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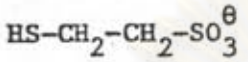
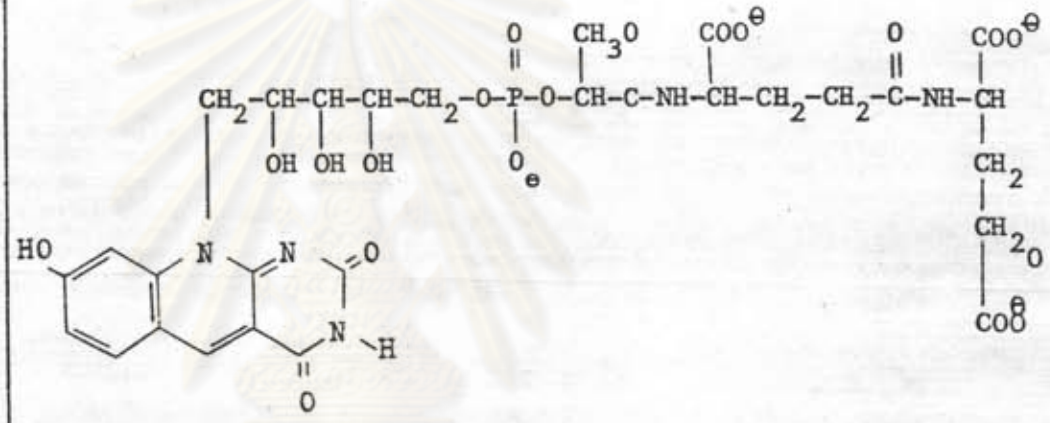
APPENDIX

APPENDIX A--STRUCTURE OF CO-M AND F 420

Enzyme	Structure
CoM	
F_{420}	

Balch et al. 1979.

APPENDIX B -- MEDIA

Formula

1. Eosin Methylene Blue Agar (EMB)

Formula in grams per liter of distilled water

Bacto-Peptone	10
Bacto-Lactose	5
Saccharose, Difco	5
Dipotassium Phosphate	2
Bacto-Agar	13.5
Bacto Eoxin Y	0.4
Bacto-Methylene Blue	0.065

Final pH 7.2

Sterilized by autoclaving

2. Nutrient Agar (NA)

Formula in grams per liter of distilled water

Bacto-Beef Extract	3
Bacto-Peptone	5
Bacto-Agar	15

Final pH 6.8

Sterilized by autoclaving

3. Salmonella-Shigella Agar (SS)

Formula in grams per liter of distilled water

Bacto-Beef Extract	5
Proteose Peptone, Difco	5
Bacto-Lactose	10
Bacto-Bile Salt No. 3	8.5
Sodium Citrate	8.5
Ferric Citrate	1
Bacto-Agar	13,5
Bacto-Brilliant Green	0.00033
Bacto-Neutral Red	0.025

Final pH 7.0

Sterilized by autoclaving

4. Triple Sugar Iron Agar (TSI)

Formula in grams per liter of distilled water

Bacto-Beef Extract	3
Bacto-Yeast Extract	3
Bacto-Peptone	15
Proteose Peptone, Difco	5
Bacto-Lactose	10
Saccharose, Difeo	10
Bacto-Dextrose	1
Ferrous Sulfate	0.2
Sodium Chloride	5
Bacto-Agar	12
Bacto-Phenol Red	0.024

Final pH 7.4

Sterilized by autoclaving

5. Mannitol Salt Agar (MS)

Formula in grams per liter of distilled water

Bacto-Beef Extract	1
Proteose Peptone No. 3, Difco	10
Sodium Chloride	75
d-Mannitol, Difco	10
Bacto-Agar	15
Bacto-Phenol Red	0.025

Final pH 7.4

Chicken york addid in the medium after antoclaving

6. Streptococcus Fecalis Agar (SF)

Formula in grams per liter of distilled water

Bacto-Trypton	20
Bacto-Dextrose	5
Dipotassium Phosphate	4
Monopotassium Phosphate	1.5
Sodium Azide	0.5
Sodium Chloride	5
Bacto-Brom Cresol Purple	0.032
Bacto-Agar	15

Final pH 6.9

Sterilized by autoclaving

7. Rumen Fluid Cellulose Agar (RFCA)

Formula in milliliter and gram per 0.5 liter

Inorganic Salt Solution	200
Resazurin (0.1 % Solution)	0.5
Rumen Fluid	75
Cellulose	7
Cystein Hydrochloride	0.05
Bacto Agar	7.5
Distilled Water	224.5

Final pH 7.0

Preparation of this medium would be discussed later

8. Rumen Fluid Cellulose Broth (RFCB)

Formula in milliliter and gram per 0.5 liter

Inorganic Salt Solution	200
Resazurin (0.1 % Solution)	0.5
Rumen Fluid	7.5
Cellulose	0.5
Cysteine Hydrochloride	0.05
Distilled Water	224.5

Final pH 7.0

Preparation of this medium would be discussed later

9. Cellulose Broth (CB)

Formula in milliliter and gram per 0.5 liter

Inarganic Salt Solution	200
Resazurin (0.1 % Solution)	0.5
Cellulose (0.1, 0.2 %X	0.5, 1
Cysteine Hydrochloride	0.05
Trace Minerals (Balch)	5
Trace Vitamins (Balch)	5
Distilled Water	289.5

Final pH 7.0

Preparation of this medium would be discussed later.

10. Inorganic Salt Solution (Hungate buffer)

Formula in gram per liter of distilled water

$(NH_4)_2SO_4$	1.25
Na_2HPO_4 12 H_2O	44.09
KH_2PO_4	11.18
$CaCl_2$	0.125
$MgSO_4$	0.125
NaCl	2.5

11. Trace Minerals

Formula in gram per liter of distilled water

Nitrilotriacetic Acid	1.5
$MgSO_4 \cdot 7H_2O$	3.0
$MnSO_4 \cdot 2H_2O$	0.5
NaCl	1.0
$FeSO_4 \cdot 7H_2O$	0.1
$CoCl_2$	0.1
$CaCl_2 \cdot 2H_2O$	0.1
$ZnSO_4$	0.1
$CuSo_4 \cdot H_2O$	0.01
$AlK(SO_4)_2$	0.01
H_3BO_3	0.01
$Na_2MaO_4 \cdot 2H_2O$	0.01

Final pH 7.0

Preparation of trace mineral would be discussed later

12. Trace Vitamins

Formula in milligram per liter of distilled water

Biotin	2
Folic Acid	2
Pyridoxine Hydrochlaride	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 - micron milipore filter

13. Balch Medium I Agar (BMA)

Formula in milliliter and gram per liter

Mineral 1	25
Mineral 2	25
Trace Minerals	5
Trace Vitamins	5
Ferrous Sulfate . 7 H_2O	0.001
Sodium Bicarbonate	2.5
Sodium Acetate	1.25
Sodium Formate	1.25
Yeast Extract	1.0
Trypticase	1.0
Cysteine Hydrochlaride	0.25
Sodium Sulfide . $9H_2O$	0.25
Agar	7.5
Distilled Water	440

Final pH 7.0

Preparation of this medium would be discussed later

14. Balch Medium I Broth (BMB)

Formula like Balch Medium I Agar but agar was omitted

15. Mineral 1

Contains 6 grams of di-potassium hydrogen phosphate per liter of distilled water.

