Report Project

Experiment 1. Isolation and characterization of proteolytic bacteria from the Sippewissett and "Dutch" sludge

Experiment 2. Isolation and characterization of methanogenic archaea from salt marsh, termite hindgut, and "Dutch" sludge

Submitted by

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Yaya Rukayadi
July 29, 1999

Project 1.

Isolation and characterization of proteolytic bacteria from the Sippewissett and "Dutch sludge

Yaya Rukayadi

Abstract

The objective of this experiment was to isolate and characterize proteolytic bacteria from environments; Sippewissett salt marsh and granular sludge from a UASB reactors. Proteolytic activity was determined for a total of 16 isolates and the three isolates (P1, P7, and UP-1) with the highest specific activity were characterized. The optimum temperatures of the proteases made by P1, P7, and UP-1 were 37°C, 25°C, and 50°C, respectively. The optimum pH of al of these proteases was approximately 7.5. After addition of 5 mM EDTA, the specific activity of P1 was described by 18.39 %, 8.85 % (P7) and no activity was detected in the UP-1 reaction. Also these enzymes seemed to be inhibited by the addition of PMSF. This result indicated that these enzymes contain serine in its active site. 16S-rRNA genes fragments from the sixteen isolates were amplified with eubacteria primers in the generation of sixteen 500 bp fragments. The comparative RFLP restriction enzymes confirmed that 7 of these 16 isolates can be classified into three distinctive distribution patterns.

Project 2.

Isolation and characterization of methanogenic archaea from the Sippewissett, termite hind gut, and "Dutch" sludge

Yaya Rukayadi

Abstract

The objective of this study was to isolate and characterize methanogenic archaea from the Sippewissett salt marsh, termite hind gut, and "Dutch sludge. Mthanogenic archaea were detected in cultures by production of methane. The samples from "Dutch" sludge produced the levels of methane, indicates that the sludge samples contained a large population of methanogenic archaea. We also did a dilution series, and found that more methane was produced in the $1{:}10^{10}$ dilution than the $1{:}\ 10^{4}$ dilution. Termite hind gut microorganism have also been shown to produce methane, and methanogenic archaea from these samples were grown on roll agar tubes and microscopically visualized. In addition, specimens from the primary enrichment of the sludge and termite hind gut were examined for methanogenic archaea by Fluorescent *in situ* hybridization (FISH). Specimens were hybridized with a rhodamin labeled archaea probe. Fluorescent cells were seen in the primary enrichment of the sludge and termite hind gut of the examined specimens, which hybridized with the archaea probe. 16S-rDNA from the 8 isolates were amplified using archaea primer and 3 PCR products (termite hind gut, sludge, and XF) were generated . The comparative RFLP analysis of the 16S-rDNA using *Hin*PI and *Msp*I restriction enzymes confirmed that all isolates have distinctive distribution patterns. This result indicated that the methanogenic archaea in the termite hind gut, Sippewissett (XF), and "Dutch" sludge are indeed different strains.

and sea water. The sample concentration of the suspension was adjusted to 10^{-4}. 100 µl of sample suspension was used to spread onto the plates. All cultures inoculated with sludge were incubated at 37°C and Sippewissett cultures were inoculated at room temperature.

Extracellular proteinase-containing cell free supernatant. Cells were grown in liquid medium with the same composition as skim milk solid medium used above. After a 24 hour incubation, the cells were centrifuged at 5000 rpm for 10 minutes. The supernatant was their diluted 10 times using Na_2HPO_4/NaH_2PO_4 buffer at pH 7.0, and was used for enzyme investigation.

Protease and protein assay. The proteolytic activity was measured according to method of Bergmeyer and Grassl (1983) using 0.2% casein (Sigma) as the protein substrate, One unit (U) of protease activity was defined as the amount of enzyme that yielded the equivalent of 1 (mole tyrosine/minute under certain conditions. Protein concentration was determined by the method suggested by Bradford (1976) with bovine serum albumin fraction V (Merck) as a standard.

Optimum pH and temperature. The optimum temperature of the proteolytic activity for each the strain was determined in 0.1M sodium phosphate buffer at pH 7. A shaking waterbath was used for incubation in the temperature range of 18-55°C. The optimum pH of the enzyme was also determined at optimum temperature in universal buffer and activity was determined for pH values ranging from 5.56 to 10.5.

Effect of protease inhibitors. Phenylmethylsulfonyl fluoride (PMSF) and EDTA in a final concentration of 2 and 5 mM was added to cell free supernatant and incubated at room temperature (25°C) for 1 hour. The remaining protease activity was measured as described previously. The protease activity without inhibitor was considered as 100% activity.

DNA extraction and PCR amplification of the 16S rDNA. PrepMan™ Method (PE Applied Biosystems a division of Perkin-Elmer) provided during the course was used to extract DNA from proteolytic bacteria. MicroSeq™ 500 16S rDNA Bacterial Sequencing Kit (PE Applied Biosystems a division of Perkin-Elmer) was used for PCR amplification of DNA extracted from proteolytic bacteria. The amplification was carried out in 50 µl reaction volume : 25 µl PCR Master Mix and 25 µl diluted genomic DNA extraction. The PCR temperature profile was as follows: 95°C for 10 min, 30 cycles of (95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec), the amplification time of each cycle was extended for 5 sec. Amplified DNA was examined by horizontal electrophoresis in 1.25% agarose with 5 µl aliquots of the PCR product.

Restriction fragment length polymorphism (RFLP). Aliquots of PCR products were mixed with the restriction endonucleases *Hin*PI and *Msp*I. Reaction mixtures of 20 µl containing: 10 µl PCR product, 2 µl restriction buffer Neb2 (New England Biolab), 8.6 µl dH_2O and 0.2 each of restriction enzyme. Restricted DNA was analysed by horizontal electrophoresis in a 2 % agarose. The resulting band patterns were used to distinguish different clusters of bacteria.

Results and Discussion

Isolation and proteolytic production. Figure 1 shows the presence of clearing zones surrounding colonies grown on the selective medium. A total of 16 isolates were analysed for their proteolytic activity and the three isolates (PI, P7, and UP-1) with the highest apecipic activity were characterized. Proteolitic activity exhibited by these isolates is shown in Table 1. In addition microscopic observation of the two isolates (UP-1 and FVA) can be seen in Figure 2.

Effect of temperature and pH. Figure 3 and 4 show the effect of temperature and pH on proteolytic activity. The optimum temperatures of the proteases made by P1, P7, and UP-1 were 37°C, 25°C and 50°C respectively. The optimum pH of the proteases was approximately 7.5.

Effect of protease inhibitors. For classification of these proteases, its activity was measured in the presence of specific protease inhibitors. Figure 5 indicated that these enzymes are metalloenzymes since EDTA at concentration of 5 mM almost totally inhibited the activity. After addition of EDTA, the specific activity of Pi was only 18.39 % (P1), 8.85% (P7) and no activity was detected in the UP-1 reaction. Also, we found that these enzymes seem to be inhibited by the addition of PMSF. The activity of P1 decreased by 67.04%, 76.21 for P7, and 78.07% for UP-1 after one hour incubation with 5 mM inhibitor. This result indicated that the enzymes contains serine in its active site.

