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Growth of Methanosarcina barkeri in Continuous Culture Systems

FINAL REPORT

Project Director

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* *This report includes a patent proposal and mostly unpublished research work.*

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INTRODUCTION

Anaerobic fermentations gained renewed interest as a technology for substitution of fossil fuels and source of raw materials. Improvement of reactors design, screening of new microbial activities, searching of new substrates are envisaged. As a result the anaerobic conversion of organic industrial and agricultural wastes to industrial chemical, biochemicals and fuels is today considered an useful alternative.

Anaerobic microorganisms were already utilized by man more than 6.000 years ago, to preserve foods through ethanol, lactic and other fatty acids formation by fermentation. It was only one century ago that Pasteur discovered and described "la vie sans air", a concept not well accepted by his colleagues. More or less by that time the first anaerobic digester was built in 1895 in Exeter, U.K., producing methane for lighting the vicinity of the plant. However, almost a century later understanding of microbial populations in anaerobic digesters is rather limited. Particularly, carbon and energy metabolism of methanogens, the "key" organisms in the prodution of methane, are not yet completely understood, remaining largely speculative.

Among anaerobic organisms, unicarbonotrophic bacteria are able to grow on one carbon compounds as the sole carbon and energy source. They are among the most metabolicaly efficient existing life forms. They perform certain chemical conversions with nearly maximal theorical yield of product and minimal energy loss.
Particularly, anaerobic fermentation systems using methanogenic bacteria result attractive if one considers that they show:

- high yields (mol. product/mol. substrate)
- low energy needs for growing
- high energy conservation in the transformation process
- simple nutritional requirements
- tolerance of high levels of sulphide
- no toxic compounds produced
- low cost of the potential substrates
- ability to fix molecular nitrogen

Biochemistry of methanogenesis involves unusual enzymatic reactions using some peculiar coenzymes. The group is unified by the common feature of forming methane to obtain ATP.

Most of the methanogenic bacteria can use H2/CO2 mixtures as carbon and energy source. Only four isolates are not able to grow on H2/CO2:Methanosarcin strain TM1, Methanotrix soehngenii, Methanolobus tindarius and a coccoid isolated by Miller.

CO, formate, methanol, acetate, methylamines are the other methanogenic substrates.

Methanosarcina barkeri is the most versatile methanogen since it is able to use most of methanogenic substrates with exception of formate.

The biochemistry of methanogenesis from H2/CO2 is better known that from other substrates. ATP is obtained by electron transport phosforilation coupled to the oxidation of H2, being CO2 the terminal electron acceptor.

As Barker proposed early in 1956 , C1 units at intermediate states of oxidation are carried as derivatives of enzymic cofactors. A set of unique coenzymes were found in methanogens, some being recognized as C1 carriers.

Recently Jones et al. (1985) discovered two new cofactors existing in nine methanogenic bacteria and absent in some other representative Archaebacteria and Eubacteria. Based on the fact that enzymes from all nine methanogens tested were able to convert derivatives of these isolated cofactors from Methanobacterium thermoautotrophicum, Jones et al. proposed a new cicling pathway to the reduction of CO_2 to CH_4.

Existing evidence corroborated by the finding that hidrogen atoms in methane come from the proton of water, point out that energy for ATP synthesis comes from a proton gradient motive force coupled to electron transport from H2 to CO2 through a closed membrane system using a chemiosmotic mechanism.

Biosynthesis was studied in M. barkeri and M. termoautotrophicum. The first step of carbon and acetate assimilation is the conversion to acetyl-CoA by an unknown mechanism. Present data sugest that carbon metabolism from all single carbon substrates begins with its conversion to acetyl-CoA. The founding in methanogens of enzymes involved in TCA cycle sugests that it may be also in operation on methanogenic bacteria, at least in part.

Since H_2/CO_2 and other substrates are at the same time the source of carbon and energy, obviously carbon metabolism and energy metabolism share, in some extent, pathways and intermediates: acetate kinase, phosphotransacetylase and COdehydrogenase possibly are enzymes both on energy and carbon metabolism.

However CO_2 is also a product of methanogenic metabolism. This is specially important in digestors where it is the precurssor of about 1/3 of methane, and at the same time it is an obligatory product of the methanogenic acetilclastic reaction which is responsable for the other 2/3. CO_2 may attain 45% of biogas composition. In anaerobic digestion the reduction of CO_2 with H_2 is essential to mantain low levels of H_2 required to assure an healthy global metabolic balance.

Methanogenic bacteria may therefore be considered a potential candidate for using CO_2 as a source of carbon in biothecnology. They will have preferential application in systems where CO2 must be used in anoxyc and highly reducing conditions competing with other autotrophic microorganisms.

In terms of applications methanogenic bacteria are most known by their fundamental role in anaerobic digestion to produce fuel gas and reducing pollution in sewage treatment and organic wastes upgrading. Design engineering of digestors is beeing improved as result of a better understanding of the biochemistryof the process. A higher efficiency is required for econnomical viability of many of biogas plants, particularly for small scale applications. Methanogens could be utilized to increase the energy contents of some gaseous products resulting from pyrolysis of coal and lignocellulosic materials by converting CO_2 and CO to CH_4. Other applications are in treating liquid industrial effluents containing acetate or methanol. This systems has unique advantages for eliminating pollution, and potential prodution of a valuable metabolites.

Corrinoids which are important metabolic constituints of methanogens and are Vitamin B12 like cofactors may account for high percentage of total cell carbon of M. barkeri. This may raise to 1% when cells are grown in methanol so B12 may be valuable sub-product of processes using methanogens.

Limitations to a widespread application of methanogens in biotechnology come from the present lack of understanding about their physiology and biochemistry, their anoxic requirements and low generation times.

We hope that knowledge coming from present experiments, being conducted in our and others laboratories, with emphasis on studies with fed-batch and continuos cultures as well immobilization techniques, will provide a basis for exploitation of methanogenic bacteria potential.

Methanogenic bacteria are living catalysts which are able to utilized CO2 and CO. It must be remembered that they were probably some of the first microorganisms to evolve on the early primitive atmosphere of the earth, where CO2 and CO had most probably an higher content than the present atmosphere composition. Therefore the potential is there and is enormous for producing either energy (CH4) either valuable compounds and at the same time on de-pollution.

On the meantime much R&D investment will be needed in this area. However it must be remembered that methane market accounts for over $12 billion US Dollars, representing the largest single market of a potential biotechnological product (source: Genex Corp. study). This study forecasts that 10 years would be needed to increase the biotechnological prodution of methane by the use of genetically engineered microorganisms.

Much knowledge must be also obtained concerning the physiology of growth of these microorganisms, understanding their growth requirements, substrate utilization and metabolic controls.

Continuous Culture theory it is a valuable technology for conducting these type of studies and they assembling of a continuous system and its application to the growth of M. barkeri was one of the main objectives of this work.

It has revealed itself as a very difficult task but the main steps were successfully achieved in order to pursue in the future some unsolved question raised in this project. Here we present the research work performed and its main results. Some of the work is still going on and the significance of it will be able to be apraised in the near future.

2

Growth of *Methanosarcina barkeri* in Batch and Continuous Culture On Single Substrates

8

1- Introduction

The study of the Methanogenic bacteria in this project aims at developing laboratory and pilot technology for the growth and study of the methanogenic bacteria. It is very important to solve problems associated with growing these bacteria in a reproducible way and in amounts which could be used for biochemical studies as well as for production. However there are technical and physiological barriers associated with the growth of these microorganisms specially with M. barkeri.

An inherent difficulty in growing these microorganisms is to use completely oxygen free medium, as well as to difficulty in measuring the biomass and product gases. A consequence it becomes difficult to accurately describe the growth of these microorganisms which is absolutely essential to study their kinetics and energetics. Studies of this type are scarce in the literature and usually they show an apparent (or real) variability with respect of the growth characteristics of M. barkeri. This could be due to two main intrinsic characteristics:

- The variable physiological answer depending on the main energetic substrate used with inoculum .
- The typical growth of methanosarcina in agglomerates.

These characteristics are responsible, we believe, for some of the variety (and discrepancy) of results found in literature requiring that care be taken when interpreting the data.

Therefore it has been particularly time consuming to obtain accurate measurements of the concentration and total amount of gas produced as well as of substrates and cell biomass.

Conditions for accurate gas and substrate measurements were improved by the acquisition of two GLC systems for our laboratory. However these apparatus only arrived on our laboratory by October 1987.

Their performance has been being evaluated but their acquisition revealed itself indispensable for speeding up of the work.

These problems have been time consuming and caused delays on some of the research lines of our project mainly for the kinetics and energetic studies.

The main purpose was no doubt the continuous fermentation of a methanogenic strain - Methanosarcina barkeri. Subsequently, it became of interest to treat the biomass in order to find some biochemical markers that could be studied by a continuous culture methodology.

Thus was started the extraction and quantification of corrinoids aiming the overproduction of vitamin B_{12} (see chapter 5). Acetokinase was the enzyme selected to start the enzymatic activity determinations in relation with the reaction conditions and with the substrates used for cell growth (see chapter 6). Lipidology was also initiated with the extraction of total lipids and its separation by thin layer and column chromatography (see chapter 7).

The work with thermophiles was conducted towards the isolation and characterization of newly isolated species. For that was done a field work collecting samples in vulcanic habitats on Açores islands (see chapter 8).

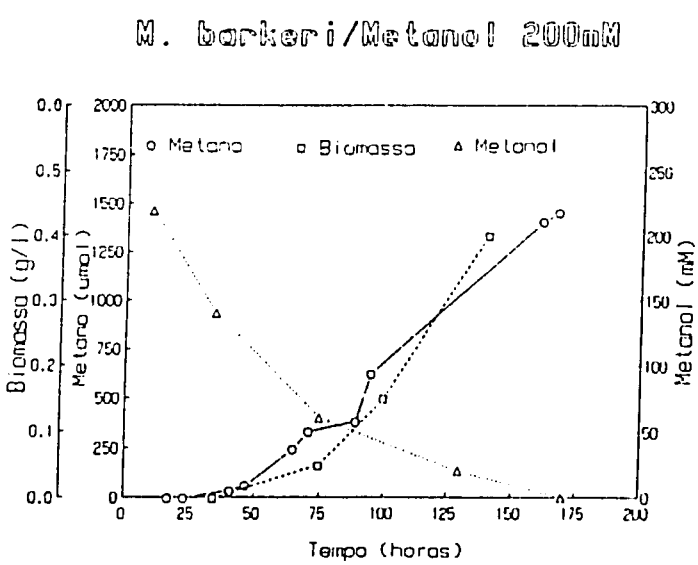
2- Experimental Work and Results

2.1- Batch studies

The analytical methodology used for these studies concerned the utilization of gas chromatography as for gases quantification, like methane and carbon dioxide, using a thermal conductivity detector, as for substrates determinations, like methanol and acetic acid, using a flame ionization detector.

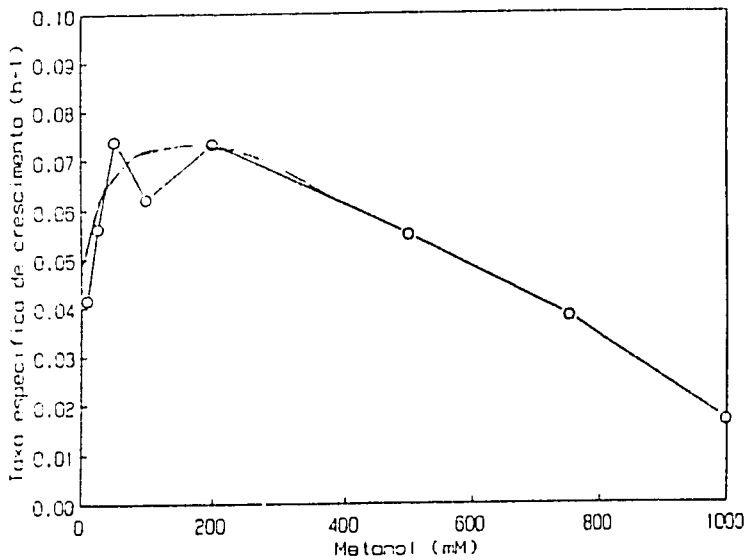
The anaerobic conditions required for manipulations and cell growth were obtained using inert gases like nitrogen, hydrogen and carbon dioxide, which were purified by passing through copper filled glass columns at 350°C. The rubber material as to be butylic or neoprene to avoid oxygen difusion.

Kinetic data were treated graphically and by linear regression (linear least squares method) what gave us kinetic parameters in accordance with those discribed in literature (Fig 1,2,3).

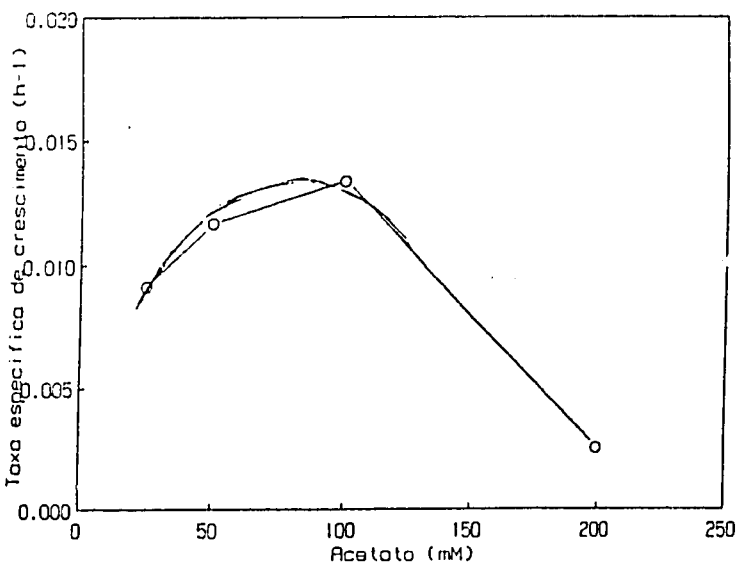


	$\mu_{máx}(h^{-1})$	K_s(mM)	K_i(mM)	S_{opt}(mM)	$Y_{x/s}$(g/mol)
Metanol	0.075	30	2000	200	2.1
Acetato	0.015	15	1000	100	-

M. barkeri/Metanol



M. barkeri/Acetato



2.2- Continuous culture

The apparatus developed for continuous culture was based in a 20 liter fermenter (Setric Genie - SGI). In the laboratory were made, developed or acquired several other equipments to implement a continuous system.

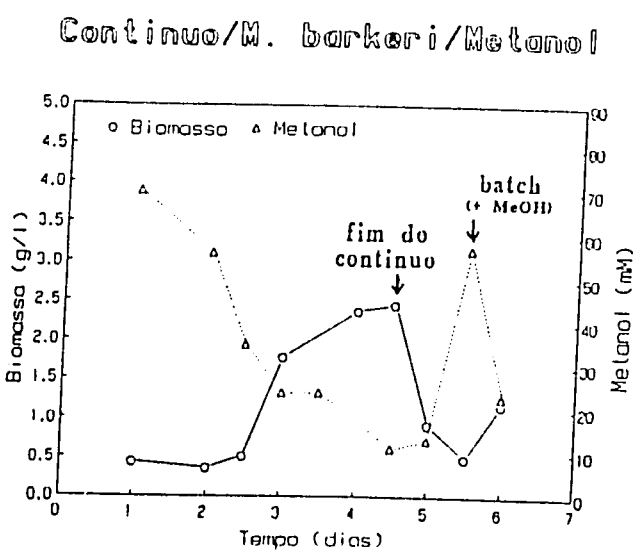
A gas flow meter device, working by liquid desplacement and connected to a microprocessor Z80 was projected and built. Since the fermenting gas output is corrosive, mainly by his high hydrogen sulfide content, as gas washing device was also made using a washing copper sulfate solution and a dehumifying silica-gel column.

To maintain anaerobiosis and a predefined up stream overpressure a combination of solenoide valves and pression sensors were acquired.

On the other hand pumping anaerobic media peristaltically envolves some problems specially at small flow rates. Thus butylic rubber tubes were used and a constantly maintained overpressure in the media reservoirs was necessary (see diagram).

As we expected specific problems arose that were meanless in the batch studies. For instance the pH drifting and the foam formation were not detected in the batch cultures.

Nevertheless, the first results (fig. 4) came out and revealed some trends for future work.



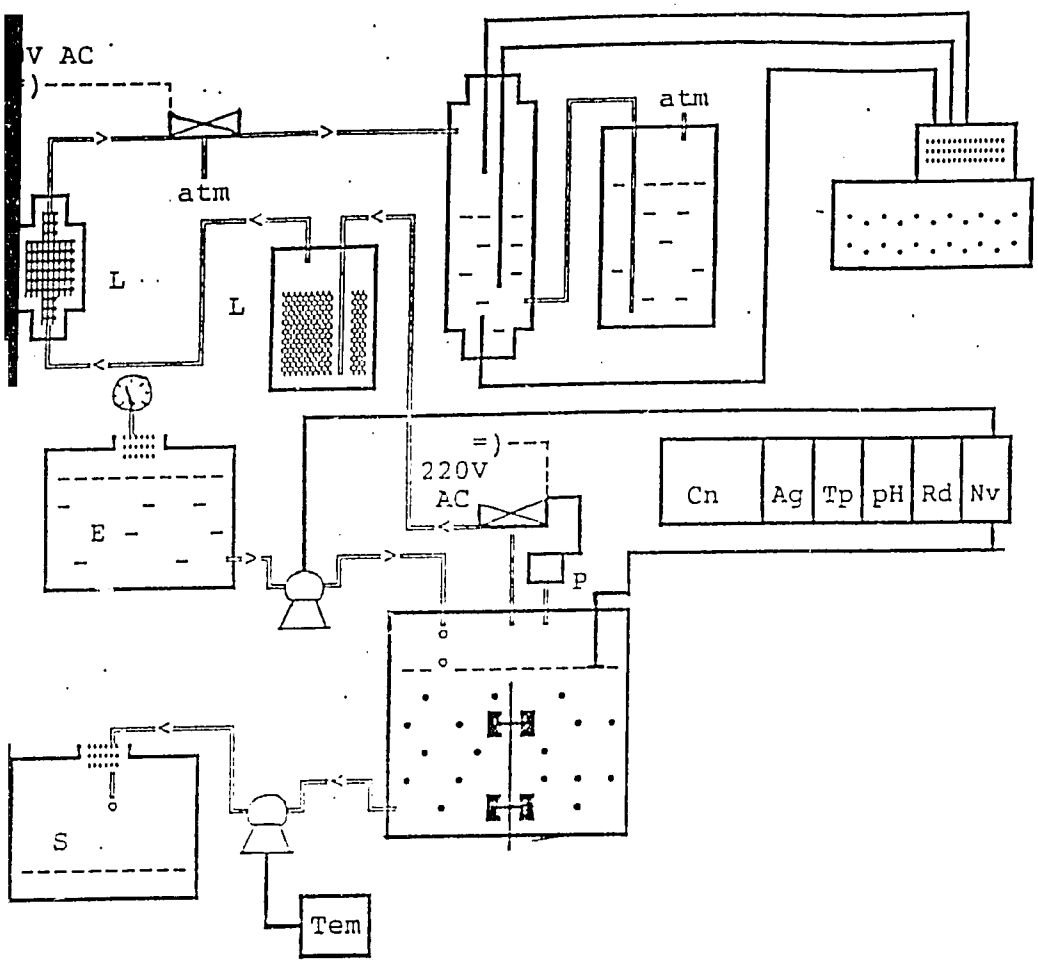


Diagram of the Continuous Culture System

L- Gas Washer
P- Pressostat valve
Cn- Control Unit
Ag- Stirring Unit
Tp- Temperature Unit
pH- pH
Rd- Redox Potential
Nv- Level Control
Tem- Timer
E- Feeding Res.
S- Output Res.

3

SUBSTRATE UTILIZATION BY *METHANOSARCINA BARKERI* IN SINGLE AND CO-SUBSTRATE CULTURE

INTRODUCTION

Among the known pure cultures of methanogens the Genus Methanosarcina is the most metabolically versatile. It can utilize H_2/CO_2, acetate, methanol and methylamines.

The single most energetic yielding substrate is H_2/CO_2, followed closely by methanol. The least energetic is acetate. The preferential methabolism of H_2/CO_2 and/or methanol followed by acetate is coincident with the difference in the availability of energy from these substrates.

Acetate is required as a cell carbon percursor in both autotrophic (Fuchs et al. 1978) and heterotrophic (Bryant et al. 1971) methanogenic bacteria.

In spite of this and the fact that 70% of the CH_4 produced in anaerobic environments is derived from acetate (McCarty 1965) still little is known about the regulation of its consumption and how is relates with other methanogenic substrates.

Schnellen first suggested that Methanosarcina grew sequentially on methanol and acetate in 1947. Latter reports of Weimer and Zeikus (1978) and Hutten et al. (1980) showed a simultaneous use of methanol and acetate, although with different rates.

However Ferguson and Mah (1983) demonstrated that when methanol and acetate were added as co-substrates, the kinetics of methanogenesis (and growth) was diauxic, indicating a sequential utilization of substrates, at least for their strain.
Some degree of metabolic interaction occurs during growth on these two substrates and pre-culture conditions strongly influence the stoichiometry of products.

In this study we calculated the specific growth rate dependence on the molar concentrations of the two substrates. This report shows the preliminary results concerning co-substrate utilization of acetate and methanol by Methanosarcina barkeri.

MATERIALS AND METHODS

We used methanol pre-grown inocula.

Anaerobic manipulation was according with Hungate technique

Growth was folowed by gas chromatography of the methane produced (Hach-Carle GC, Porapak-QS column, TCD)

Growth medium contained Yeast extrate trypticase, Mah's trace minerals solution, bicarbonate buffer and rezasurin.