16. Mineral 2

Formula in grams per liter of distilled water

Potassium Di-hydrogen Phosphate	6.0
Ammonium Sulfate	6.0
Sodium Chloride	12.0
Magnesium Sulfate . H_2O	2.6
Calcium Chloride . 2 H_2O	0.16

Preparation

1. Preparation of Medium number 7, 8, 9, 13 and 14

In a 1-liter ground joint round bottom flask, ingredients and 500-ml of distilled water were added, and heated. During boiling, cysteine hydrochloride and/or sodium sulfide were added, then the flask was suddenly stopped with a rubber stopper, covered with cloth and tied tightly and autoclaved. After transferring into the anaerobic chamber, trace vitamin solution was added. Before used, any plate or tube contained certain medium should be incubated over night.

2. Prepartaion of Trace Minerals

Nitrilotriacetic acid was firstly dissolved in 50-ml distilled water which KOH solution had been gradaully added until pH 6.5, then the preparation was proceeded by adding other minerals and making final volume to be 1 liter with distilled water.

APPENDIX C--CONDITION OF CHROMATOPAC, STANDARD CURVE AND CALCULATION METHOD

1. The Condition of Chromatopac were :

Column : Porapak QS, 80-100 mesh, 2m. long, inside diameter 3 mm, outside diameter 4 mm.

Detector : Thermal conductivity detector

Temperature : Column temp 50°C

Injector temp 100°C

Detector temp 150°C

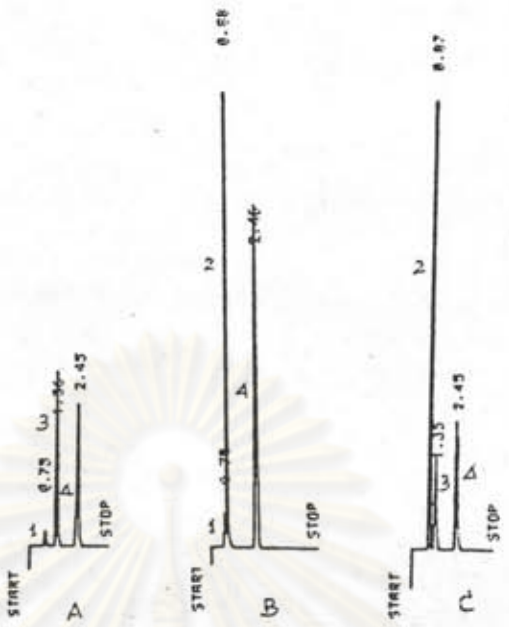
Current : 110 mA

Flowrate : 30 ml per min

Carrier Gas : He with pressure 1 kg per cm^2

Record with attenuate 32

2. Gas Chromatographic Curves were recorded by Porapak QS, 80/100, 2m x 3mm ID, 100°C, 30 ml/min, He, TCD.



1. H_2 2. N_2

3. CH_4 4. CO_2

A. Standard gas

B. Gas sample drawn from a fermentation tube inoculated with a cellulotytic bacteria

C. Gas sample drawn from a fermentation tube inoculated with a cellulotytic bacteria and a methanogenic bacteria or co-culture

3. Calculation Method of Gas analyzed from Gas Chromatographic Method

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

while

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

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

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

M_p = the sum of micromeles removed after equilibration

M_p = $\{ \Sigma (\% \text{ Gas} \times V_r)/2.24 \}$

APPENDIX D--PREPARATION OF CARRIER PLATES, SOLVENTS SPRAY REAGENTS AND METHOD OF CALCULATION

1. Preparation of Carrier Plates

In a dry porcelain mortar contained 30g of silica gel 60 G, 40 ml of distilled water were gradually added and the mixture was stirred thoroughly in lowspeed until the entire mass had a uniform consistency, and also entirely free of air bubbles. Twenty milliliters of distilled water were added. The slurry was poured into the spreading device and coated nn the 200 x 200 mm glas plates with the thickness of 0.25 mm. The coated plates were left to air-dry and activated in a drying cabinet at 105°C for 60 minutes before used.

2. Preparation of Solvent

The mixture of chloroform, isobutanol, methyl ethyl ketone, methanol, ammonia solution (S.G. 0.88) and distilled water were prepared under the ratio of 35, 35, 15, 20, 1 and 2 parts, respectively in 150 ml erlen-mayer flask and poured into the separating chamber. The solvent should be prepared before used.

3. Preparation of Spray Reagent

3.1 pH indicator :

Four parts of BDH universal indication were mixed with one part of 0.5 N KOH.

3.2 Ammonical Silver Oxide Solution :

One gramm of silver oxide was disolved in ammonia solution with the final volume of 100 ml.

4. Calculation Method of Unknown Acids

(From Updegraff 1969, 133)

Equation of Purdy : $\sqrt{\text{spot area}}$ α log of the material weight

$$K = \frac{\text{log weight}}{\sqrt{\text{area}}}$$

$$W = KA$$

when W = log of unknown acid weight

A = square root of unknown acid spot area

K = constance

$$\text{Acid production in culture tube} = \frac{\text{Anti log } W}{V \times 0.6}$$

v = volume of spotted samples

Figure D.1-D.6 showed the relationships of Purdy's equation by the technique of continuous thin layer chromatography.

