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ISOLATION AND CHARACTERIZATION OF XYLANOLYTIC BACTERIA.

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Introduction

Hemicelluloses are one of the most abundant types of plant polysaccharides. One type of hemicelluloses is xylan, which has B-1,4 linked-xylose backbone and varying amounts of side groups (arabinose, 4-o-methylglucuronic acid, acetate).

Since xylan is not degraded by the digestive enzymes of mammals, the utilization of this polysaccharide is mediated by the microbial communities that inhabit the gastrointestinal tract of these animals. The predominant species of xylanolytic ruminal bacteria include *Butyrivibrio fibrisolvens*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, *Eubacterium ruminantium*, *Prevotella ruminicola* (Cotta and Zeltwanger, 1995) and *Bacteroides xylanolyticus* (Scholten-Koerselman et al., 1986)

Environments different from the gastrointestinal tract of mammals are also populated by xylanolytic bacteria. *Cytophaga xylanolytica*, an anaerobic gliding bacterium, was isolated from methanogenic and sulfidogenic enrichments from freshwater sediments (Kidd and Breznak, 1993) and *Clostridium xylanolyticum* from decayed *Pinus patula* wood chips (Rogers and Baecker, 1991).

Nutritional studies have shown that at least half of all cellulose and xylan ingested in the human diet disappears during passage of food through the colon. No one has tried an enrichment approach to isolating xylanolytic colonic bacteria and only a few of the known species of colonic anaerobes can utilize xylan.

In this project the isolation of xylanolytic bacteria from colon and freshwater sediments was attempted. Morphological and metabolic characterization of isolates was done.

Materials and Methods.

Media. The media used in this study contained per liter: 0.5 g of yeast extract, 50 ml of 1M potassium phosphate buffer, 40 ml of salts solution, 5 ml of 0.1 % hemin solution, 1 ml of 0.8 % CaCl_2 solution, 0.5 g of cysteine hydrochloride and 5 g of xylan from oat spelts. The final pH was 7.0-7.2. Salts solution contained per liter: 0.5g MgCl_2 , 2 g NaCl , 0.4 g $\text{MnCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and 10 g NaHCO_3 . 1.7% of acetone and water washed Bacto Agar was added to solid media. Alternatively 20 % sea water was used.

Anaerobic medium was prepared by boiling for a few minutes and then cooling under Oxygen-free $\text{N}_2\text{-CO}_2$ (80-20) flow. Cysteine was added in this step. Liquid medium was distributed in 60 ml vials previously purged with the gas mixture. Butyl rubber stoppers and aluminum caps were utilized. Solid medium was poured in aerobic conditions and plates were preincubated in anaerobic chamber 24 hs. before inoculating.

Liquid aerobic medium with D-xylose replacing xylan was also utilized. Growth in casaminoacids (2 % casaminoacids, 0.1 % K_2HPO_4 and 0.05 % of yeast extract, CAA) and casaminoacid-sea water (20 %) medium was also tested.

Inoculum. Two different freshwater sediments (Cedar Swamp and Mill Pond) and one colon inoculum from rectal swab were tested.

Enrichments. Enrichments were performed on liquid media by adding 5 % inoculum (by volume) and incubating them in aerobic or anaerobic conditions. Also mesophilic (37C) and thermophilic (55C) conditions were tested in anaerobic enrichments. Controls without xylan were performed. After comparing with controls and observing macro and microscopic growth, the supernatant was removed and replaced by fresh medium. This was done to enrich for bacteria that adhered to xylan particles.

Isolation. Particulate material was streaked on agar plates under aerobic and anaerobic conditions. Anaerobic plates were incubated in an anaerobic jar. Colonies which produced clearing zones of xylan degradation were removed and restreaked to obtain pure cultures.

Morphology and growth conditions. Cultures were examined by phase contrast microscopy. Shape, motility and spore formation were determined. Gram and oxidase test were performed. Anaerobic and aerobic growth was tested. D-xylose and casaminoacids were tested as alternative substrates. Growth in xylose liquid medium containing 5 % NaCl was determined.

rDNA Probes hybridization. Isolates were tested by hybridization with fluorescent rRNA gene probes. Alfa, Beta and Enteric Proteobacteria probes were tested for Gram negative bacteria and High G+C and Low G+C probes were tested for Gram positive bacteria. Positive controls with the Universal probe and negative controls without probes were performed. Coumarin was included in all the probe solutions tested. A fluorescence (Axioscop, Zeiss) microscope was used.

Fermentation end product analysis. Fermentation end products from xylan were determined by high pressure liquid chromatography. End products were separated by a Waters LC Module 1 liquid chromatograph equipped with an anionic exchange column (RSpak KC-811, Shodex), a precolumn (RSpak KCG, Shodex) and a Refractive Index Detector (RID-6A, Shimadzu). Elution was performed at room temperature with a 0.5 ml/min flow of 0.2 % H₃PO₄. Standards were D-xylose, lactate, formate, acetate, propionate, isobutyrate, butyrate, ethanol, 1,2-butanediol and 2,3-butanediol were utilized.

rDNA Banding profiles. PCR amplification of 16S rDNA from the isolates was done and amplicons were analyzed by performing restriction digests on the products. Digestion were performed with 1 unit of each of the follow enzymes: Hae III, Msp I and BstU I (Sigma Biosciences). Separation of the products was carried out by running a 1.5 % Agarose gel in 0.5M TBE buffer.

Results.

Morphology and growth conditions of the isolates.

Microcolonies of bacteria adhering to particulate xylan were observed in the enrichments. Many different bacteria were present and clear differences between adherent and free microorganisms were observed (Fig. 1, 2 and 3).

4 isolates from colon contents and 4 from fresh water sediments were obtained. All of them, except one colonic isolate, were Gram positive spore-forming rods. Two of them were strict anaerobes. No strict aerobes were isolated.

Source and morphology of the colonic isolates are summarized in Table 1 and growth conditions are summarized in Table 2.

Strain	Shape	Gram	Spores	Motility	Enrichment conditions
C1	rod	-	-	+	aerobic/37C/SW
C2	rod	+	+	+	aerobic/37C/SW
C3	rod	+	+	-	anaerobic/55C/SW
C4	rod	+	+	+	anaerobic/55C/SW

TABLE 1.

Strain	Aerobic growth	Anaerobic growth	Substrates utilized	
			Xylose	CAA
C1	+	+	+	nd
C2	+	+	+	+
C3	+	+	**	+
C4	+	+	+	+

TABLE 2.

(*) Note: 3 days lag phase.

The Gram negative strain gave an oxidase negative tests indicating that it belongs to the Enterobacteriaceae family.

The isolates C2, C3 and C4 are probably *Bacillus* species. Clear morphological differences were observed between the strains C3 and C4 and the strain C2 (Fig. 4). Slime, big colonies and central spores were formed by C2 isolate, whereas small irregular flat colonies and terminal spores were observed in C3 and C4 isolates.

Although C3 and C4 strains were isolated at 55C, better growth was observed when incubated at 37C.

Abundant growth but no clearing zones in xylan agar plates were detected from anaerobic mesophilic enrichments.