DNA extraction, PCR amplification of the 16S rDNA, and Restriction fragment length polymorphism (RFLP). 16S-rDNA genes from the sixteen isolates have been amplifield resulting in the generate on of sixteen 500 bp fragments (Figure 6). The comparativeRFLP analysis of the 16S-rDNA using *Hin*fI and *Msp*I restrictions enzymes confirmed that 7 of the 16 isolates can be classified into three distinctive distribution patterns (Figure 7). Group I includes only isolate P2. P4,P7, P9, and UP-1 isolates can be found in group II and , PG into group III category.

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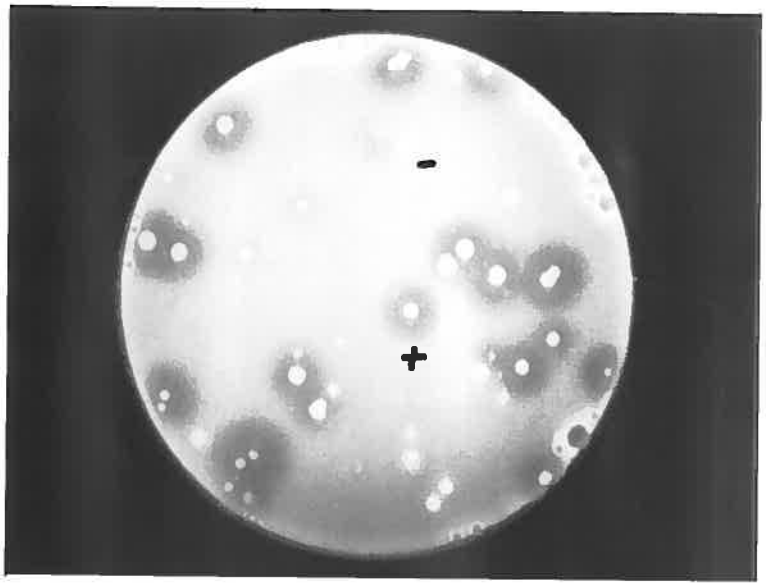
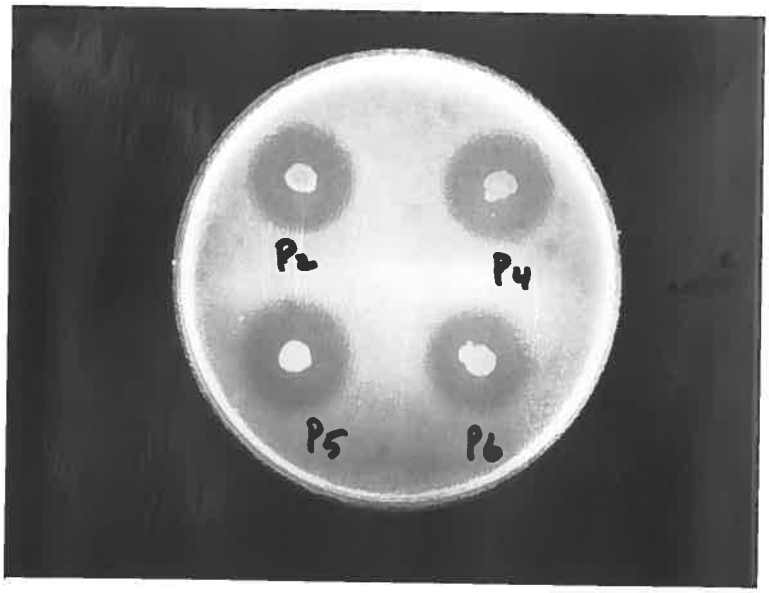
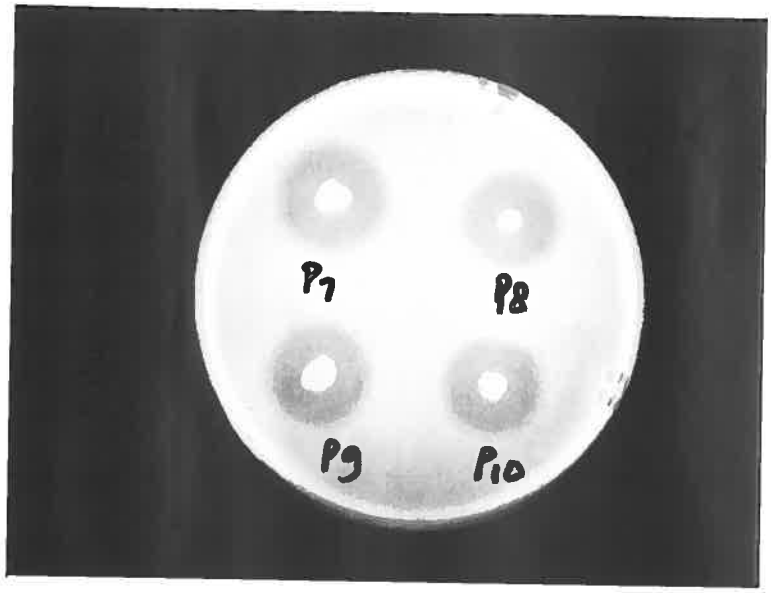
Table 1.

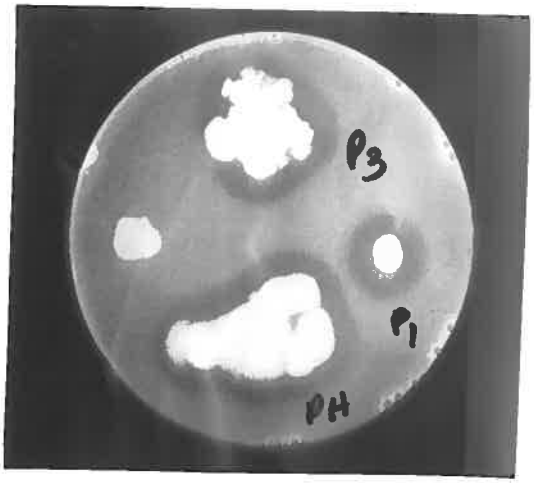
No.	Isolates	Proteolytic activity (Units/ mg protein)
1	P1 (VFA sludge)	13.590
2	P2 (XF-Sippewissett)	7.378
3	P3 (Aviko sludge)	5.364
4	P4 (UP-2 Sippewissett)	12.125
5	P5 (C1-Sippewissett)	3.827
6	P6 (C1-Sippewissett)	6.239
7	P7 (C1-Sippewissett)	60.070
8	P8 (HF-Sippewissett)	18.470
9	P9 (HF-Sippewissett)	3.550
10	P10 (C1-sippewissett)	5.945
11	PD (HF-Sippewissett)	11.416
12	PE (C1-Sippewissett)	13.455
13	PF (Aviko sludge)	4.638
14	PG (C1-Sippewissett)	4.035
15	PH (VFA-Sludge)	4.461
16	UP-1 (Sippewissett)	14.925