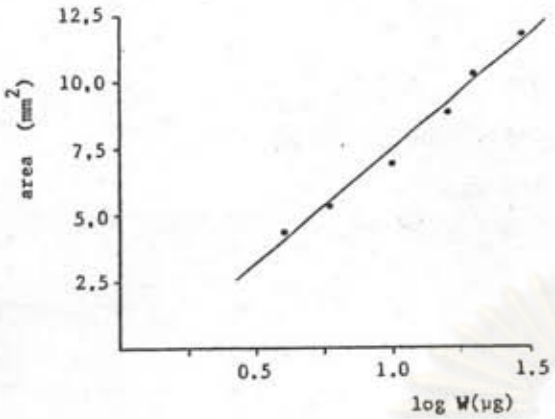


Figure D.1 Butyric acid

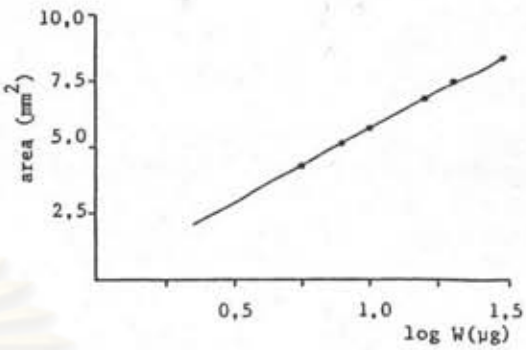


Figure D.2 Formic acid

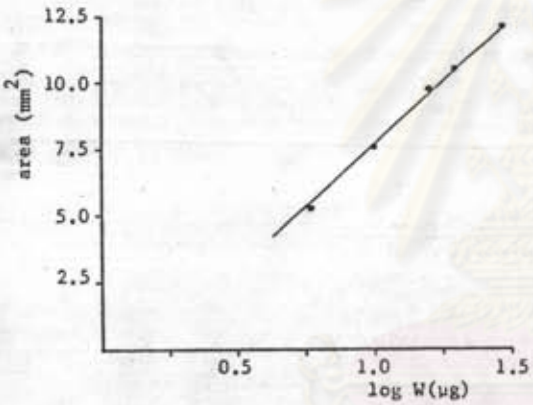


Figure D.3 Propionic acid

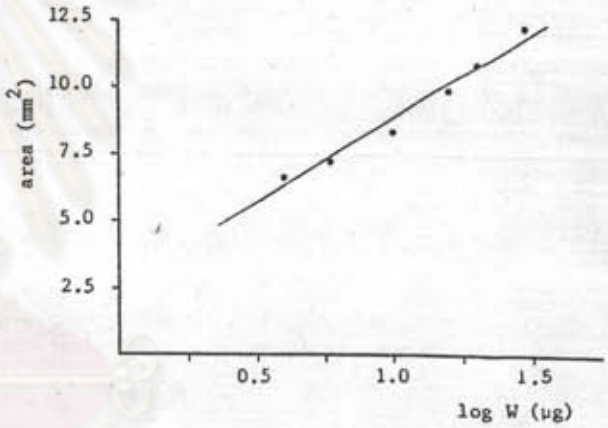


Figure D.4 Acetic acid

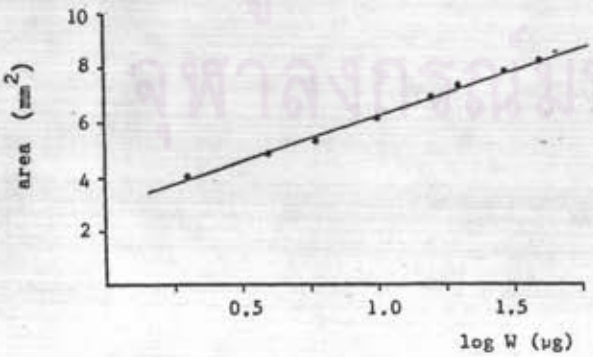


Figure D.5 Lactic acid

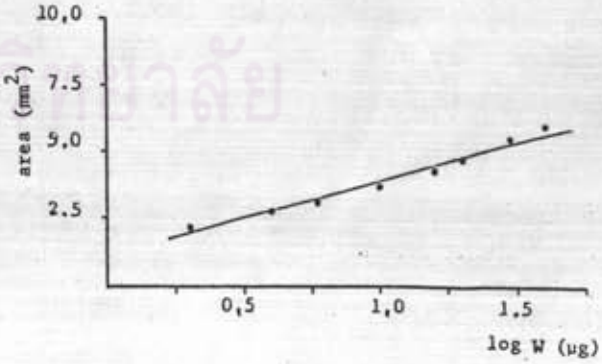


Figure D.6 Succinic acid

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

1. Preparation of Reagent

Acetic/Nitric reagent

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

Anthrone reagent

Dissolved 0.2 g anthrone p.a. in 100 ml concentrated sulfuric acid, prepared fresh daily. Chill about 2 hours in refrigerator prior to use.

2. Preparation of Standard Curve

To prepare the stock standard dissolved 50.00 mg pure cellulose (Alpha cellulose fiber 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 this solution to 500 ml with distilled water to contain 100 µg cellulose per ml. Analyzed 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 ml stock standard, corresponding to 10, 20, 30, 40, 50, 100, 150, 200, 250 and 300 µg of cellulose as in step xiii of the procedure

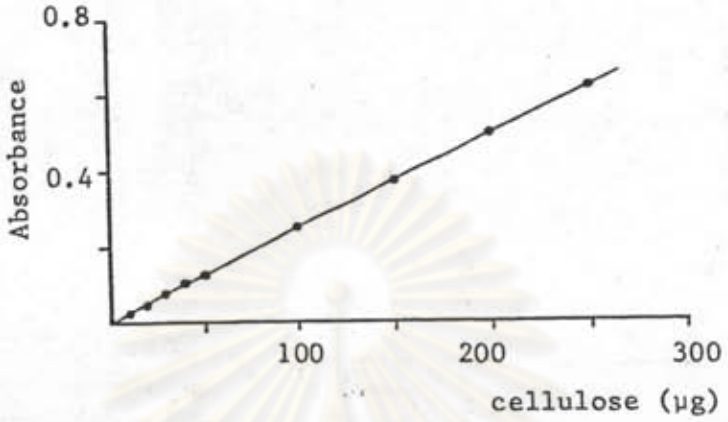


Figure E.1 A linear standard curve of cellulose detected by this method.

3. Calculation Method

my cellulose = k x 0.2

k = microgram of cellulose read from standard curve which prepared by running together with the unknown

APPENDIX F--FERMENTATION OF CELLULOSE BY CO-CULTURE

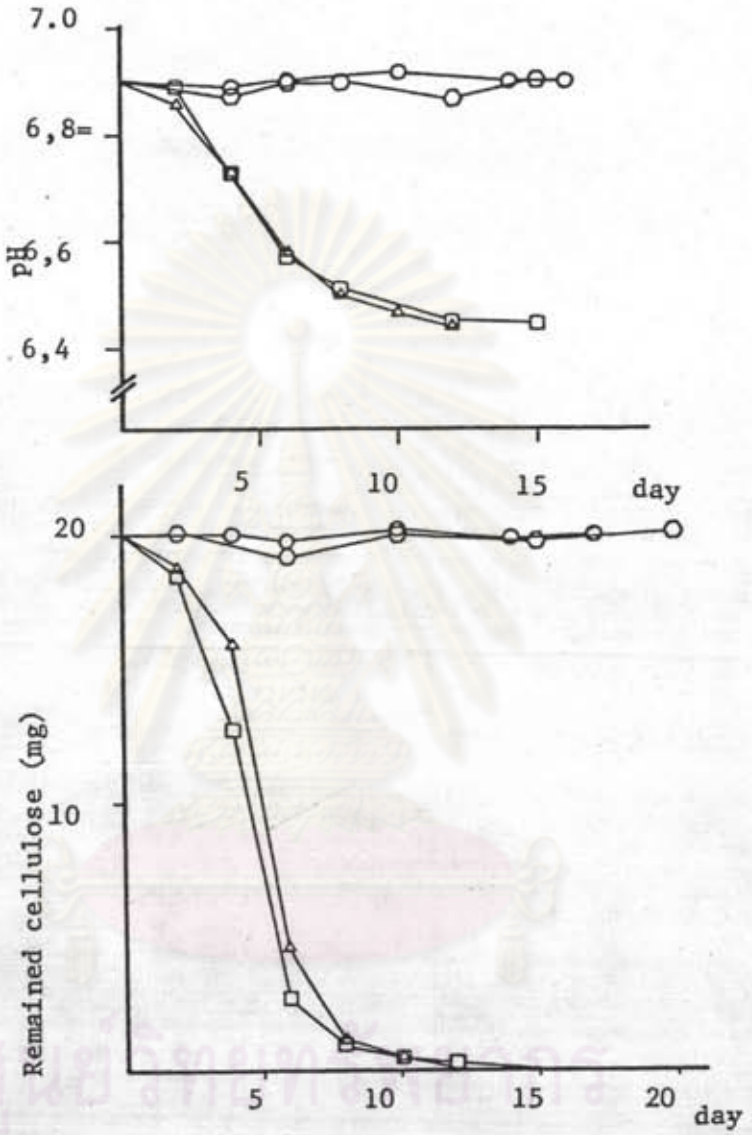


Figure 4.19 Remained cellulose and pH reduction in cellulose fermentation by cellulolytic bacteria CU1, methanogen Sc1; CU1 (△): methanogen Sc1 (o), CU1+Sc1(□) and mixed culture (○)