Isolate C3 was able to growth in fresh water xylan agar medium but a long lag phase was observed when this strain was cultivated in fresh water xylose liquid medium. Also, a lag phase was showed by the C4 strain when it was cultivated in 5 % NaCl medium from a fresh water medium.

Spore-forming Gram positive rods were isolated from freshwater sediments. Motility, growth conditions and source of the isolates are summarized in Table 3.

Strain	Moti- lity	Aerobic growth	Anaerobic growth	Substrates utilized		Enrichment conditions
				Xylose	CAA	
F1	-	+	+	+	+	aerobic 37C/FW
F2	+	-	+	+	nd	anaerobic 37C/FW
F5	-	+	+	+	+	anaerobic 55C/FW
F7	+	-	+	+	nd	anaerobic 37C/SW

TABLE 3.

Terminal spores were observed in isolates F2, F5 and F7, whereas central or subterminal spores were formed by isolate F1 (Fig. 5 and 6). Although F5 was isolated by incubation at 55C, all the isolates are mesophilic.

Aerobic and anaerobic growth allowed me to classify tentatively the isolates into the Genera *Clostridium* (F2 and F7) and *Bacillus* (F1 and F5).

rDNA Probes hybridization.

Fresh cells to perform rDNA probes hybridization were obtained by cultivating the isolates in xylose liquid medium to avoid xylan fluorescence.

C1 strain hybridized with the Enteric Proteobacteria probe (Fig. 7) and did not hybridized with alfa or beta Proteobacteria probes.

The hybridization pattern for the Gram positive isolates are summarized in Table 4 and some results are shown in Fig. 8 and Fig. 9. No autofluorescent bacteria were observed and all the tested microorganisms hybridized with Universal probe. Three strains that had been tentatively identified as *Bacillus* species hybridized with the Low G+C probe. Neither the Low G+C nor the High G+C probes gave a positive reaction for the other isolates.

Strain	Low G+C probe	High G+C probe
C2	+	-
C3	-	-
C4	+	-
F1	-	-
F2	-	-
F5	+	-
F7	-	-

TABLE 4.

Fermentation products.

Fermentation products from the anaerobic xylan degradation by C2, C3 and C4 strains were measured.

Formate, ethanol and acetate were the main metabolites produced. Neither higher fatty acids nor higher alcohols were formed.

The fermentation patterns determined for strains C3 (Fig. 10) and C4 (Fig. 11) were similar, although metabolite accumulation, presumably oligosugars, was observed in the C3 strain fermentation. This strain was isolated and always cultivated in sea water medium. This result suggests that under this condition, limited degradation of xylan is performed.

In spite of the low product concentration measured for C2 strain fermentation, profuse growth was observed. Metabolite accumulation, presumably oligosugars, was measured at 60 hrs. incubation. Prolonged incubation resulted in xylose production and reduced amounts of oligosugars (Fig. 12).

rDNA banding profiles.

Banding profiles from 16S rRNA gen was performed to compare the three colonic strains belonging to the genus *Bacillus*. Results are shown in Fig. 13.

C2 was clearly distinguished from the two other strains. Different restriction fragments were produced by the three restriction enzymes.

C3 and C4 amplicons gave a similar patterns for Hae III digestion, and BstU I produced equal number and size fragments but with different yield. Msp I digestion of the C4 amplicon generated one light band a few higher than 300 pb which was not observed on the digestion products for C3 strain.

Discussion.

Since lignocellulose is an abundant and renewable resource with high potential for production of industrially useful compounds, the biodegradation of lignocellulose has interested researchers and technologists in many fields. Xylanases have been reported in numerous microorganisms, including fungi, Actinomycetes and aerobic and anaerobic bacteria.

As expected from the structural diversity of the substrate, xylan degradation by microorganisms is a complex biochemical process. It appears that xylosidic linkages in lignocellulose are not all equivalent and equally accessible to xylanolytic enzymes. The accessibility of some linkages also changes during the course of hydrolysis. The production of a system of enzymes, each enzyme with specialized functions, is one strategy that microorganisms may use to achieve superior xylan hydrolysis (Wong et al., 1988).

Several xylanases have been characterized from *Bacillus* sp. (*B. circulans*, *B. coagulans*, *B. pumilus*, *B. subtilis*) and from *Clostridium acetobutylicum* and *C. stercorarium* although it has not been determined how they are functionally distinct. No well characterized analysis of products has been performed (Wong et al., 1988).

High xylanolytic activity was observed from both colon and fresh water sediments bacteria and 8 isolates were obtained. The bacteria isolated were classified into the Enterobacteriaceae family, and into the *Bacillus* and *Clostridium* genera.

It is remarkable that from the colon habitat, where most of the microbial population consist of strict anaerobes, just facultative microorganism were isolated. Also, even though mesophilic facultative bacteria were present, the isolates were exclusively obtained from aerobic mesophilic and anaerobic thermophilic enrichments. Many different bacterial morphotypes with no xylanolytic activity were observed in plates inoculated with the mesophilic anaerobic enrichments. These bacteria were not present in controls without xylan. That suggests that many of these bacteria could growth from the metabolites produced by xylan degradation and overgrew the xylanolytic microorganisms. An analogous situation has been described in ruminal contents, where the xylan degradation products can be utilized by a group of bacteria that outnumber xylanolytic microorganisms (Cotta and Zeltwanger, 1995). Different types of enrichments to improve the growth and the selection of xylan-sticked bacteria could probably result in a larger and more diverse number of isolates.

Since that xylan degradation in humans occurs in the lower gastro intestinal tract, bacterial characterization was focused on the colonic microorganisms, particularly the Gram positive sporeformers.

At least two different *Bacillus* species were isolated from colon contents according to the rDNA banding profiles. Characterization was facilitated because a small number of *Bacillus* species grows in anaerobic conditions (Sneath, 1986).

Morphological characteristics suggest that the C2 isolate was *Bacillus polymyxa*. This strain grew as mucoid colonies with matte surface and amoeboid spreading. Ellipsoidal spores with swollen sporangium were observed. However there was a difference in the spore position. Mostly central spores were observed in the C2 isolate, whereas *B. polymyxa* has terminal spores (Sneath, 1986).

The hybridization of C2 strain with Low G+C probe gives one more evidence of the analogy between this isolate and *B. polymyxa* which has a G+C content of 44.3 % (Sneath, 1986).

Fermentation product correspondence between both strains was also observed. Most bacilli carry out incomplete oxidations when growing on carbohydrates. The substrate is partially converted to acetate, pyruvate, acetoin and 2,3-butanediol. In aerobic conditions TCA enzymes are synthesized and these products are further oxidized to CO₂ to produce the energy for the sporulation (Gottschalk, 1986). Anaerobic growth of the C2 strain on xylan rendered small amounts of ethanol, acetate, formate and lactate and prolonged incubations allowed detection of xylose in the medium. No butanediol was detected. Anaerobic fermentation of glucose by *B. polymyxa* produces butanediol and ethanol and trace levels of acetate, formate and lactate, but no butanediol was produced from xylose (Hespell, 1996).

Even if a considerable growth from xylan was observed, only a low concentrations of products was detected in early steps of incubation. From such a complex substrate it probably would be difficult to establish which was in fact the substrate that supported the growth during the earlier steps of the growth.