Figure 1






P7
P8
P9
P10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P1
PH

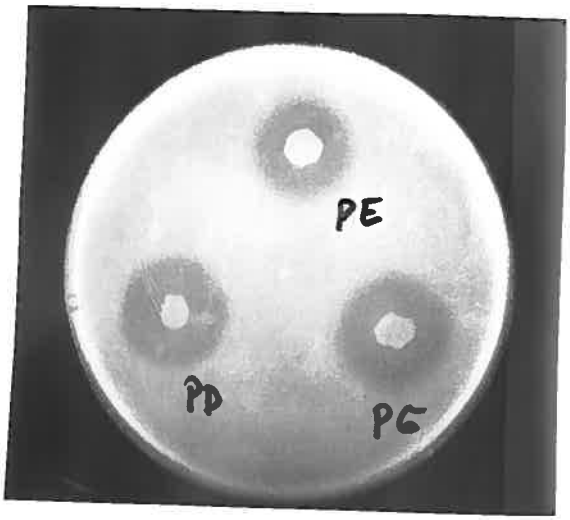
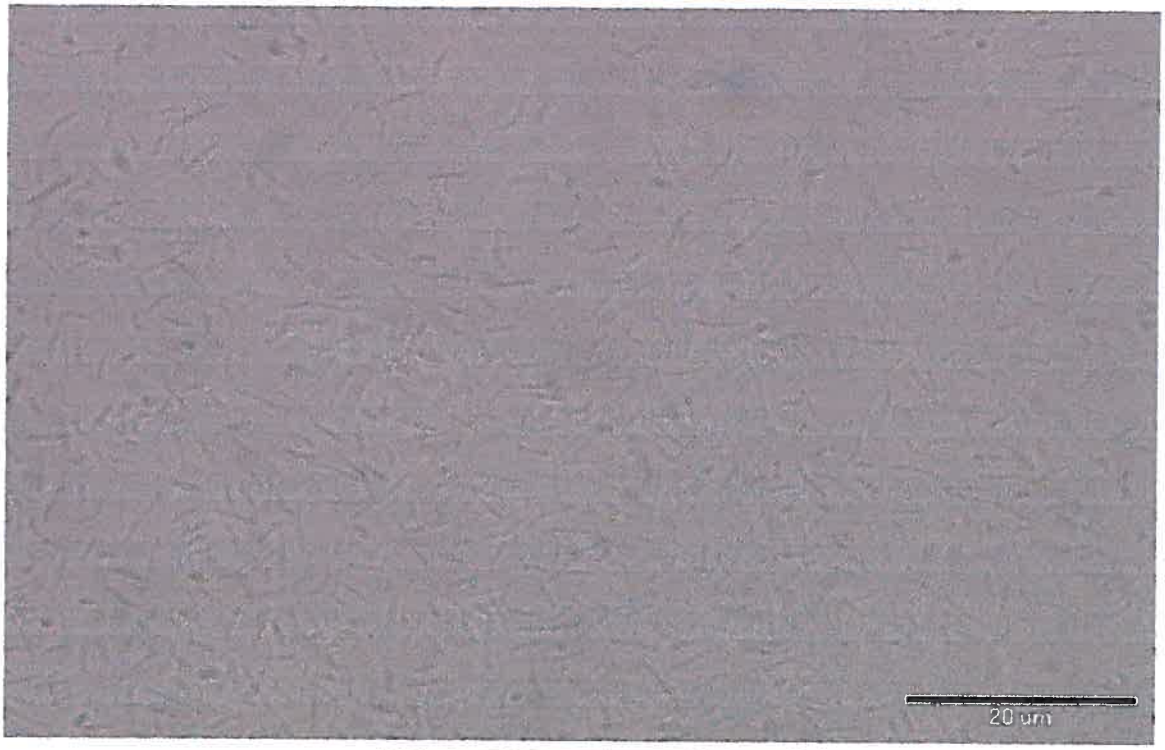
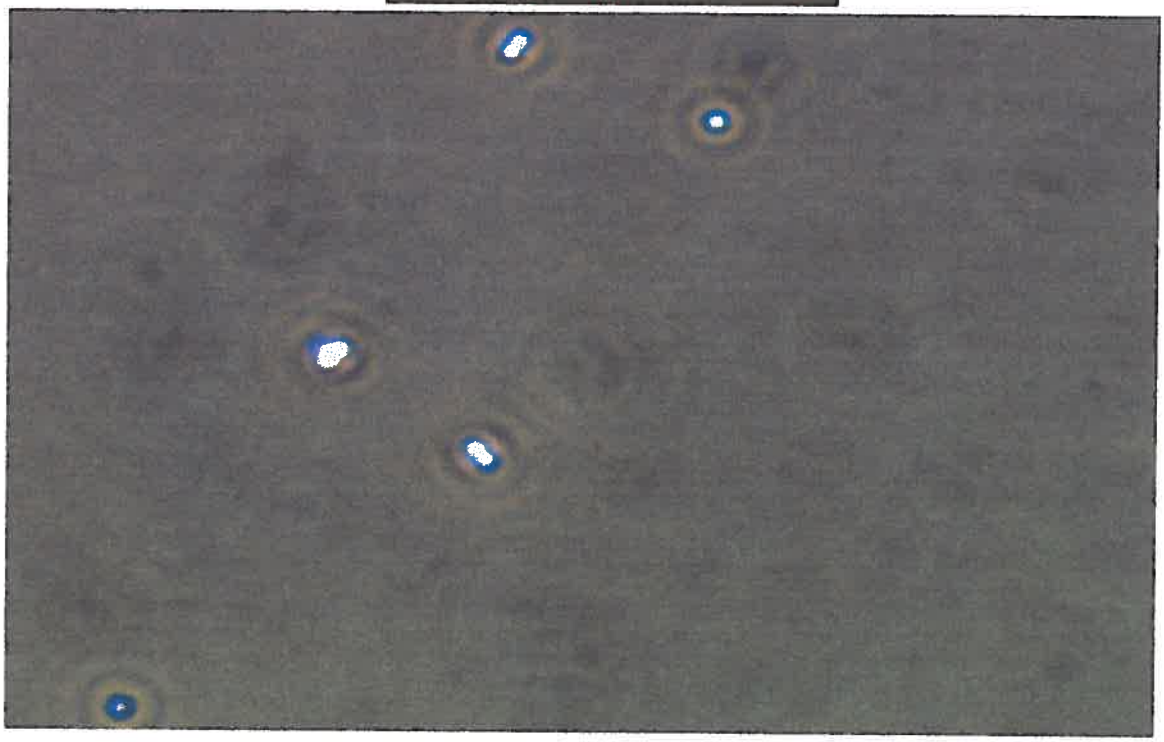

PE
PD
PG

Figure 2. **Proteolytic bacteria**



UP-1 (Sippewissett)



