" EFFECTS OF ELEMENTAL SULPHUR IN CULTURES OF

Methanosarcina acetivorans "

and

" ISOLATION OF METHANOGENIC BACTERIA ABLE TO GROW

IN HIGH SALT CONCENTRATION "

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From the begining , investigators have considered biological life as a dicotomy. For this reason organisms were divided into animals and plants. Nowdays we know that there are structures cells which are impossible to include in one of the groups cited. Something similar has occured with cell type since some time ago, it was thought that there were only two cell types:.Eukariotics and Prokariotics. Actually, it is well known that there are 3 cellular types: Archaebacteria, Eubacteria and Eukariotics.

Comparatives studies of 16S and 18S rRNAs have confirmed the existence of a large differences between archaebacteria and all the other, which now are called eubacteria.[1,2] The following table shows some of the differences among the 3 cellular types.

	EUBACTERIA	ARCHAEBACTERIA	EUKARIOTICS
Celular structure	Single	Single	Complex
Cell wall	All with muramic acid	Different kinds, none have muramic acid	None in animals cells. Differents kinds in other phyla.
Membrane lipids	Ester bind	Ether bind	Ester bind
tRNA			
timin in common branch	Presence in most species	Absence	Presence in all the species
Promoter aminoacid	N-formil metionin	Metionin	Metionin
Ribosoms			
Cloramfenicol sensitivity	Sensitive	Not sensitive	Not sensitive
Kanamicin	"	"	"
Genetic organitation			

Archaebacteria is composed basically of three groups: methane producing bacteria, termophilic bacteria and halobacteria. These microorganisms have been able to live to date due to the fact that their habitats are very extreme, so competition with other bacteria is very improbable. The first group is able to grow in a reduced atmosphere using very reduced substrates to make CH_4 under strictly anaerobic conditions [3]. Most of the members of the second group have been isolated from continental volcanic areas and grow optimally between 70 and 85°C [4]. During growth, H_2S is formed by sulphur reduction. The last group grows optimally between 25% and 30% salt concentration at 40°C [5].

Phylogenetically speaking, there are more similarities between methanogens and halobacteria than either of these two groups have with termophilics. However, it seems that some methanogens are able to grow in presence of S° forming H_2S which is speculated to be useful in the creation of their own anaerobic environment [6]. Through their ability to reduce sulphur methanogens are related to the aerobic sulphur-reducing bacteria, the metabolism of which is thought to be rather primitive. It remains unclear, however, whether H_2S formation or methanogenesis was the primeval means of energy conservation during evolution of the archaebacteria. Possibly both metabolic systems are related to each other and methanogenesis could have developed from the energetically less efficient sulphide-forming metabolism. Comparasion of both metabolic types at the molecular level may shed further light on the evolution of these two groups of organisms.

The other group will talk is about halobacteria, which are closely related at level of proteins, lipids and cell wall to the methanogens [7]. These two groups seem to have the same ancestor, which should be a halophilic methanogenis bacteria or some organism very closely related with this. I have done

1) EFFECTS OF S° IN CULTURES OF Methanosarcina acetivorans

The first project was to elucidate if M. acetivorans (isolated from marine sediments [8]) was able to grow in the presence of S°. The production of CH_4 and H_2S is competitive. In presence of S° no methanogenesis occurs. The metabolics implications would be as follows:

- No S°

$CO_2 + (CH_3)_2 NH^+$ ↕ $6e^-$ $(CH_3)_3 NH^+$

$3\,(CH_3)_3 NH^+ \xrightarrow{6e^-} 3CH_4 + 3(CH_3)_2N$

SUM: $4\,(CH_3)_3 NH^+ \longrightarrow 3\,CH_4 + CO_2 + 4\,(CH_3)_2 NH_2$

- With S°

$CO_2 + (CH_2)_2 NH_2^+$ ↕ $6e^-$ $(CH_3)_3 NH^+$

$35° \xrightarrow{6e^-} S^=$

SUM: $35° + (CH_3)_3 NH^+ \longrightarrow (CH_2)_2 NH_2^+ + 3S^= + CO_2$

The first question to answer was; could methanogens grown despite of the fact that methanogenesis does not occur?