Substantial differences were not found between C3 and C4 isolates except that there was no hybridization of the C3 isolate with the Low G+C probe. However this test was not performed in the best conditions for this strain. Because of its slow growth in xylose medium, an old xylan culture was employed.

Two *Bacillus* species, *B. coagulans* and *B. licheniformis*, have a low G+C content, can grow in anaerobic conditions, tolerate 55C, and produce acids from xylose (Sneath, 1986). These species can be differentiated by their tolerance to NaCl. *B. licheniformis* grows in medium with up to 10% NaCl whereas *B. coagulans* does not tolerate higher NaCl concentrations than 2%. Fast growth by the C3 isolate occurred in 5% NaCl medium. Differences in time response for these two isolates probably are due to the previous culture conditions. C3 strain was isolated and cultivated in sea water medium whereas C4 was cultivated in freshwater medium.

These results indicate that C3 and C4 isolates could be assigned to the *B. licheniformis* species for which until now no xylanolytic activity has been reported.

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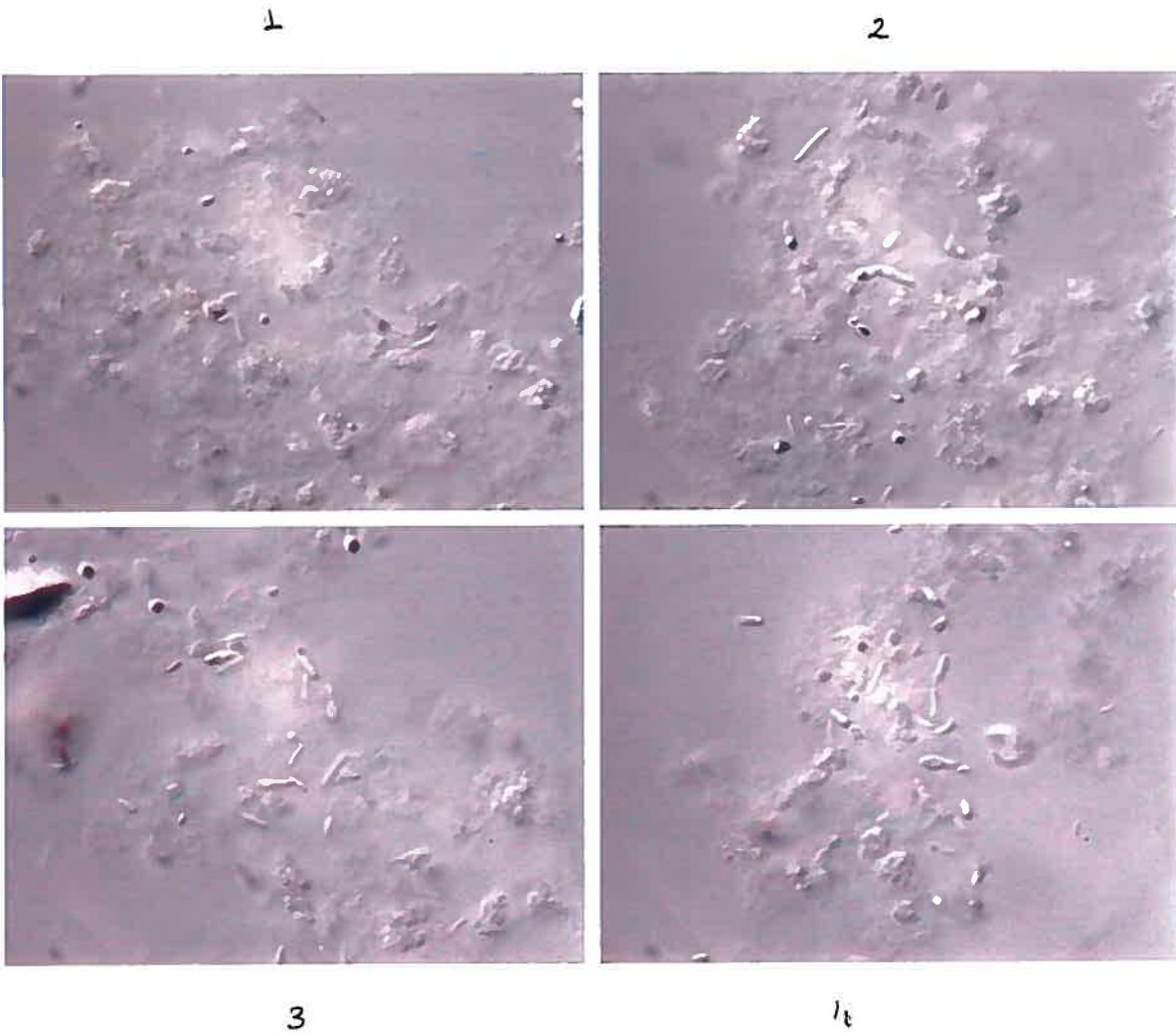


Figure 3

- 1: Enrichment from colon in Sea water, anaerobic, 37°C
2, 3 and 4: Enrichment from fresh water in sea water medium, anaerobic, 37°C