RESULTS

1. Influence of the inocula pre-growth substrate.

Growth in acetate of the methanol pre-cultured cells started after a lag of 8 to 16 days and was faster than the normal growth in acetate. (Fig. 1)
This pattern occured only with acetate concentrations lower than the methanol ones. With a 1:1 ratio the specific growth rate was much lower but still higher than the acetate pre-grown one

2. Influence of methanol/acetate ratio:

a) Plotting Specific Growth Rate versus Acetate at different Metahnol concentrations (Fig. 2, 3 and 4).

b) Plotting Specific Growth Rate versus Acetate at different Acetate concentrations (Fig. 5, 6 and 7).

DISCUSSION

- Specific growth rates are dependent not only on the ratio of methanol and acetate concentrations but also on the absolute value of each substrate present in the medium.

- Specific growth rates dependence on co-substrates ratio vary from the one for growth on pure acetate to the higher one for methanol alone. (Fig. 8).

We did not observe bifasic growth curves characteristic of diauxic growth. But as Mah showed (Mah, 1983) it is necessary to follow the evolution of $^{14}CH_4$ from CH_3OH and [2-^{14}C]-acetate to precisely determine whether the substrates consumption or simultaneous or not.

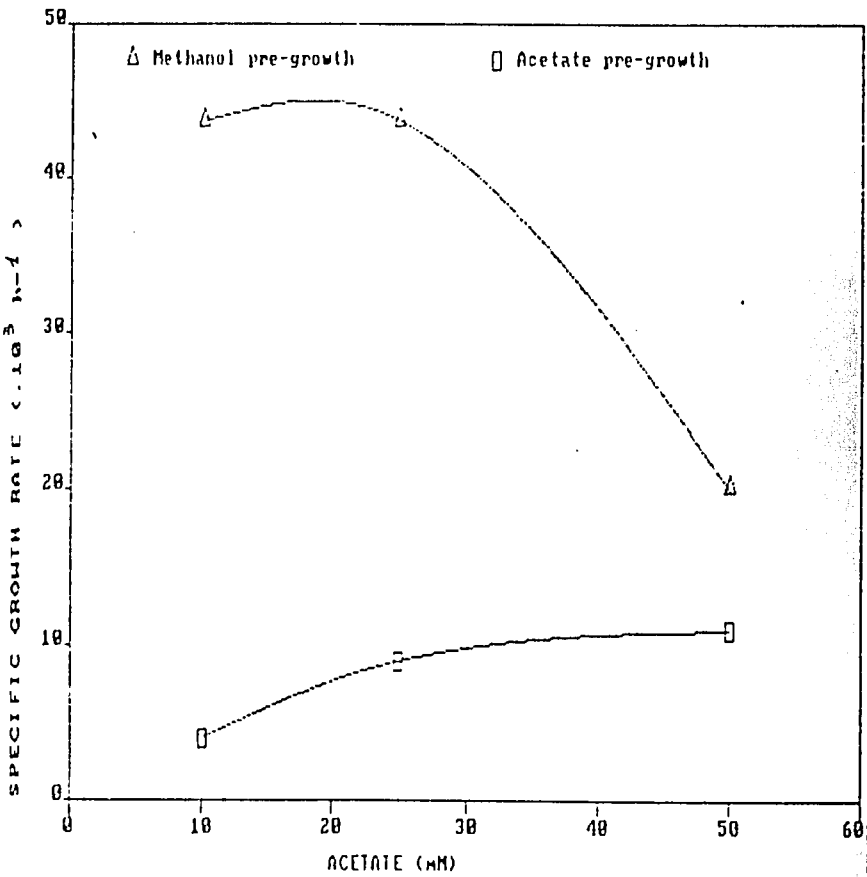

Δ Methanol pre-growth
□ Acetate pre-growth
SPECIFIC GROWTH RATE (.10³ h⁻¹)
ACETATE (mM)
0
10
20
30
40
50
60

FIG 1.

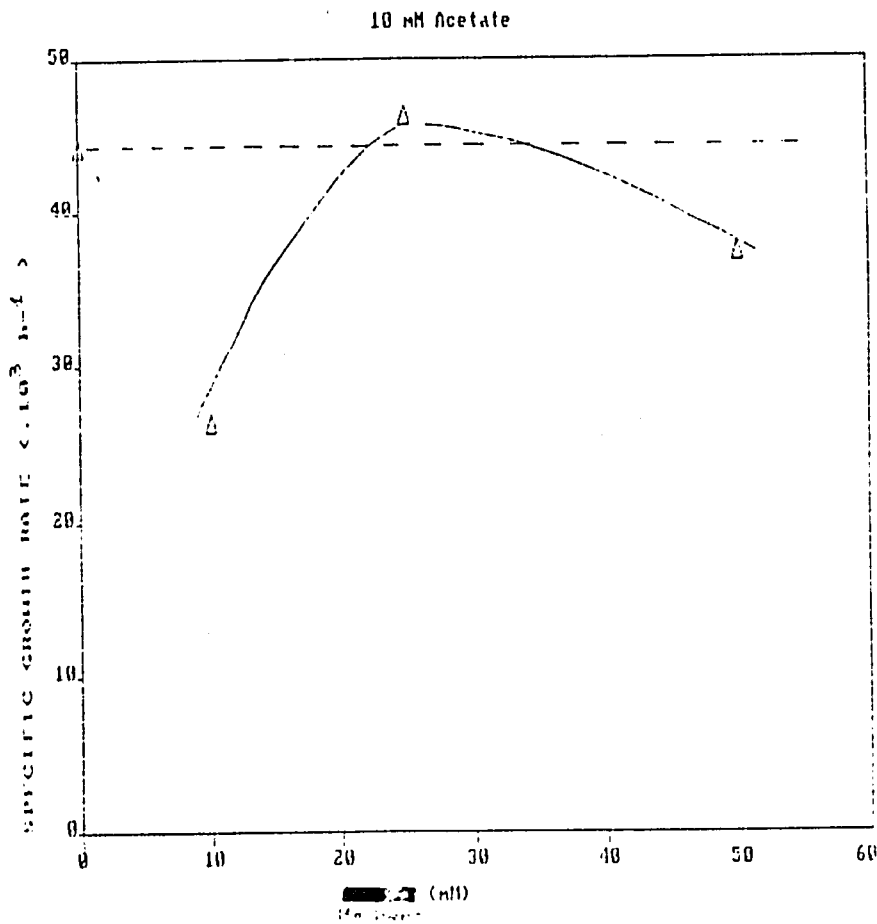


FIG 2.

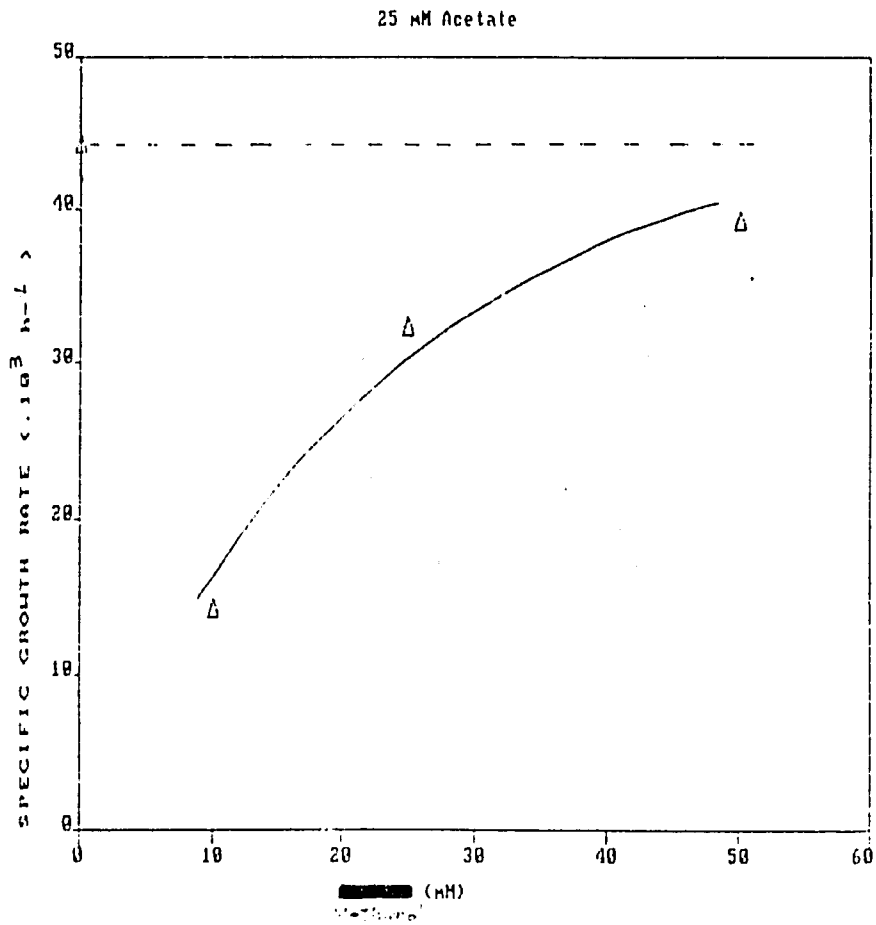

25 mM Acetate
SPECIFIC GROWTH RATE (.10³ h⁻¹)
0
10
20
30
40
50
0
10
20
30
40
50
60
(mM)

FIG. 3

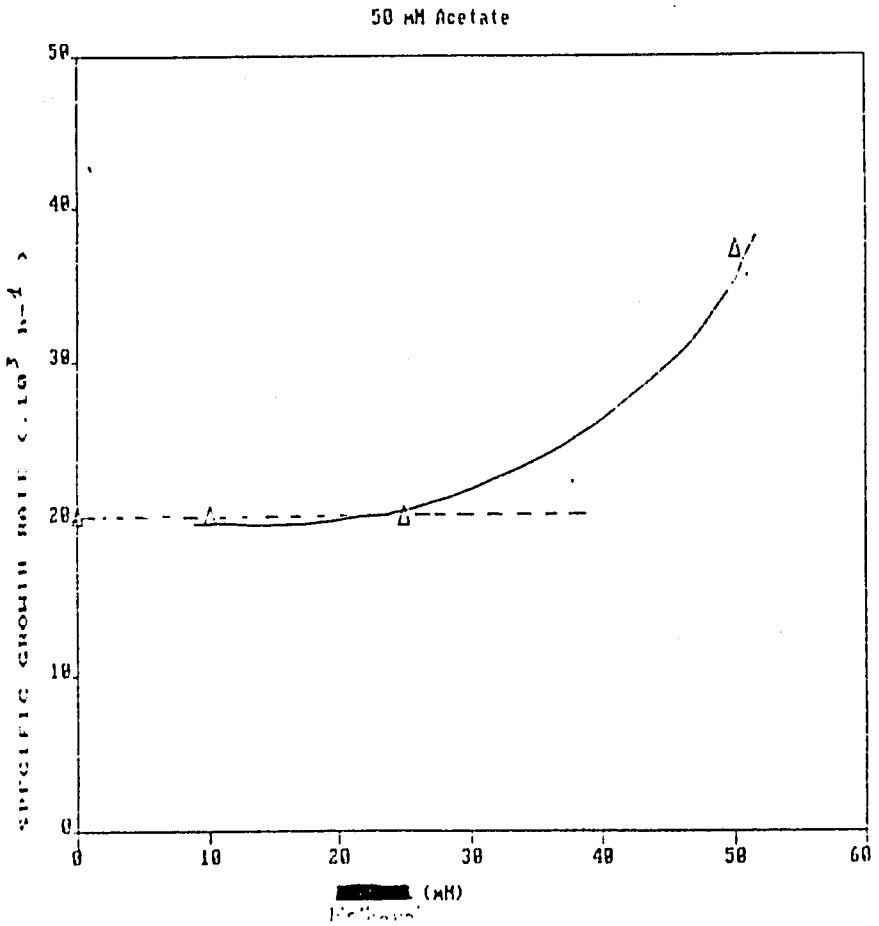


FIG 4

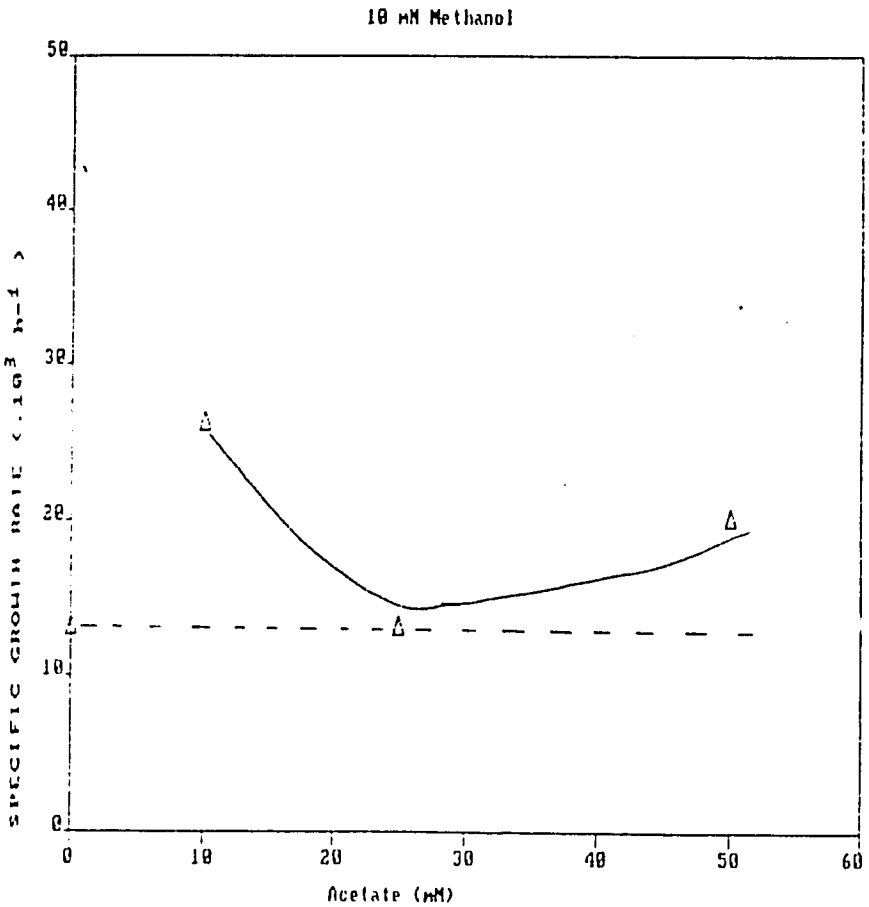

10 mM Methanol
SPECIFIC GROWTH RATE (.10³ h⁻¹)
0
10
20
30
40
50
0
10
20
30
40
50
60
Acetate (mM)

FIG.5

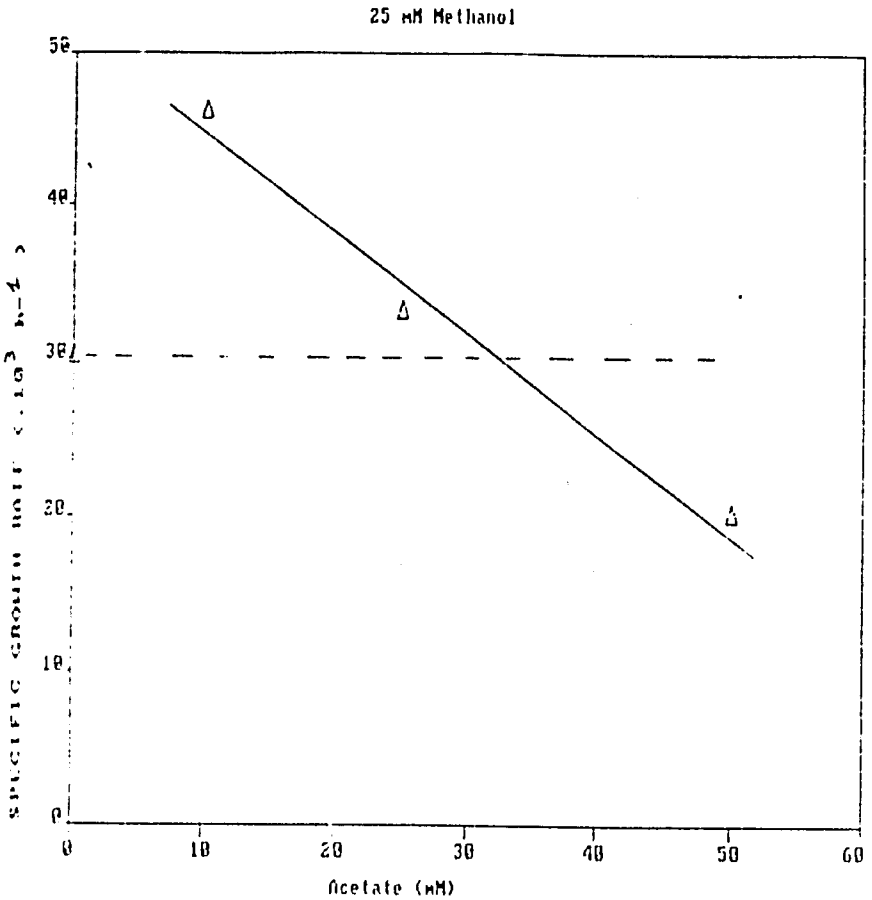


FIG.6

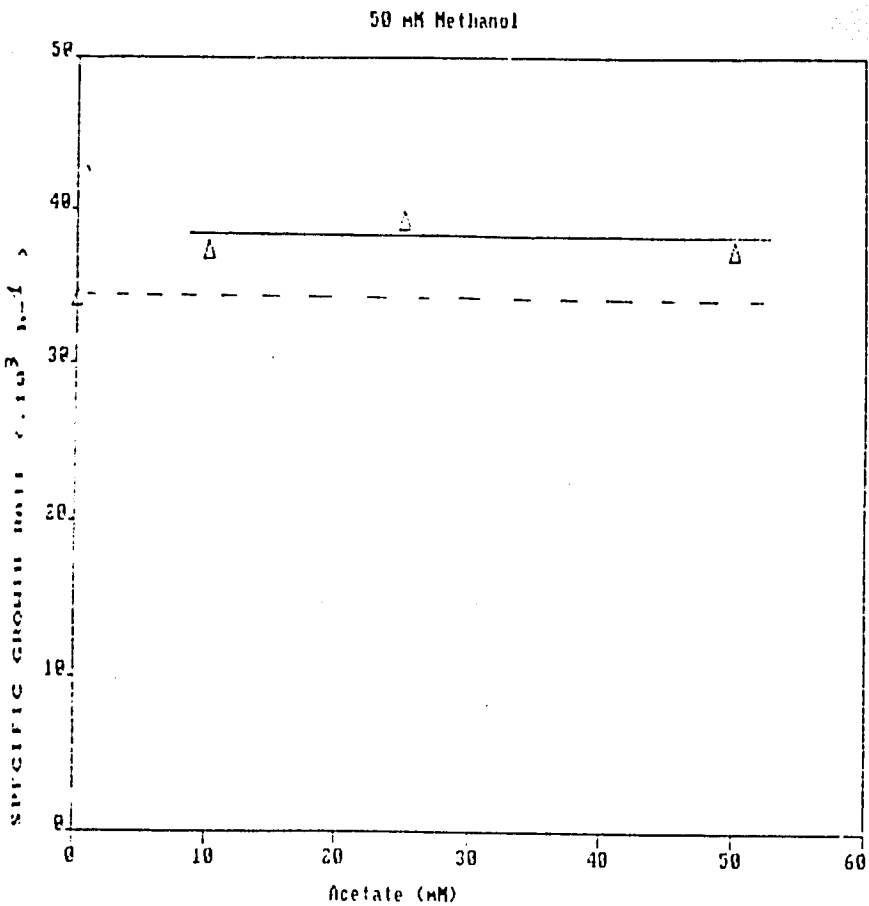


FIG.7.

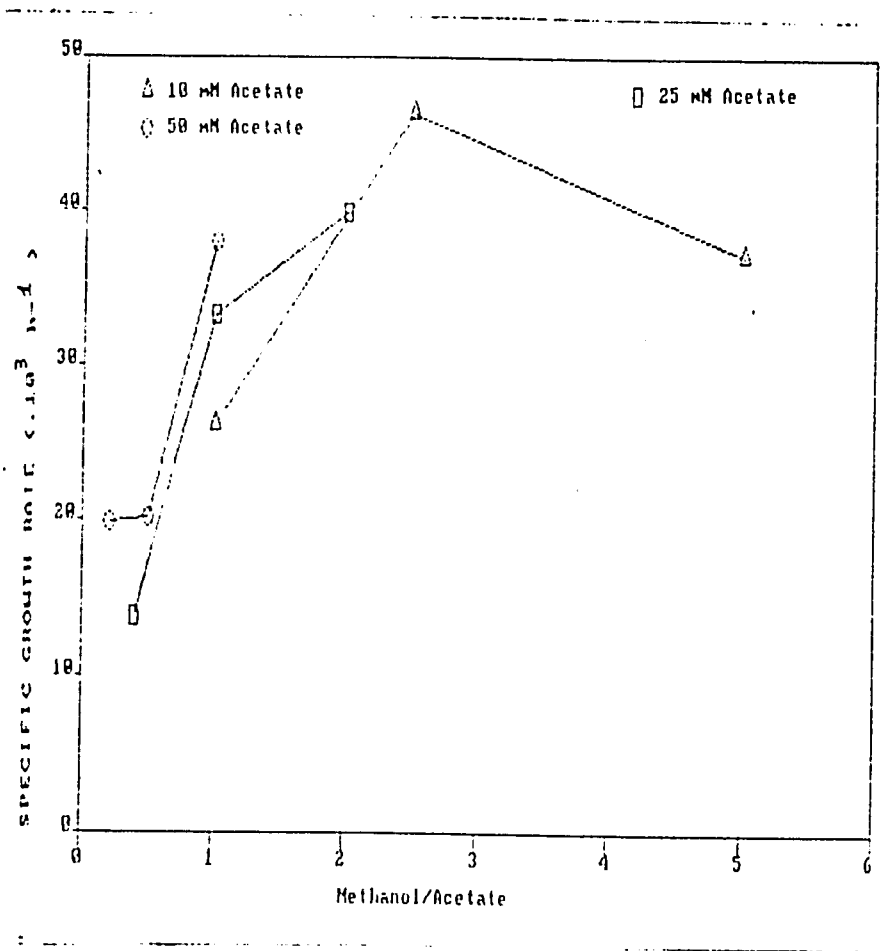

10 mM Acetate
50 mM Acetate
25 mM Acetate
SPECIFIC GROWTH RATE (.10^3 h^-1)
0
10
20
30
40
50
0
1
2
3
4
5
6
Methanol/Acetate

Fig. 8

4

Methanosarcina barkeri Fluoroacetate Resistant Mutants with Restored Capacity to Metabolize Exogenous Acetate

INTRODUCTION

The obtention of mutants in metabolic pathways and resistance to analogues or antibiotics in Archaebacteria in general and in the Methanogenic branch of this Urkingdom has been scarce(8,11,12).Many of the known antibiotics effective against eubacteria have little or no effect in methanogens(1). Amino-acid and other small molecules important in the intermediary metabolism are prevented to diffuse through the special array of the archaebacterial ether-linked lipids membrane (1) hence diminishing the practical possibilities of isolation of a number of auxotrophic mutations, especially those affecting the synthesis of polar and charged molecules (8).