P1 VFA sludge

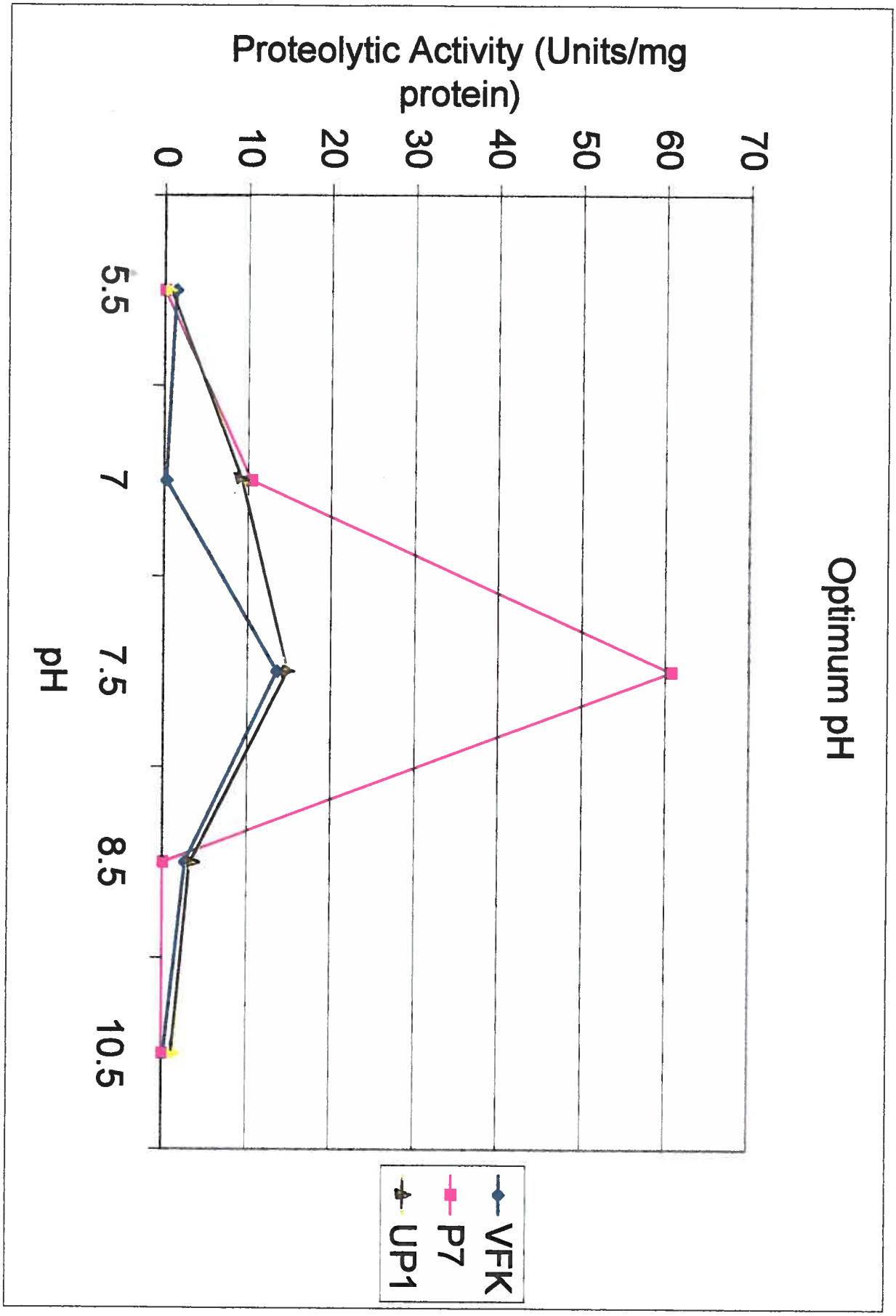
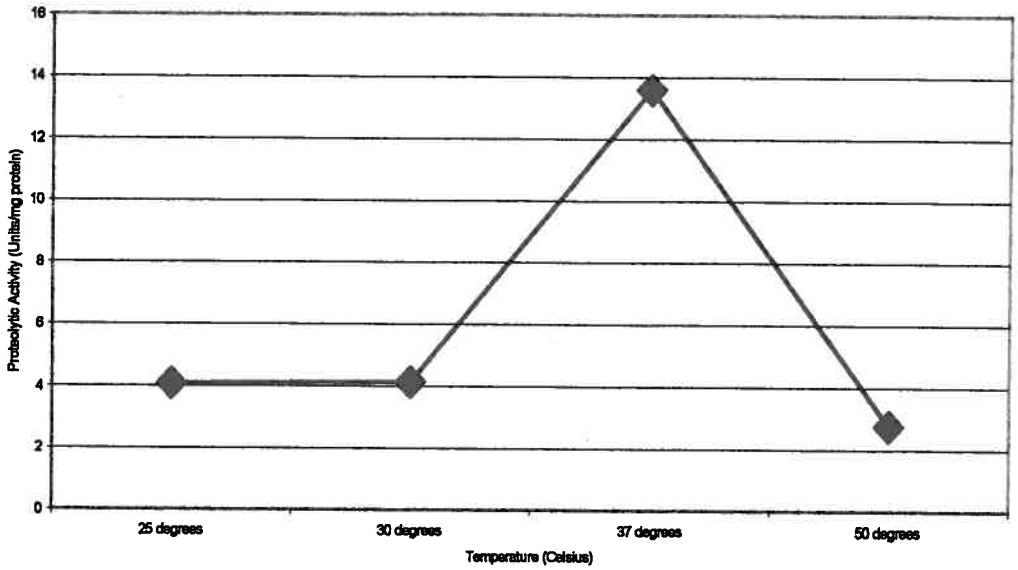
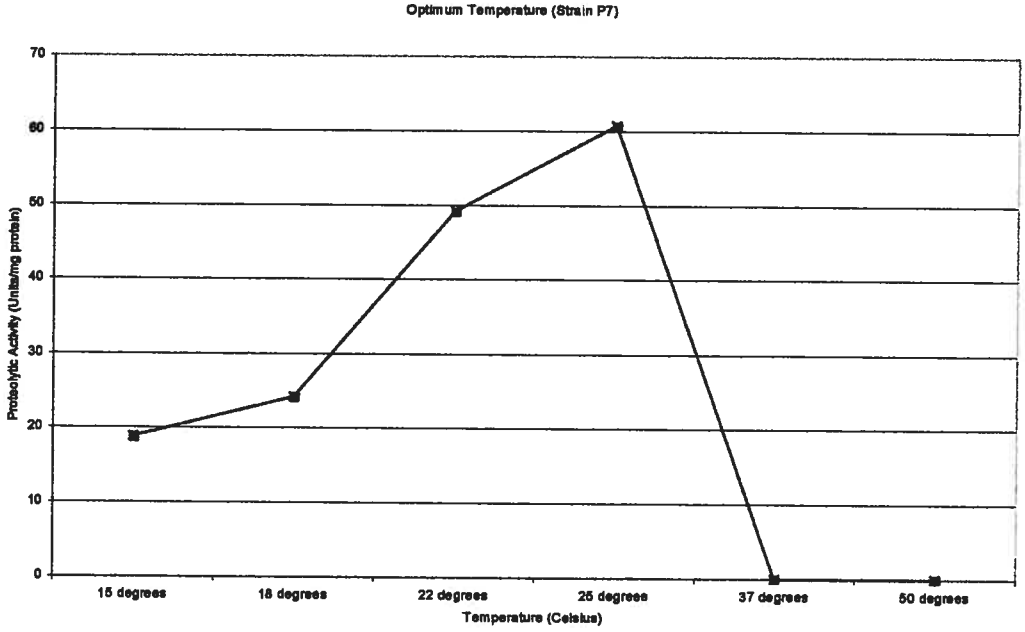
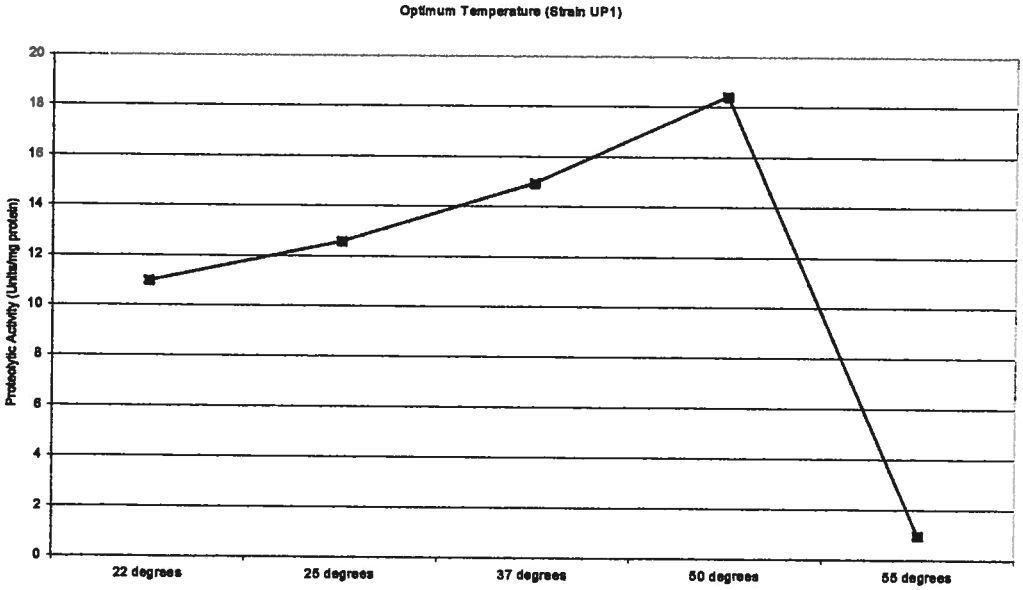