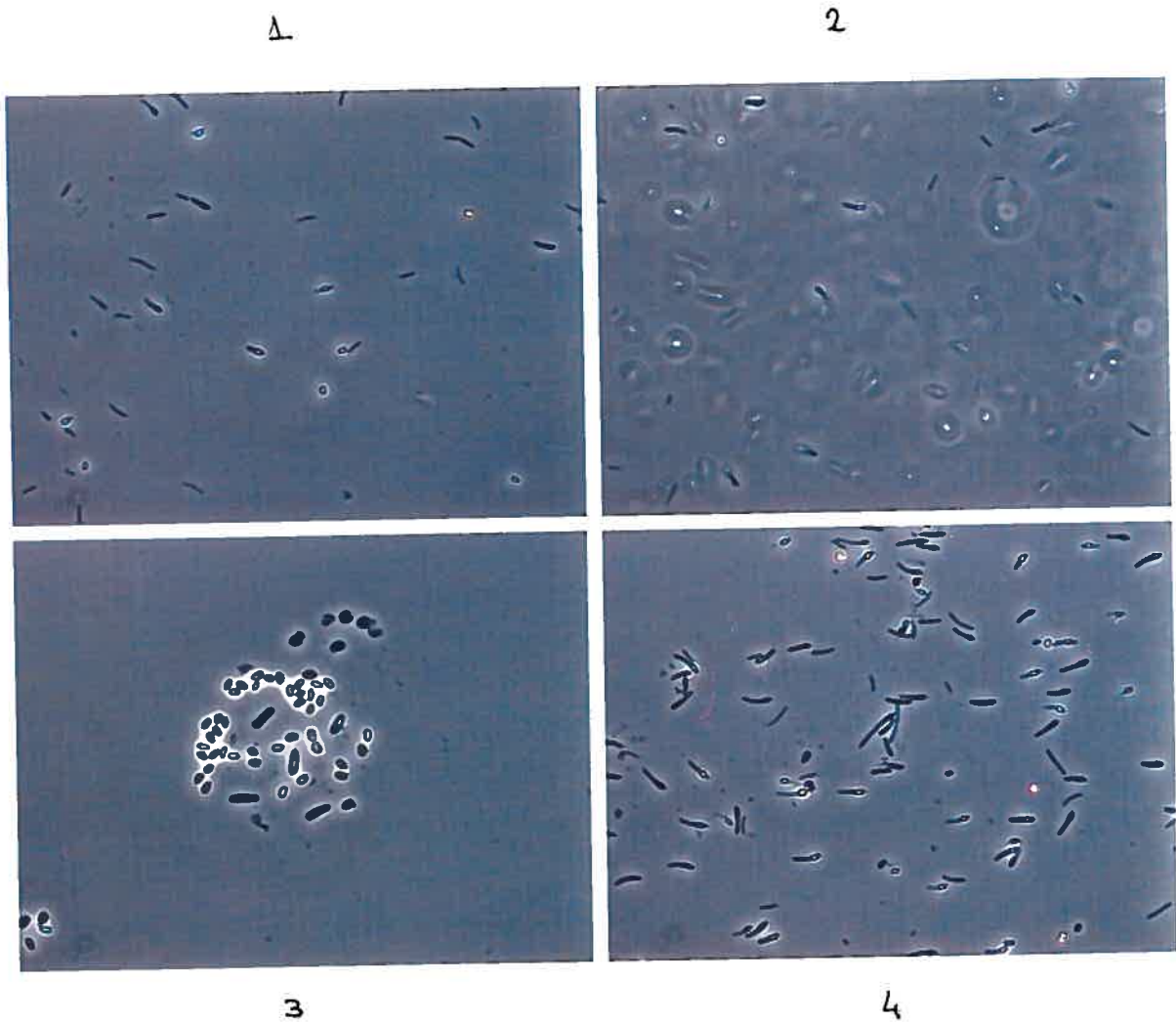


Figure 4

1 and 4 : C₄ strain

2 : C₃ strain

3 : C₂ strain

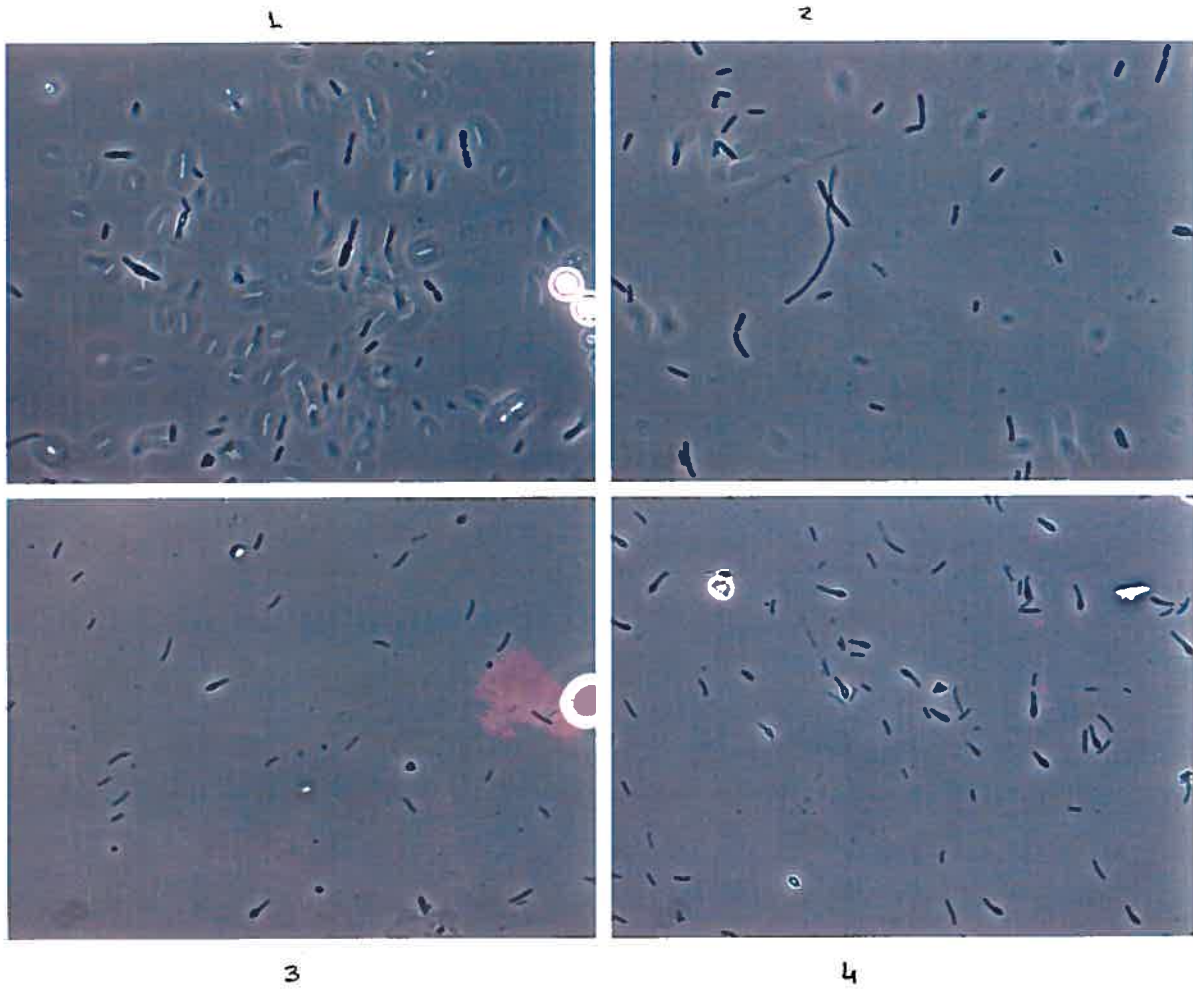


