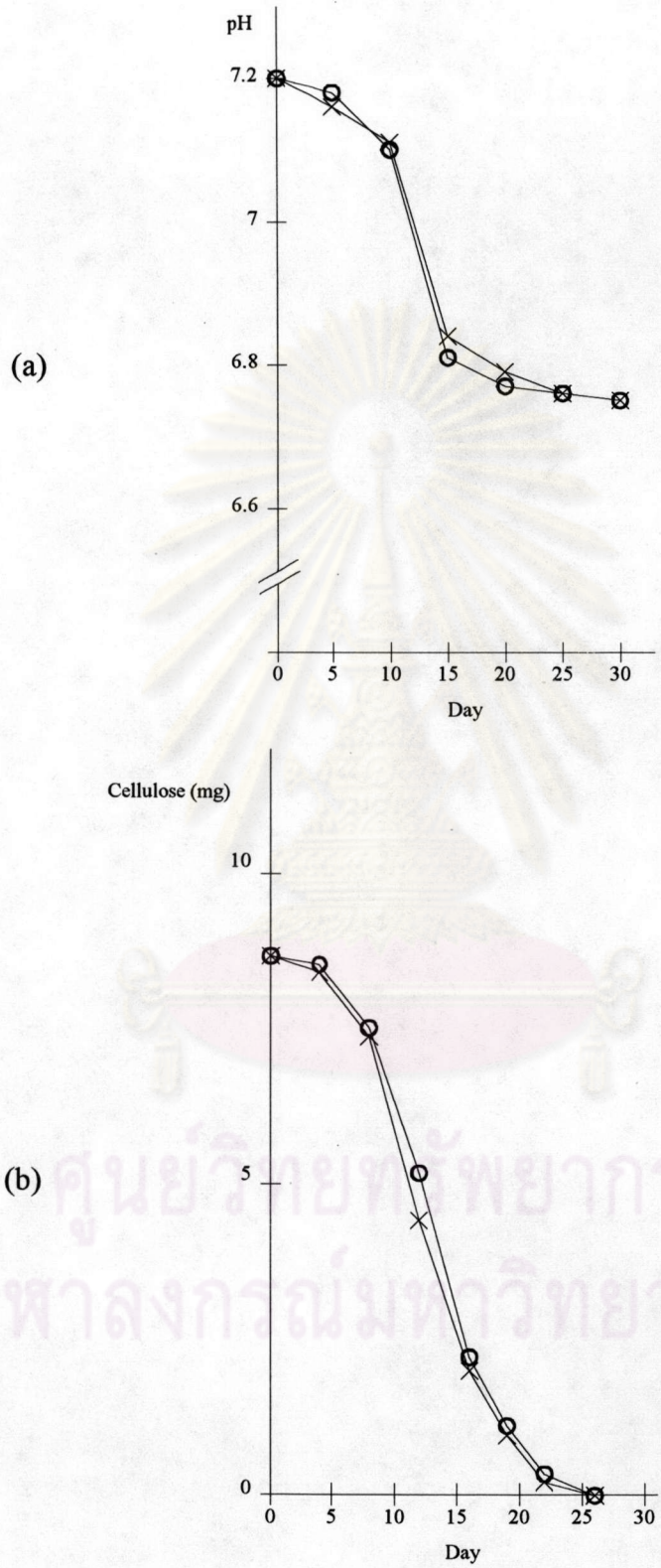


Figure G-3 pH reduction (a) and remained cellulose (b) in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M48: C23 (O) and C23 + M48 (×)