Proteolytic Activity (Units/mg protein)
0
10
20
30
40
50
60
70
5.5
7
7.5
8.5
10.5
pH
Optimum pH
VFK
P7
UP1

Figure 3.

Figure 4.


Optimum Temperature (Strain VFK)
Proteolytic Activity (Units/mg protein)
16
14
12
10
8
6
4
2
0
25 degrees
30 degrees
37 degrees
50 degrees
Temperature (Celsius)


Optimum Temperature (Strain P7)
Proteolytic Activity (Units/mg protein)
70
60
50
40
30
20
10
0
15 degrees
18 degrees
22 degrees
25 degrees
37 degrees
50 degrees
Temperature (Celsius)


Optimum Temperature (Strain UP1)
Proteolytic Activity (Units/mg protein)
20
18
16
14
12
10
8
6
4
2
0
22 degrees
25 degrees
37 degrees
50 degrees
55 degrees

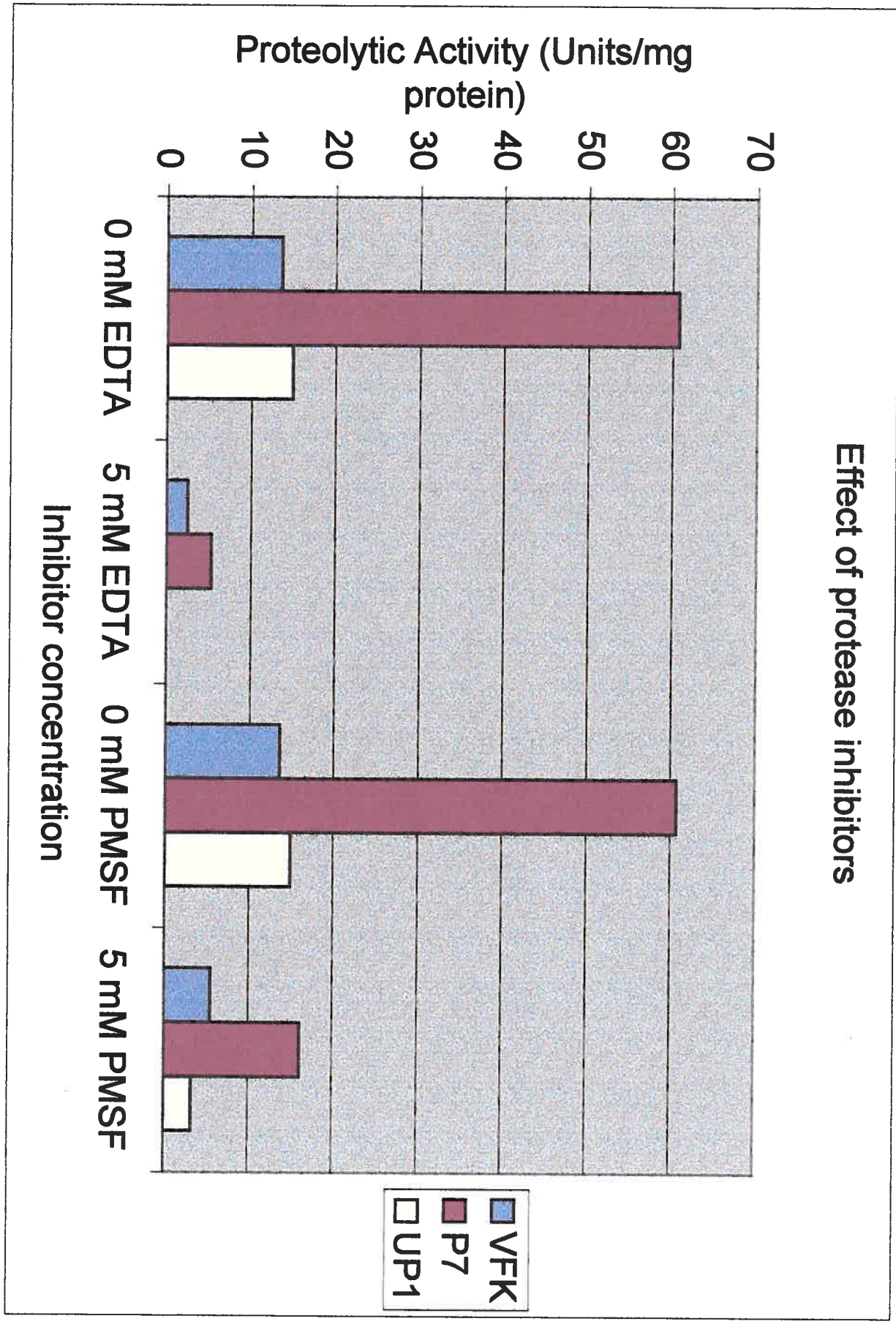
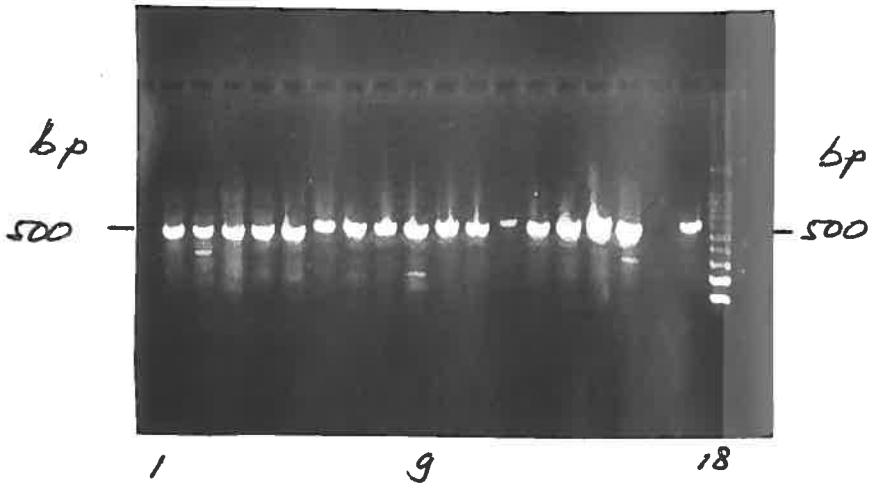