The second question would be; does this organisms have different from other methanogens cytochromes which use electrons from either S° or $(CH_3)_3$ NH ?---

The medium that I used was marine media [8]:

- Na Cl 2.3%
- KCl 0.08%
- NH_4 Cl 0.05%
- $MgCl_2$ 1%
- $CaCl_2$ 0.014 %
- Na_2HPO_4 0.06%
- Cystein 0.025%
- Na_2CO_3 0.2%
- Na_2S 0.025%
- Mineral Mix [9] 1%
- Resarzurin 2% (from stock solution 0.01%)

and trimethylamine (50 mM) as substrate. All compounds were dissolved in distilled water, minus Na_2S. The pH was adjusted to 6.5 and then the medium was boiled to remove the oxygen and the resarzurin turned pink. Once the medium was cold Na_2S was added to reduce it. The medium was then autoclaved at 121°C for 20 minutes. S° was added in one of the medium (pea size) [10].

The growth was followed by measuring the amount of protein, using the following formula [11]:

$$[\text{Protein}] = 183 \times A_{230} - 75.8 \times A_{260} = [\quad] \mu gr/ml$$

A_{230}= Absorbance at 230 nm

A_{260}= " " 260 nm

To calculate the absorbance a Beckman DU-7 spectophotometer was used.

A basic gas-cromatograph (Carle 9500) was used to measure the methane production.

Crude cells extracts fractions were obtained (after lyses) by runing the sample through cellulose diethylaminoethyl ether column. The first fraction was used to calculate the spectra of the cytochromes and the second one was used for F_{420} characterization. Both fractions were suspended in HEPES buffer (pH 7) with 3% NaCl and 50 mM $MgSO_4$ for the assay.

man DU-7 spectophotometer. For recording reduced minus oxidized difference spectra. Samples were reduced with a few grains of $Na_2S_2O_4$ and measured against an air-oxidized reference [12].

Results and discussion

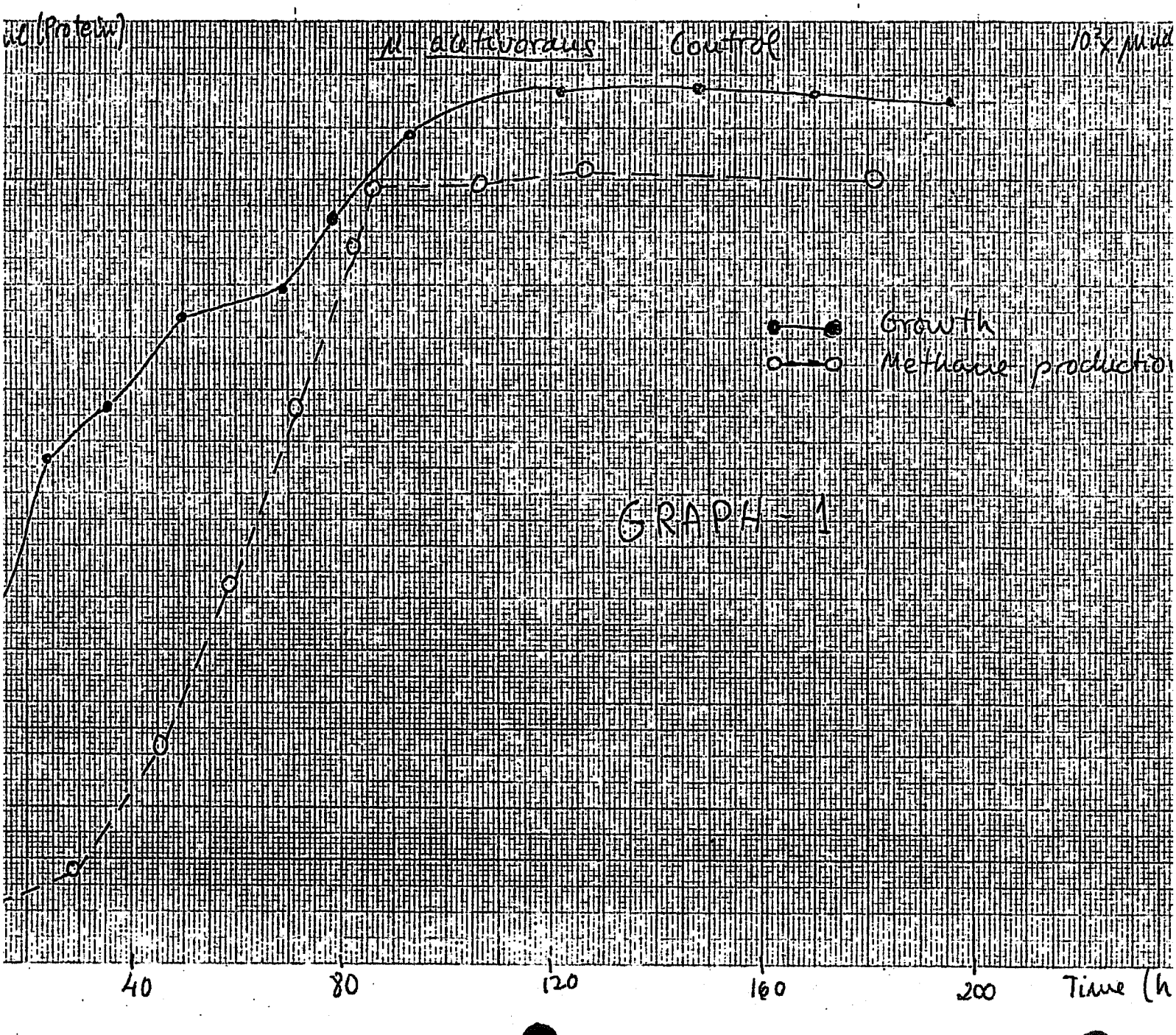
Two serum bottles (120 ml) were filled with 60 ml of marine medium. After inoculation, samples of methane and medium were taken periodically. One of the cultures had S° and the other one was used as control without S°.