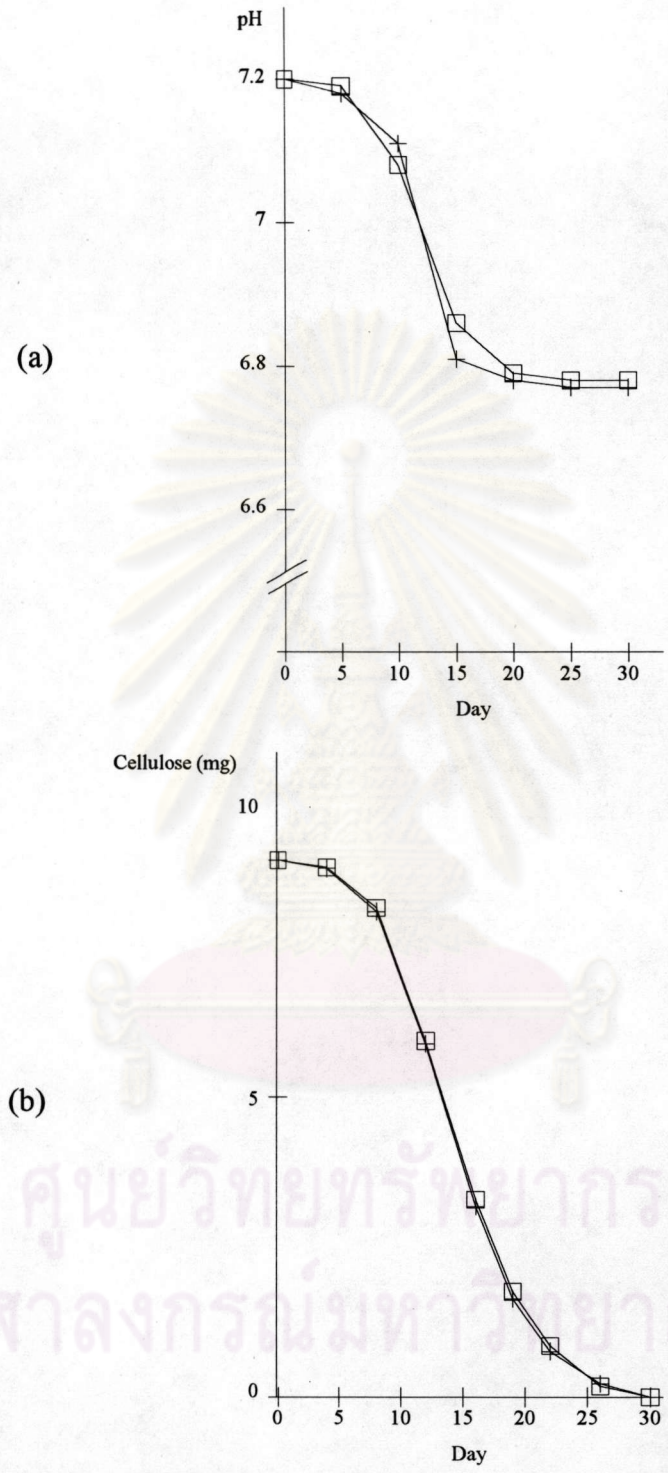


Figure G-4 pH reduction (a) and remained cellulose (b) in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M38: C73 (□) and C73 + M38 (+)