Effect of protease inhibitors
Proteolytic Activity (Units/mg protein)
0
10
20
30
40
50
60
70
0 mM EDTA
5 mM EDTA
0 mM PMSF
5 mM PMSF
Inhibitor concentration
VFK
P7
UP1

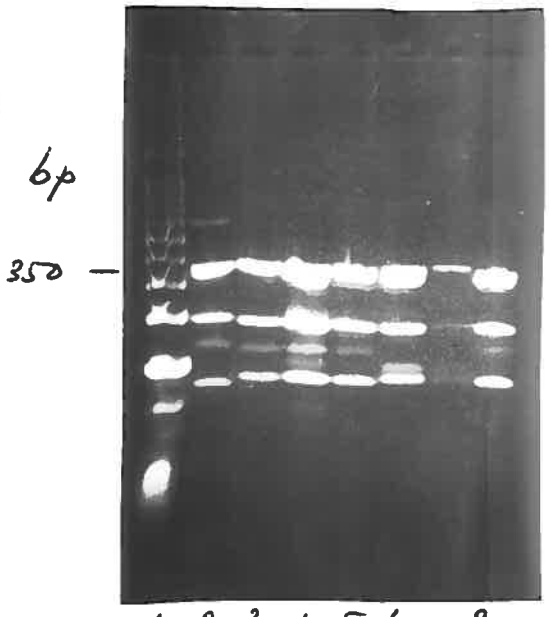
Figure 5

Figure 6. PCR Product



1. P_1
2. P_2
3. P_3
4. P_4
5. P_5
6. P_6
7. P_7
8. P_8
9. P_9
10. P_{10}
11. PD
12. PE
13. PF
14. PG
15. PH
16. UP-1
17. negative control
18. positive control
19. 100 bp DNA Ladder.

Figure 7. RFLP



1. 100 bp DNA Ladder
2. P_2
3. P_4
4. P_7
5. P_9
6. P_{10}
7. PG
8. UP-1

Group I = P_2

Group II = P_4
P_7
P_9
UP-1

Group III = PG

Experiment 2. Isolation and characterization methanogenic archaea from the Sippewissett salt marsh, termite hind gut, and "Dutch" sludge

Introduction

The methanogenic archaea produced large quantities of methane as the major product of their energy metabolism and are strictly anaerobic. Methanogenesis is the terminal step in the carbon flow in many anaerobic habitats. Biogenic methane formation occurs in a wide variety of environments such as, ruminants, termites, cockroaches, mammals, lakes, wetland, sludge, landfills, oceans, tundra fields and human-made waste water treatment plants and other systems for bioremediation. The methanogenic archaea produce methane primaily from H_2-CO_2 and in some cases, from formate, acetate, methanol or methylamine.

The main goals of this study were to isolate and characterize methanogenic archaea from the Sippewissett salt marsh, termite, and granular sludge from a UASB reactors from The Netherland.

Materials and Methods

Primary enrichment. Enrichment cultures for each sample were maintained in 60-ml serum bottles containing liquid medium. Granular sludge from UASB reactors (The Netherland), termite hind gut, and soil from both fertilized and unfertilized plots from Sippewissett were examined. The granular sludge and termite hind gut samples were first diluted in phosphate buffer, and the Sippewissett samples were diluted in sea water. Concentration of all samples was adjusted to 10^{-4}. 100 µl of sample suspension

was used to inoculate all of the bottles. All cultures were incubated in the dark, sludge was incubated at 37°C, termite hind gut cultures at 30°C, and Sippewissett cultures were incubated at 22°C. Before inoculation, the bottles contained 18 ml standard medium, gassed with either nitrogen or H_2-CO_2 (80:20) and pressurized to 0.5 atm. Methanol was used as carbon source for Sippewissett and sludge cultures, and H_2-CO_2 was carbon source for termite hind gut cultures..

Isolation of methanogenic archaea. Roll agar tubes (3%) were used to isolated methanogenic archaea. The roll agar tubes were inoculated aseptically under nitrogen or H_2-CO_2 and then were gassed, pressurized, and inoculated as described above.

Secondary enrichment. A single colony from the roll agar tubes, was transferred into liquid medium aseptically under nitrogen or H_2-CO_2 and was then gassed, pressurized, and inoculated as described above.

Methane analysis. Gas chromatography (GC) was used to determine methane production in the primary and secondary enrichment cultures and roll agar tubes cultures. The gas chromatograph (Varian 3800) was fitted with a CP-Poroplot U column (Chrompach/Varian) and a flame ionization detector (FID). The chromatographic conditions were: 50°C running temperature and 80°C detection temperature. A standard of 1 % of methane in air was used to calibrate the gas chromatograph.

Microscopic observation. A Zeiss microscope was used to determined whether coenzyme-420 which is found in methanogenes could e detected in net mounts made from the enrichment cultures. The microscope was arranged so that this coenzyme could be observed by epifluorescence microscopy. In addition, , the microscope was attached to a video camera, a monitor and a computer to enable image capture and processing.

Fluorescent *in situ* hybridization (FISH). A rhodamine labeled archaea probe was used to detect methanogenic archaea in enrichments, and the termite hind gut. Treatment of specimens and hybridization reaction protocols were carried out as described in the workshop manual provided by Scott Davson.

DNA extraction and PCR amplification of the 16S rDNA. PrepMan™ Method (PE Applied Biosystems a division of Perkin-Elmer) provided during the course was used to extract DNA from both secondary enrichment and a single colony selected from roll agar tubes containing methanogenic archaea. Universal archae primers, 21F forward (position 21) 5'-TTCCGGTTGATCCYGCCGGA-3' and 915AR reverse (position 915) 5'-GTGCTCCCCCGCCAATTCCT-3' were used for PCR amplipication of DNA extracted from the secondary enrichment and the single colony in roll agar tube. The amplification was carried out in a 50 µl reaction volume : 1 µl of template DNA with 49 µl of polymerase reaction mixture. Each reaction mixture

received one bead containing the *Tag* polymerase. The PCR temperature profile was as follows: 95°C for 10 min, 30 cycles of (95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec), the amplification time of each cycle was extended for 5 sec. Amplified DNA was examined by horizontal electrophoresis in 1.25% agarose with 5 µl aliquots of the PCR product.