The metabolism of acetate assimilation and methanisation has been until now studied at enzymatic level. It has been shown (4,7,9,10) that methanogenesis from acetate in Methanosarcina spp. proceeds trough (a) activation steps mediated by acetate kinase (AK) and phosphotransacetylase (PTA) and (b) cleavage of the acetyl moiety of acetylcoenzyme-A to a methyl group bound to coenzyme M and a C_1 unit at the oxidation level of CO which is then converted to CO_2 (9,11,14). In this later step the enzyme carbon monoxide dehydrogenase (CODH) plays an essential role. The CODH is required for assimilation of C_1 compounds into cell carbon through acetyl-coA formation from free coenzyme A, carbon monoxide and a corrinoid-bound methyl group (7). Also there is some evidence for CODH catabolic and anabolic activities being performed by different proteins (14). The induction of AK and PTA enzimes from the acetate activating pathway, along the shift from methanol to acetate growth has also been reported (15).

Fluoroacetate is a toxic analogue of acetate very adequate to select mutants with altered exogenous acetate metabolism. The toxicity of fluoroacetate (FAc) is due to the incorporation of FCH_2- group into cellular components (3) namely inhibiting the tricarboxilic acids cycle. The resistance towards FAc can only be due either to an impairement of uptake and/or of activating enzymes AK and PTA or to an increased rate of cleavage of acetate and it's toxic analogue sufficient to prevent a lethal incorporation of fluoride-bound groups in the intermediate metabolism molecules and strutural components.

The FAc resistant mutant described by M. Smith and J.L. Lequerica (12) was deficient in an early step of acetate mobilization having one fourth of the PTA activity, half of the AK activity and about the same CODH activity of the initial FAc sensitive strain. The authors favored the hypothesis of such FAc resistance being due to an acetate permease defect, although there is no clear evidence on acetate uptake depending on any specific transport protein. However, the new mutants and revertants we described here could bring new insights to the study of methanogenesis from acetate in Methanosarcina barkeri.

Materials and Methods

Microbial Strains

Methanosarcina barkeri 227	FAc^S , ac^+	(ATCC)
Methanosarcina barkeri FR42	FAc^r , ac^-	(our study)
Methanosarcina barkeri FR45	FAc^r , ac^-	(our study)
Methanosarcina barkeri REV42	FAc^r , ac^+	(our study)
Methanosarcina barkeri REV45	FAc^r , ac^+	(our study)

+

ac^+=growing with acetate as sole carbon and energy source;

ac^-=unable to grow at the expense of acetate alone;

FAc^S=sensitive to fluoroacetate concentrations under 0.1mM

FAc^r=resistant to fluoroacetate concentration of 1 mM

Culture Media and Conditions

The complex medium(1)contained (grams per liter): NH_4Cl 1.0; K_2HPO_4 0.4; $MgCl_2.6H_2O$ 0.1; yeast extract 2.0; Trypticase peptones 2.0; resazurin 0.001. It was suplemented with 10 ml of trace minerals solution (1) per liter. After boiling under 80% N_2 - 20% CO_2 this gas mixture was flushed into the baloon until room temperature was attained then, 0.5 g of cysteine hydrochloride and 2.4 g $NaHCO_3$ were added anaerobically. The medium was dispensed in 50 ml portions to 160 ml serum flasks or in 10 ml portions to 20 ml tubes flushed with N_2:CO_2 gas mixture and sealed with butyl rubber stoppers secured by aluminium crimps.After autoclaving 20 min. at 120 C the growth substrates

and 2.5% $Na_2S.9H_2O$ stock solutions (anaerobically prepared) were added to give a 100 fold dilution in the final medium. The pH was 7.0-7.2.

The minimal medium (13) contained (grams per liter): Na_2HPO_4 0.6; NH_4Cl 0.5; $MgCl_2.6H_2O$ 0.1; $CaCl_2.2H_2O$ 0.07; resazurin 0.001 and 10 ml per liter of trace minerals solution. Cysteine hydrochloride (0.25 g per liter, final concentration) and $NaHCO_3$ (2.4 g per liter, final concentration) were added after boiling the medium under 80% N_2 - 20% CO_2. Stock solutions of growth substrates and a 2.5% $Na_2S.9H_2O$ solution were added after autoclaving to give a 100 fold dilution. The pH was 6.8-7.0.

Sodium monofluoroacetate (FAc) was added to the final media anaerobically to obtain 0.01, 0.1 or 1 mM final concentration.

In experiments with H2 this gas was flushed through a sterile needle until the desired pressure was attained.

The rolled tubes were prepared according to Hungate (5).

Both liquid cultures and rolled tubes were statically incubated at 37 C. Liquid media with H_2 were incubated with agitation at low speed on a rotatory shaker at 37 C.

The growth was monitored either by measuring CH_4 percentage in 0.5ml samples of the headspace in Hach-Carle 100 GC or by measuring the total gas production with a 20 ml glass syringe.

Results and Discussion

1. Obtaining FAc resistant mutants

Methanol grown **Methanosarcina barkeri** 227 showed a high proportion of spontaneous FAc resistant mutants. The decrease in survival of FAc treated cultures was estimated through a series of rolled tubes with or without 0.1 mM FAc inoculated with aliquots from cultures pre-grown in the presence or absence of the toxic analogue. The results are listed in Table 1. The results obtained in the selection for FAc resistance showed that the lethal effect of this toxic analogue of acetate was much decreased (about 50% survival) in cell lines growing during consecutive transfers in methanol media. The lethality was more severe if FAc was present in agar media for in such rolled tubes 0.2 to 1% survival was observed.

Samples taken from these rolled tubes were used to inoculate liquid media with 50 mM methanol and 0, 0.1 and 1 mM FAc.The gas produced (see figure 1) showed a transient lag in the 0.1 mM FAc concentration for the non-treated inocula followed by an exponential increase to reach the same or higher levels of the equivalent cultures of FAc resistant isolates. This growth inhibition with different extent from culture to culture of the non-treated inocula was due to the selection in the initial population of the FAc resistant cells. This was confirmed with 1mM FAc where the inhibition effect was more severe but nevertheless allowed the growth of the few 1 mM FAc resistant cells present in the non-treated inocula. The FAc resistant isolates showed a different behaviour upon exposure to 1 mM FAc:

L3 showed a significant decrease in the rate and final levels of gas production while L4 had only a slight initial delay followed by a very high increase and a total gas production level higher than the other cultures.From L3 and L4 ten independent FAc resistant cell lines where obtained trough serial dilutions in rolled tubes with 0.1 mM FAc added being chosen the tubes with a single colony. Those showed a very high stability towards FAc resistance noticeable by the loss of their capacity to shift to acetate metabolism even after a number of cell generations without selective agent.

2. Assaying for reversion of FAc resistant mutants

The above mentioned ten cell lines were transferred through three consecutive cultures in 50 mM acetate/20 mM methanol to acetate liquid media. None has shown significant growth in acetate medium even after one month incubation. To test further the possibility of direct revertion in agar medium the ten cell lines were transferred to acetate rolled tubes from cultures exponentially growing in methanol media. Small size visible colonies were formed in some acetate rolled tubes but this growth must have been due to the methanol present in the inocula medium because the transfer of these colonies to acetate liquid media was unsuccessfull and they kept their growth capacity in methanol media with or without FAc added. These results pointed to the high stability of the FAc resistance conferring mutations in all the cell lines and showed a need for an enrichment protocoll to obtain acetate catabolising revertants of these FAc resistant mutants.

3.Enrichment for acetate catabolising revertants in two FAc resistant strains

The independent FAc resistant strains FR42 and FR45 were chosen for enrichment studies. These were maintained through three sequential transfers either in 50 mM acetate/ 20 mM methanol liquid media (FR 45) or in 50 mM methanol/ 1 mM FAc media (FR 42). When inoculated to serum flasks with different substrate combinations (see figure 2) both strains showed an unexpected behaviour. With the simultaneous presence of acetate, metanol and FAc a 6 to 8 days lag was observed, followed by growth to the same total gas production as the control cultures with acetate and methanol. Rolled tubes with 50 mM acetate as sole methanogenic substrate and parallel controls with 50 mM acetate/ 20mM methanol were inoculated with the above-mentioned liquid cultures of FR42 and FR45. The resulting countings (see Table 2) show that the yield of revertants is higher with FR42 if pre-cultured in liquid media in the presence of 1 mM FAc than when this toxic analogue is absent from the inoculum culture. The results with FR45 are quite different showing that the release of the selective pressure exerted by FAc is beneficial (the opposite for FR42) for the isolation of acetate catabolising revertants. The fact that these strains show a very significant difference on the basis of the revertants yield in contrasting selective conditions is an indication that the FAc resistance conferring mutations are not in the same gene or at least, if located in the same gene, are not in the same position. Two independent revertant strains, named REV 42 (derived from FR42) and REV 45

(derived from FR45) were isolated in serial dilutions of liquid enrichment cultures to rolled tubes with single colonies.

One should expect that upon enrichment in methanol/acetate medium the restoration of acetate catabolism would be correlated to the loss of FAc resistance. This was not the case namely with REV45 derived from acetate/methanol enrichment of FR45. Even more unexpected was the high efficency for selecting revertants of a methanol/acetate/fluoroacetate medium.The process itself leading to the isolation of REV42 allow us to postulate that the FAc resistance could not in such case be accounted by an inactivation of a gene coding an enzyme in acetate mobilization. Preliminary results (A. Eusebio, personal communication) indicate that REV42 growing in acetate medium has a significantly lower AK (acetate kinase) activity when compared to the wild type but it is present and anyhow such activity must be high enough in acetate media to account for the vigorous growth of this strain.

4.Characterization of the revertant strains

Both REV42 and REV45 strains kept FAc resistance when cultured in a complex or mineral medium with methanol. Transferring acetate pre-grown cultures of both strains to methanol/acetate media only once and then immediatly to acetate media again was enough to induce long lag periods of 2-3 weeks before noticeable increase in methane production. As the wild type both strains formed aggregates with the pseudo-sarcina morphology but REV42 forms in acetate liquid cultures aggregates of a smaller size than equivalent cultures of the wild type. The main phenotypical

distinct character between the two revertant strains was the great difference in growth capacity: REV42 had as high or higher growth capacity in acetate as the wild type but REV 45 had about the half. Acetate pre-grown inocula from REV42 showed no lag period when transferred to methanol +1 mM FAc liquid media growing at the same rate as control cultures without FAc. Moreover the same strain grew to some extent in acetate media in the presence of 1 mM FAc although it did not grow in a subsequent transfer to a medium with the same composition.

5.H_2 effects in acetate growth in REV42 and 227

The growth pattern in acetate media from REV42 relativelly to 227 (see figure 3A) and their response to the presence of H_2 at 33, 66 and 100 kPa hydrogen gas levels (see figure 3 B, C and D) exhibited some marked differences. In acetae media REV42 cultures from acetate pre-grown inocula (fig. 3A, open circles) grew rapidly and without any initial lag to reach within two weeks 40% CH_4 in the gas phase; in contrast to this pattern the strain 227 had an initial short lag and then grew slowly to reach 6-7% CH_4 within two weeks. When the inocula were pre-grown in H_2/CO_2 the transfer to acetate medium caused a much more severe growth inhibition in REV42 relatively to strain 227. The behaviour of strain 227 in the presence of H_2 (see figure 3 B, C, D open and closed squares) either acetate or H_2/CO_2 pre-grown showed at all H_2 concentrations a very significant stimulation of the methane production from acetate; also the methane levels were allways higher in strain 227 H_2/CO_2 pre-grown. With strain REV42 the

situation was quite different: compared to unitrophic growth on acetate, the methane final levels were lower in the presence of H_2 irrespectively of the growth substrate of the inocula; furthermore H_2/CO_2 pre-grown REV42 cultures (closed circles)had lower methane levels than acetate pre-grown (open circles) at all time-points samplings.

The absence of enhancement of acetate consumption with the addition of H_2, an effect observed in strain 227 (2,6), is also pointing to an altered regulation of the acetoclastic methanogenesis process.

All these phenotypical traits depict an image for FR42 and REV42 incompatible with an acetate permeation/acetate activation defect as postulated for the previously described FAc resistant mutant (12). It is possible that other mutant cell lines obtained in this study are resistant to FAc because of defective enzymes or permease in acetate mobilization process. Should it be the case of RF42 and RF45 too, one has to postulate three independent mutational events to account for the revertant phenotypes of these strains, namely (1) the abolition of acetate uptake/activation leading to FAc resistance, (2) the restoration of the exogenous acetate metabolism and (3) the arising of a new kind of FAc resistance which doesn't destroy the unitrophic acetate growth capacity. The second and third events had to occur at the same time in the case of REV42 because of enrichment process itself leading to it's isolation.

Independently of speculative assumptions on the molecular mechanism underlying such phenomenon the fact remains that FAc

resistance doesn't imply the loss of exogenous acetate uptake and therefore in strain REV42 the cause for FAc resistance should be searched at the level of acetoclastic methanogenesis regulation genes rather than at the level of it's structural genes. The precise location of such mutation(s) and the understanding of the physiological properties making possible the restoration of acetate catabolism keeping FAc resistance is only possible with the development of genetic analysis in these and other Methanosarcina spp. strains.

Table 1: Estimation of spontaneous FAc resistance in methanol grown cultures of Methanosarcina barkeri 227

Methanol grown cultures with or without 0.1 mM FAc (5 independent cultures from each) were inoculated (at 5% density) to rolled-tubes in duplicates. The countings of visible colonies were performed after 10 to 12 days incubation.

	non treated inocula		. FAc treated inocula	
rolled-tubes composition	-	FAc 0.1 mM	-	FAc 0.1 mM
mean c.f.u./ml	920	2	440	8

Table 2: Effects of enrichment media composition on the revertant yields with strains FR42 and FR45

Four flasks for each medium composition were used to inoculate acetate rolled-tubes in duplicates. The same number of acetate/methanol rolled tubes was used to evaluate the total viable cells from each flask culture.

Strains	FR42		FR45	
Enrichment liquid media	acetate methanol	acetate 1mM FAc methanol	acetate methanol	acetate 1mM FAc methanol
Mean c.f.u./ml in acetate rolled-tubes	10	376	144	20
% of acetate growing to total viable cells	1	87	25	2

Figure 1: Total gas formation in strain 227 (A), isolate L3 (B) and isolate L4 (C). All flasks had 50 mM methanol as growth substrate. ( )without aditions; ( )with 0.1mM FAc; ( )with 1mM FAc. Each point represents the mean obtained with 4 culture flasks.

Figure 2: Gas production of FR42 (A) and FR45 (B) with the following media compositions: ( )50 mM methanol + 1 mM FAc; ( )50 mM methanol + 50 mM sodium acetate;( )50 mM methanol + 50mM sodium acetate + 1 mM FAc. Each point is the mean obtained from 4 culture flasks.

Figure 3: Methanogenesis from acetate in strains 227 and REV42 in the presence or absence of H_2.
(A) 50 mM acetate with no H_2 added; (B) 50mM acetate +33 kPa H_2 (C) 50mM acetate + 66 kPa H_2; (D) 50 mM acetate + 100 kPa H_2.
Symbols:( ) REV42 acetate pre-grown; ( )REV42 H_2/CO_2 pre-grown; ( )strain 227 acetate pre-grown; ( )strain 227 H_2/CO_2 pre-grown.
Each point represents the mean value from 4 culture flasks.

FIGURE 1: Total gas formation [A] strain 227; [B] isolate L3; [C] isolate L4. Culture conditions (●) methanol alone; (▲) methanol with 0.1 mM FAc; (▼) methanol with 1 mM FAc.

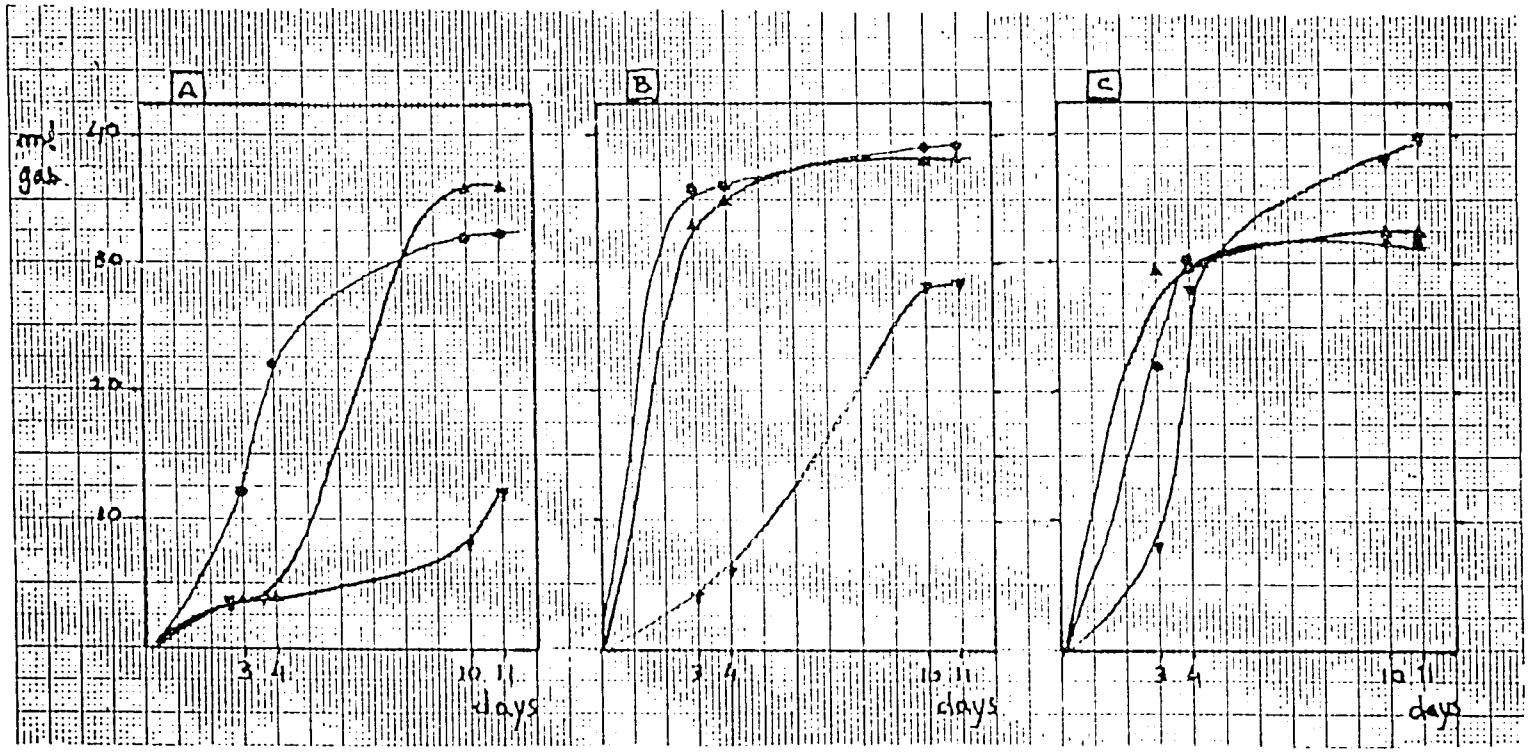


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FIGURE 2: Gas production of FR42 (A) and FR45 (B) with different media compositions: close circles (●) 50 mM methanol + 1 mM FAc; open circles (o) 50 mM methanol + 50 mM sodium acetate; closed triangles (▲) 50 mM methanol + 50 mM sodium acetate + 1 mM FAc.