As the graph-1 shows, methanogenesis and growth occur normally in M. acetivorans. However, as shown in graph-2 methanogenesis decreases rapidly in a few hours after inoculation due to the presence of S°, according to previous papers and with the equations proposed above [6]. On the other hand, growth it seems to be unaffected by the presence of S° and this is an unexpected results, since the previous reports say that both methanogenesis and growth stops when S° is added in the medium.

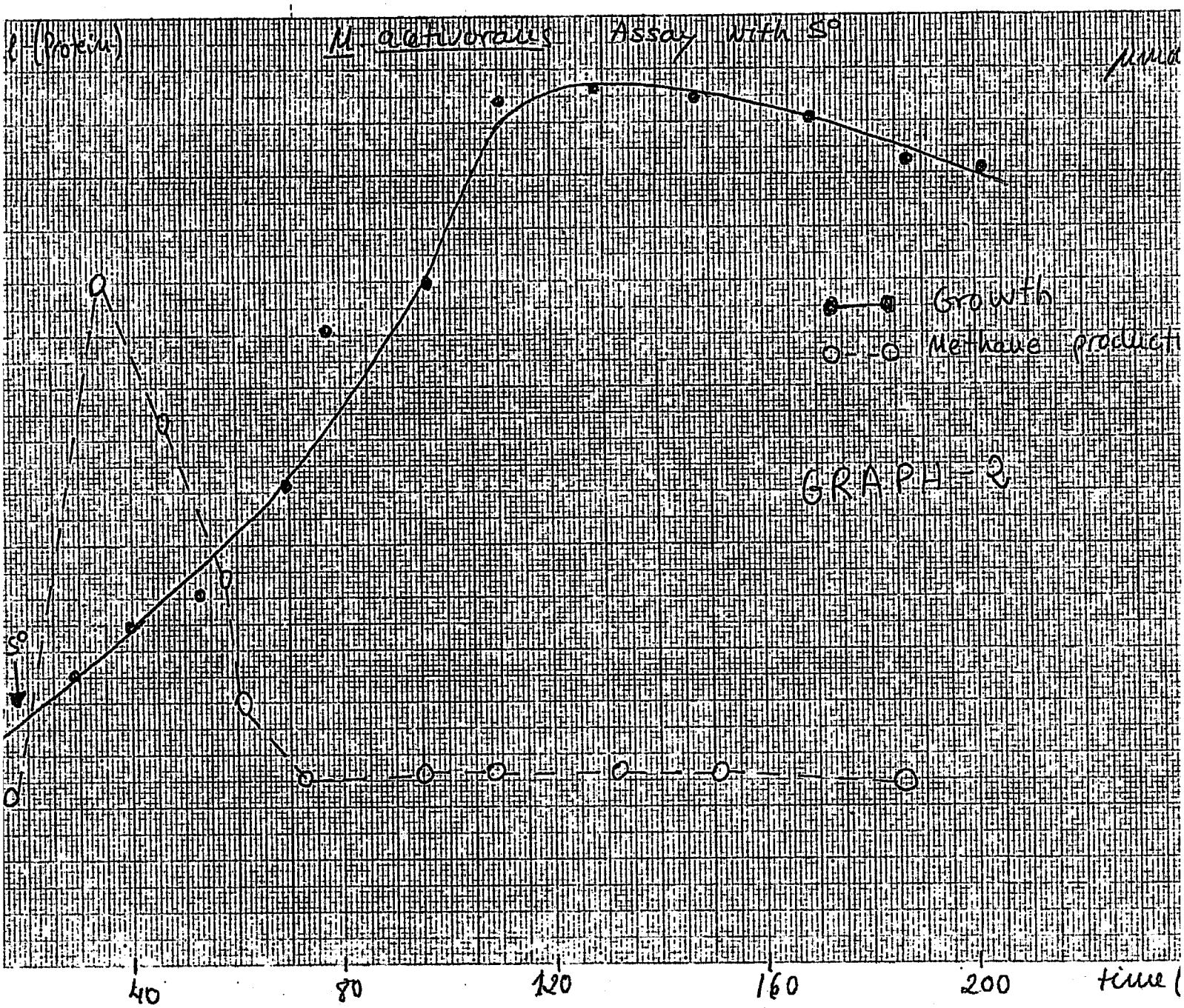
It is relevant to say that a higher gas pressure was detected in the control bottle than in the assay bottle, according with the equations cited before. The presence of H_2S was also detected and it seems to be that, in spite of the fact that large amounts of H_2S kill the cells, this species can tolerate the presence of H_2S.