Restriction fragment length polymorphism (RFLP). Aliquots of PCR products were mixed with the restriction endonucleases *Hin*PI and *Msp*I. Reaction mixtures of 20 µl containing: 10 µl PCR product, 2 µl restriction buffer Neb2 (New England Biolab), 8.6 µl dH_2O and 0.2 each of restriction enzyme. Restricted DNA was analyzed by horizontal electrophoresis in a 2 % agarose. The resulting band patterns were used to distinguish between different species of bacteria.

Results and Discussion

Methane production. Production of methane was used as indicator of methanogenic activity. Methane production was detected in the primary and secondary enrichments and roll agar tubes (Table 1).

Table 1. Methane production from the primary and secondary enrichments and roll agar tubes

Sample		Methane (%)	
	Week 1	Week 2	Week 3
"Dutch" sludge (primary enrichment-roll agar tube) 1. Aviko a. high dilution	1.69	59.46	448.28

b. low dilution	ND	0.13	0.09
2. CSM			
a. high dilution	0.61	7.98	120.81
b. low dilution	ND	0.09	0.16
3.VFA			
a. high dilution	76.80	556.77	365.04
b. low dilution	0.61	6.66	6.74
"Dutch" sludge (secondary enrichment-liquid medium)			
1. Aviko	0.04	0.04	
2. CSM	0.04	0.04	
3. VFA	0.46	0.04	
Sippewissett (primary enrichment-liquid medium)			
C	1.03	0.02	
UP	0.43	0.04	
HF	0.19	0.02	
XF	3.96	ND	
Sippewissett (primary enrichment-roll agar tube)			
C	0.58	2.34	
UP	0.08	2.45	
HF	1.17	72.44	
XF	0.92	1.55	
Termite hindgut (primary enrichment-roll agar tube)	0.07	2.04	

ND : non-detection
C : control or unfertilized
UP : fertilized using urea and phosphat
HF : high ferlilized
XF : extra fertilized
High dilution ($1 : 10^{10}$)
Low dilution ($1 : 10^{4}$)

Table 1 shows that the samples from "Dutch" sludge produced the highest levels of methane, suggesting that the sludge samples contained the largest concentrations of methanogenic archaea. We also found that methanogenic archaea

activity in high dilution was higher than low dilution. This low value of methane found in the high dilution might be explaned by competition between methanogenic archaea with an other microanaerobic organism. Termite hindgut microorganisms have also been shown to produce methane, and methanogenic archaea have been visualized from some clonies in roll agar tube.

Isolation and microscopic observation. Methane production was detectable in the roll agar tubes and fluorescence was observed. This result indicates that methanogenic archaea were sucessfully isolated. Methanogenic archaea from "Dutch" sludge can be seen in Figure 1.

Fluorescent *in situ* hybridization (FISH). Specimens from primary enrichment of sludge and termite hind gut were examined for methanogenic archaea by Fluorescent *in situ* hybridization (FISH). Specimens were hybridized with a rhodamin labeled archaea probe. Cells that hybridized with the archaea probe were seen in the primary enrichment of sludge and the termite hind gut. The result of FISH can be seen in Figure 2.

DNA extraction, PCR amplification of the 16S rDNA, and Restriction fragment length polymorphism (RFLP). 16S-rDNA squences from the 8 isolates have been amplifield using archaeal primers and 3 PCR products were generated (Figure 3). The comparative RFLP analysis of the 16S-rDNA using *Hin*PI and *Msp*I restriction

enzymes confirmed that all isolates can be classified into different distinctive distribution patterns (Figure 4). This result indicates that the methanogenic archaea in the termite hind gut, Sippewissett, and "Dutch" sludge are different strains.

Suggested References

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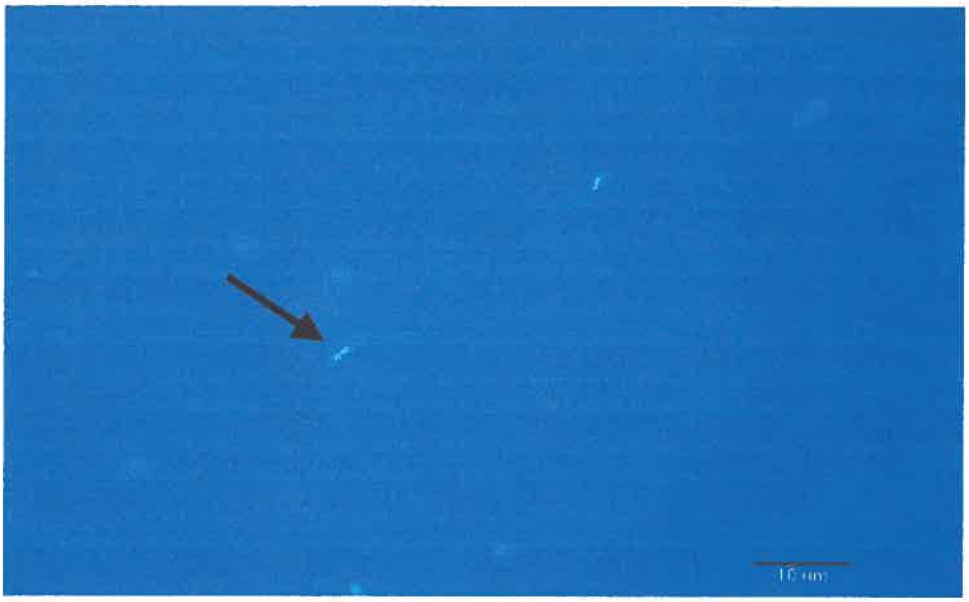
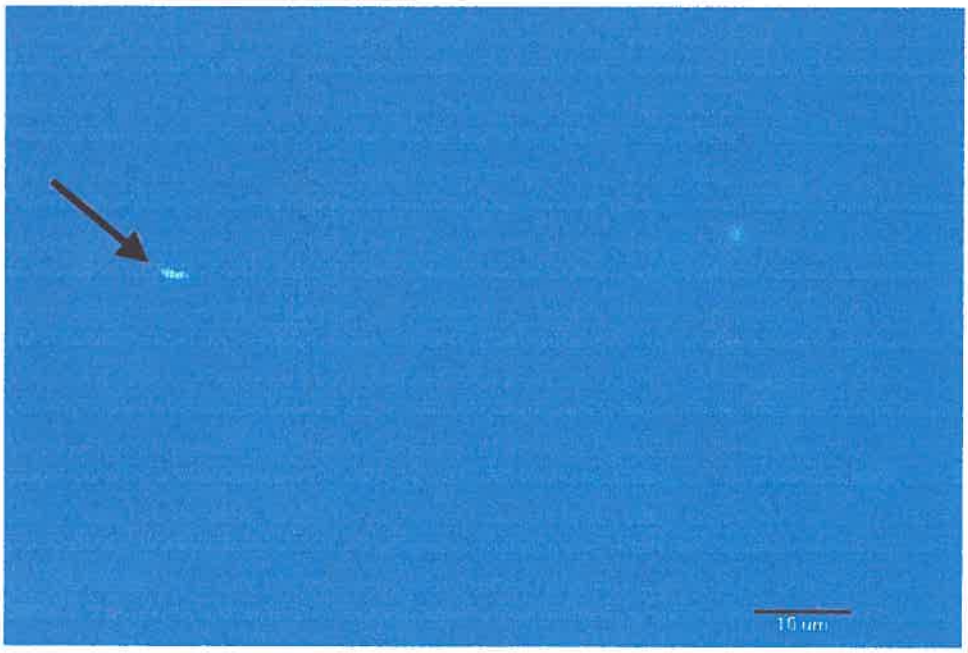
Figure 1. Methanogenic Archaea