Figure 5

1 and 2 : F₁ strain

3 and 4 : F₅ strain

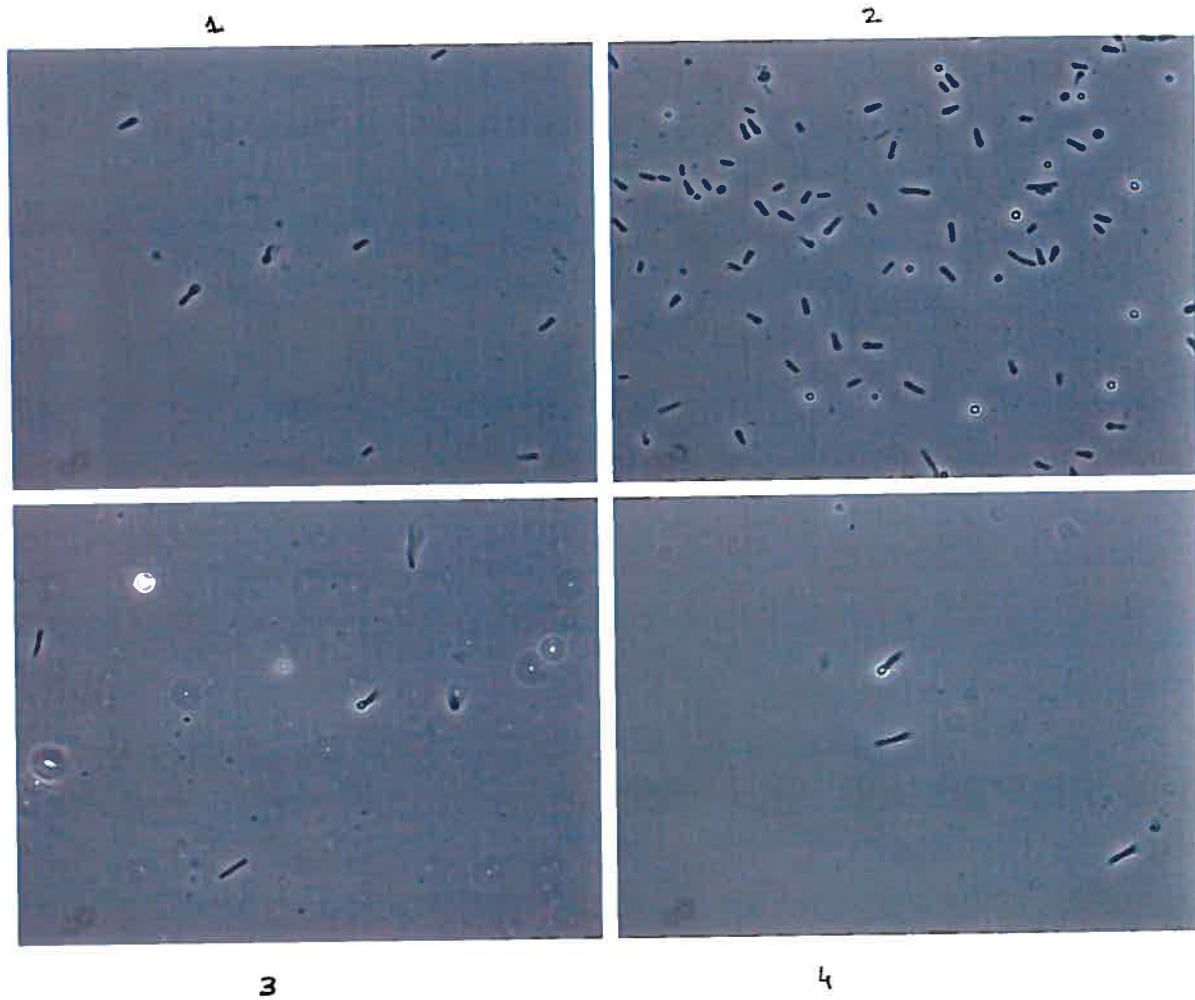
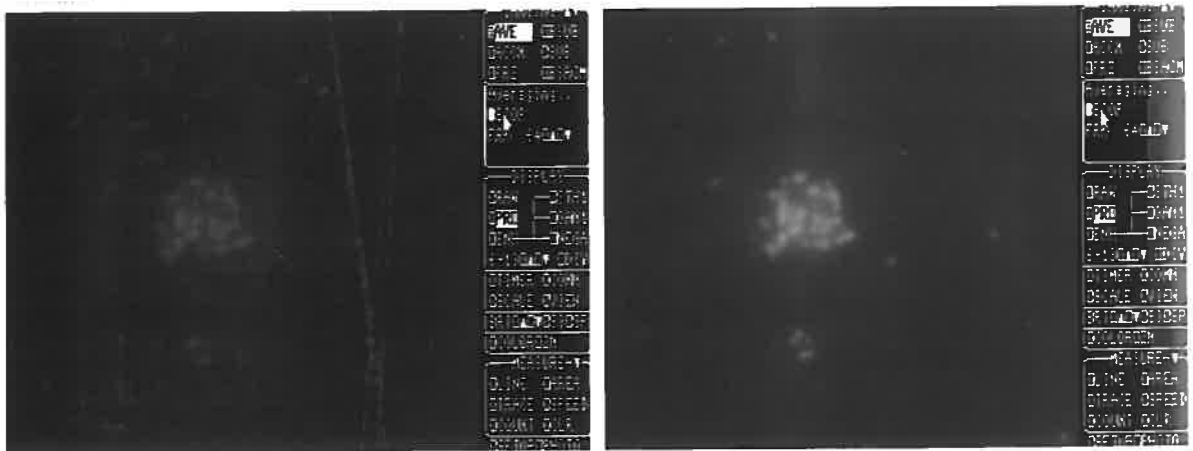