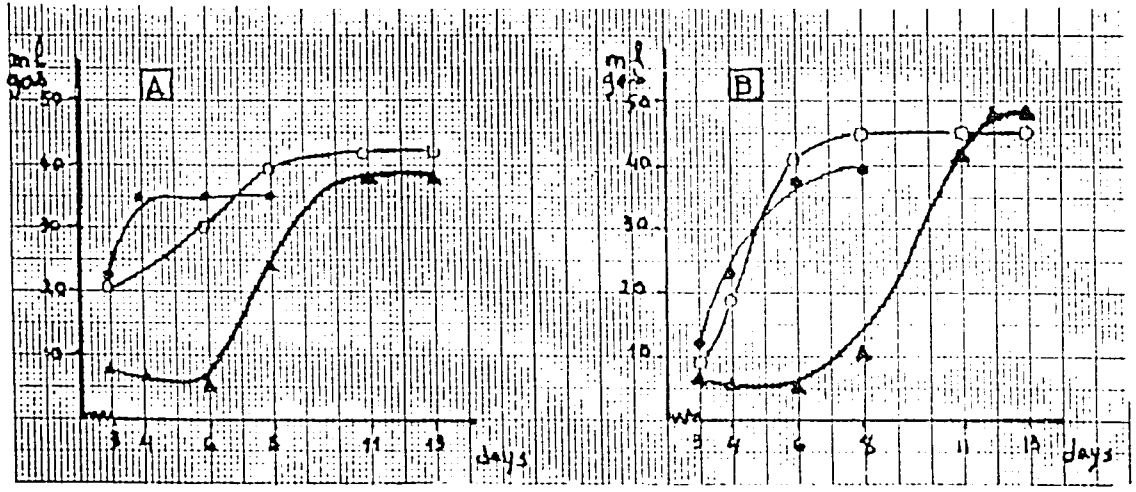

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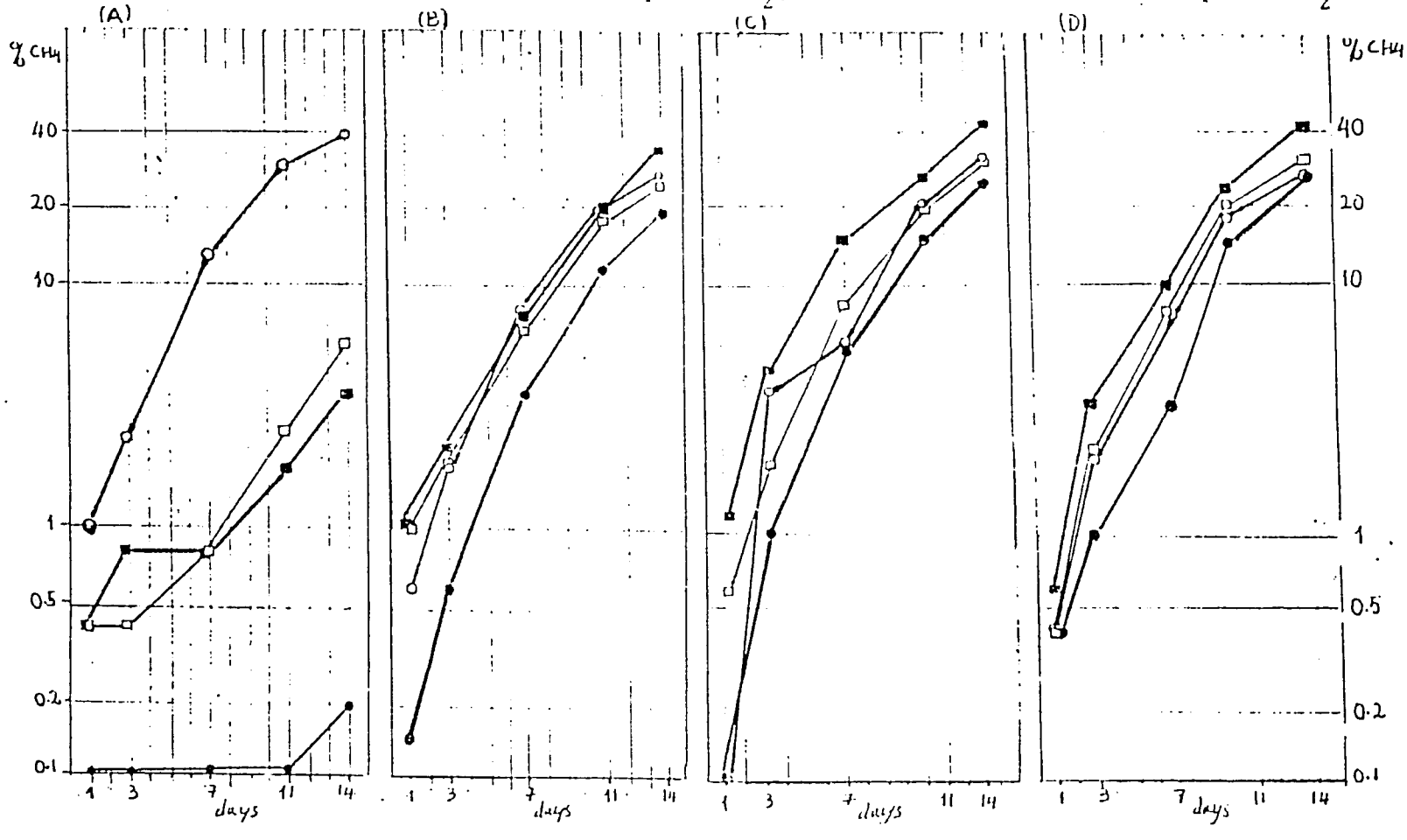
FIGURE 3: Methanogenesis from acetate in strains 227 and REV42
(A) 50 mM acetate with no H_2 added; (B) 50 mM acetate with 5 p.s.i. H_2;
(C) 50 mM acetate with 10 p.s.i. H_2; (D) 50 mM acetate with 15 p.s.i. H_2.



SYMBOLS: open circles (○) REV42 acetate pre-grown; closed circles (●) REV42 H_2CO_2 pre-grown;
open squares (□) 227 acetate pre-grown; closed squares (■) 227 H_2CO_2 pre-grown.

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LITERATURE

1.Balch, W.E., G.E. Fox, L. J. Magrum, C. R. Woese and R.S. Wolfe
Methanogens:Reevaluation of a unique biological group.
Microbiol. Rev. 43: 260-296 (1979)

2.Boone, D.R., J.A.G.F.Menaia, J.E. Boone and R. A. Mah
Effects of hydrogen pressure during growth and effects of pregrowth with hydrogen and acetate degradation by Methanosarcina species
Appl. Env. Microbiol. 53, 83-87 (1987)

3.Brown, T.D.K., M.C. Jones-Mortimer and H.L. Kornberg
The enzymic interconvertion of acetate and acetyl-coenzyme A in Escherichia coli.
J.Gen.Microbiol. 102, 327-336 (1977)

4.Fischer, R. and R.K. Thauer
Methane formation from acetyl phosphate in cell extracts from Methanosarcina barkeri; dependence of the reaction on coenzyme A.
FEBS Lett.228, 249-253 (1988)

5.Hungate, R.E.
A roll tube method for cultivation of strict anaerobes p.117-132.
In J.R. Norris and D.W. Ribbons (ed.). Methods in microbiology vol. 3B. Academic Press,Inc. New York (1969)

6.Kemner, J.M. and J.G. Zeikus
Comparision of soluble and membrane bound hydrogenase activity in Methanosarcina grown on acetate versus hydrogen
Abstr. Annu. Meet. Am. SOc. Microbiol. p.181 (1988)

7. Kenealy,W.R. and J.G. Zeikus
One-carbon metabolism in methanogens: evidence for synthesis of a two-carbon cellular intermediate and unification of catabolism and anabolism in Methanosarcina barkeri.
J. Bacteriol. 151, 932-941 (1982)

8.Kiener, A., C. Holliger and T. Leisinger
Analogue-resistant and auxotrophic mutants ofMethanobacterium thermoautotrophicum.
Ach. Microbiol 139 , 87-90 (1984)

9. Nelson, M.J.K. and J.G. Ferry
Carbon monoxide-dependent methyl coenzyme M methylreductase in acetotrophic Methanosarcina sp.
J. Bacteriol. 160, 526-532 (1984)

10. Peinemann, S., V. Müller, M. Blaut and G. Gottschalk
Bioenergetics of methanogenesis from acetate by Methanosarcina barkeri
J. Bacteriol. 170, 1369-1372 (1988)

11. Smith, M.R. and R.A. Mah
2-Bromoethanesulfonate: A selective agent for isolating resistant Methanosarcina mutants.
Curr. Microbiol. 6, 321-326 (1981)

12. Smith, M.R. and J.L. Lequerica
Methanosarcina mutant unable to produce methane or assimilate carbon from acetate
J. Bacteriol. 164, 618-625 (1985)

13. Sowers, K.R. and R.P. Gunsalus
Adaptation for growth at various saline concentrations by the Archaebacterium Methanosarcina thermophila
J. Bacteriol. 170 , 998-1002 (1988)

14.Terlesky, K.C., M.J.K. Nelson and J.G. Ferry
Isolation of an enzyme complex with carbon monoxide dehydrogenase activity containing corrinoid and nickel from acetate grown Methanosarcina thermophila
J. Bacteriol. 168, 1053-1058 (1986)

15.Zinder, S.H. and A.F. Elias
Growth substrate effects on acetate and methanol catabolism in Methanosarcina sp. strain TM1
J. Bacteriol. 163, 317-323 (1985)

5

VITAMIN B12 FROM CORRINOIDS OF *Methanosarcina barkeri* 227

Methanosarcina barkeri 227 was grown in a complex culture medium with methanol as the sole carbon and energy source. Cysteine was used as the sole sulfur source allowing to increase the soluble cobalt chloride concentration up to six fold (6 x 2.7 mg/l) in the growth medium, without precipitation. With the increase in the cobalt concentration there was a simultaneous increase in the corrinoids quantity and in their conversion in vitamin B12, attaining maximal values at 10.8 mg/l of $CoCl_2$. In the absence of inorganic sulfide as reducing agent, the corrinoids conversion in vitamin B12 is not dependent of the cobalt concentration in the growth medium. After two transfers to a culture medium supplemented with an appropriate base moiety (5,6= dimethylbenzimidazole 200 μM), the cellular concentration of total corrinoids was quadruplicated and there were significative results for their conversion in vitamin B12.

INTRODUCTION

Vitamin B12, also named by cyanocobalamin or 5,6= dimethylbenzimidazolylcyanocobamide, was isolated in 1948 by Folker and Smith. It is an important cofactor to the carbohydrates, lipids, amino acids and nucleic acids metabolism, therefore becaming in an important additive to the human and animal nutrition. Furthermore, is considered to have a number of important applications in the field of medicine.

Many reports indicate that microorganisms could produce high quantities of these compounds but only vitamin B12 and riboflavin are produced at a commercial level, by processes of microbial fermentation that could compete with the chemical synthesis. For the industrial prodution of vitamin B12, wich concentration in the medium could be higher than 50 mg/l, sugar fermenting cultures of Propionibacterium spp. and Pseudomonas denitrificans are normally used.

Vitamin B12 like compounds are natural cofactors of methanogenic bacteria. These organisms, particularly M.barkeri, have a high level of corrinoids when they are cultivated in methanol.

Biosynthesis of corrinoids (Figure 1), by methanogenic bacteria, involve one step where occur the insertion of cobalt in the molecule. In this basis, it was studied on this report the corrinoids level and its conversion in vitamin B12, with cells of M.barkeri grown on a medium supplemented with different concentrations of cobalt. Methanogenic bacteria need reduced= sulfur compounds, e.g. inorganic sulfide, to maintain an appropriate redox potential to the anaerobic growth and for the synthesis of organic compounds that contain sulfur, like coenzyme M. Usually, it is used one mixture of Na_2S and cysteine to cultivate methanogenic bacteria.

However, an high concentration of sulfide is toxic for methanogenics and precipitate in the culture medium with essential minerals f· · the growth, for example, in the presence of a concentration cobalt too high. So, it was studied the growth of M.barkeri and the conversion of corrinoids in vitamin B12, in a culture medium with cysteine as the sole sulfur source and reducing agent.

Methanogenic bacteria could also synthesized vitamin B12 by incorporation of the respective base moiety, 5,6=dimethylbenzimidazole (DBI) in the molecule, if that was supplemented to the culture medium. It was studied the growth of M.barkeri and the conversion of corrinoids in vitamin B12 after two transfers to the culture medium supplemented with 5,6=DBI.

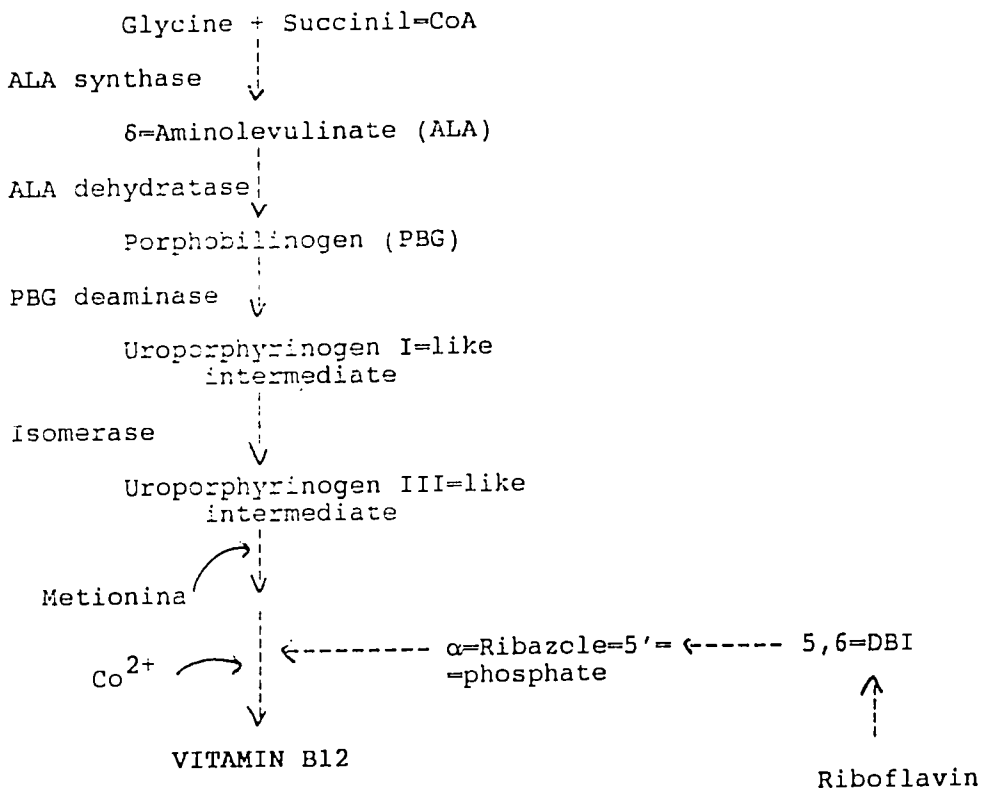


Fig.1. Biosynthesis of vitamin B12.

MATERIALS AND METHODS

BACTERIAL STRAIN:
Methanosarcina barkeri 227

GROWTH MEDIUM AND CULTURE TECHNIQUE:
All manipulations of media and solutions were carried out under O_2 = free atmosphere of N_2 gas. A modified Hungate technique was used. The culture medium (pH 7.0) contained the following materials (per liter of deionized water): yeast extract, 2g; tripticase, 2g; K_2HPO_4, 0.4g; NH_4Cl, 1g; $MgCl_2.6H_2O$, 0.1g; $CaCl_2$, 0.1g; resazurin, 0.1mg; $NaHCO_3$, 5.6g; Mah's trace minerals, 10ml; methanol as sole carbon and energy source, 200mM. L=cysteine hydrochloride, 0.56g and $Na_2S.9H_2O$, 0.25g; or L=cysteine alone, 1.12g, as the sulfur source and reducing agent. The basal medium without methanol and reducing agents was boiled and then cooled with continuous flashing of N_2CO_2 (70:30). The medium was prepared in a 2000 ml bottles wich were sealed with black butyl rubber stoppers, and autoclavated at 120°C, 20 min. Methanol and sulfide were injected into the basal medium as concentrated aqueous solutions by using a hypodermic syringe. Media were supplemented with a $CoCl_2.6H_2O$ anaerobic solution to final concentrations of 5.4, 10.8 and 16.2 mg/l, respectively. When required, media were supplemented with a 5,6=DBI anaerobic solution to a final concentration of 200µM. The culture of *M.barkeri* was inoculated with 5% (vol/vol) of a culture grown in the same medium containing either cysteine or cysteine plus sulfide as the sulfur source. Cultures were then incubated at 37°C without shaking. Cell growth was followed by methane production analysis by gas chromatography (Porapak=QS column, TCD), since the formation and deposition of *M.barkeri* cell aggregates difficults the growth visualization by usual methods of turbidity. Pure cultures were visualized by optical in simultaneous with fluorescence microscopies (Olympus BH=2).

The growth rates (µ) were obtained from the values of methane production during the log phase of growth, and doubling times were obtained using the following equation: $T_d(h)=\ln 2/\mu(h^{-1})$.

EXTRACTION AND PURIFICATION OF COBAMIDES:
At the end of the log phase, cells were harvested by centrifugation of 4,000 rpm, 10 min.(Burkard, rotor 009), washed with deionized water and resuspended in a 50% methanol aqueous solution containing 1%KCN and were refluxed for 4 hours. After centrifugation at 10,000 rpm, 20 min. (Burkard, rotor 001), supernatants were applied to a chromatograph column (0.5 x 7.0 cm) filled with neutral aluminum oxide. Corrinoids were eluted in a red fraction with deionized water.

ANALYTICAL PROCEDURES:
Red fraction was monitorized in a Hitachi spectrophotometer. UV/VIS absorption spectra were obtained in a range of wavelength from 200 to 600 nm. Corrinoids were quantified by HPLC with a reverse phase system, on a Spherisorb C_{18} column = RP18 (LDC/ Milton Roy Co.). Chromatograms were obtained with a linear gradient elution of methanol 0 to 50% in acetate buffer 25 mM (pH 6.0) within 10 min. 550 nm was the wavelength used to quantified corrinoids against a cyanocobalamin standard.

CHEMICALS:

RESULTS AND DISCUSSION

INFLUENCE OF COBALT CONCENTRATION AND ABSENCE OF SULFUR:
In presence of sulfide and cysteine as sulfur sources, it was observed an gradual obscuration of the culture media simultaneous with the increasing of cobalt chloride concentration. This means a slightly precipitation due to the excess of cobalt in presence of inorganic sulfur. Mountfort and Asher (1979) repport that redox potential of culture medium, without sulfide as reducing agent, was about -300 ± 10mV, indicating that the medium was sufficiently reduced to support the growth of M.barkeri.Our results suggest that methanogenesis and cell growth of M.barkeri were not inhibited by the presence of cysteine as the sole sulfur source, even after one transfer to the same medium. Under these conditions, it was possible to increase the soluble cobalt concentration in the culture media up to 16.2 mg/l, avoiding the precipitation observed in presence of inorganic sulfide. In terms of growth study, there were no significative differences among the different media. The values obtained for the cell biomass, growth rates and doubling times were about 10 g, 0.05 h^{-1} and 14 h, respectively (TABLE I).

Figure 2 represents a chromatogram obtained by the HPLC method, allowing to distinguish the peaks of the extracted corrinoids from cell biomass of M.barkeri, grown in culture media with different cobalt concentrations, in presence or in absence of sulfide as reducing agent. In this example, chromatogram B represents the eluted red fraction obtained from the medium with 16.2 mg/l of cobalt chloride, in presence of inorganic sulfide and cysteine as sulfur sources. The chromatogram A represents one elution of standard vitamin B12 wich have a retention time of 11.48 min. In chromatogram B, the eluted peak with a retention time of 11.41 min. was considered like vitamin B12. The high "basal noise" inherent to the instrument, wich is verified in chromatogram B, is due to the fact that corrinoids were at a concentration too reduced in the sample; therefore, the

detections were analyzed using an AUFS (absorbance units full scale) too low, 0.01. Standard solutions of vitamin B12 were analyzed using an AUFS of 0.05, because they were much more concentrated. Chromatogram B indicates the existence of corrinoids other than vitamin B12, at much more high concentrations, namely the peaks with retention times of 4.19, 5.94, 6.06, 7.36, 7.51 and 10.73 min.

TABLE I. Growth study of cells of M.barkeri 227 grown at different cobalt concentrations and in presence or absence of sulfide in the culture medium.

SULFUR SOURCE	COBALT CONC. (mg/l)	CELL BIOMASS [g(ww)/l]	GROWTH RATE (h^{-1})	T_d (h)
Sulfur + Cysteine	2.7	9.025 (3.25)	0.052	14
	5.4	10.755 (3.47)	0.045	15
	10.8	10.180 (1.12)	0.052	13
	16.2	9.515 (0.51)	0.049	14
Cysteine	2.7	7.000 (1.01)	0.044	16
	5.4	9.640 (0.46)	0.049	14
	10.8	9.280 (0.72)	0.051	14
	16.2	13.290 (5.32)	0.048	15

The results were obtained from quadruplicated samples.
Values in parentesis represents standard deviations.

FIG.2. Separation of corrinoids by HPLC (detection at 550 nm). (A) Standard solution of vitamin B12, 10.0 µg/ml. AUFS = 0.05. (B) Red fraction with corrinoids extracted from medium with 16.2 mg/l of cobalt, in presence of inorganic sulfide. AUFS = 0.01.