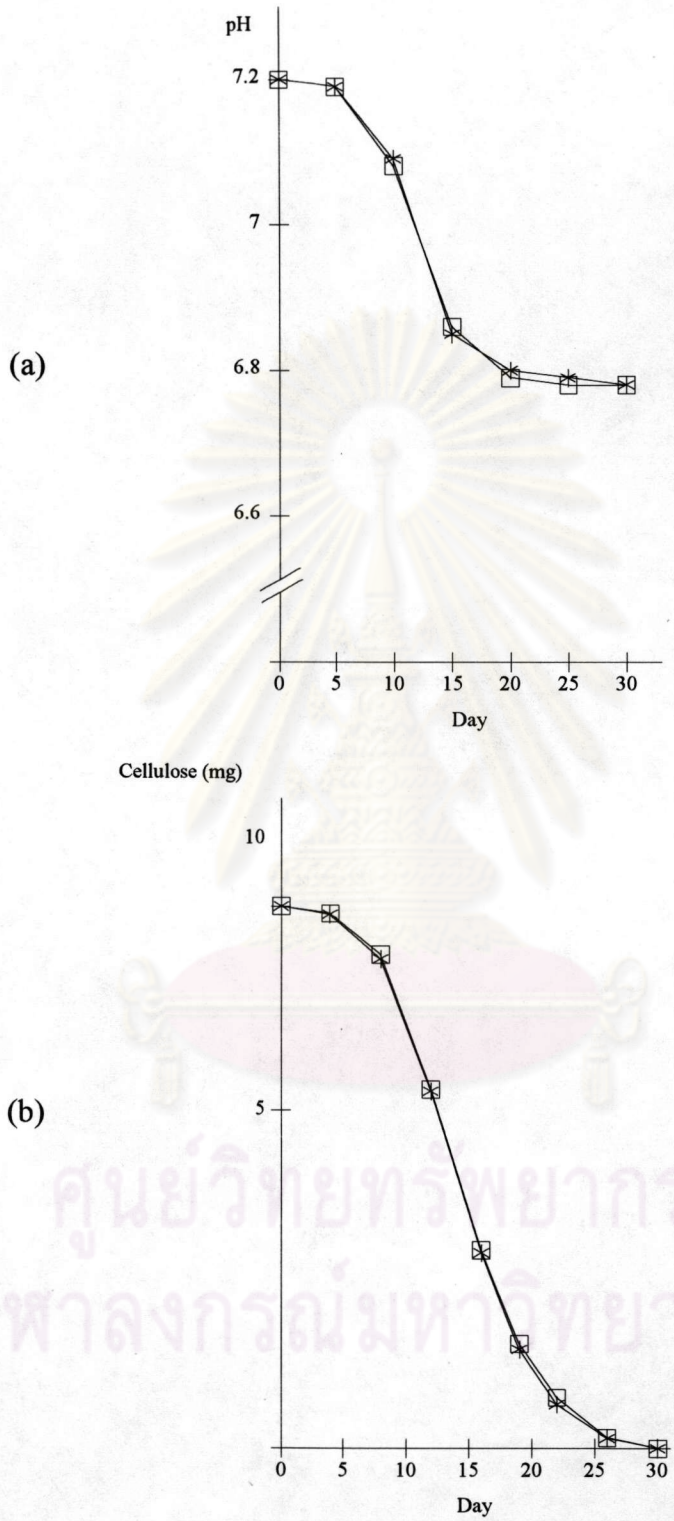


Figure G-5 pH reduction (a) and remained cellulose (b) in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M47: C73 (□) and C73 + M47 (*)

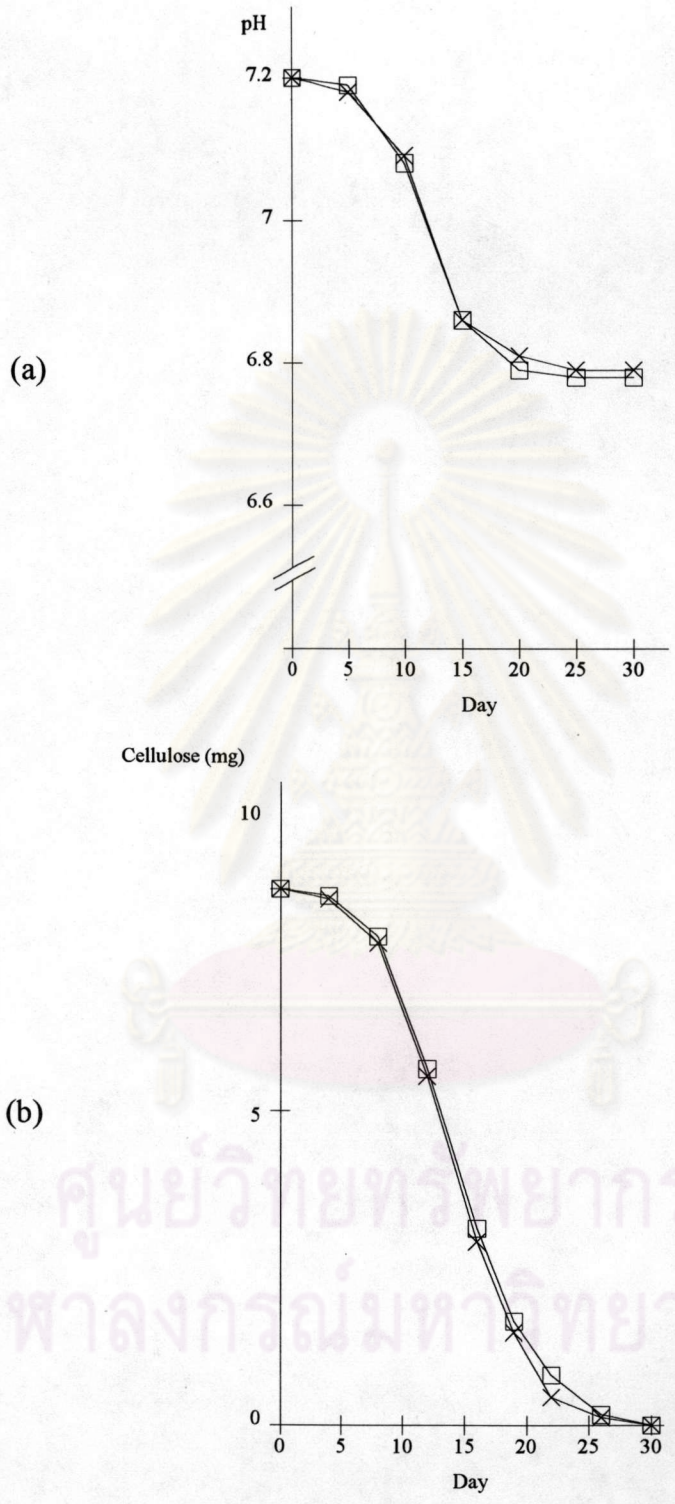


Figure G-6 pH reduction (a) and remained cellulose (b) in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M48: C73 (□) and C73 + M48 (×)