Figure 6

1 and 2: F₂ strain

3 and 4: F₇ strain



C₁ + Enteric Proteobacteria probe

C₁ + Coumarin

Figure 7

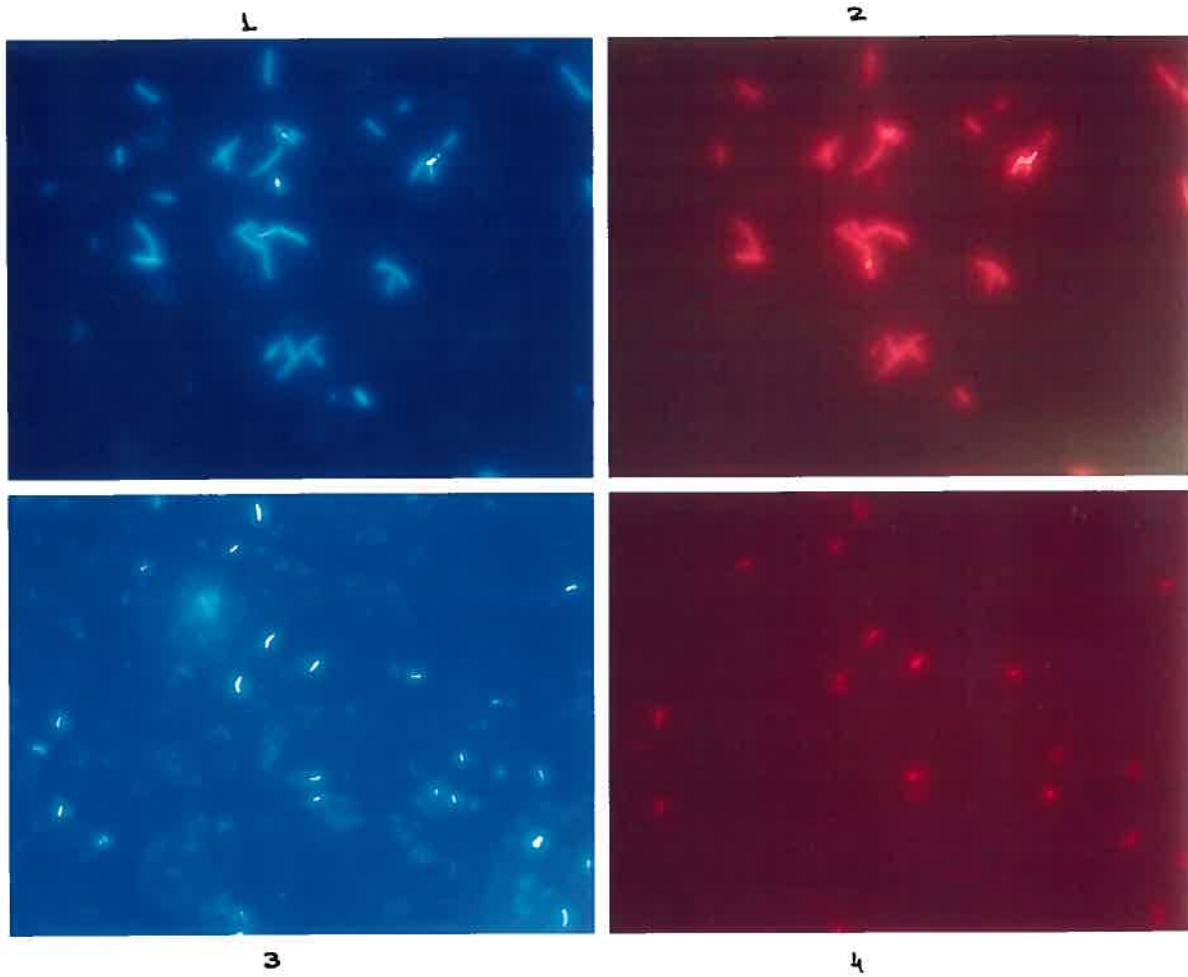
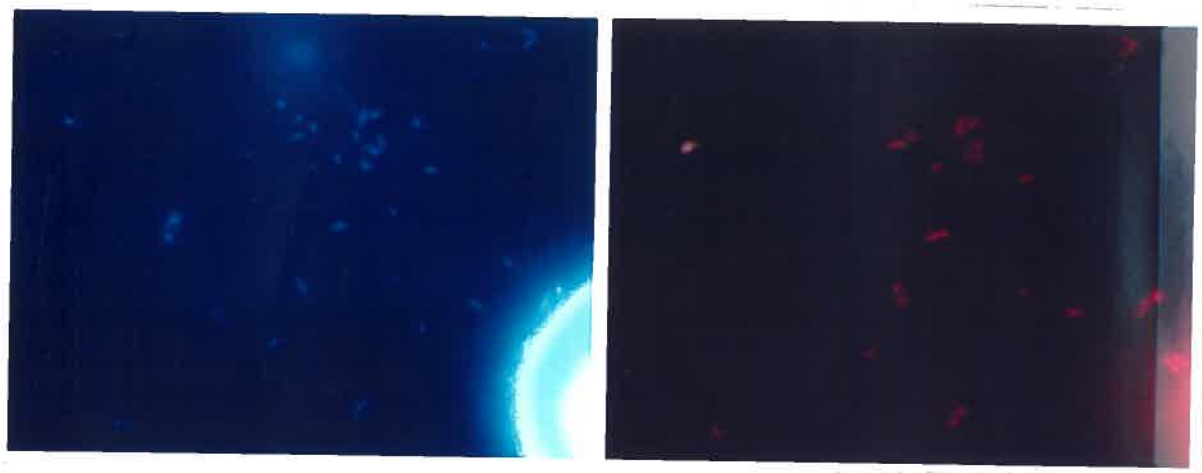