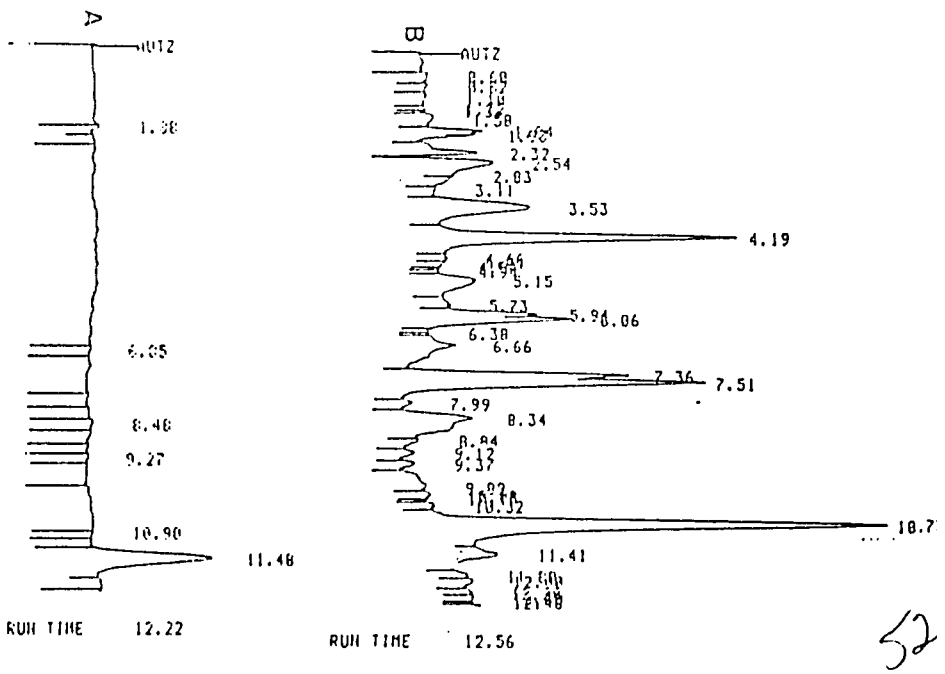


TABLE II. Conversion of corrinoids into vitamin B12 by M.barkeri.

SULFUR SOURCE	COBALT CONC. (mg/l)	TOTAL CORRINOIDS (%)	VITAMIN B12 (% OF TOTAL CORRINOIDS)
Sulfur + Cysteine	2.7	100	--
	5.4	228	5.8
	10.8	821	10.0
	16.2	656	6.5
Cysteine	2.7	40	--
	5.4	127	--
	10.8	66	--
	16.2	210	4.3

INFLUENCE OF THE SUPPLEMENTATION WITH 5,6-DBI:

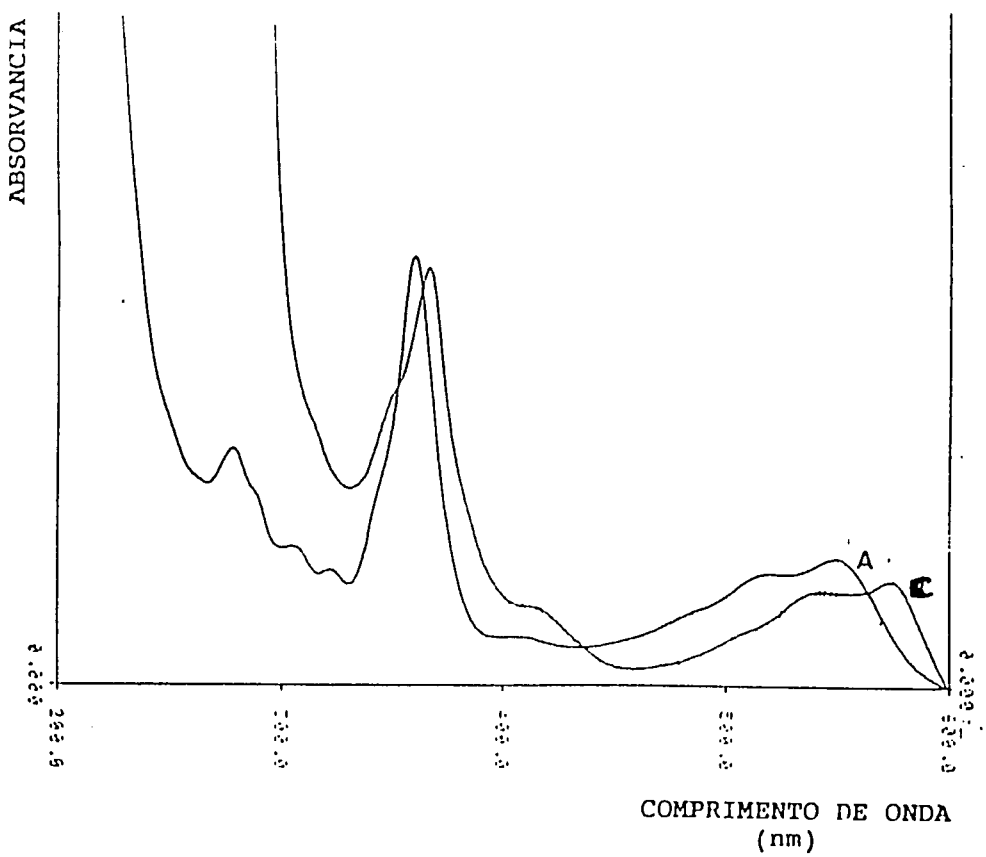
The values obtained for the biomass weight, growth rates and doubling times of M.barkeri for each transfer performed to the medium supplemented with 5,6-DBI 200 μM are represented in Table III. The supplemented medium with 5,6-DBI origins a slightly diminution in the growth rate values and, consequently, in the doubling time of M.barkeri 227. The cell biomass shows a significative decrease, probably due to a need of previous adaptation of the cell metabolism to the supplementation. During the next transfers, in similar conditions, exists one tendency for the restablishement of the initial values. Therefore, it is possible to perform the experiments of 5,6-DBI supplementation without a significative change in the energetic metabolism (methane production) and in the cell carbon synthesis (cell biomass production).

The red fraction with corrinoids obtained from the reflux method of cells grown in a 5,6-DBI supplemented medium was submetted to an analysis by HPLC method (Figure 4). Chromatogram B represents the separation of corrinoids of the red fraction obtained after the second transfer to the supplemented medium. After one dilution (1:1) of this sample in equal volume of a standard vitamin B12 solution, it was obtained the chromatogram C. Indeed, the peaks areas were reduced to one half of the initial ones and it was not observed no additional peaks. Commercial vitamin B12 was eluted with a retention time of 10.98 min. and the red fraction contains a predominant corrinoid eluted with a retention time of 10.97 min. The analysis of the chromatogram C is conclusive once the peak was eluted with a retention time identical, and the presence of standard vitamin B12 in the sample didn't origins an additional peak, after the total elution.

Figure 3 represents the absorption spectra of standard cyanocobalamin (A) and the red fraction obtained from cells of M.barkeri grown in a medium with 16.2 mg/l of cobalt chloride and sulfide plus cysteine as reducing agents (B). It could be observed, in this case, one deviation of spectrum B to higher wavelengths. It is possible that the form of vitamin B12 extracted with the reflux method was the dicyano form (dicyanocobalamin), because it was performed in presence of cyanide in excess, justifying the deviation observed in spectrum B.

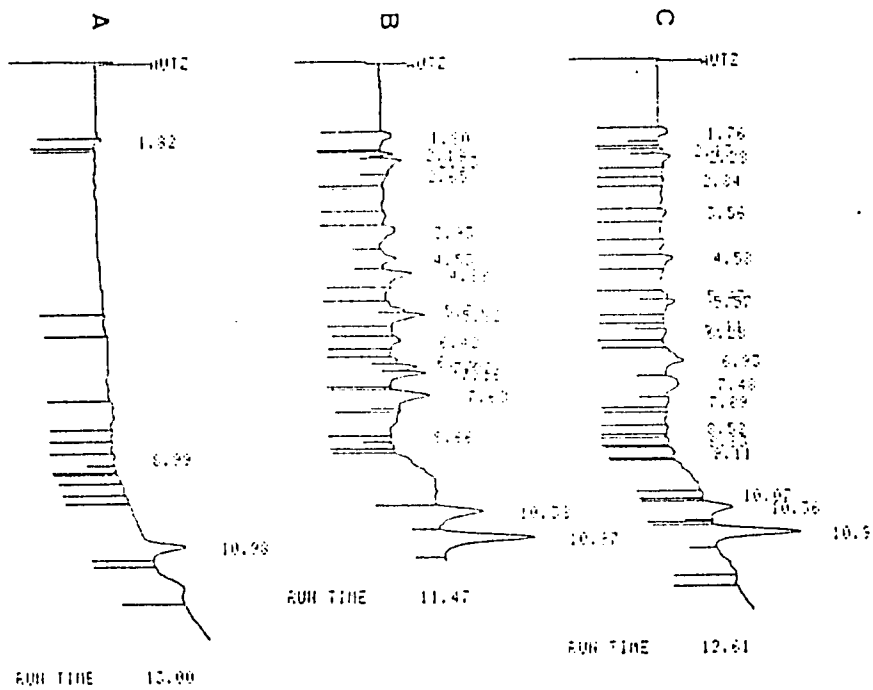
Table II indicates the results obtained for the conversion of corrinoids into vitamin B12 and the influence of cobalt concentration in the content of these compounds, extracted from cells grown in a medium with sulfide plus cysteine or sole cysteine as sulfur sources and reducing agents. These data show a parallel increase with the cobalt chloride concentration, attaining the maximal values (82% of total corrinoids and 10% for their conversion into vitamin B12) at 10.8 mg/l of cobalt. However, with cysteine as the sole reducing agent, the corrinoids content and their conversion into vitamin B12 were higher at 16.2 mg/l of cobalt chloride.

FIG.3. UV/VIS spectra. (A) Standard solution of vitamin B12 (cyanocobalamin). (B) Red fraction with corrinoids obtained from culture media with 16.2 mg/l of cobalt in presence of sulfide. (C) Red fraction with corrinoids extracted in the final of the 2nd transfer to a medium supplemented with 5,6=DBI.



N.D.- Not determinated.

FIG.4. Separation of corrinoids by HPLC (detection at 550 nm, AUFS = 0.05). (A) Standard solution of vitamin B12. (B) Red fraction containing corrinoids, obtained after the 2nd transfer to 5.6-DBI supplemented medium. (C) Sample of (B) diluted in standard vitamin.



The base shift is caused by the change in the mobile phase due to the linear gradient of the elution.

FIG.5. Cellular content of vitamin B12 after each transfer to the supplemented medium.

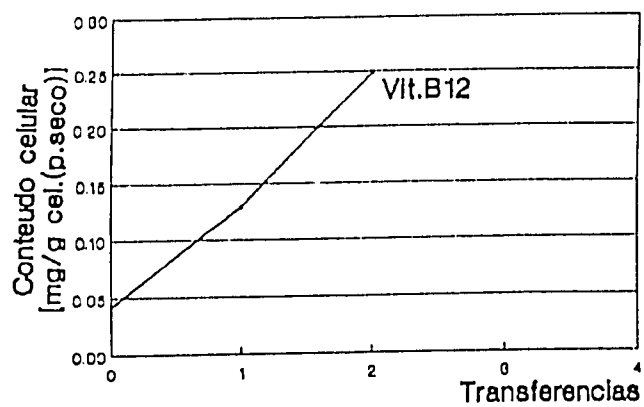


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Figure 5 represents a variation of the cell content in vitamin B12 after each transfer to a new 5,6-DBI supplemented medium. The concentration of vitamin B12 extracted from the cells increase significantly after each transfer.

The UV/VIS absorption spectra obtained for the red fractions, containing corrinoids, presents deviations of the wavelenghts of the maximal peaks for higher values than those of the standard solution of vitamin B12 (cyanocobalamin), like the example of Figure 3. The dicyano form of vitamin B12 (dicyanocobalamin) probably is the predominant form in the red fraction extracted by the reflux method in presence of cyanide in excess from cells grown in the supplemented medium, as it could be evidenced by the analysis of Table IV and Figure 3. The hypothesis that the presence of Factor III could origins the deviation of wavelenghts observed must be excluded by the analysis of Table V because the maximal absorption peaks of this corrinoid are lower than those presented by vitamin B12, either in the cyano or in dicyano forms, and by the red fraction, contaning corrinoids. Values obtained for the conversion of corrinoids in vitamin B12, after each transfer to the supplemented medium could be compared through the analysis of Table IV. This analysis of Table IV indicates the content of total corrinoids and their conversion in vitamin B12 increase significantly with the transfers to 5,6-DBI supplemented medium, attaining the maximal values of 401% and 28%, respectively. Figure 5 confirms these results giving evidence to the increase of the cellular vitamin B12 content. Conversion of 28% of total corrinoids in vitamin B12 is too higher when compared to the values obtained for the cobalt supplementation, suggesting that 5,6-DBI supplementation will be more effective and that bacteria grown in a supplemented medium with a base moiety tends to incorporate that base for the biosynthesis of cobamides, in general.

TABLE III. Cell biomass recovered after each transfer to the medium supplemented with 5,6-DBI 200 μM, growth rates and doubling times (T_d).

5,6-DBI CONC. (μM)	TRANSFERS	CELL BIOMASS [g(ww)/l]	GROWTH RATE (h^{-1})	T_d (h)
0	--	5.0	0.042	17
200	0	2.0	N.D.	N.D.
	1	4.0	0.029	24
	2	5.0	0.031	23

TABLE IV. Conversion of corrinoids in vitamin B12 by M.barkeri and the respective content after each transfer to the 5,6-DBI supplemented medium.

5,6-DBI CONC. (μM)	TRANSFERS	TOTAL CORRINOIDS (%)	VITAMIN B12 (% OF TOTAL CORRINOIDS)
0	--	100	--
200	0	133	14
	1	326	18
	2	401	28

TABLE V. Maximal absorption peaks relative to the red fraction, containing corrinoids, to the standard solution of vitamin B12 (cyanocobalamin), to the cyano (Bzm,CN^-) and dicyano (CN^-,CN^-) forms, and to Factor III, repported in literature.

CORRINOID	MAXIMAL ABSORPTION PEAKS (nm)		
Red fraction	574	540	365
Vit. B12 (Standard)	550	518	360
Vit. B12 (Dicyano)	580	540	367[a]
	580	540	368[b]
Vit. B12 (Cyano)	550	520	361[a]
Factor III	525	486	311[b]

[a] Lino,A.R. (1986)
[b] Friedrich,W. (1975)

CONCLUSIONS

Methanogenesis and growth cell of M.barkeri 227 were not inhibited when inorganic sulfide was eliminated and cysteine was duplicated in the culture medium. Cysteine could be used to substitute completely for sulfide. The use of cysteine as the sole sulfur source allowed to increase the soluble cobalt concentration in the media up to 6 fold (6 x 2.7 mg/l) without precipitation.

The increase of cobalt in the culture media caused an increase in the total corrinoids content and in their conversion in vitamin B12 attaining the maximal values at 10.8 mg/l of $CoCl_2$. Without inorganic sulfide, the corrinoids conversion into vitamin B12 don't depend of the cobalt concentration in the growth medium.

The vitamin B12 contents, very low with the basal medium, increased up to 0.249 mg/g dry cell at the end of the second transfer on 5,6-DBI-supplemented medium. DBI-supplementation is effective for the synthesis of vitamin B12 suggesting that the dimethylated benzimidazole ring is directly incorporated into the corrin ring. The capacity of DBI-incorporation is increasing from one cell generation to the other. This phenomena means that the cell is "overproducing" vitamin B12 under the experimental conditions.

The UV/VIS spectra suggested that the corrinoid extrated are mainly in the much more stable dicyano form. In the HPLC analysis the identification of the vitamin B12 peak was done by the retention time and confirmed by the addition of external standard to the example.

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BIOCHEMISTRY OF ACETATE AND CO_2 ASSIMILATION IN M. barkeri

I. INTRODUCTION

The study of the regulation mechanisms at the level of the CO_2 fixation by methanogenic bacteria is important. They could be considered as depolluting agents due to the fact that either CO as CO_2 are polluting sources that are routinely evolved to the atmosphere and they could be considered as growth substrate for these organisms. In addition, methane production by methanogenic bacteria is an important chemical energy source because of its direct combustion and food value: some microorganisms able to metabolize methane are considered as important protein and carbohydrates sources.

Methanogenic bacteria, specially Methanosarcina barkeri, produce a high level of corrinoids, such as vitamin B12, but its biotechnological application has been limited by the scant experimental research in fundamental areas of physiology and biochemistry. A pre-requisite to study the biotechnological potential of M.barkeri is the basic knowledge of its metabolism.

Methanogens may derive the cell carbon biosynthesis from precursors of the methanogenesis. However, although many of the methanogens are autotrophs, they do not fix CO_2 by the Calvin cycle unlike most photosynthetic and chemotrophic organisms, and they have a modified reversed citric acid cycle (Daniels et al., 1984), known as the activated acetic acid pathway or acetyl-CoA pathway (Fuchs & Stupperich, 1986; Kräutler, 1988).

A metabolically versatile methanogenic bacteria, M.barkeri, is able to disproportionate acetate or methanol to CO_2 and CH_4. There is good evidence that acetate is activated and then cleaved

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to a bound CO and a bound methyl group. The oxidation of the carbonyl to CO_2 is coupled to the reduction of the methyl group, which is transferred to coenzyme M prior to reduction. Acetate in Methanosarcina may be activated to acetyl-CoA by the combined action of acetate kinase (EC 2.7.2.1) and phosphotransacetylase (EC 2.3.1.8), which requires ATP consumption. Acetyl-CoA is an intermediate, formed from one CO_2 via a tetrahydropteridine-bound 1-carbon unit (methyl group of acetate), and from another CO_2 via a bound carbon monoxide (carboxyl group of acetate). The most characteristic and complex enzyme involved in acetyl-CoA synthesis is carbon monoxide dehydrogenase ('acetyl-CoA synthase'). The enzymes of this acetyl-CoA pathway not only participate in (1) acetate synthesis but also mediate (2) acetyl-CoA oxidation; (3) acetate disproportionation to CO_2 and CH_4; (4) autotrophic CO_2 fixation; (5) assimilation and/or dissimilation of 1-carbon compounds; (6) CO oxidation to CO_2 (Fuchs, 1986).

Acetyl-CoA is reductively carboxylated to pyruvate, a reaction catalyzed by pyruvate synthase and requiring coenzyme F_{420} as electron donor. The carbon source for the amino acids biosynthesis consists of intermediates of the incomplete tricarboxylic acid (TCA) cycle, as oxaloacetate (OAA) and α-ketoglutarate, being glutamine synthase a key-enzyme submitted to regulation by covalent modification or by feedback inhibition of another biosynthetic pathways products. Hence, autotrophic CO_2 fixation involves three CO_2 fixing enzymes in methanogens: CO_2 reduction to CO (CO dehydrogenase), reductive carboxylation of acetyl-CoA to pyruvate (pyruvate synthase EC 1.2.7.1 and EC

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1.2.99.1), and phosphoenolpyruvate (PEP) carboxylation to OAA (PEP carboxylase EC 4.1.1.31 or other enzyme).

Kenealy & Zeikus (1982) suggest that (i) reactions of energy metabolism and cell carbon synthesis are unified in methanogens; (ii) the synthesis of a two-carbon cell intermediate is derived from a C-1 + C-1 condensation reaction involving a methyl-corrinoid; (iii) M.barkeri synthesizes acetate from CO_2 or methanol or both, and it is readily interconvertible with acetyl-CoA; and (iv) carbon assimilation mechanisms, in M.barkeri, share a common pathway involving one-carbon carrier-bound intermediates for the synthesis of methane and a two-carbon cell precursor. So, M.barkeri displays high metabolic efficiency in cell carbon synthesis because common biochemical intermediates are used in both catabolic and anabolic one-carbon transformations. Fuchs & Stupperich (1986) suggested three different mechanisms: (1) CO transfer to the cobalt-bound methyl via CO insertion; (2) CH_3 migration to CO bound to the nickel (CO dehydrogenase) and addition to CO. (3) Both CO and CH_3 are combined at a third center. In any case an activated acetate molecule must be formed which is released by thiolysis with CoASH, rather than by hydrolysis, to give acetyl-CoA. The intermediate C_2 compound and the mechanism of its formation are a matter of speculation.

The role of ATP in this process is unknown. It is probably required only in catalytic rather than in stoichiometric amounts. Under standard conditions, the reduction of $2CO_2$ with $4H_2$ to acetic acid is strongly exergonic and may allow for the synthesis of ATP. It is possible that CO dehydrogenase is membrane-associated and electron transport in CO metabolism involves a

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chemiosmotic mechanism. The electron carriers involved in the CO dehydrogenase reaction may depend on whether the physiological role of CO dehydrogenase is to catalyze acetyl-CoA synthesis or oxidation and which exogenous electron donor/acceptor is available (Fuchs, 1986).

The acetyl-CoA pathway involves the participation of electron and 1-carbon units carrying coenzymes. In methanogenic bacteria several one-carbon carriers and electron carriers have been discovered recently and some structures have been elucidated, such as methanofuran, methanopterin which is the counterpart of folate in methanogens, and coenzyme M. All 3 play a role in CH_4 formation and in acetate disproportionation to CO_2 and CH_4, and methanofuran and methanopterin function in autotrophic acetyl-CoA synthesis in these bacteria. Little information is known about the cellular localization, organization and regulation of this complex acetyl-CoA synthesizing system (Fuchs, 1986).

The acetyl-CoA pathway is a major pathway for CO_2 assimilation in acetogens, methanogens and perhaps some sulfate reducers. The role of the acetyl-CoA pathway in biological processes apparently is large. On a molar basis, about 5% of the carbon fixed by photosynthesis is converted to atmospheric methane. Much of the methane produced in swamps, sediments in the ocean and in the forest beneath the surface is reoxidized by aerobic organisms to CO_2 when the methane reaches aerobic conditions at the surface. Therefore, the role of methanogens in recycling carbon via acetate and C_1 compounds is much larger than

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indicated by the methane content of the atmosphere (Wood, Ragsdale & Pezacka, 1986). The course taken by the ("ancient enzymatic machinery" of the) "acetyl-CoA pathway" thus also exemplifies a remarkable and efficient path for the reduction of carbon dioxide, of interest in view of the ongoing search for ways to synthesize basic organic chemicals and "fuels" from carbon dioxide in an economic way (Kräutler, 1988).

The main study of this report involves the enzymology of the acetate activation and CO_2 assimilation by M.barkeri 227.

II. EXPERIMENTAL METHODS

II.1. CELL GROWTH

Methanosarcina barkeri strain 227 was the microorganism used in this work. A revertent strain (REV 42) was obtained from a mutant. This one was isolated from a culture medium with fluoroacetate and used methanol as growth substrate. The revertent strain isolated was capable of utilize acetate as growth substrate and was resistant to fluoroacetate.