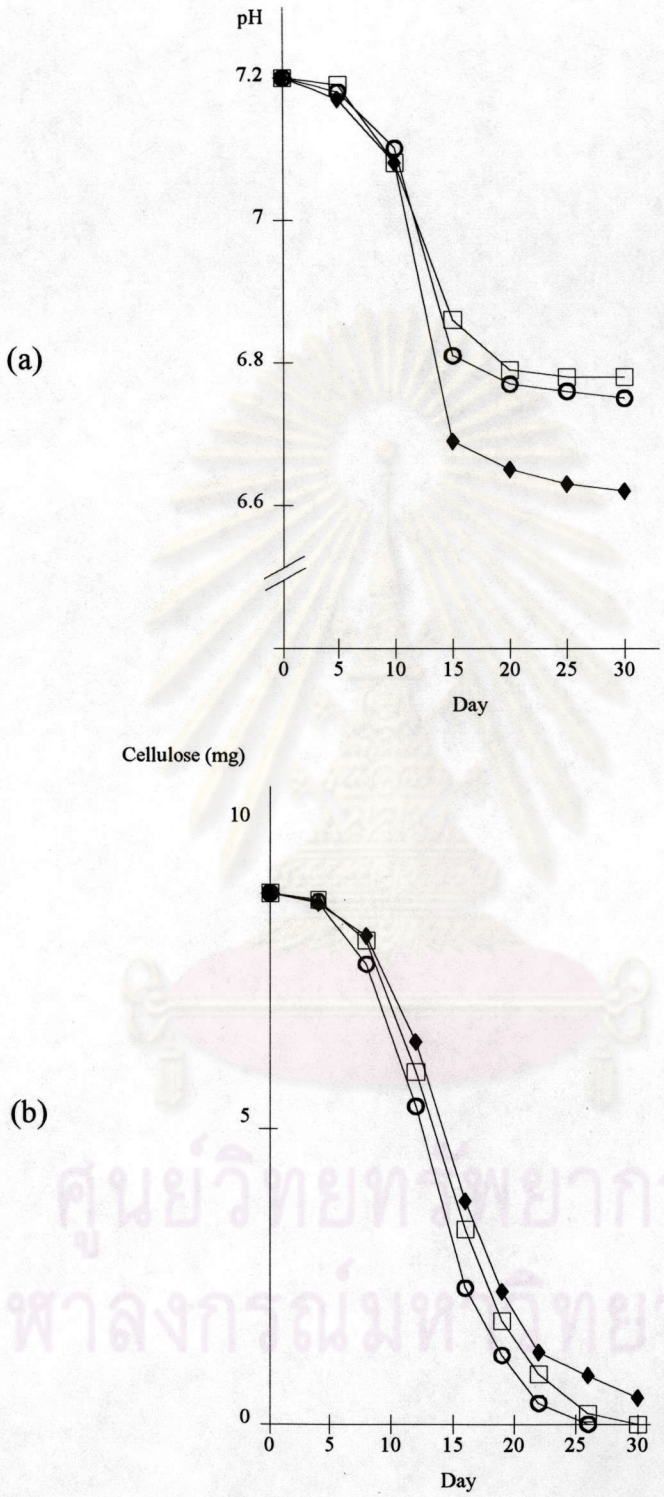


Figure G-7 pH reduction (a) and remained cellulose (b) in paper waste fermentation by a thermophilic mixed culture: C23 (○), C73 (□) and mixed culture (♦)