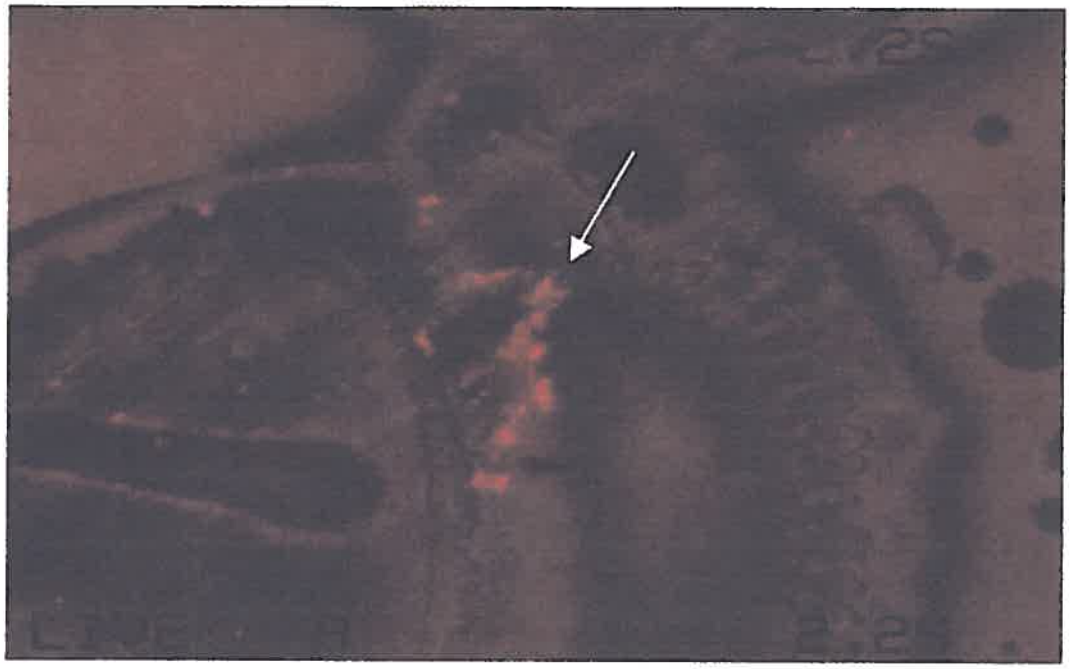


A

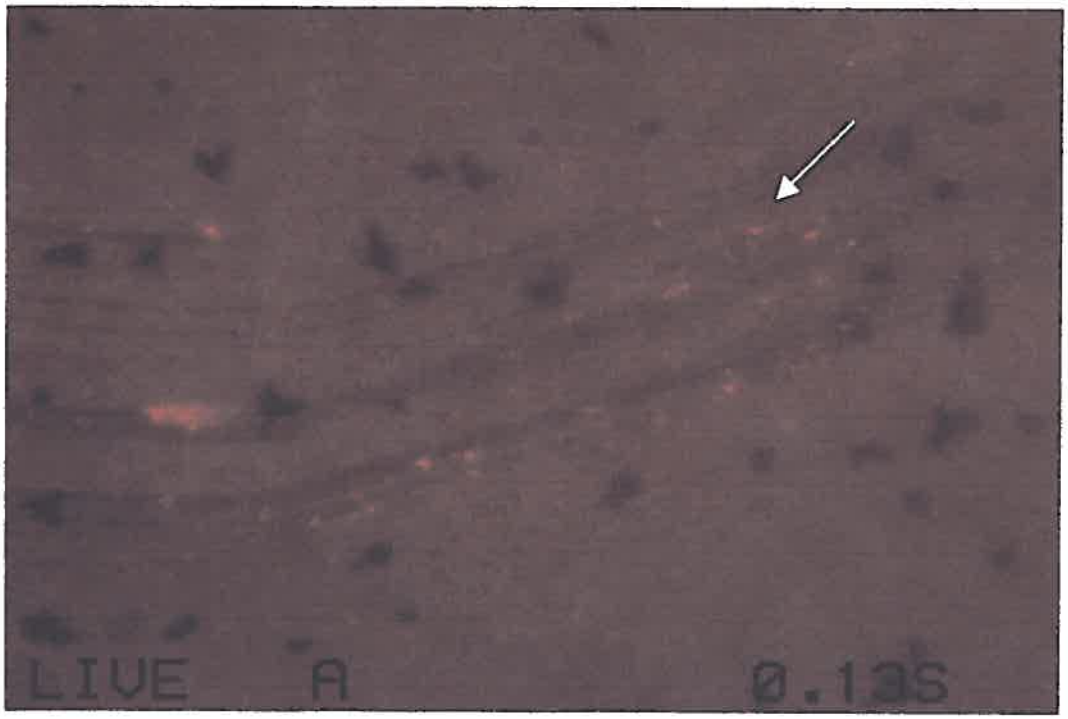


B

Figure 2. **Fluorescent in situ hybridization (FISH) (Rhodamin labeled archaea)**

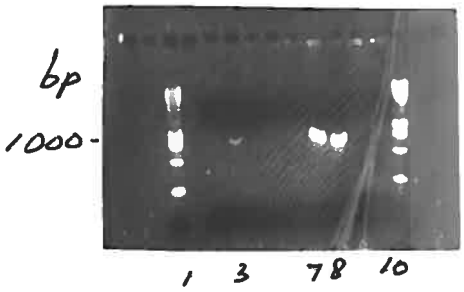


A. Aviko sludge enrichment



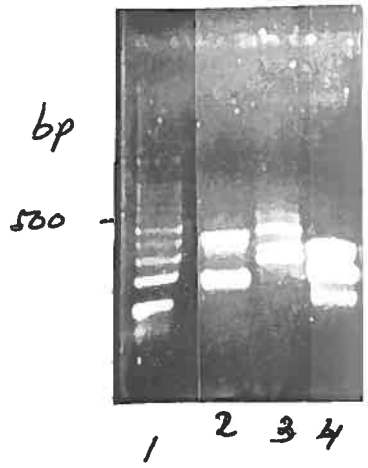
B. Termite hind gut

Figure. 3 PCR Product



3. DNA (Termite hindgut)
7. XF (Sippewissett)
8. AVIKO Sludge
10. 1kb DNA Ladder

Figure. 4. RFLP



1. 100 bp DNA Ladder
2. DNA (Termite hindgut)
7. XF (Sippewissett)
8. AVIKO (Sludge)