Growth of M.barkeri 227 was followed by methane production analysis by gas chromatography (Porapak-QS column, Thermical Conductivity Detector - TCD). Pure cultures were monitorized by light microscopy simultaneously with microscopy of fluorescence (Olympus BH-2).

Cells were grown on a complex medium adaptated from Mah (1980), at 37°C and pH 7.0. Culture media contained yeast extract (OXOID), Trypticase peptone (BBL), Bicarbonate buffer, Mah's trace minerals solution and L-cysteine hydrochloride (MERCK) plus sodium sulfide (M&B) as the sulfur source and reducing agents.

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Cells were grown on methanol 200 mM, acetate 50 mM or H_2/CO_2 (80:20) as the carbon and energy source. All manipulations of media and cultures were carried out under O_2-free atmosphere of N_2 (100%) gas, as the modified technique of Hungate (Wolfe, 1971). Gas phase was N_2/CO_2 (70:30), except for autotrophically grown cells (H_2/CO_2).

Cell biomass was obtained by centrifugation of the growth medium at 4,000 rpm for 10 min. (Burkard, rotor 009) and was freezed at -20°C for further utilization.

II.2. CELL RUPTURE

Cell suspensions were passed two times through a French-pressure cell at 20,000 psi, under an atmosphere of N_2 (100%). The protein extracts were obtained by centrifugation at 18,000 rpm, for 20 min. at 4°C (Sorvall RC5C).

Protein levels in cell-free extracts were quantified by the dye - binding procedure of Bradford (1976).

II.3. ENZYME ASSAYS

Acetate kinase (EC 2.7.2.1) activity in the direction of acetyl-P, depends on the coupling to the NADH oxidation reaction by pyruvate kinase and lactate dehydrogenase activities. Enzymatic activity of acetate kinase was corrected for the background ATPase activity before the addition of the substrate, acetate, to the reaction mixture. NADH oxidation was monitorized at 340 nm with a spectrophotometer (Spectronic 601, Milton Roy Co.), under anaerobical conditions.

Acetate kinase activity was studied with protein extracts

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obtained from cultures of M.barkeri 227 grown on H_2/CO_2, acetate or methanol as carbon and energy sources. NADH oxidation was followed under an atmosphere of N_2 100% or under an atmosphere of H_2/CO_2 (80:20).

Acetate kinase activity was studied on a revertent strain of M.barkeri (REV 42) grown on a complex medium, containing acetate as energy and carbon source.

Phosphotransacetylase (EC 2.3.1.8) activity was followed by the acetyl-CoA synthesis from acetyl-P plus coenzyme A at 233 nm (Spectronic 601, Milton Roy Co.), under anaerobical conditions at 25° and 37°C. Protein extracts were obtained from cultures of M.barkeri 227 grown on methanol as carbon and energy source.

The phosphotransacetylase capacity to synthesize acetyl-CoA without addition of coenzyme A to the reaction mixture was assayed, under an N_2 (100%) and an H_2/CO_2 (80:20) atmosphere, at 25° and 37°C.

Synthesis of acetyl-CoA was followed without addition of acetyl-P and coenzyme A to the reaction mixture, under an N_2 (100%) and an H_2/CO_2 (80:20) atmosphere, at 25°C.

A last analysis was assayed following the NADH oxidation at 340 nm from the acetyl-P addition to the reaction mixture, under an N_2 (100%) and an H_2/CO_2 (80:20) atmosphere, at 25°C.

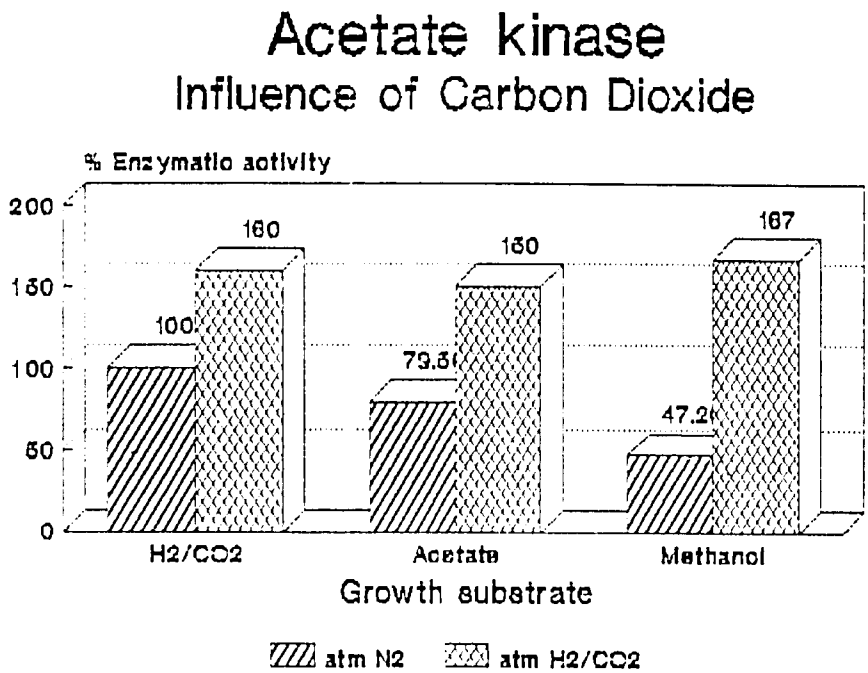

FIG.III.1. Acetate kinase activity relative to growth substrate of M.barkeri 227, at 37°C. N_2 H_2/CO_2 atmosphere of reaction mixture.

TABLE 1. Specific activities, at 37°C, of acetate kinase on two strains of M.barkeri.

GROWTH SUBSTRATE	PROTEIN EXTRACT	ACETATE KINASE ACTIVITY (nmolNADHox./min./mg prot.)
Acetate	REV 42	0.451
	227	3.382

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TABLE 2. Phosphotransacetylase activity, at 25° and 37°C, in crude extracts obtained from cultures of M.barkeri 227 grown on methanol. AcP, acetyl-P; CoA, coenzyme A.

REACTION MIXTURE	PHOSPHOTRANSACETYLASE ACTIVITY (%)	
	25°C	37°C
+AcP/+CoA	66.98	100.00
+AcP/-CoA	1.40	1.02
+AcP/-CoA/+CO_2	5.02	5.03
-AcP/-CoA	0.76	N.D.[a]
-AcP/-CoA/+CO_2	6.80	N.D.
+AcP/-CoA/+NADH	0.35	N.D.
+AcP/-CoA/+NADH/+CO_2	0.47	N.D.

[a] N.D., not determinated.

IV. DISCUSSION

Analysing Table 1 is possible to observe that acetate kinase activity on the strain revertent of M.barkeri (REV 42), grown on acetate and obtained from a mutant which is not able to metabolize that substrate, is lower than the one presented by the wild strain of M.barkeri 227. Smith & Lequerica (1985) have shown that the mutation has multiple effects on acetate utilization and the properties of the mutant are consistent with a loss of permeability of M.barkeri to acetate or with a defect in an activation step required for methanogenesis and carbon assimilation from acetate, involving acetate kinase. In addition, acetate diffusion into the cells is the principal means of entry, and loss of permeability to acetate suggests that diffusion is

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facilitated in strain 227 but not in fluoroacetate-resistant strain.

One mechanism proposed to explain the increase on specific enzymatic activities in presence of CO_2 is presented in figure IV.1.

Since the enzymatic extract used is a crude extract all enzymes and cofactors are present, and with a CO_2 atmosphere we could expect that phosphotransacetylase converts acetyl-P into acetyl-CoA and this molecule is carboxylated into pyruvate by the pyruvate synthase activity with the subsequent oxidation of a NADH molecule. In addition, we have observed the formation of acetyl-CoA in a reaction mixture which consists only of crude extract and a H_2/CO_2 atmosphere (Table 2), being this fact one evidence for the CO dehydrogenase activity.

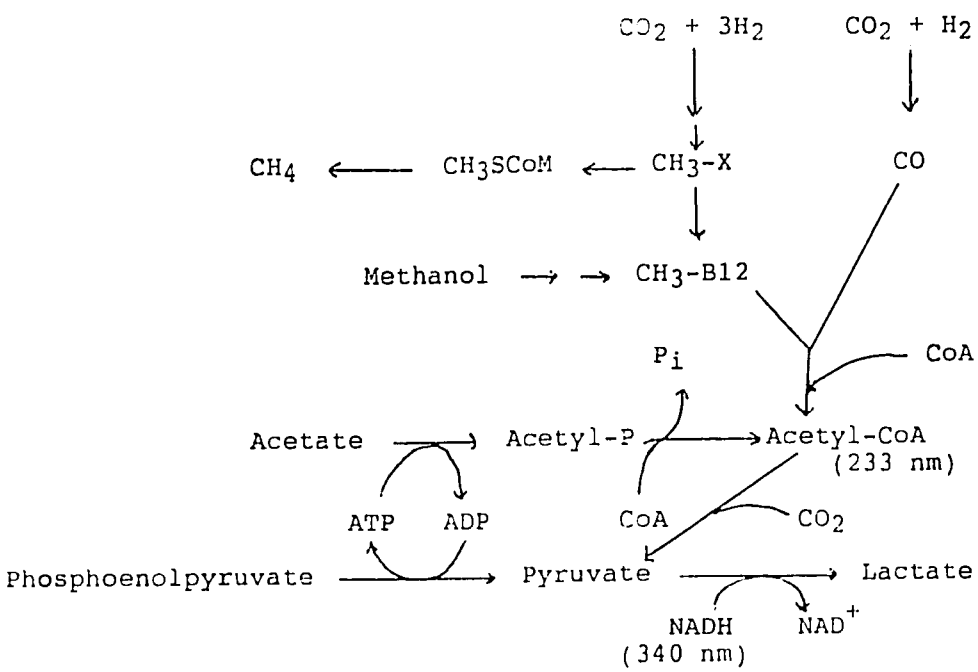

FIG.IV.1. Proposed mechanism for acetyl-CoA synthesis by CO_2 activation (Duarte & Eusébio, 1989).

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Analysis of figure IV.1 indicates that acetate kinase levels found were highly on extracts obtained from cultures of M.barkeri 227 grown on H_2/CO_2 as carbon and energy source. Identical results were obtained by Kenealy & Zeikus (1982) for acetate kinase activity between different growth substrates. It could be observed a significantly increase on the levels of acetate kinase activity when H_2/CO_2 is added to reaction mixtures. These data represent the important physiological role of carbon dioxide on cell carbon synthesis pathways, which involves CO_2 fixation enzymes of M.barkeri, as CO dehydrogenase and pyruvate synthase. concentrações não saturantes. Table 2 indicates that synthesis of acetyl-CoA is still 1% without addition of coenzyme A. With CO_2 pyruvate and lactate were formed, with concomitant NADH oxidation. Under an atmosphere of CO_2, CO dehydrogenase synthesizes acetyl-CoA, increasing 5x NADH oxidation values. On this way, it could be observed that phosphotransacetylase levels were almost absent when there is not any addition of coenzyme A and acetyl-P to the reaction mixture, but increase in presence of CO_2. In addition, there is also NADH oxidation, but these values are too reduced.

These results confirm the existence of an enzymatic complex of carbon monoxide dehydrogenase that synthesizes acetyl-CoA, which must also act as the methyl-corrinoid binder.

V. REFERENCES

Bradford, M. (1976). Anal. Biochem. 72:248-254.

Daniels,L.; Sparling,R.; Sprott,G.D. (1984). B.B.A. 768:113-163.

Duarte,J.C. & Eusébio,A. (1989). 19th FEBS Meeting, Rome.

Fuchs,G. (1986).FEMS Microbiol Rev., 39:181-213.

Fuchs,G. & Stupperich,E. (1986). Syst.Appl.Microbiol.,7:364-369.

Kenealy,W.R. & Zeikus,J.G. (1982). J.Bacteriol., 151(2):932-941.

Kräutler,B. (1988). Chimia 42(3):91-94.

Mah,R.A. (1980).Proceedings of International Symposium on Biogas, Microalgae & Livestock Wastes.

Smith,M.R. & Lequerica,J.L. (1985). J.Bacteriol., 164(2):618-625

Wolfe,R.S. (1971). Advances in Microbial Physiology, vol.6. Academic Press, New York.

Wood,H.G.; Ragsdale,S.W.; Pezacka,E. (1986). TIBS 11:14-18.

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7
ISOLATION AND IDENTIFICATION OF LIPIDS

INTRODUCTION

The membrane lipids of Archaebacteria represent a new class of lipids and because of that they are important for classification and characterization of this kingdom. They reflect the unique composition and metabolism of the diverse genera of the Archabacteria.

The group of the methanogens seems to be the one with greater diversity of structuires of these lipid molecules. They englobe the diether lipids typical from the halophiles (see fig. 1a; 1b; 1c) as those from the thermophile (tetraethers) (see fig. 2). They include still the tetritol (substituting for glycerol) detected on Methanosarcina (see fig 1e), and that of the macrocyclic diether of glycerol (see fig 1d) which is only found on Methanococcus jannaschii.

Therefore we considered of importances to develop methodologies for lipid isolation and characterization.

Details on their isolation, identification, synthesis and metabolism have been recently reviewed (in M. De Rosa and A. Gambacorta; 1988; Prog. Lipid Res.; Vol 27; pg 153-175).

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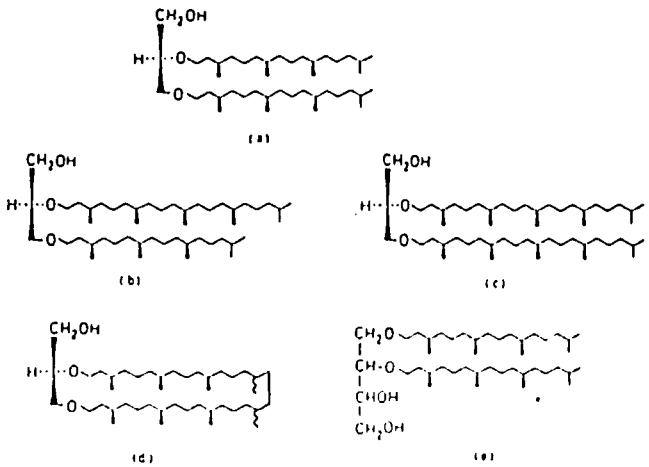

FIG. 1. Isoprenoid diethers basic components of some complex lipids of archaebacteria. a: 2,3-di-*O*-phytanyl-*sn*-glycerol; b: 2-*O*-sesterpanyl-3-*O*-phytanyl-*sn*-glycerol; c: 2,3-di-*O*-sesterpanyl-*sn*-glycerol; d: macrocyclic diether; e: tetritol-dyphytanyl-diether.

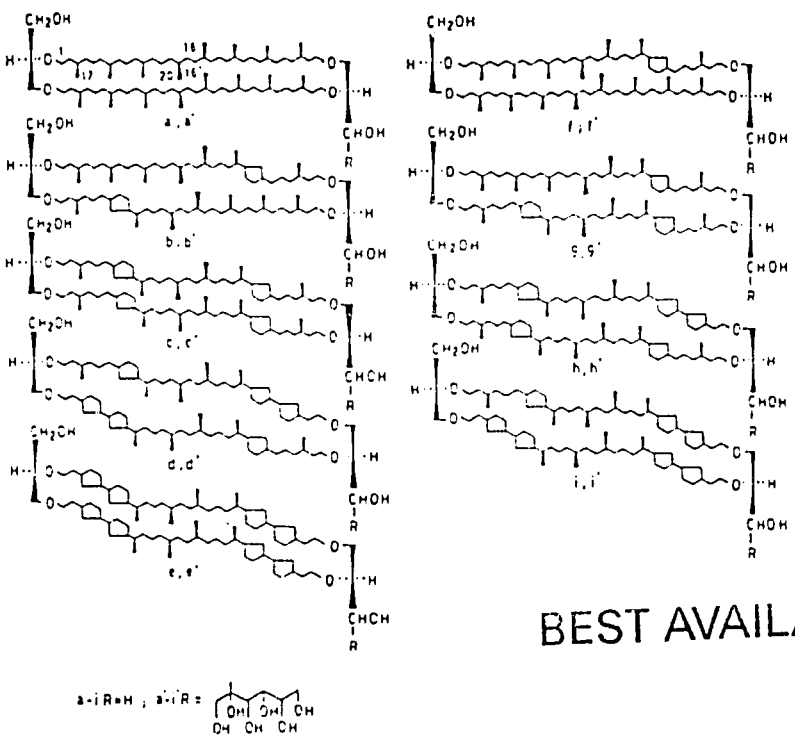

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FIG. 2. Isoprenoid tetraethers basic components of some complex lipids in archaebacteria. a-i: Glycerol-dialkyl-glycerol tetraethers (GDGT); a'-i': Glycerol-dialkyl-ponitol tetraethers (GDNT).

Materials and Methods

The initial step was the production of biomass of *Methanosarcina barkeri* 227. For this purpose we cultivated the cells in cultures of 1500 ml of α medium supplemented with 15 ml of Na_2S solution (2.5%) and 200 mM of methanol. The inoculae was added in a proportion of 1:100.

The cells were collected at the stationary phase; washed with desionized water and centrifuged (3000 r.p.m. for 10 min.). After this, the biomass was freeze-dried and weighted.

Two processes of lipid extraction were tested:

a) By soxhlet extraction;

b) By the method of Blight and Dyer.

The soxhlet extraction method was the most used. The extration solvent was trichloromethane/methanol (2:1 v/v) for 12-24 hours.

The lipid extract was then washed with water in a proportion of 1/4 for the total volume of the extraction solvent. The apolar phase was collected and the solvent evaporated.

The total lipids extracted (T.L.E.) was weighted and analysed with spectrometry of infra-red (I.R.).

We tryed to separate the polar lipids from the apolar lipids using solvents of diferent polarity. The polar lipids were colected in nethanol and the apolar lipids in n-hexano. The two fractions were than dried and weighted.

The extract was treated with methanolic HCl (0.7) for 6h under reflux, and dried in vacuum.

The chloroform-soluble fraction of the methanolised lipid was fractionated in a silica-gel column of 20cm length and 1cm inner diameter. The sequence of eluents is in table I.

ELUENT	VOLUME (ml)	FRACTIONS (number)
Petroleum benzine	63	1 - 24
Petroleum benzine/ /Diethylether (85:15)	120	25 - 50
" (6:4)	90	51 - 67
" (1:1)	480	68 - 150
" (3:7)	20	151 - 154
Petroleum benzine/ /Methanol (98:2)	50	155 - 169
" (95:5)	5	170
Methanol	Column wash	171

TABLE I. Eluent variation used in column chromatography.

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The analysis of fractions collected in the column was performed by thin layer chromatography (T.L.C.). The fractions were joined by the similarities in the thin layer chromatogram.

Two fractions that seems to be of great purity was selected for partial characterization.

The characteristics includes:

1) Determination of R_f by T.L.C.

2) Nuclear Magnetic Ressonance (NMR) Spectrometry

RESULTS

The characterization of the M.barkeri 227 cultures was made by their growth graphic. The specific growth rate (μ) and duplication time (T_d) were calculated.

$\mu = 5.74 \times 10E\text{-}02\ h^{-1}$ $T_d = 12.07\ h$

The average of biomass produced in each culture is 1.3136 g (d.w.), about 25% of the non-dryed biomass (5.1934 g).

The extraction by soxhlet gave the best rates of extraction (Table II).

	1	2	3	4	5
Extraction Rate (%)	5.50	3.55	2.30	1.43	8.73
Extraction time (h)	12	24	±24	24	24

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TABLE II. Rates of lipid extraction by soxhlet refluxe for different times of extraction.

The average rate of extraction (% of dry cells) was 4.302 ± 2.603.

One extraction by the method of Blight and Dyer gave a rate of 1.91% (% of dry cells). However, more assays must be performed to have conclusive results.

The infra-red spectrum of the T.L.E. showed the bands in the Table III.

Absorption frequencies (cm^{-1})	Chemical functions
3400 - 3200	Hydroxyl (OH)
2960, 2920, 2850, 1460	Saturated hydrocarbon chains (CH_3, CH_2)
1740 - 1720	C=O
1375 - 1365	Isopropyl [C-$(CH_3)_2$] or methyl (C-CH_3)
1260	P=O
1110 - 1010:	
i) 1100 - 1110	i) Aliphatic ether (C-O-C)
ii) 1100	ii) P-O^-
iii) 1090 - 1060	iii) P-O-C
iv) 1045	iv) Primary hydroxyl (C-OH)

TABLE III. Characterization of TLE infra-red absorption bands, before and after methanolysis.

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The spectrum is very similar to that described by many authors (1,2,3,4,5,6), revealing the presence of lipid components constituted by a long-chain hidrocarbon joined to an alcohol (glicerol) by ether bond. The presence of phosphate groups on acidic groups is probably due to substituents of some polar lipids.

The unique separation of polar lipids from the apolar ones gave the next results: polar fraction = 54.1% (d.w.); apolar fraction = 54.5% (d.w.).

After the fractionation of the TLE in the column two fractions were selected and designated F 15 and F 17. The TLC of these fractions gave the following Rf (the optimized system of eluents was: petroleum benzine (60 - 80°C) / diethyl ether (1 : 1 v / v) and trichloromethane / diethyl ether (4 : 6, v / v):

F 15: Rf = 0.61

F 17: Rf = 0.41

Due to the low sensitivity of the I.R. spectrophotometer and the very small amount of each fraction the I.R. spectrum was not performed.

The results of the NMR spectrum of F 15 are given in table IV.