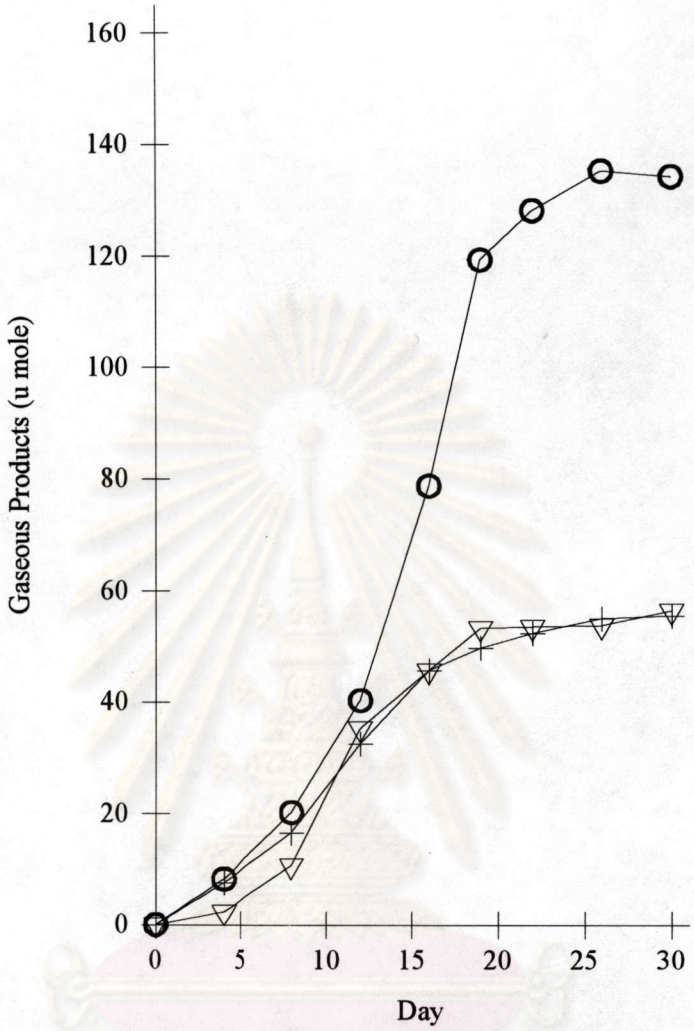


Figure G-8 Gas production in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M38: C23 (O = CO_2) and C23 + M38 (+ = CO_2 , ∇ = CH_4)

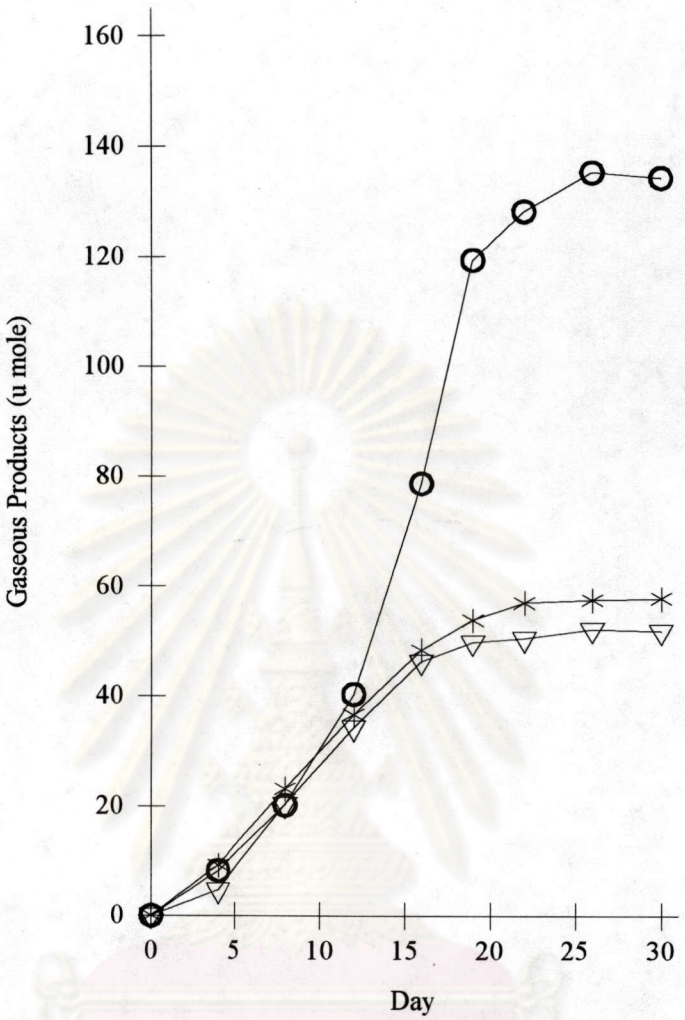


Figure G-9 Gas production in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M47: C23 (O = CO_2) and C23 + M47 (* = CO_2 , ∇ = CH_4)