Figure 8

1. C_2 + Coumarin
2. C_2 + low G+C probe
3. C_4 + Coumarin
4. C_4 + low G+C probe



F₅ + Coumarin

F₅ + Low G+C probe

Figure 9

FERMENTATION PRODUCTS FROM XYLAN BY C3 ISOLATE

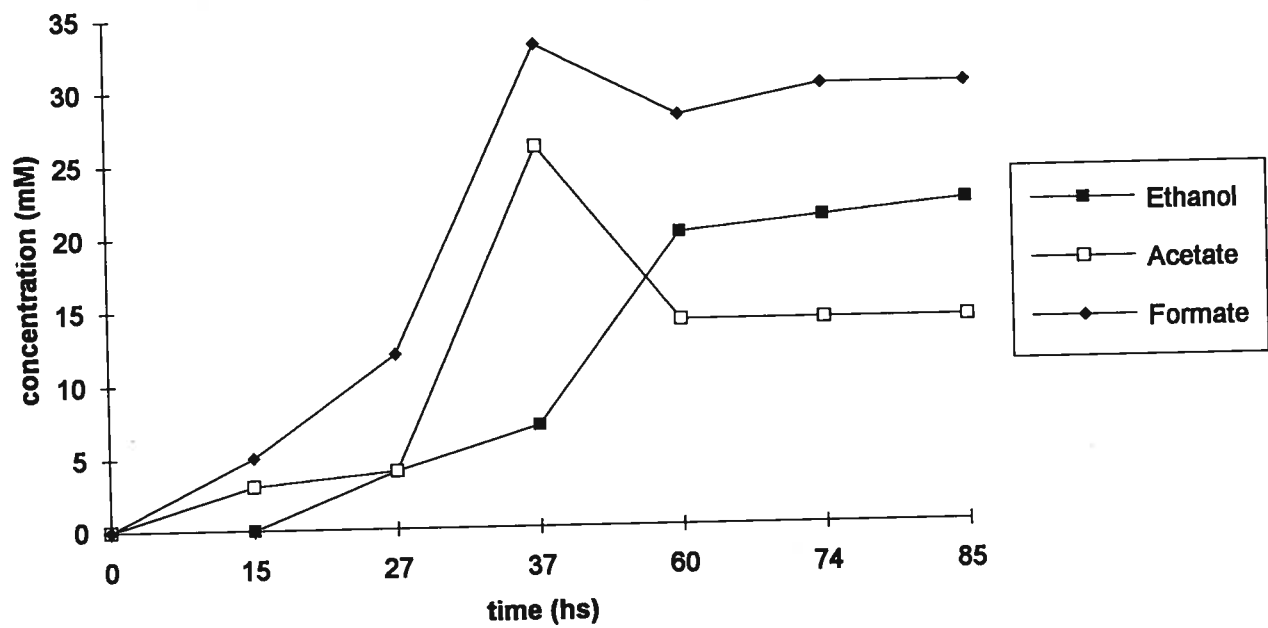


Figure 10

FERMENTATION PRODUCTS FROM XYLAN BY C4 ISOLATE

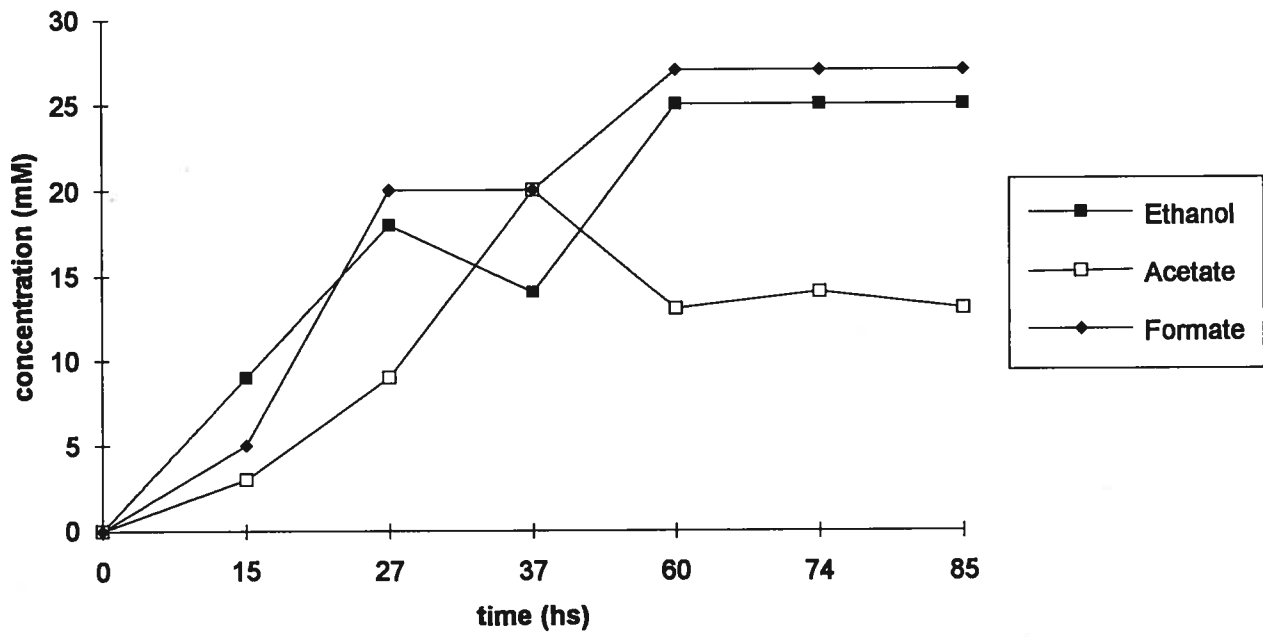


Figure 11

FERMENTATION PRODUCTS FROM XYLAN BY C2 ISOLATE

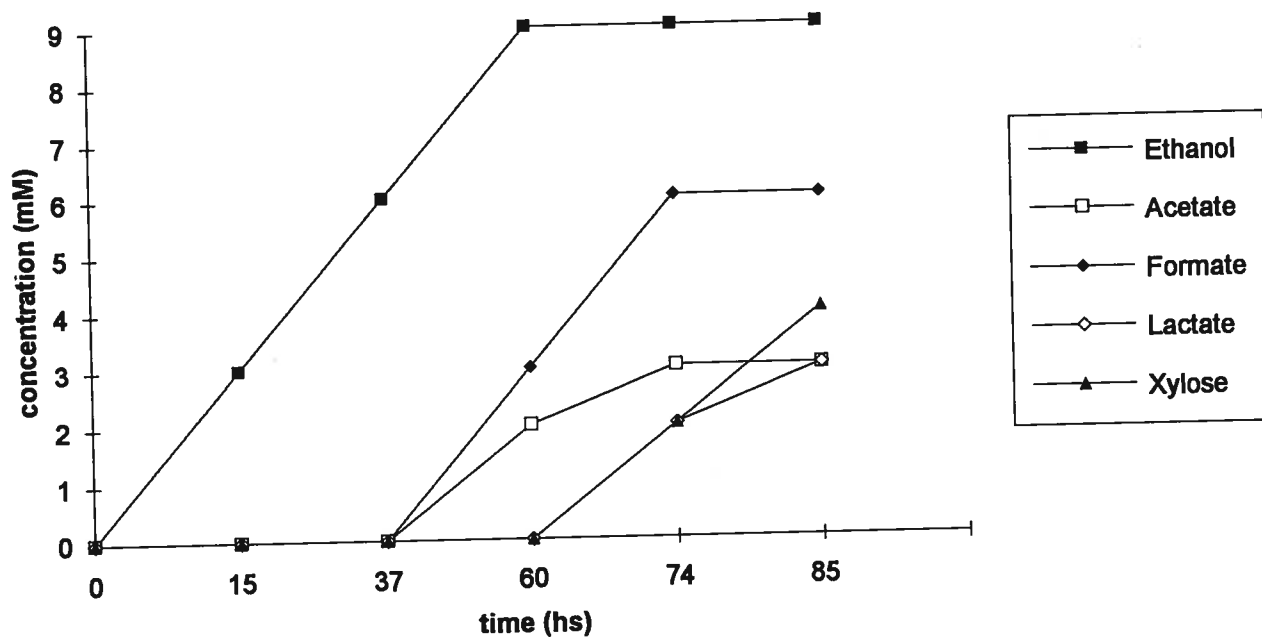
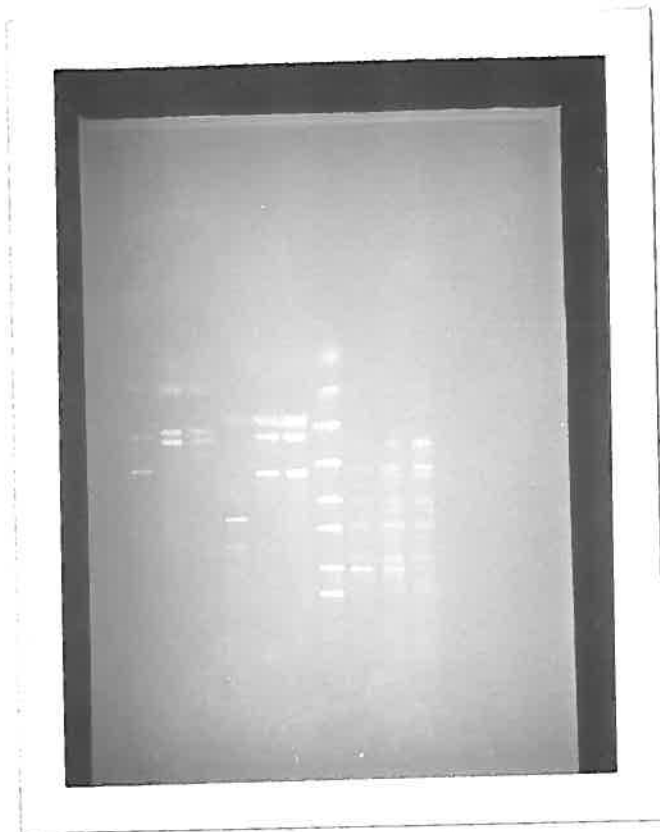


Figure 12



1-2-3-4-5-6-7-8-9-10

Figure 13: rDNA Banding profiles of 16S rRNA.

Line	Strain	Restriction enzyme
1	C2	Msp I
2	C3	Msp I
3	C4	Msp I
4	C2	Hae III
5	C3	Hae III
6	C4	Hae III
8	C2	Bst UI
9	C3	Bst UI
10	C4	Bst UI

Line 7: Molecular Weight Marker: 50, 150, 300, 500, 750, 1000, 1500 and 2000pb.