Chemical shift δ	Multiplicity	Group	Found integral	Calculated integral
0.83-0.88	Triplet	CH_3	22	22
1.04-1.40	Multiplet	CH_2;CH	52	5
2.20-2.40	Triplet	H_2C-0H	1	1
3.40-3.76	Multiplet	CH_2-O-R; HC-O	11	11

TABLE IV. Analysis of ^{1}H-NMR spectrum of F 15 isolated from M. barker. 227.

This results when compared with that of others authors (1,3,4,6) seems very closely with them. We assumed that the component of F_{15} is a glycerol diether (24 -CH_3; 52H -CH_2,-CH; 1H -C-OH; 9H -HC-O, CH_2-O-R).

The F_{17} gave a NMR spectum very poor and because of that little information is obtained about the component. We only observe the presence of the bands 0.83-0.88δ (-CH_3); 1-1.4 (-CH_2; -CH) and a little multiplet at 3.4-3.6 δ (HC-O-R; H_2C-O-R).

No solid conclusions are possible about the true structure of this compound.

In general this work must be considered as a starting point for the elucidation of the lipids from our strain.

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REFERENCES

1. De Rosa, M. Gambacorta, A.; Lanzotti, V.; Trincone, A.; Harris, J.E. and Grant, W.D.; 1986; Biochimica et Biophysica Acta; Vol 875; 487-492.

2. De Rosa, M. Gambacorta, A; 1988; Prog. Lipid Res.; Vol 27; 153-175.

3. Kushwaha, S.C.; Kates, M.; Sprott, G.D. and Smith, I.C.P.; 1981; B.B.A.; Vol. 664; 156-173.

4. Langworthy, T.A.; 1977; B.B.A.; Vol 487; 37-50.

5. Makula, R.A. and Singer, M.E.; 1978; Bioch. and Bioph. Res. Comm.; Vol 82; Nº 2; 716-722.

6. Morii, H.; Nishihara, M.; Ohga, M. and Koga, Y.; 1986; J. of Lipid Res.; Vol 27; 724-730.

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METHANOGENIC THERMOPHILIC BACTERIA FROM FURNAS-AÇORES

The ecology of volcanic sulfatara environments is now being widely studied and many new microorganisms from these habitats were found (1, 2). Microorganisms from these habitats are quickly enlarging the Archaebacteria Kingdom.

S. Miguel island in Açores is widely known for its Furnas, with its hot emanations of sulfurous gases and water vapours. Samples have been collected there with the objective of isolating thermophilic methanogenic bacteria.

From the pool of samples collected four methanogenic strains were already isolated in pure culture growing in H_2/CO_2. All the isolates are thermophilic with optimal growth on the 65-70°C range. The optimal pH is 7.0 to 7.5. They are autotrophic, utilizing so far only H_2/CO_2 as energy and carbon sources.

An enrichment culture growing on acetate was also isolated. This showed to be a consortium of a methanogenic and a non-methanogenic bacterium (non-fluorescent). In pure culture the methanogen use only H_2/CO_2. The physiological relationships within the consortium are now under investigation.

INTRODUCTION

Archaebacteria predominance on the living world of high temperatures (above 80°C) is almost absolute. Considering this extraordinary feature it is reasonable to expect that this group of bacteria may be the base of new developments in biotechnological applications.

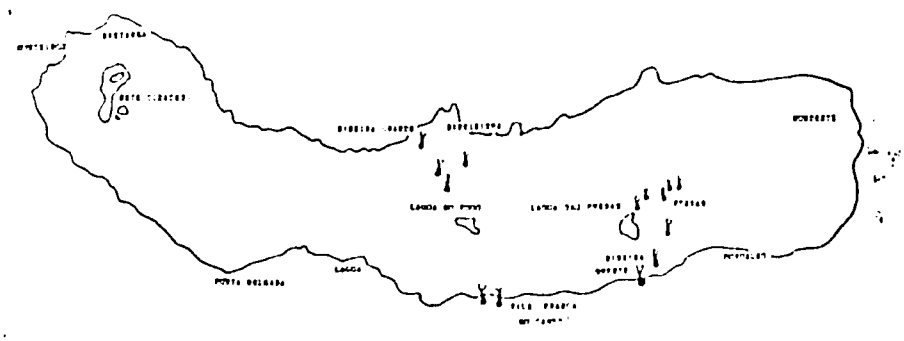


Figure 1. S. Miguel island and its furnas

S. Miguel island in Açores has a few sulfatara vents and hot ponds locally known as "Furnas" (fig. 1).
It is assumed that these archaebacteria, a little existing all over are in some way the remainders of an ancient micropopulation that once began life on Earth. In the same atmosphere, with the same temperatures, they kept for us to learn ancient ways of stabilizing proteins in "extreme" conditions, of degrading "toxic" agents, of living symbiotically, of performing life 4 billion years ago in a way that we could not imagine in the twentieth century.

Being aware with the sampling requisites and developing new anaerobic techniques for isolating and culturing these bacteria authors are in the last decade discovering large amounts of entirely new microorganisms.

METHODS

The samples were anaerobically collected in several locations where the temperatures varied between 65-100°C. We used serum bottles which were completely filled and butyl rubber stoppered. To maintain the low redox potential we added to each flask a sodium sulfide solution to a final concentration of 1,5mM. The normal Hungate technique, modified by Balch, was used to enrich, isolate and cultivate the different strains.

Enrichment cultures were done with 1% horse cecum fluid, acetate and H_2/CO_2 as main substrates, which were passed to roll tubes after serial dilutions till only one type of colonies was obtained. Then one was picked to inoculate in liquid medium.
To detect the presence of methanogenic bacteria was used the methane quantification by gas chromatography (TCD detector, Porapak column) and the epifluorescence

microscopy both to detect fluorescent colonies in the roll tubes and to check for the purity of the liquid cultures.

RESULTS

From the pool of samples collected were isolated already four methanogenic strains considered to be in pure culture and growing in H_2/CO_2.

The isolates are fast growing thermophilic methanogens. All bacilli, appearing as medium size, thick rods, green fluorescent at 420 nm excitation wavelength.

They exhibit different morphologies depending on the cultural conditions and age of the cultures. In the early stages of growth they appear like small rods as an homogeneous population while in the late growth phases they become more heterogeneous in size. Increasing temperature causes changes from linear rods to somewhat curved or even twisted forms.

Kinetic studies were done to determine the optimal growth temperature and the optimal growth pH, and the results obtained so far indicate a range of 65-75°C and a neutral pH for optimum growth. The Marburg strain of Methanobacterium thermoautotrophicum was used as control because of the morphologic resemblance with the methanogenic isolates, although they exhibit different optimal temperatures for growth.

Isolates which were growing on acetate alone were also obtained. However, purity tests revealed that we were dealing with a consortium of two strains, one being a non-methanogenic bacterium, rod shaped and non fluorescent and the other a methanogenic one, green fluorescent.

On the examination of the colonies it was observed that they consisted of a large and translucid colony with the other showing as a spot in the center.

From this consortium, a methanogenic bacterium was isolated which only grew on H_2/CO_2, with an optimum growth temperature of 65°C and an optimum growth pH of 7.5 .

Consortia of this type have only been described before by Zinder S.H. (3) where the acetate utilization by the nonmethanogenic bacterium was used to produce the H_2/CO_2 that the methanogen needed.

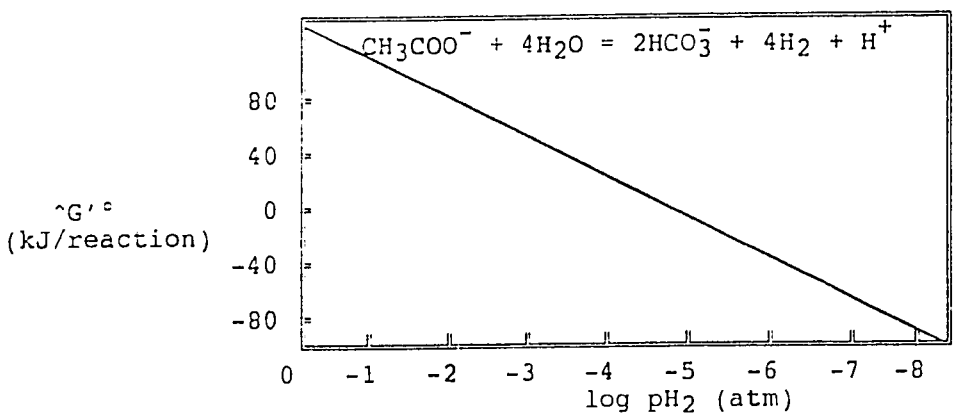
There have been two mechanisms proposed for anaerobic acetate degradation. One utilized by the aceticlastic bacteria (Methanosarcina, Methanotrix) as demonstrated with isotope labeling.

Another, in two steps, first purposed by Barker and Van Niel (1936), which was questioned till a few years ago but now being claimed as a possible mechanism for acetate metabolism on thermophilic syntrophic cocultures.

Mechanism I : Acetate "splitting"	^G'°(kJ/reaction)
$CH_3COO^- + H_2O = CH_4 + HCO_3^-$	-31.0
Mechanism II : Acetate oxidation	
$CH_3COO^- + 4H_2O = 2HCO_3^- + 4H_2 + H^+$	+104.6
$4H_2 + HCO_3^- + H^+ = CH_4 + 3H_2O$	-135.6
$CH_3COO^- + H_2O = CH_4 + HCO_3^-$	-31.0

When acetate was not present the methanogen grew very poorly in contrast with what happens when it is present even if H_2 or CO_2 lacks. The methanogen alone grew well in pure culture with H_2/CO_2.

However thermodynamically unfavourable the conversion of acetate into H_2 and CO_2 could raise the possibility that the nonmethanogenic bacterium was using some other substrate from the complex medium we used, perhaps aminoacids from the yeast extract or the peptonated casein digest.



On the other hand partial pressure strongly influences the free energy available from the first reaction, according with the Le Chatelier principle. For instance with a H_2 partial pressure of 4.10^{-5} atm the free energy available to the methanogen would be 16 kJ/reaction while with 1 atm of H_2 would be 135 kJ/reaction.

Since H_2 partial pressure in anaerobic habitats are rarely greater than 10^{-3} atm (except perhaps in certain geothermal waters) it is understandable that a methanogen might participate in a mechanism like this.

DISCUSSION

Anaerobic thermophily is in some extension related with autotrophy. Oxygen is little soluble in hot water and hydrogen and carbon dioxide are the most stable energy and carbon sources at high temperatures (and pressures).

Strain	Substrate	Temperature	pH
JL17	H_2/CO_2		
JL20	H_2/CO_2		
JL22	H_2/CO_2		
JL23	H_2/CO_2	65°C	7.0

Perhaps the way that these bacteria could live as the temperatures went down was to maintain close symbiotic relationships with other strains that in a way protect them and provide them the conditions they needed.

It is still uncertain the interspecies relationship concerning the utilization of C_1 compounds. Thus we think this new consortium now isolated can give some answers in that area. Next step will be the identification of the non-methanogenic bacteria and the establishment of the basis of the metabolic and physiological relationships among the two microorganisms.

REFERENCES

(1) Stetter, K.O.; König, H.; Stackebrandt, E. (1983) "Pyrodictium, a New Genus of Submarine Disc-Shaped Sulfur Reducing Archaebacteria Growing Optimally at 105°C", System. Appl. Microbiol. 4:535-551

(2) Stetter, K.O.; Thomm, M.; Winter, J.; Wildgruber, G.; Huber, H.; Zillig, W.; Janecovic, D.; König, H.; Palm, P,; Wunderl, S. (1981) "Methanothermus fervidus, a Novel Extremely Thermophilic Methanogen Isolated from an Icelandic Hot Spring", Zentralbl. Bakteriol. Mikrobiol. Hyg. 1 Abt. Orig. C 2:166-178

(3) Zinder, S.H.; Koch, M. "Non-aceticlastic Methanogenesis from a Acetate: Acetate Oxidation by a Thermophilic Syntrophic Coculture", Arch. Microbiol. (1984) 138:263-272

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PARAMETER ESTIMATION OF KINETICS AND MODELLING IN METHANOGENIC FERMENTATION OF *Methanosarcina barkeri*.

The mathematical modelling of anaerobic digestion has reached a high level of sophistication in the last twenty years. In spite of this, little is known about the kinetics of the growth of pure cultures of methanogens. The growth of Methanosarcina barkeri 227 in an anaerobic environment is studied herein in batch and fed-batch culture[1]. In particular the interest on methanol utilization as substrate over a wide range of concentrations was studied. A non-structured model with enzyme inhibition kinetics is proposed to fit the experimental kinetic data of bacterial growth since an inhibitory effect at high methanol concentrations is observed. Estimated model parameters were obtained by employing nonlinear optimizing routines over a classical least squares objective function.

INTRODUCTION

A large number of publications have appeared on mathematical modelling of substrate inhibition. In most cases the equations are derived from the theories of the inhibition of a single enzyme. Andrews[2] proposed that the effect of substrate concentration on specific growth rate could be governed by the equation

$$\mu = \frac{\mu_m S}{K_s + S + S^2/K_i} \qquad (5)$$

where μ is the specific growth rate, μ_m is the maximum specific growth rate, K_s is the substrate saturation constant, S is the substrate concentration and K_i is the substrate inhibition constant. This equation is of the same type of the Haldane's equation[3] used to describe enzyme inhibition by the formation of an inactive complex of the enzyme with two substrate molecules. Edwards[4] compared five different equations with reference to experimental results from literature. He pointed out that many other inhibitory models could be borrowed from enzyme kinetics to fit kinetics data. There are many other mathematical models (linear, exponential, Tessier) that provide an adequate fit of growth data at high substrate levels.

MODEL OF BATCH GROWTH

The kinetics of bacterial growth, substrate consumption, and product formation are formulated in the following state equations:

$$\frac{dX}{dt} = (\mu - k_d)X \tag{2}$$

$$-\frac{dS}{dt} = \frac{\mu X}{Y_x} - mX \tag{3}$$

$$\frac{dP}{dt} = -Y_p \frac{dS}{dt} \tag{4}$$

where k_d is cell decay rate, m is the specific maintenance rate, Y_x is cell growth yield, Y_p is product (methane) yield, X is cell concentration and P is the amount of CH_4 produced at time t referred to the liquid volume of growth medium.

It was decided to utilize the kinetic model proposed in eq. (1), since Yang et al[5] have already used it in a kinetic study of methanogenesis of acetate with a pure culture of methanogens and it has also been used by Andrews[6] in a kinetic study of anaerobic digestion. The determination of model parameters from batch culture data is made by integration of the differential equation resulting from transformations in eqs. (1) to (4). In the case of negligible k_d and m (a reasonable assumption in the exponential growth phase) the following differential equation is obtained:

$$-\frac{dS}{dt} = \frac{\mu_m SX}{Y_x(K_s+S+S^2/K_i)} \tag{5}$$

X is related to S by the cell growth yield. If Y_x could be considered constant the following equation will relate the two variables:

$$X = X_o + Y_x(S_o-S) \tag{6}$$

The same is valid for the production of methane gas:

$$P = P_o + Y_p(S_o-S) \tag{7}$$

Finally, the following integrated model is obtained:

$$\mu_m t = \left(1+\frac{K_s}{S_o+X_o/Y_x}+\frac{S_o+X_o/Y_x}{K_i}\right)\ln\frac{X}{X_o} - \frac{K_s}{S_o+X_o/Y_x}\ln\frac{S}{S_o} + \frac{S-S_o}{K_i} \qquad (8)$$

The constants μ_m, K_s, and K_i are estimated using nonlinear regression methods to fit the experimental data. Since only one of the three time-dependent variables and the initial conditions are needed to use eq. (8), methane gas formation (P) was selected because it is monitored more easily and precisely.

MATERIALS AND METHODS

M. barkeri strain 227 was used from a methanol pre-grown inocula. The bacteria were grown in 25 cm3 serum bottles containing 10 cm3 culture medium. Cultures were incubated at 37°C without shaking. The growth medium contained yeast extract, trypticase, Mah's trace minerals solution, bicarbonate buffer and rezasurin. Anaerobic manipulation was made according to Hungate technique[7]. Bacterial growth was followed by gas chromatography of the methane produced (Hach-Carle GC, Porapak-QS column, TCD).

RESULTS

Data from batch fermentations of M. barkeri 227 were fitted to eq. (8) using a nonlinear least-squares regression technique, the finite difference Levenberg-Marquardt algorithm[8,9]. The estimated model parameters μ_m, K_s, and K_i, and also the optimal initial methanol concentration and the maximum specific growth rate are listed in Table 1. The optimal methanol concentration can be estimated by making $d\mu/dS$ equal to 0 in eq. (1).

Table 1. Model parameters

	μ_m (hr^{-1})	K_s (mM)	K_i (mM)	S_{opt} [a] (mM)	μ_{max} [b] (hr^{-1})
eq. (8)	0.030	26.7	2208	240.0	0.024
eq. (1)	0.032	29.2	2110	248.0	0.026

a) $S_{opt} = (K_s K_i)^{1/2}$
b) $\mu_{max} = \mu_m S_{opt}/(2K_s+S_{opt})$

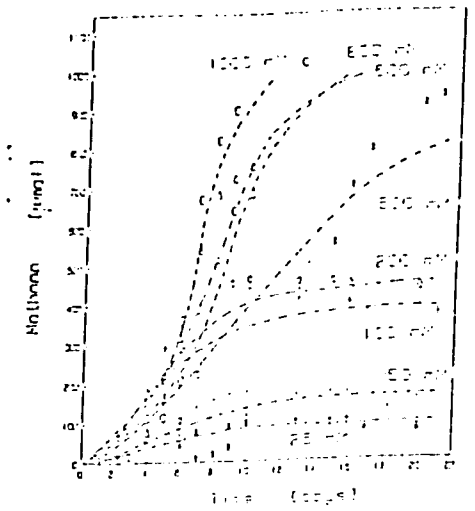

Figure 1. Methane formation for M. barkeri 227 grown on various initial concentrations of methanol. (symbols: data, dotted lines: model predictions)

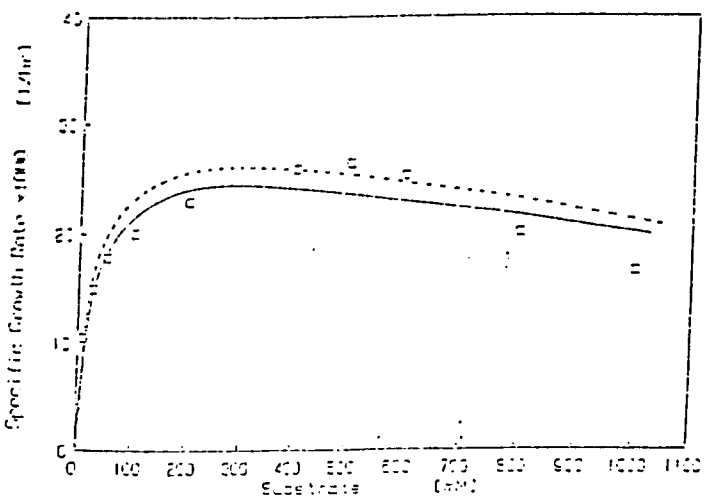

Figure 2. Effect of methanol concentration on the specific growth rate. (squares:data, dotted line: prediction of eq. (1), line: prediction using the parameters fitted with eq. (8))

The comparison of the proposed model and the experimental data is illustrated in Fig. 1. The kinetic data obtained in this study were reasonably well represented by the proposed model.

Values of the model parameters for Andrews equation are also listed in table I. A graphical method was used to estimate μ from batch culture data, by plotting the methane gas produced against time on the semi-log paper for each initial methanol concentration. This procedure is only valid when Y_x and Y_p are constant and X_o very small. These data were then fitted to eq. (1) by the Levenberg-Marquardt algorithm for estimation of the kinetic constants. Values obtained differ only slightly from the integral method (eq.8). Some errors due to the graphical estimation of the slopes may contribute to this difference. In Figure 2 are plotted the observed specific growth rates and the model predictions.

This work was supported by JNICT - contract n. 87/246 and A.I.D. grant no. 936-5542-G-SS-5005-00

REFERENCES

[1] J.A.G.F. Menaia; L. Pereira; E.C. Ferreira; J.C. Duarte (1987) in Proc. of 4th European Congress in Biotechnology, 3, 548, Amsterdam, Elsevier.
[2] J.F. Andrews (1968) Biotechnol. Bioeng. 10, 707
[3] J.B.S. Haldane (1930) Enzymes, Longmans, N.Y.
[4] V.H. Edwards (1970) Biotechnol. Bioeng. 27, 679
[5] S.T. Yang, M.R. Okos (1987) Biotechnol. Bioeng. 30, 661 [6] J.F. Andrews (1969) J. Sanit. Eng. Div. Am. Soc. Civ. Eng., SA1, 95
[7] R.E. Hungate (1969) Methods Microbiol., 3B(4), 117-132
[8] Levenberg, K. (1944) Quart. Appl. Math., 2, 164-168
[9] Marquardt, D.W. (1963) J. SIAM, 11(2)

10

FERMENTATION DATA ACQUISITION AND CONTROL WITH MICROCOMPUTERS

INTRODUCTION

Biochemical processes are highly sensitive even to small changes in operating conditions, such as temperature, pH, or concentration of substrate. Deviations from optimum may have detrimental consequences for microbial cultures.

Real-time computer applications to fermentation processes (on-line data acquisition, parameter monitoring and process control) in order to improve product quality or to optimize production efficiency are becoming more significantly in the last years.

The process control it will be made by Adaptive controllers *(1,2)*. These by definition, perform two simultaneous functions in that they learn about the process under control whilst at the same time controlling it. Process wich are nonlinear and time variant may be successfully controled by adaptive controller as a result of their learning ability.

The aim of this research is the establishment of stable algorithms for parameter and state estimation and regulation of fermentation processes and to implement them on a computer coupled fermentation system.