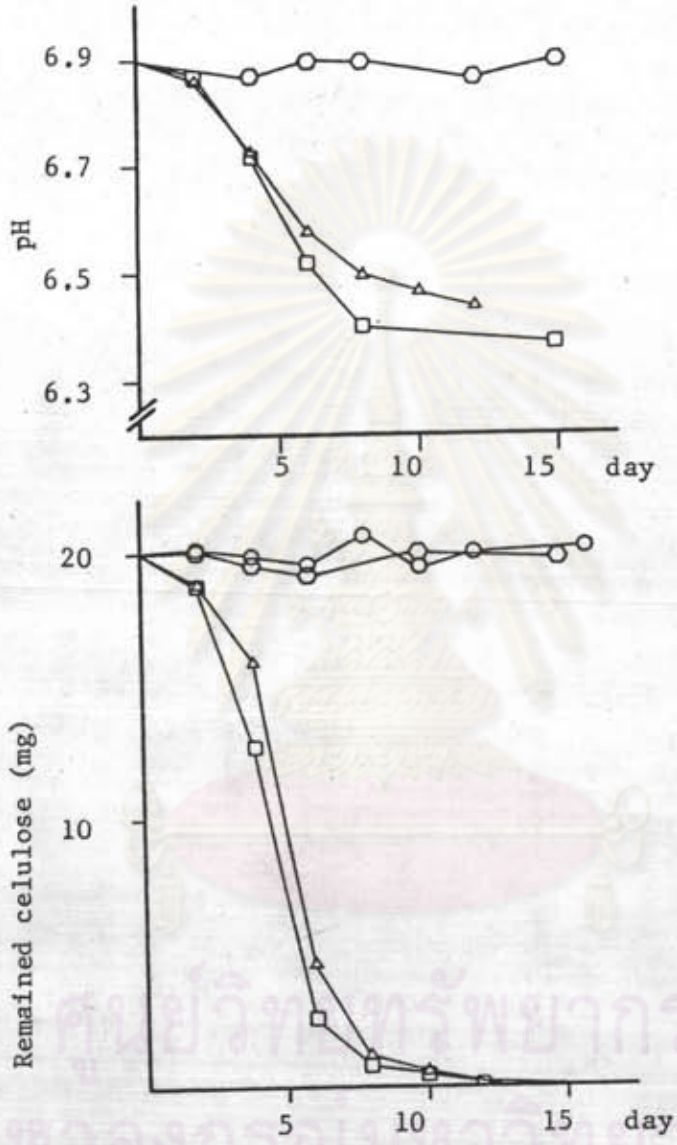


Figure 4.20 Remained cellulose and pH reduction in cellulose fermentation by cellulolytic bacteria CU1 (△); methanogen Sc4 (○), CU1 + Sc4 (□) and mixed culture (○)

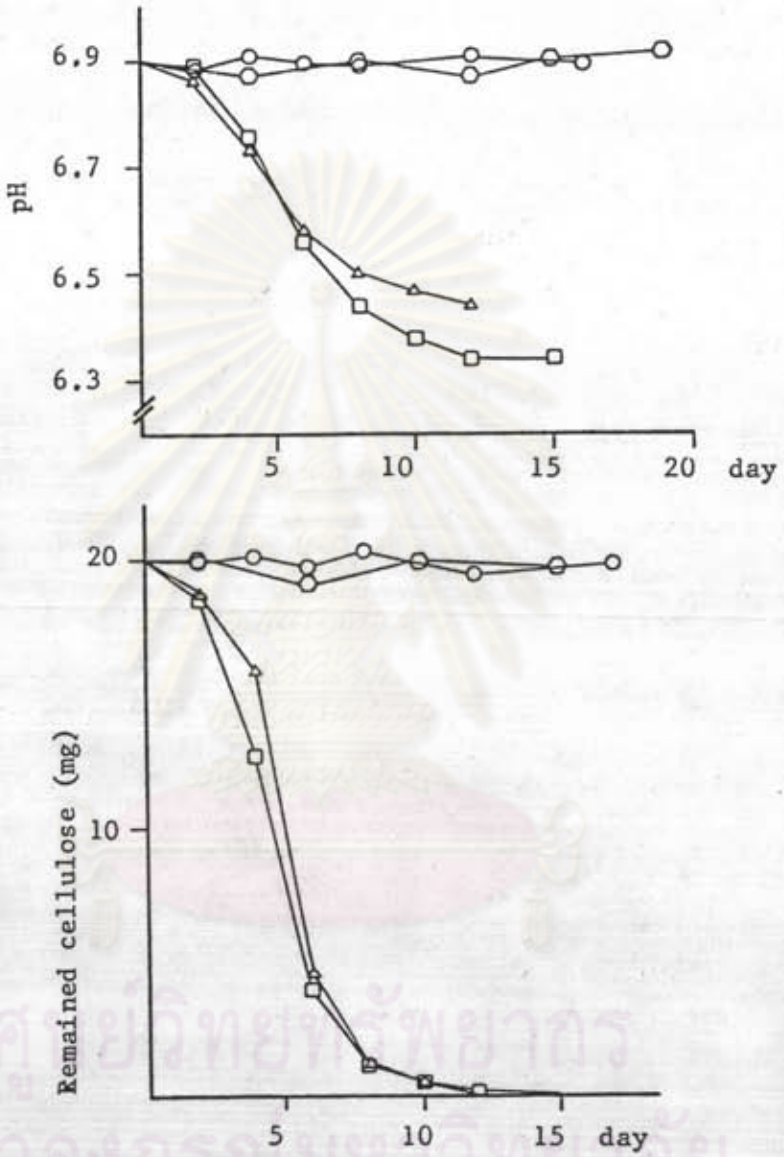


Figure 4.21 Remained cellulose and pH reduction in cellulose fermentation by cellulolytic bacteria CU1 (▲) ; methanogen Sc5 (○), CU1 + Sc5 (□) and mixed culture (○)