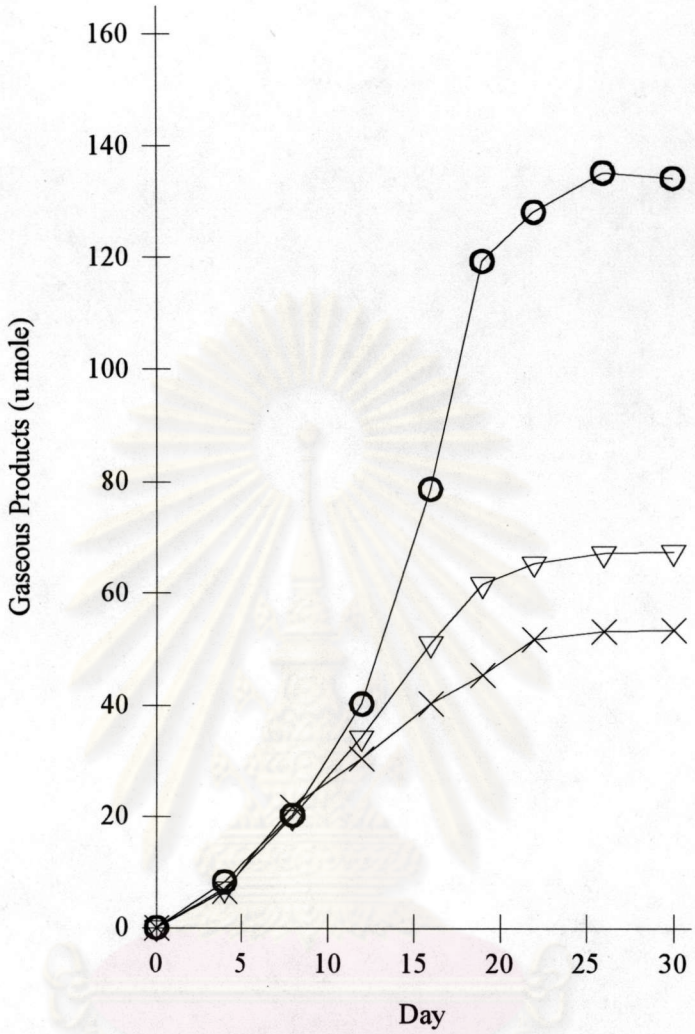


Figure G-10 Gas production in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C23 and thermophilic methanogen M48: C23 (O = CO_2) and C23 + M48 (× = CO_2 , ∇ = CH_4)

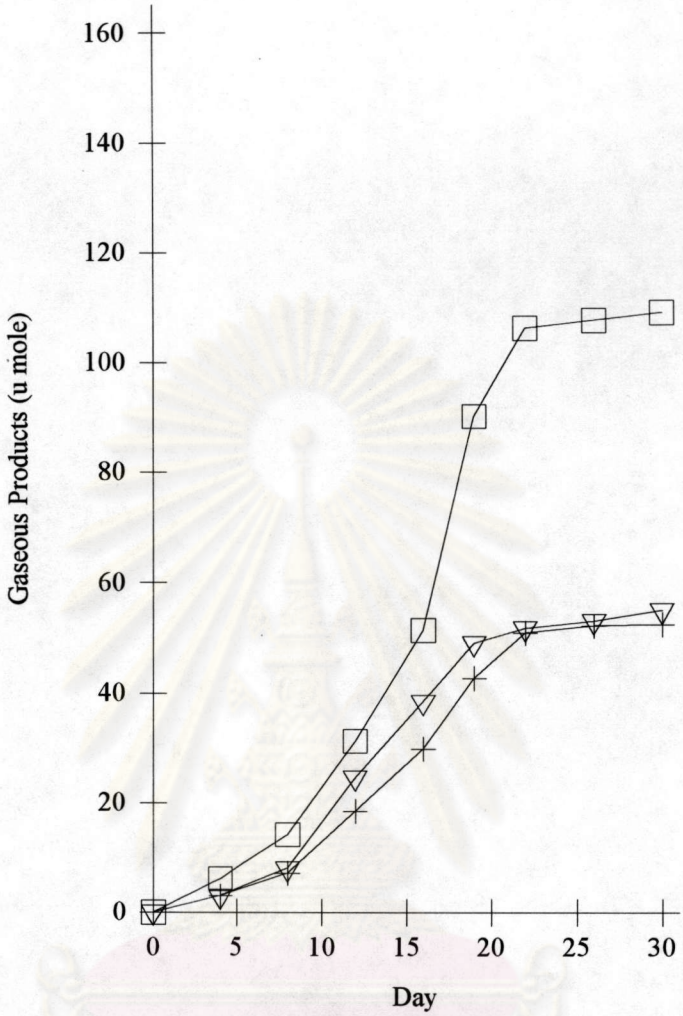


Figure G-11 Gas production in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M38: C73 (□ = CO_2) and C23 + M38 (+ = CO_2 , ∇ = CH_4)