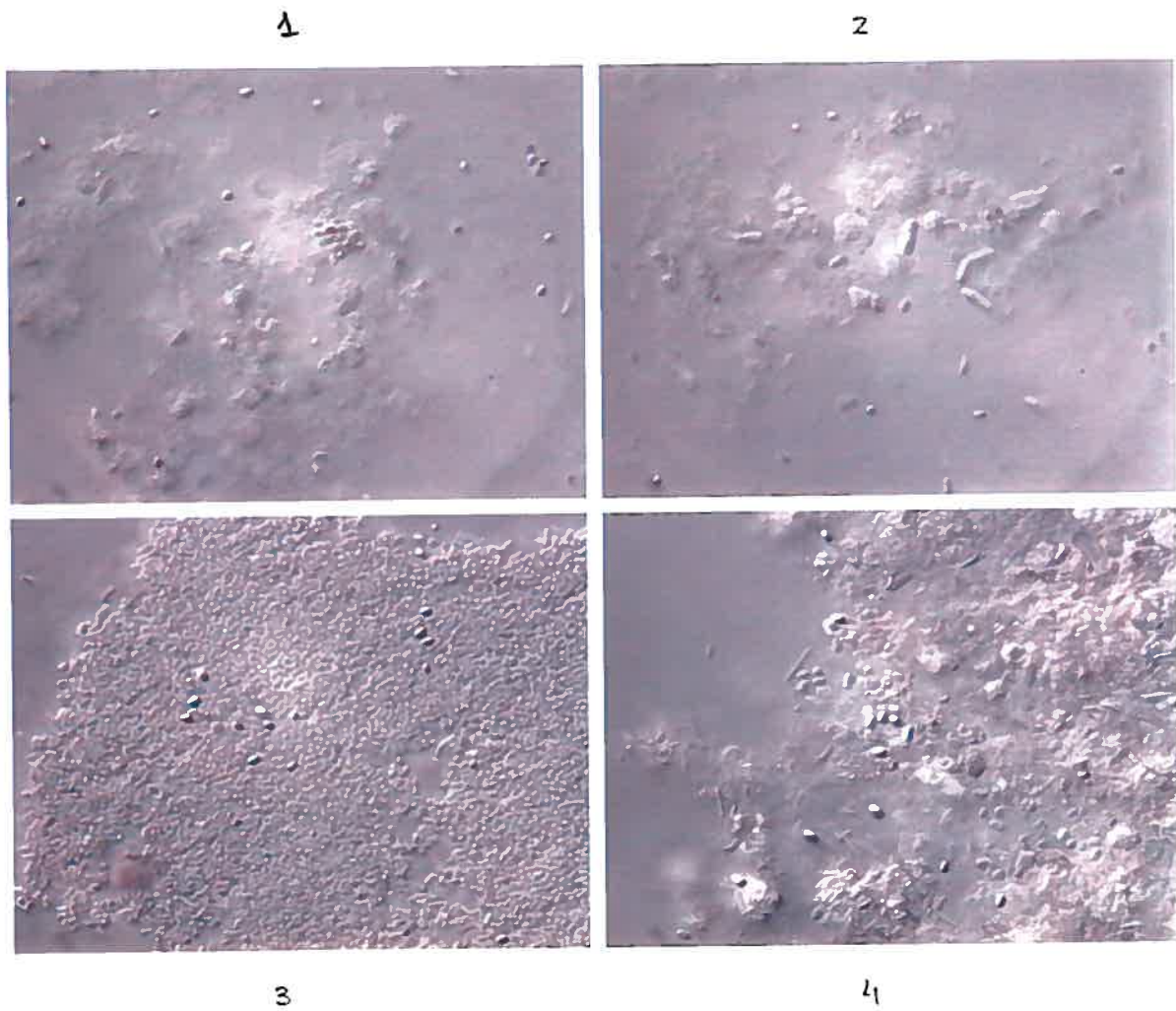


Figure 2

1 and 2: Enrichment from colon, aerobic, 37°C

3 and 4: Enrichment from colon in sea water medium,
aerobic, 37°C.

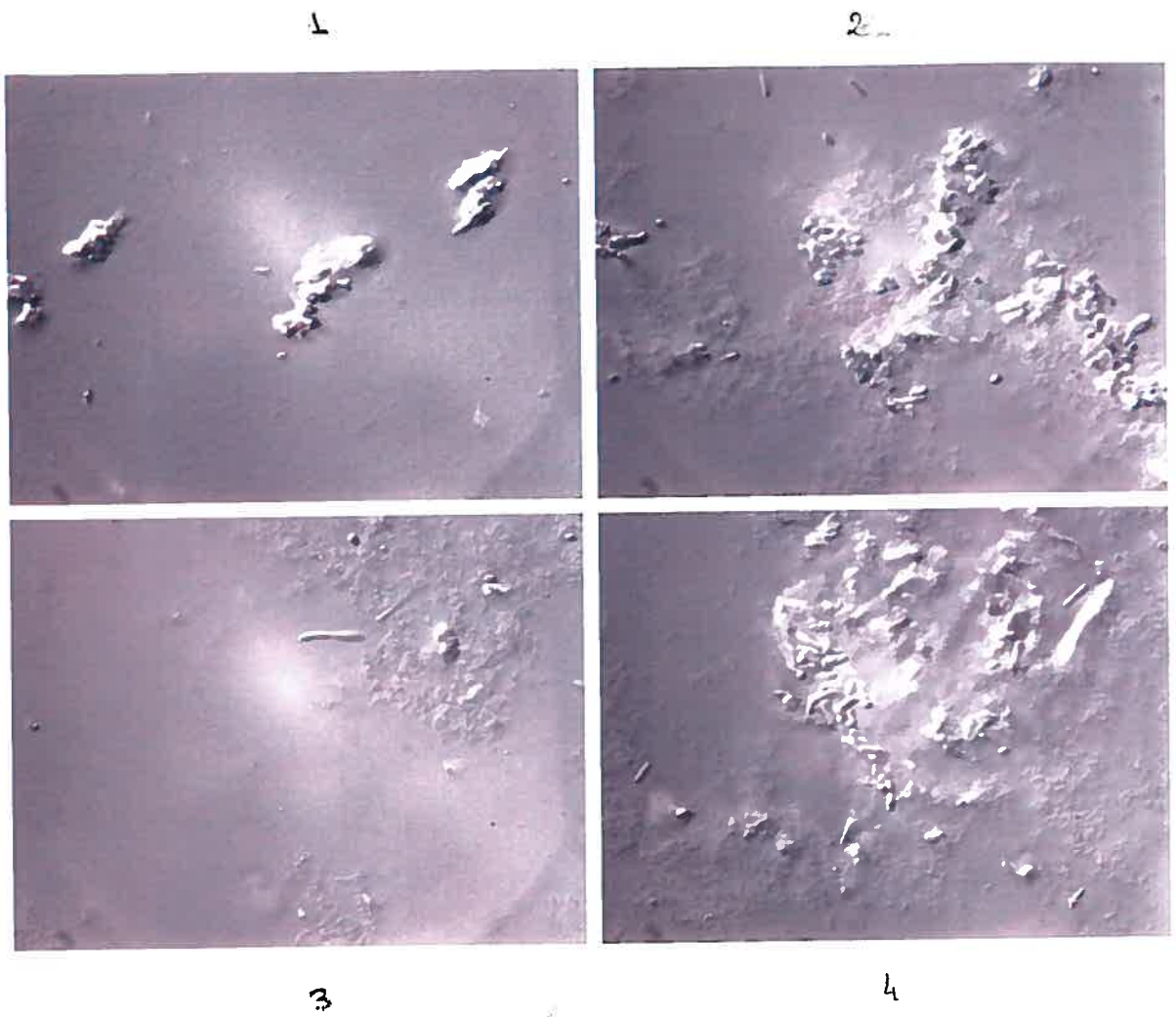


Figure 1

1: Basal medium without inoculum.

2, 3 and 4: Enrichment from colon, anaerobic, 37°C