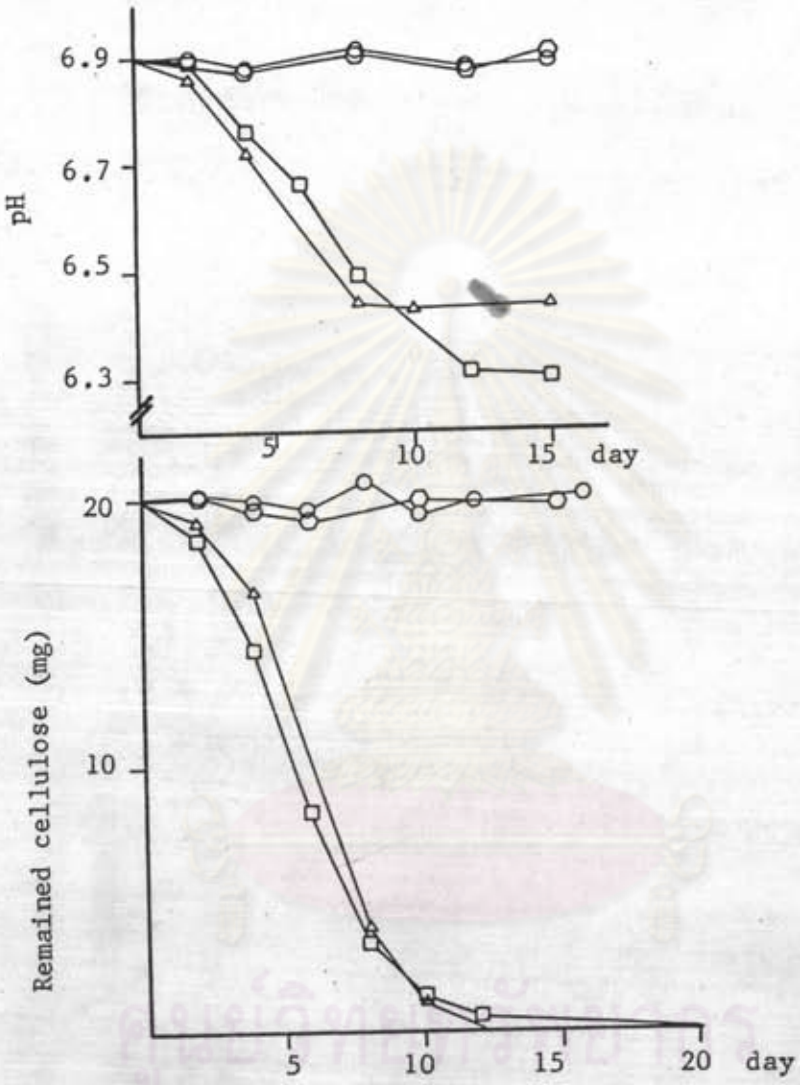


Figure 4.22 Remained cellulose and pH reduction in cellulose fermentation by cellulolytic bacteria CU3 (△) ; methanogen Se4 (○), CU3 + Sc 4(□) and mixed culture (○)

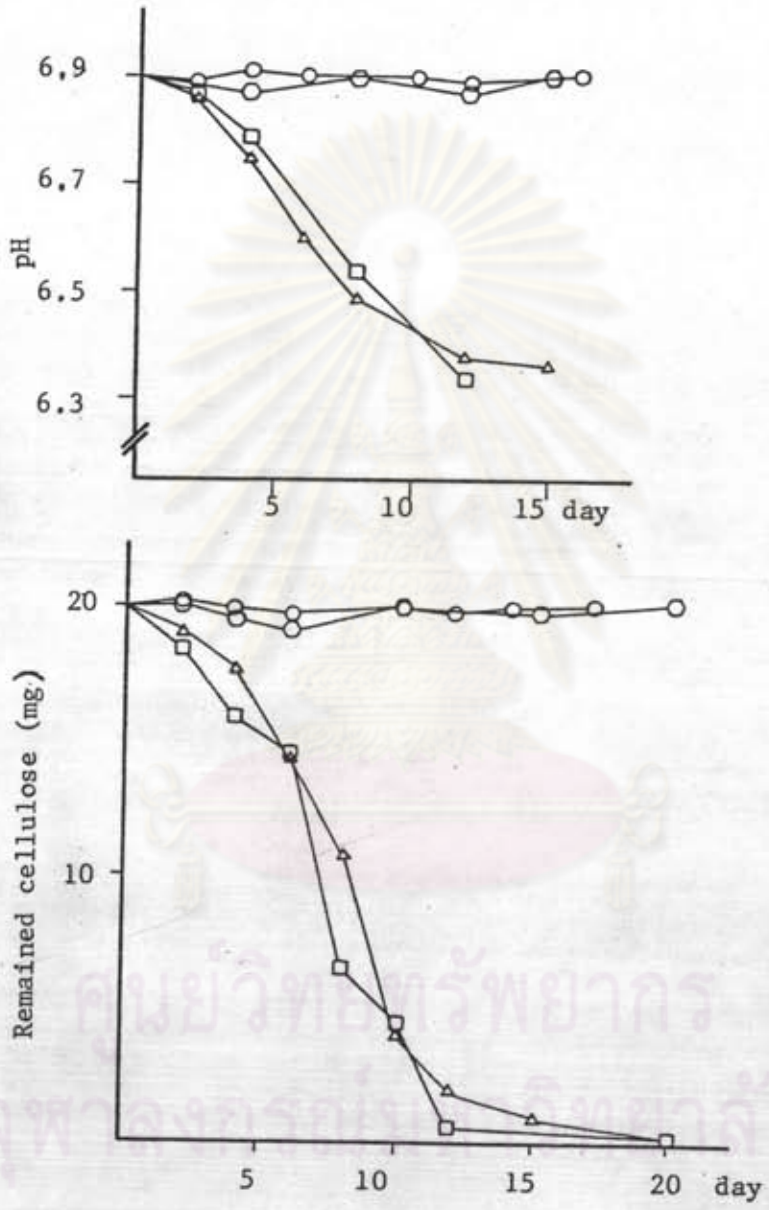


Figure 4.23 Remained cellulose and pH reduction in cellulose fermentation by cellulolytic bacteria CU 4 (△), methanogen Sc 2 (○), CU4 + Sc2 (□) and mixed culture(○)