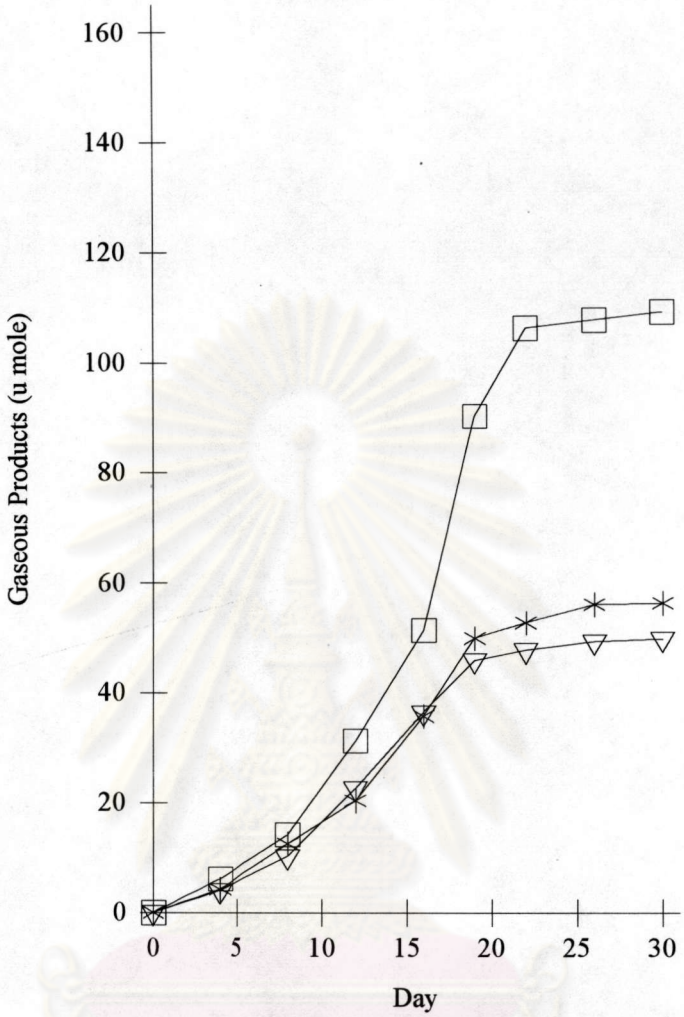


Figure G-12 Gas production in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M47: C73 (□ = CO_2) and C23 + M47 (∗ = CO_2 , ∇ = CH_4)