I also scaned the crude cell extract fractions and looked for the maximun peaks of absorbance, due to cytochromes.

As shown in graph-3, the maximun peaks were found at 555 nm (α), 523 nm (β) and 427 nm (γ). All the scaning were done at room temperature. All the papers reviewed have shown differents peaks of absorbance comparing with these peaks. May be this organism has different cytochromes as electron carriers which permits take the electrons from S°. This could be an explanation for use


(Protein)
M. acetivorans
Control
10% mud
Growth
Methane production
40
80
120
160
200
Time (h

GRAPH - 1


M. methanica Assay with So
Growth
Methane production
GRAPH-2
So
40
80
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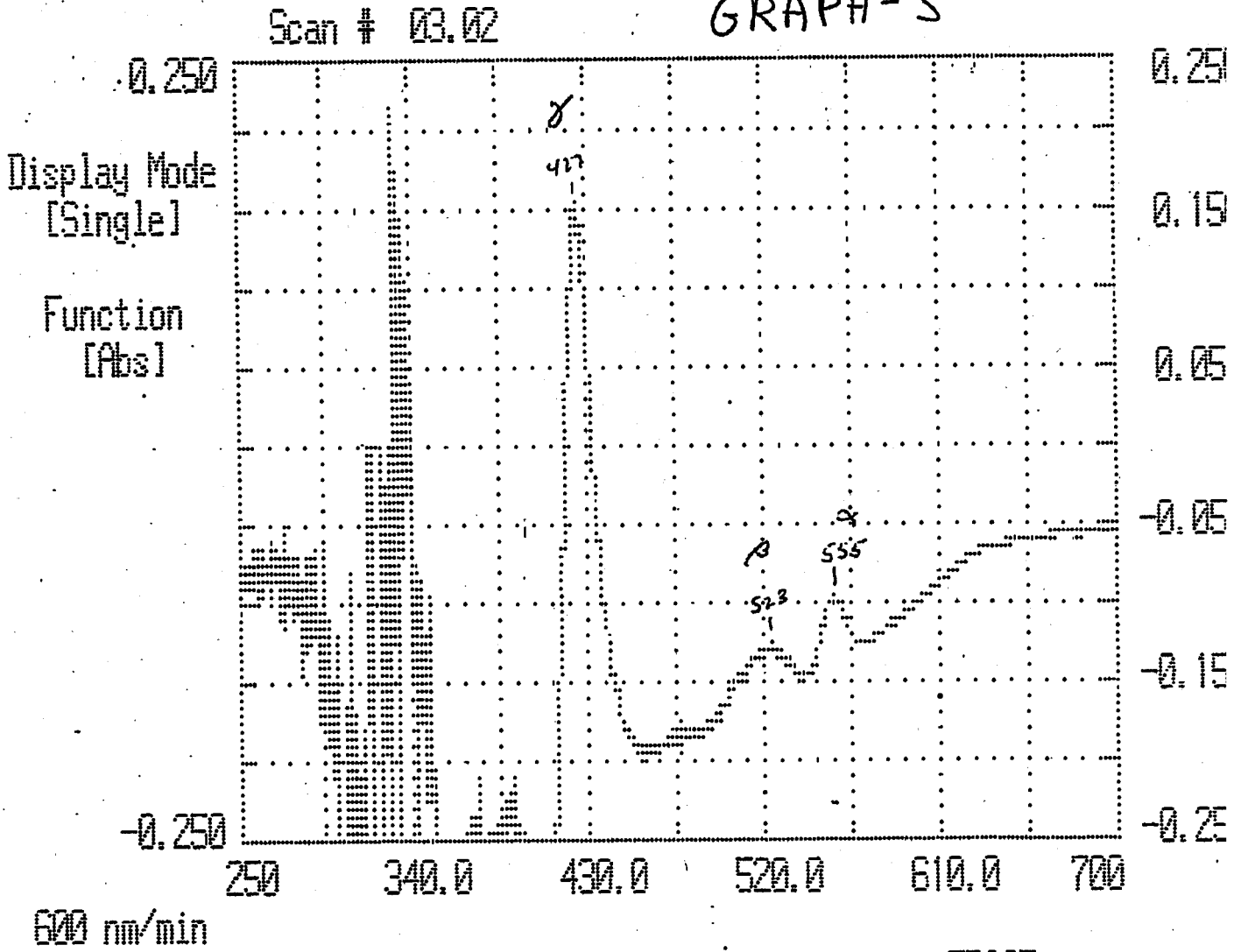
160
200
time (

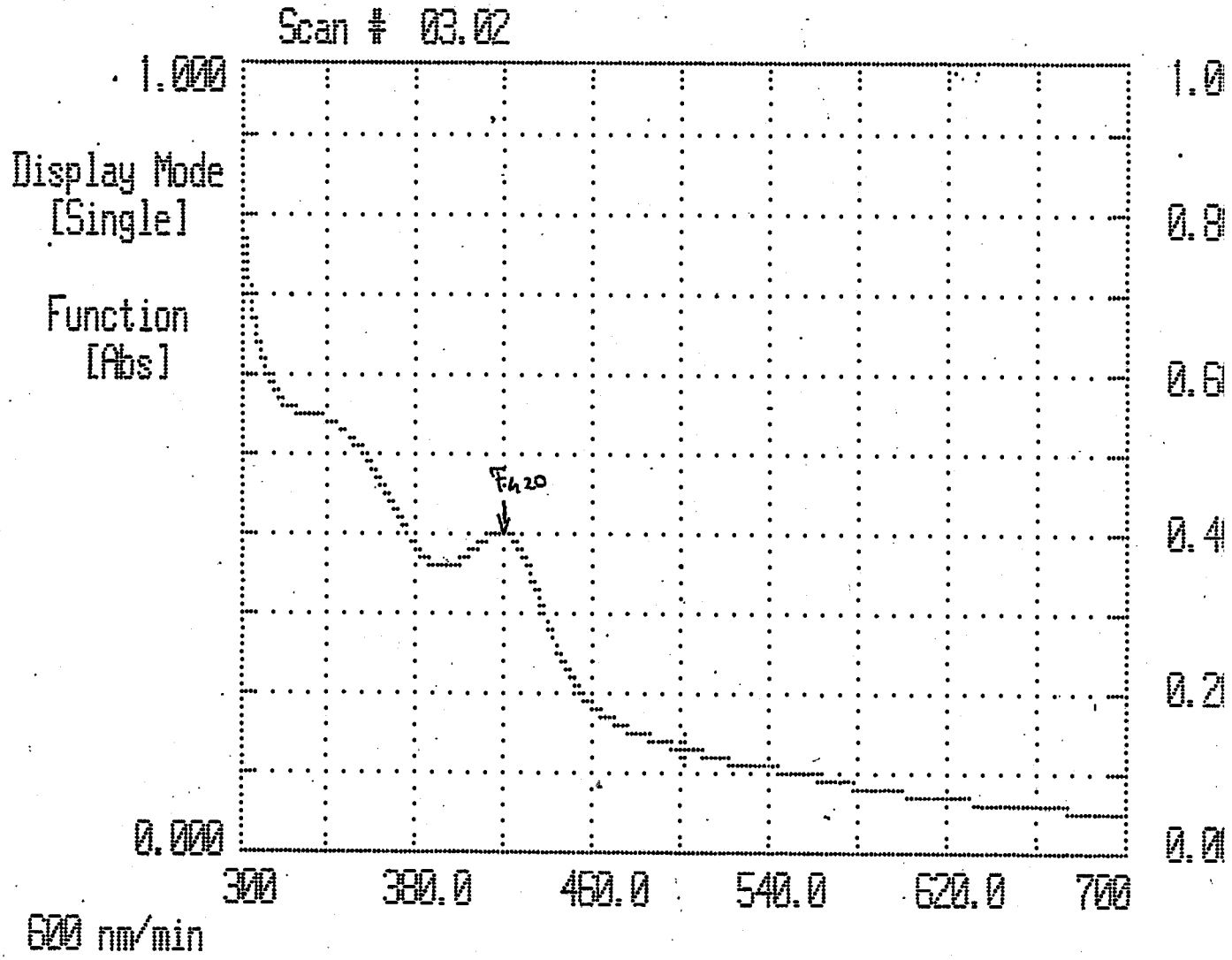
scanning of a second fraction have shown the presence of F_{420} activity (graph-4).