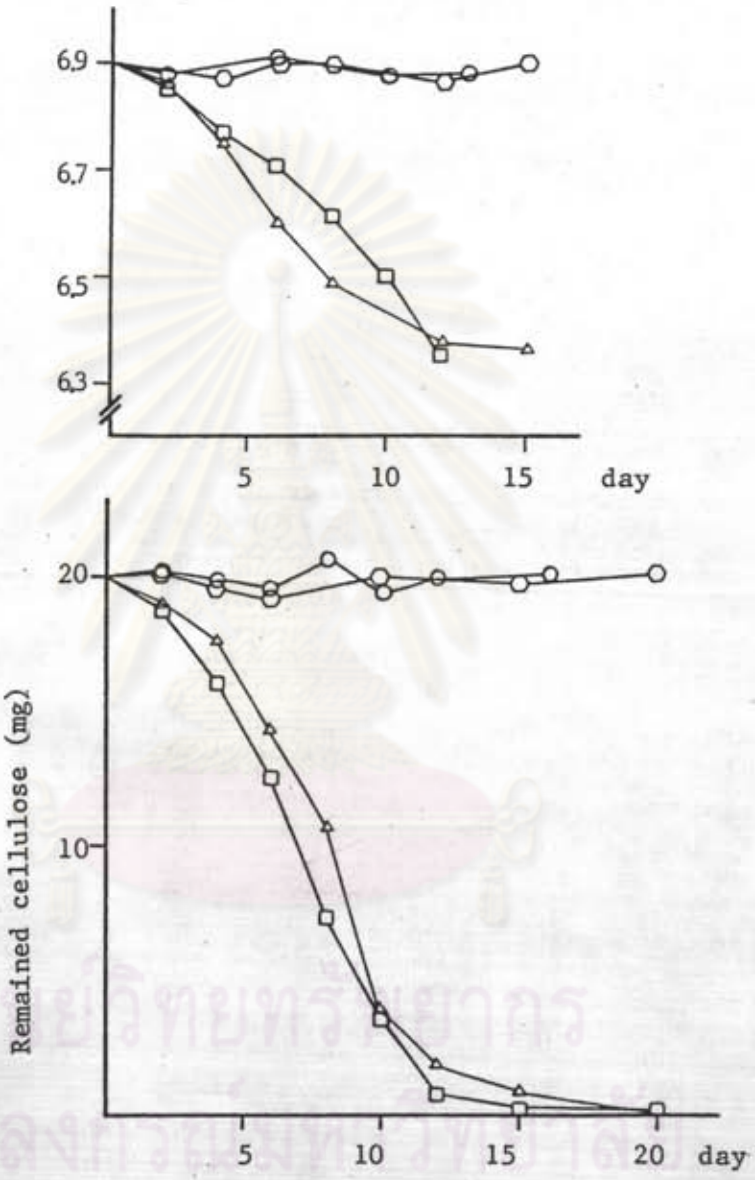


Figure 4.24 Remained cellulose and pH reduction in cellulose fermentation by cellulolytic bacteria CU4 (△), methanogen Sc4 (○), CU4 + Sc4 (□) and mixed culture (○)

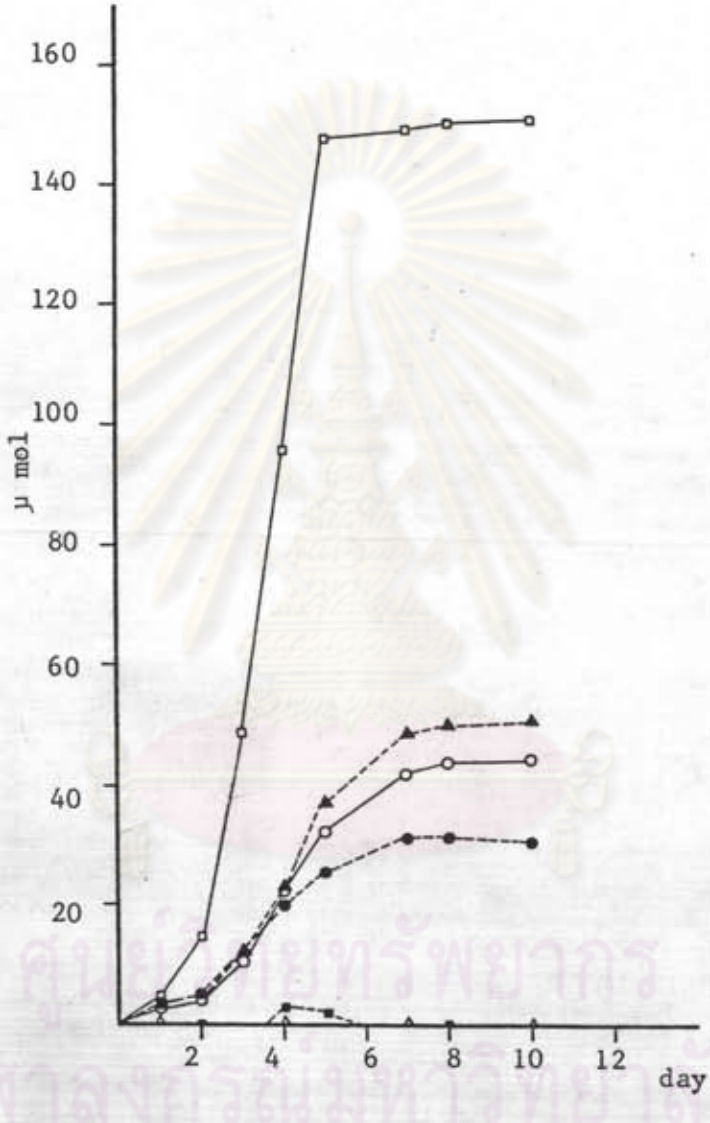


Figure 4,25 Gas production in cellulose fermentation (20 mg) by cellulolytic bacteria CU1 (▫ = H_2, ○ = CO_2, △ = CH_4) and CU1 + methanogen Sc1 (▪ = H_2, ● = CO_2, ▲ = CH_4)

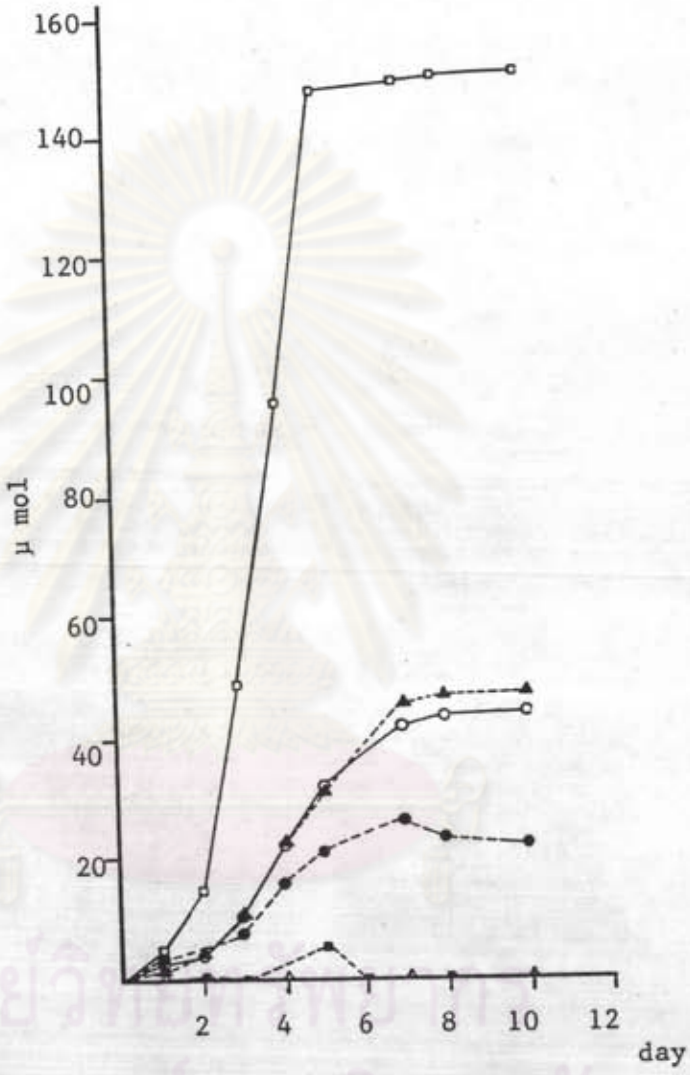


Figure 4.26 Gas production in cellulose fermentation (20 mg) by cellulolytic basteria CU 1 (▫ =H_2, ○ =CO_2, △ = CH_4) and CU1+methanogen Sc 4 (▪ =H_2, ● = CO_2, ▲ = CH_4)