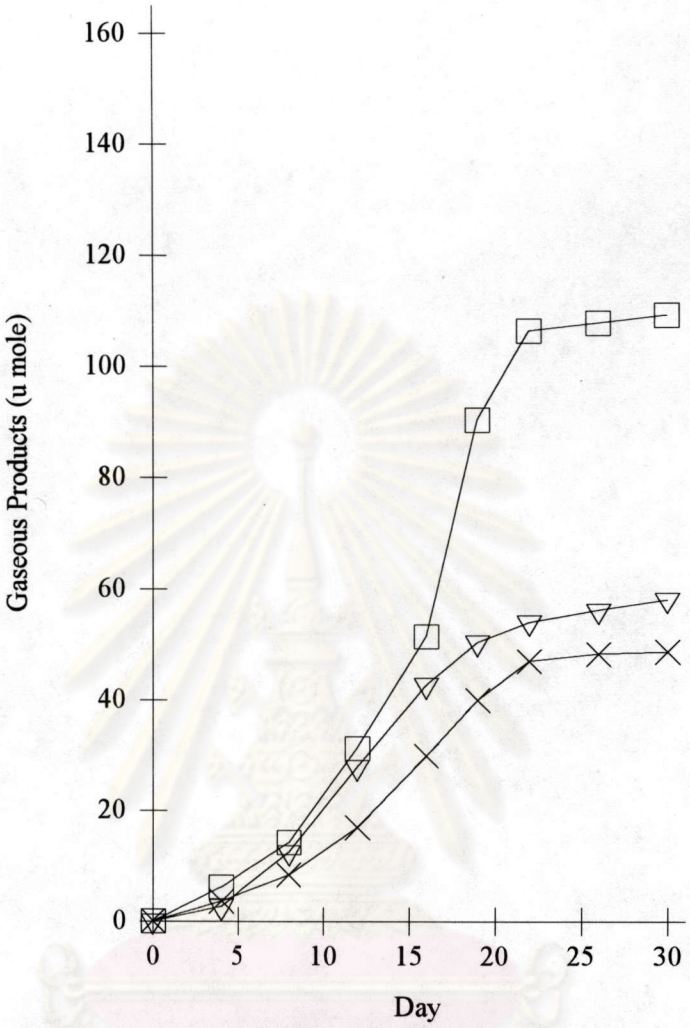


Figure G-13 Gas production in paper waste fermentation by a coculture of thermophilic cellulolytic bacteria C73 and thermophilic methanogen M48: C73 (□ = CO_2) and C23 + M48 (× = CO_2 , ∇ = CH_4)

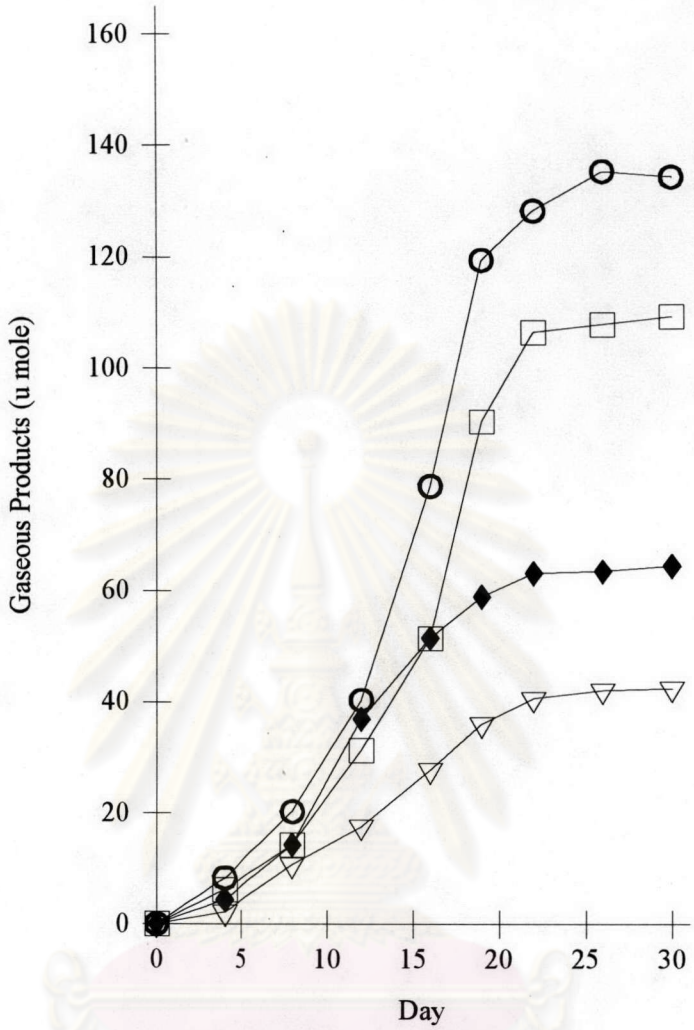


Figure G-14 Gas production in paper waste fermentation by a thermophilic mixed culture: C23 (○ = CO_2) , C73 (□ = CO_2) , and mixed culture (♦ = CO_2 , ∇ = CH_4)

BIOGRAPHY

Mr. Supawin Watcharamul was born in Bangkok on the 11th of June, 1970. He entered Chulalongkorn University in 1988 as a freshman and graduated a Bachelor of Science (General Science) in 1991 from the Faculty of Science. The degradation of cellulosic wastes by alkalophilic bacteria was the topic of his senior project. Then, he furthered his education at the Inter-department of Environmental Science, Graduate School of Chulalongkorn University, in 1992. A year later, he was awarded a two-year scholarship by the University Development Committee (UDC), Ministry of University Affairs. After his graduation, he serves as a full-time lecturer at the Department of General Science, Faculty of Science, Chulalongkorn University.