GRAPH-3



TRACE

λ	ABS	SOURCE	MODE	CELL	λ	ABS
700.0	-0.0598	Vis/UV	Scan	1	475.0	-0.1822



TRACE

λ	ABS	SOURCE	MODE	CELL	λ	ABS
700.0	0.0521	Vis/UV	Scan	1	500.0	0.133

The presence of gaseous bubbles on the surface of solar salt ponds sediments induced me to look for halophilic methanogenic bacteria.

I brought the samples from Alicante (Spain) and they were taken from a depth of 20 cm under the sediment surface. After this, they were spurged with N_2 to keep anaerobic conditions until assays procedures.

Materials and methods

The total salt concentration of the water from the samples was calculated by drying the water and by weighting the remaining salt.

The salt water used had the following composition [15]:

- NaCl	23.4%	- $MgCl_2$	1.95%	- $MgSO_4$	2.9%
- $CaCl_2$	0.11%	- KCl	0.06%	- $NaHCO_3$	0.02%
- NaBr	0.08%				

This solution had a total salt concentration of 30% (the same as the sample tested). The basic medium had the salt water plus 0.02% yeast extract as aminoacids and oligoelements requirements, 1% mineral mix, and 2% resarzurin (from 0.01% stock solution). Different substrates were added in each experiment.

After several days of growth, serial dilutions were done in roll tubes which also had 2% agar and vancomycin (100 mg/L) to avoid the presence of another microorganisms [8]. The serial dilutions were done from 10^{-1} to 10^{-5} with H_2/CO_2 (80:20) as energy and carbon source.

Some slides were done from differents media to test the presence of methanogenic bacteria since one of the properties of these microorganisms is that fluoresces under 420 nm (UV light) [16] due to the presence of the F_{420} coenzyme. Some of the colonies were tested under scaner electron microscope and photographs were taken.

	Growth	Methanogenesis
H_2/CO_2	+	+
Methylamines	+	±
Na Formate	±	-
Na Acetate	-	-