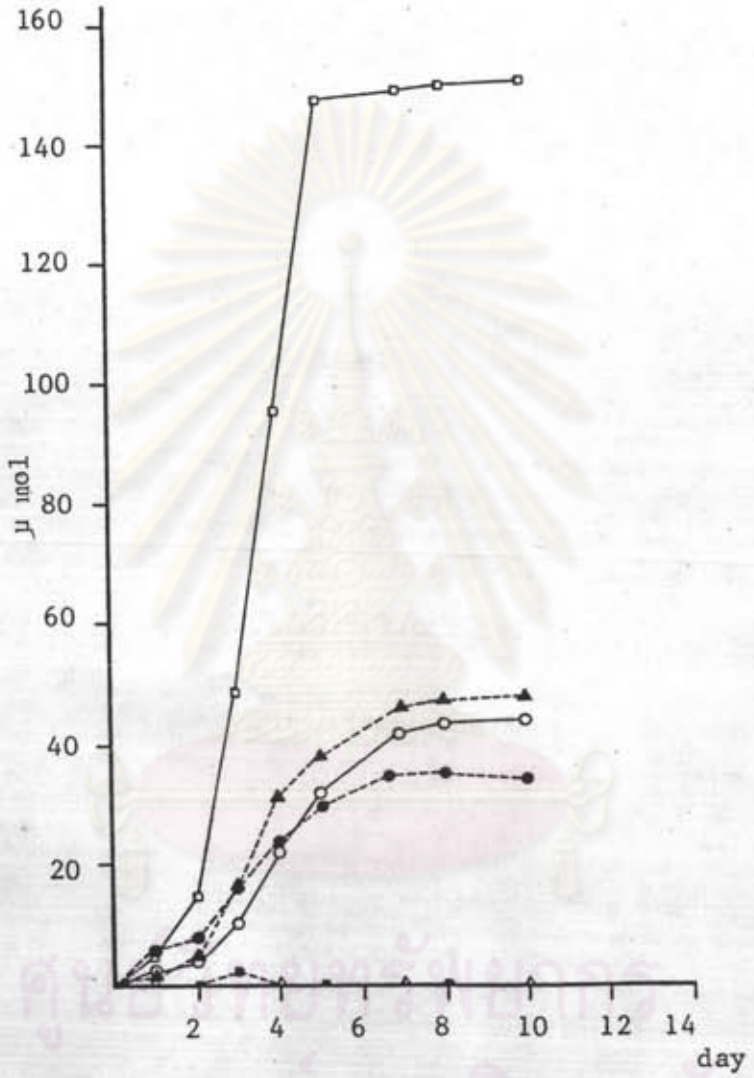


Figure 4.27 Gas production in cellulose fermentation (20 mg) by cellulolytic bacteria CU1 (▫ = H_2, ○ = CO_2, △ = CH_4) and CU_1 + methanogen Sc 5 (▪ = H_2, ● = CO_2, ▲ = CH_4)

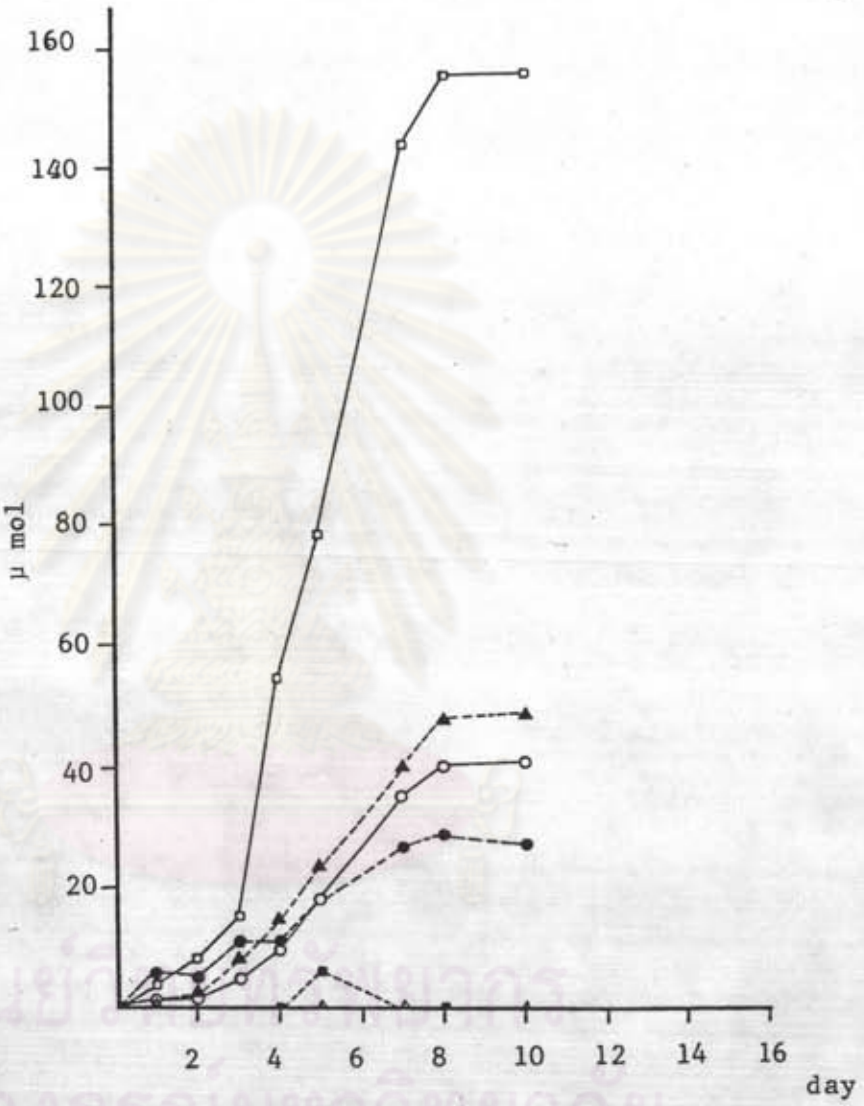


Figure 4.28 Gas production in cellulose fermentation (20 mg) by cellulolytic bacteria CU$_3$(○ =H_2, ○ = CO_2, △ = CH_4) and CU 3+ methanogen Sc 4 (▪ = H_2, ● = CO_2, ▲ = CH_4)