+ = Significant ; ± = Very low ; - = No detected

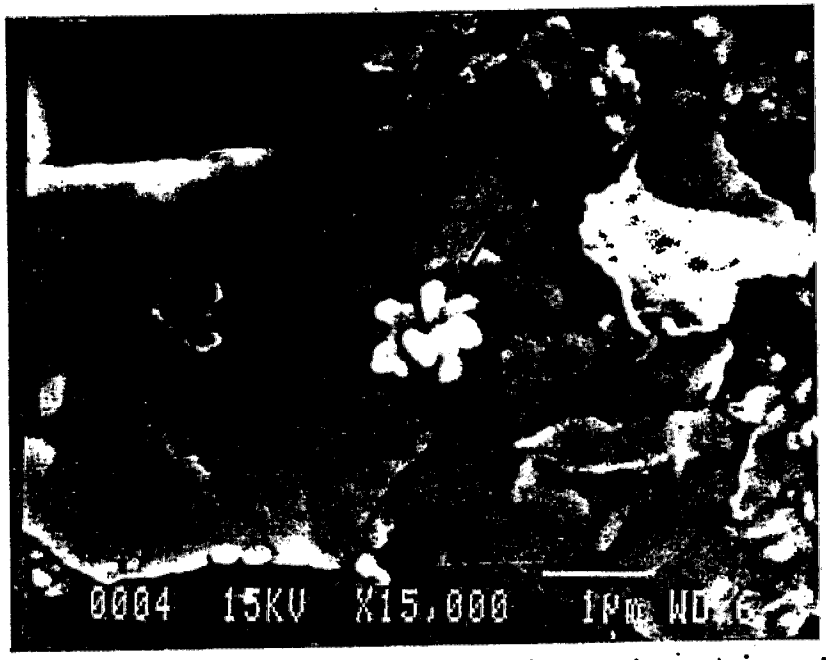
As shown in this table, growth and methanogenesis were obtained with H_2/CO_2 much better than with the other substrates.

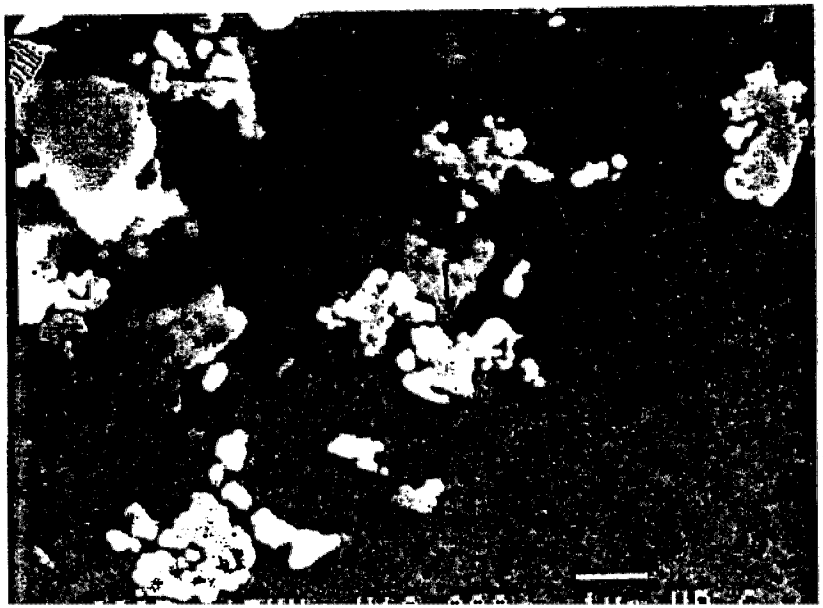
It is important to say that in all the media the growth was very slow due to the low water activity which is very low in saline environments [17,18,19].

Colonies have been got (after 30 days) in the roll tubes dilutions and some of these were observed under scaner electron microscope (next page). These organisms have a regular size (0.2 µm) but irregular shape. Also, these bacteria form clumps and therefore it is difficult to follow the growth with O.D.

To summarize, new obligate halophilic methanogenic bacteria have been isolated from solar salt pond sediments, able to grow at 30% salt concentration (no growth was detected at 12%), and with an optimal growth at 40°C. The growth was really slow with an approximate generation time of 20 days. The cells are very small with a regular size of 0.2 µm, which are able to form clumps.

Halophilic Methanogenic bacteria (...





These two projects have risen differents questions such as:

1) Can another species of methanogenic bacteria grow in presence of S°?

2) Is it possible to find another kind of cytochromes?

3) Is there a strong competition between methanogens and S° reducing bacteria?

4) How many strains of halophilic methanogenic bacteria are involved into the salt sediments?

5) What is the optimal pH, salinity, temperature,etc?

6) What kind of lipids, proteins have these bacteria compared to the lipids, proteins founded in methanogens and halobacteria?

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