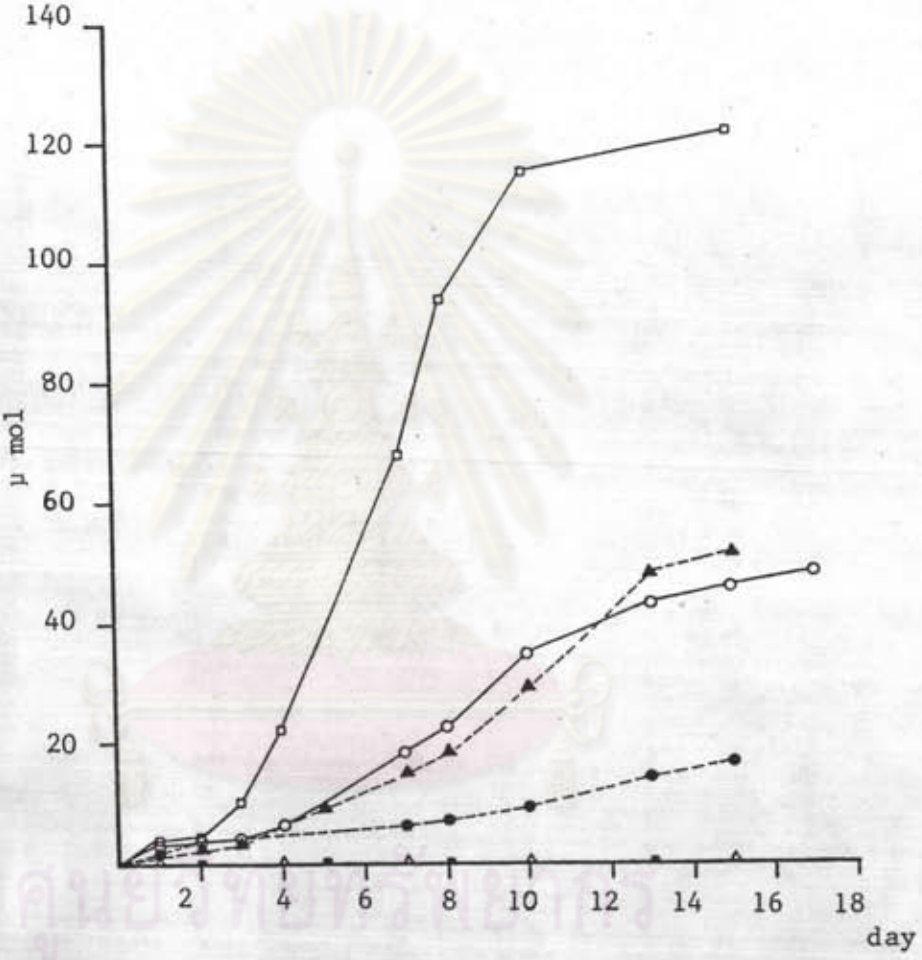


Figure 4.29 Gas production in cellulose fermentation (20mg) by cellulolytic bacteria CU4 (▫ = H_2 ○ = CO_2, △ = CH_4) and CU 4 + methanogen Sc2 (▪ = H_2, ● = CO_2, ▲ = CH_4)

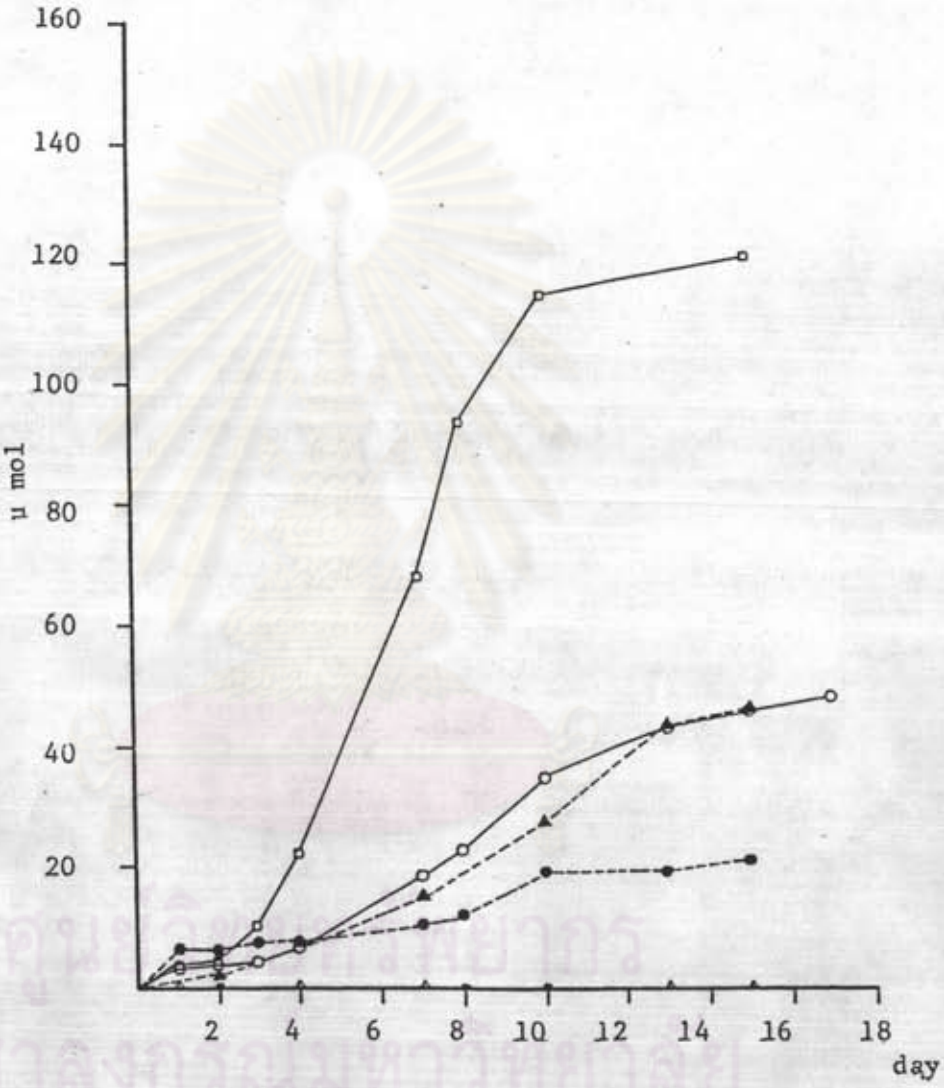


Figure 4.30 Gas production in fermentation (20mg) by cellulolytic bacteria CU_4 (▫ = H_2, ○ = CO_2, △ = CH_4) and CU 4 + methanogen (▪ = H_2, ● = CO_2, ▲ = CH_4)

BIOGRAPHY

Mr. Pipat Sribenjalux was born in Bangkok Thailand, on March 15, 1955. He was graduated with a B.Sc. in Medical Technology from Faculty of Medicine, Chulalongkorn University in 1978.