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CASE STUDY

The present case study is the methanogenic fermentation in an anaerobic environment with *Methanosarcina bakeri* (3). The reaction vessel is a SGI-SETRIC 20 dm^3 continuous stirred tank reactor (CSTR) with baffled type heat exchanger.

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HARDWARE

- **Microcomputer Sperry µIT from UNISYS:**

  - a 1Mb RAM capacity
  - mass memory: 40 Mb hard-disk; 1.2 floppy diskette
  - mathematic coprocessor
  - communications ports: RS 232-C; Centronics

- **Process-System Interface THINKLAB from 3D-Digital,Design and Development with the following plug-in boards:**

  - 8 instrumentation amplifiers followed by multiplexer and gain programmable amplifier
  - 12 -bit fast Analog to Digital Convertor plus sample/hold 25 µsec conversion time
  - 8 channel solid-state mains relay, rating 2.5 Amp at 240 V AC
  - Real-time clock/calender with battery back-up
  - Bi-directional RS 232-C interface with 512 byte buffer

- **Unit of Analog Control MDD7F from SGI-SETRIC with the following modules:**

  - agitation speed
  - pH
  - temperature
  - redox potential

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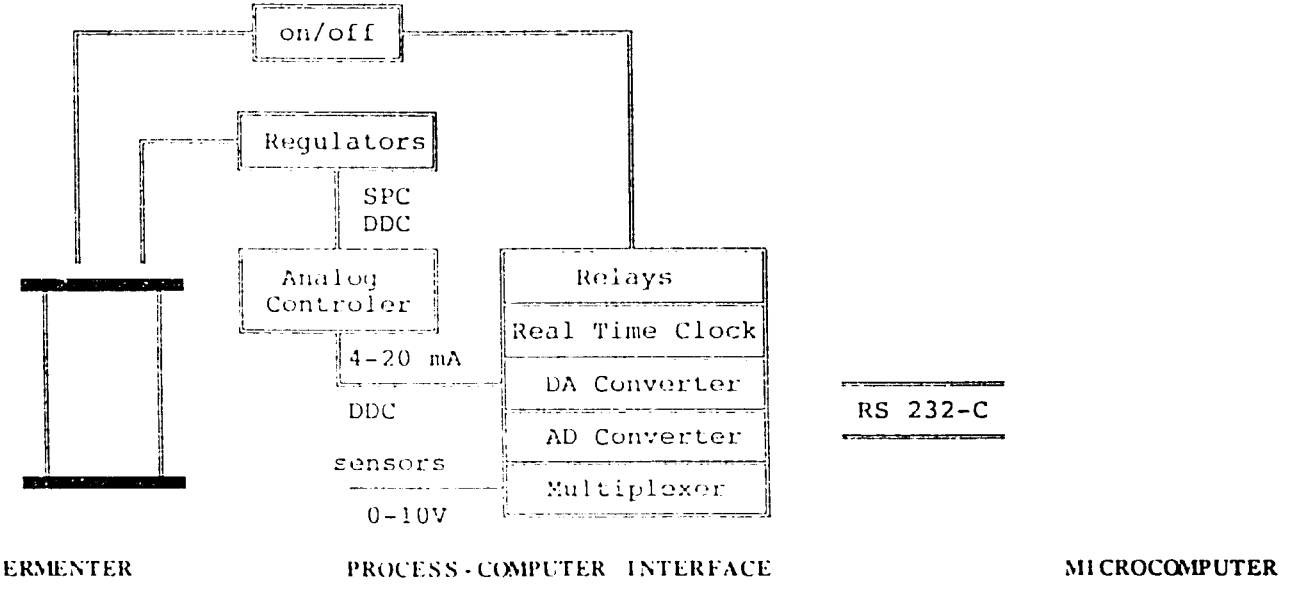
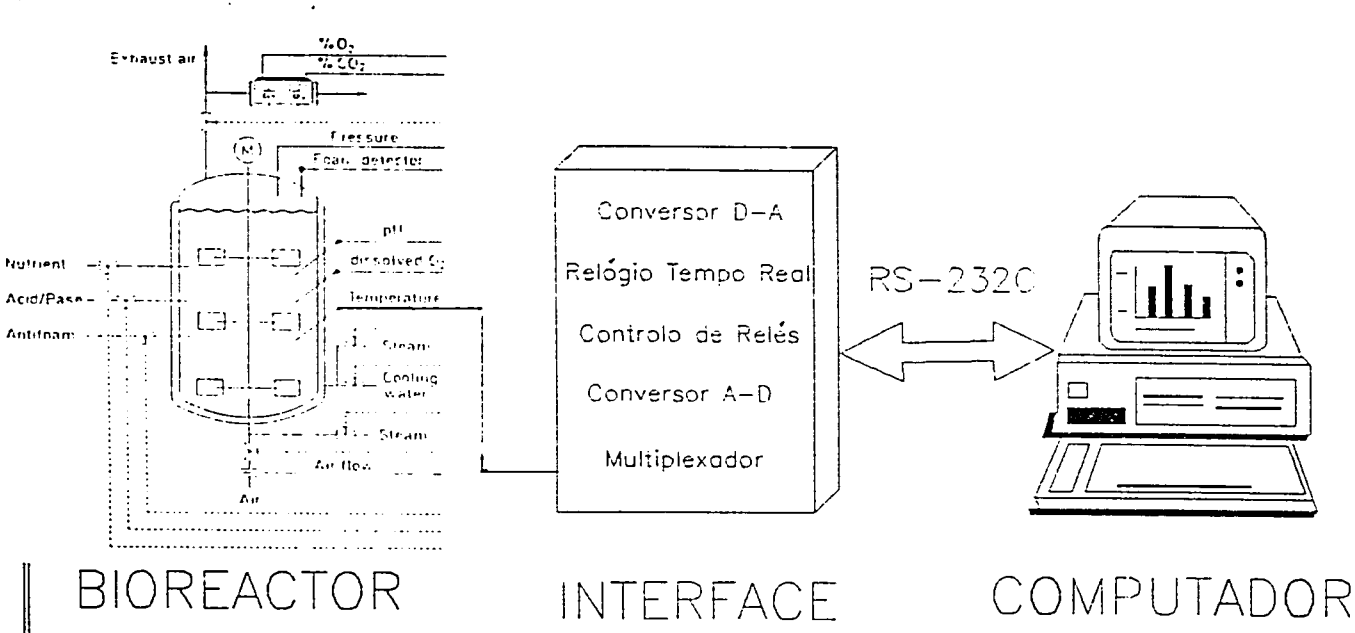


Fig : Hardware configuration.

Legend:

SPC - Set-Point Control
DDC - Direct Digital Control
AD - Analog-Digital
DA - Digital-Analog



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SOFTWARE

On software development we are currently considering:

- **DATA ACQUISITION AND TREATMENT:**

  - initialization and parameter set-up
  - data filtering
  - on-line data logging
  - off-line data input/output

- **CONTROL:**

  - graphics and chart aid: operational trends; process perfomance (per unit of time and over a period of time)
  - replacement of analogue control by digital control (DDC)
  - on-line modeling and optimization; adaptive control

Main objective is to be able to implement adaptive control strategies.

References:

1. Dochain,D.; Bastin,G.(1984) Adaptive identification and control algorithms for nonlinear bacterial growth systems. Automatica,20:5 621-634
2. Renard,P.;Dochain,D.;Bastin,G.;Naveau,H.;Nyns,E.-J.(1987) Adaptive control of anaerobic digestion processes. To appear in Biotechnol. Bioeng.
3. Pereira,L.M.;Ferreira,E.;Duarte,J.C. Kinetics of co-substrate cultures of *Methanosarcina Barkeri*. Presented at 8º Congresso Nacional de Bioquímica Póvoa de Varzim 28 Nov.-1 Dez. 1987

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11

Proposal for a patent to be submited to the Portuguese and USA patent Offices

Activation of Methanogenesis by "Agribyeprod" (AGP) with *M. barkeri* 227 Growing on Methanol or Acetate

ACTIVATION OF METHANOGENESIS BY "AGRIBYEPROD" (AGP)
WITH M. BARKERI 227 GROWING ON METHANOL OR ACETATE

Francis Cladipo Olatunji,.....
and Jose Cardoso Duarte assignors to DTIQ/LNETI,
2745 Queluz, Portugal.

14 Graphs as Drawings attached. 8 claims.

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ABSTRACT OF THE DISCLOSURE:

The present invention relates to the activation of methanogenesis with a pure strain of M. barkeri 227 growing on methanol or acetate using an agricultural bye-product locally found in Portugal named "Agribyeprod". This material, "Agribyeprod", herein known also as "activator" and abbreviated as AGP, was found to increase the rate of production of methane in anaerobic fermentations when compared with a control not containing the said material. The kinetic characteristics of methanogenesis with AGP were investigated and are herein reported together with the methods of activation while effort at identifying the chemical components of the activation has begun.

BACKGROUND OF INVENTION:

It has long been recognised that under anaerobic conditions, the production of methane and carbon dioxide in natural environments is usually the terminal step in the mineralization of organic compounds[1,2]. Also, many specific anaerobes, such as Methanosarcina barkeri, have been used to carry out this last step in the anaerobic digestion process, known as methanogenesis. The major problem in anaerobic digesters is the rate-limiting step in the process viz, the methanogenesis.

It is, therefore, not surprising that a lot of work have been reported[3,4,5] in an attempt to improve on this rate-limiting step. It was thus of practical interest to us when we stumbled on the activation of methanogenesis by an agricultural-based product and we decided to investigate its full potential. The AGP is a bye-product of an agricultural produce of Portugal. It is of interest to note that the activating effect of AGP was found by chance during our attempt

to develop an immobilised material for M. barkeri methanogenesis of methanol or acetate.

An object of the present invention is to determine the activating effect of AGP on methanogenesis and to present results that would be useful in the overall design and operation of an anaerobic digestor. The invention relates to the improvement of methanogenesis as a rate-limiting step in the overall mineralization of organic compounds.

After extensive fermentation experiments, it was discovered that AGP, when prepared in various ways and at varying environmental conditions, showed this activating effect when M. barkeri was grown on either methanol or acetate, thus opening up a potentially-improved anaerobic digestion process.

SUMMARY OF INVENTION:

Methanosarcina strain 227 used was the one isolated from a Barker-type acetate enrichment culture as described by Mah et al (1978)[3], and the base culture medium used were the same as reported by the original authors, Mah et. al. (1978)[3] and later used by Menaia et al (1986)[6] in our laboratory. For most of the experiments, 50 mmolar methanol or acetate were used at optimum pH 6.8 as earlier reported by Mah et. al. All the special precautions of anaerobic fermentation experiments as stated by previous workers were carried out. The control experiments which contained no AGP or its suspension or filtrate confirmed that the above precautions were maintained. For example, all media were prepared under an atmosphere of oxygen-free 100% N_2 or oxygen-free mixture of CO_2 and N_2. Culture volumes of 50ml were employed for experiments performed in 125ml serum bottles. Serum bottles were stoppered with butyl rubber stoppers.

Methane was determined by gas chromatography. Total gas production was determined as earlier reported[3] at known periods by measuring the volume of gas produced at room temperature of about 23°C. This was achieved by displacement of the glass-tipped plunger of an unlubricated glass syringe. Micromoles of methane were computed from

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the gas composition determined by gas chromatography and from the measured volumes of gas phase in the vessel and syringe. The method of calculation is as reported by Mah et. al (1978), viz: micromoles of CH_4 formed = $((\%CH_4 \times (V_f+V_r))/2.24) + M_p$ where $\%CH_4$ is the volume (millilitres) per 100ml of gas phase, V_f is the volume (millilitres) of gas phase removed by syringe following equilibration to atmospheric pressure; and M_p is the sum of micromoles removed after daily equilibration, e.g.

$$M_p = ((\%CH_4 \times V_r)/2.24)$$

The following methods were used to prepare the various AGP and its derivatives used in the experiments:

i) Cold-soaked AGP:- AGP was immersed in deionised water for about 4 hours. The water was then decanted off and the wet AGP was used as cold-soaked AGP.

ii) Boiled AGP:- AGP was immersed in deionised water and then boiled at atmospheric pressure for about 4 hours. The coloured suspension was decanted off and the wet AGP used as boiled AGP.

iii) Dried AGP:- AGP was prepared as in (ii) above. The boiled AGP was then dried in an oven at given temperatures of 50°C overnight or 200°C for about 5 hours.

iv) Milled AGP:- Dried AGP was prepared as in (iii) above and then milled to either 2mm or 0.75mm mesh size.

v) Aqueous suspension of AGP:- In the preparation of boiled AGP, the coloured suspension obtained was used as 1st aqueous suspension of AGP. Where boiled AGP is reboiled with fresh deionised water, the aqueous suspension of AGP is referred to as the 2nd suspension.

vi) Filtrate from aqueous suspension of AGP:- 1st and 2nd filtrates were obtained from 1st and 2nd suspensions using Whatman filter paper 1 for filtration.

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Most of the fermentation experiments were carried out without agitation except where otherwise stated. All samples were sterilised at 121°C for 15 mins and cooled before the start of any fermentation. Methanol, being volatile, was sterilised separately and added aseptically and anaerobically to the rest of the medium.

Results showed that AGP increased the rate of production of methane when compared with the control containing no AGP. But there appeared to be little or no effect on the thermodynamic equilibrium of the reaction as there was no change in the maximum concentration of methane produced for a given concentration of substrate. Therefore, the effect of AGP on methanogenesis appears to be catalytic. When varying concentration of AGP was used, only a marginal increase in the rate of methanogenesis was observed with increase in AGP indicating that activation was not dependent on a proportional increase of activator concentration. The suspensions from boiled AGP (1st & 2nd suspensions) also showed similar activating effect to the AGP itself. When boiled AGP was oven-dried at 50°C overnight, there was similar activating effect to the boiled but non-dried AGP. However, when the boiled AGP was dried at 200°C for about 5 hours, the sample lost its activating effect even though there was no inhibitory effect. Also, the 2nd filtrate from the 2nd suspension appeared to have lost the activating effect of AGP.

An optimum temperature between 37 - 43°C was obtained for both the control and the activator. However, the rate of methanogenesis was much higher with AGP at any given temperature. The results appeared to suggest that while both 20 - 23°C and 50°C showed an intense inactivation of methanogenesis 20 - 23°C showed a time-dependent inactivation whereas 50°C showed a time-independent inactivation. This activating phenomenon was also observed with increasing methanol substrate concentration. When AGP was milled to two different sizes, viz: 2mm and 0.75mm mesh sizes, there was apparently no difference in the activating effect confirming an earlier result that there was little or no increase in activation with increasing concentration or particle size distribution of the material. Results, with and without agitation, showed that shaking did not enhance the rate of methanogenesis

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was enhanced with shaking.

When acetate substrate was used, the organism showed similar activating effect with AGP or its suspension although at much longer time or lower rate. A pre-grown culture of the organism using the activator was used as a seeding culture with methanol as substrate. There was only slight increase in the rate of methanogenesis with AGP and its suspension when compared with control, suggesting that a partial induction of the cells may have occurred during the pre-incubation with the activator.

EXAMPLE 1

An anaerobic fermentation of M. barkeri on 50mmolar methanol was carried out with 2 concentrations of boiled AGP and an aqueous suspension of AGP together with a control having no AGP or its suspension. The reactor bottles contained the standard medium and one of the following:

i) 10% ($\frac{v}{v}$) boiled AGP;

ii) 20% ($\frac{v}{v}$) boiled AGP; and

iii) 2nd aqueous suspension.

An inoculum of 5% was used. Temperature was at 37°C and initial pH was 6.8. The result is as shown in Fig. 1.

EXAMPLE 2

Similar anaerobic fermentation was carried out as above at varying temperatures of 23°C, 37°C and 50°C in one experiment and 20°C, 30°C, 37°C and 43°C in another experiment. 2 concentrations of boiled AGP were used, viz: 10% and 20% ($\frac{v}{v}$) in the 1st experiment and only 20% ($\frac{v}{v}$) AGP in the 2nd experiment. 50mmolar methanol was used as substrate with initial pH at 6.8. An inoculum of 5% was used. The results are as shown in Figs. 2a, 2b and 3.

EXAMPLE 3

Similar anaerobic fermentation was carried out at varying methanol substrate concentrations of 20, 40, 50 and 100 mmolar. 20% ($\frac{v}{v}$) boiled AGP was used and compared with a control. An inoculum of 5% was used. Temperature was 37°C and initial pH 6.8. The results are as shown in Figs. 4a and 4b.

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EXAMPLE 4

Other sets of anaerobic fermentations were carried out with 2% ($\frac{W}{V}$) dried AGP; 2% ($\frac{W}{V}$) milled AGP at both 0.75mm and 2mm mesh size; 2nd aqueous suspension of AGP; 2nd filtrate from aqueous suspension of AGP and control without AGP or its suspension or filtrate. 50mmolar methanol was used as substrate with 5% inoculum. The temperature was at 37°C and initial pH 6.8. The results are as shown in Figs. 5a and 5b.

EXAMPLE 5

Again, similar anaerobic fermentations of M. barkeri were carried out with 20% ($\frac{V}{V}$) boiled AGP; 20% ($\frac{V}{V}$) cold-soaked AGP; 2% ($\frac{W}{V}$) dried AGP at 50°C; 2% ($\frac{W}{V}$) dried AGP at 200°C; 1st and 2nd aqueous suspensions of AGP; 1st and 2nd filtrates from suspensions; and control not containing AGP or suspension or filtrate. 50mmolar methanol was used as substrate with 5% inoculum. Temperature was at 37°C and initial pH 6.8. The results are as shown in figs. 6a and 6b.

EXAMPLE 6

A step-up anaerobic fermentation of M. barkeri was carried out by preculturing the organism with 20% ($\frac{V}{V}$) boiled AGP using 50mmolar methanol as substrate. At the exponential phase, the culture was used as seed culture for an anaerobic fermentation containing 20% ($\frac{V}{V}$) boiled AGP; aqueous suspension of AGP; and control containing no AGP or liquo[illegible] An inoculum of 5% was used. Temperature was at 37°C and initial pH 6.8. The result is as shown in Fig. 7.

EXAMPLE 7

Similar anaerobic fermentation of M. barkeri was carried out with methanol as substrate and 5% inoculum. One batch of the experiment was carried out with agitation while another batch was carried out without agitation. The total volume of product gases removed by syringe after equilibration to atmospheric pressure was measured. This was approximately proportional to the methane product formed. (Graph not shown). Temperature was at 37°C and initial pH was 6.8. The result is as shown

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EXAMPLE 8

An anaerobic fermentation of M. barkeri was carried out with acetate as substrate. AGP of varying concentrations, 6, 20, 40% ($\frac{v}{v}$) as well as aqueous suspension of AGP and a control containing no AGP or suspension were tried. 5% inoculum was used. Temperature was at 37°C and initial pH was 6.8. The result is as shown in Fig. 9.

What we claim is:

1. A method of activation of methnogenesis which comprises anaerobically culturing M. barkeri 227 on methanol substrate with 10% ($\frac{v}{v}$) and 20% ($\frac{v}{v}$) boiled AGP and other concentrations as well as aqueous suspension of AGP. 5% inoculum was mostly used.
2. A method as set forth in claim 1, wherein activation of methanogenesis by M. barkeri is highest at 43°C, followed by 37°C, then 30°C, and finally 20 - 23°C and 50°C.
3. A method as set forth in claim 1, wherein increased concentration of methanol substrate produced correspondingly higher concentration of methane, and for each concentration of methanol, there was an activation of methanogenesis in the presence of AGP.
4. A method as set forth in claim 1, wherein boiled AGP, dried AGP at 50°C, and some AGP later milled to 2mm mesh size and some milled to 0.75mm mesh size showed increased activation of methanogenesis over such samples as 2nd aqueous suspension of AGP and filtrate from the suspension when compared with the control which contained no AGP or suspension or filtrate.
5. A method as set forth in claim 1, wherein cold-soaked AGP, boiled AGP, and dried AGP at 50°C gave the highest activation of methanogenesis; followed by 1st aqueous suspension from 1st AGP boiling, 2nd aqueous suspension from 2nd AGP boiling, and 1st filtrate from 1st aqueous suspension; and no activation by 2nd filtrate from 2nd aqueous suspension as well as dried AGP at 200°C.

6. A method as set forth in claim 1, wherein an AGP-activated M. barkeri culture gave only a marginal increase in total methane production in the presence of boiled AGP and aqueous suspension of AGP over a control containing no AGP or aqueous suspension.

7. A method as set forth in claim 1, wherein boiled AGP was found to give increased activation of methanogenesis with no agitation, whereas agitation had the effect of increasing the rate of methanogenesis in the control more than in AGP-containing fermentation.

8. A method as set forth in claim 1, wherein acetate was used as substrate instead of methanol, and similar but lower activation of methanogenesis was observed with different concentrations of AGP as well as with aqueous suspension of AGP.

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REFERENCES CITED

1. Mah, R. A. et. al: Annu. Rev. Microbiol. "Biogenesis of methane" 31: 109-131 (1977).

2. Zeikus, J. G. Bacteriol. Rev. "The biology of methanogenic bacteria". 41: 514-541 (1977).

3. Mah, R. A. et. al: Appl. Environ. Microbiol. "Studies on an Acetate-Fermenting Strain of Mehanosarcina" 35, 1174-1184 (1978).

4. Smith, M. R. and Mah, R. A: Appl. Environ. Microbiol. "Growth and Methanogenesis by Methanosarcina Strain 227 on Acetate and Methanol". 36, 870-879 (1978).

5. Bhadra, A. et. al: Biotechnol. Bioeng., "A kinetic Model for Methanogenesis of Acetic Acid in a Multireactor System", 26, 257-264, (1984).

6. Menaia, J. et. al: Proc. 4th Eur. Congr. Biotechnology, Amsterdam". Substrate utilisation by Methanosarcina barkeri in single - and co-substrate cultures" (1986).

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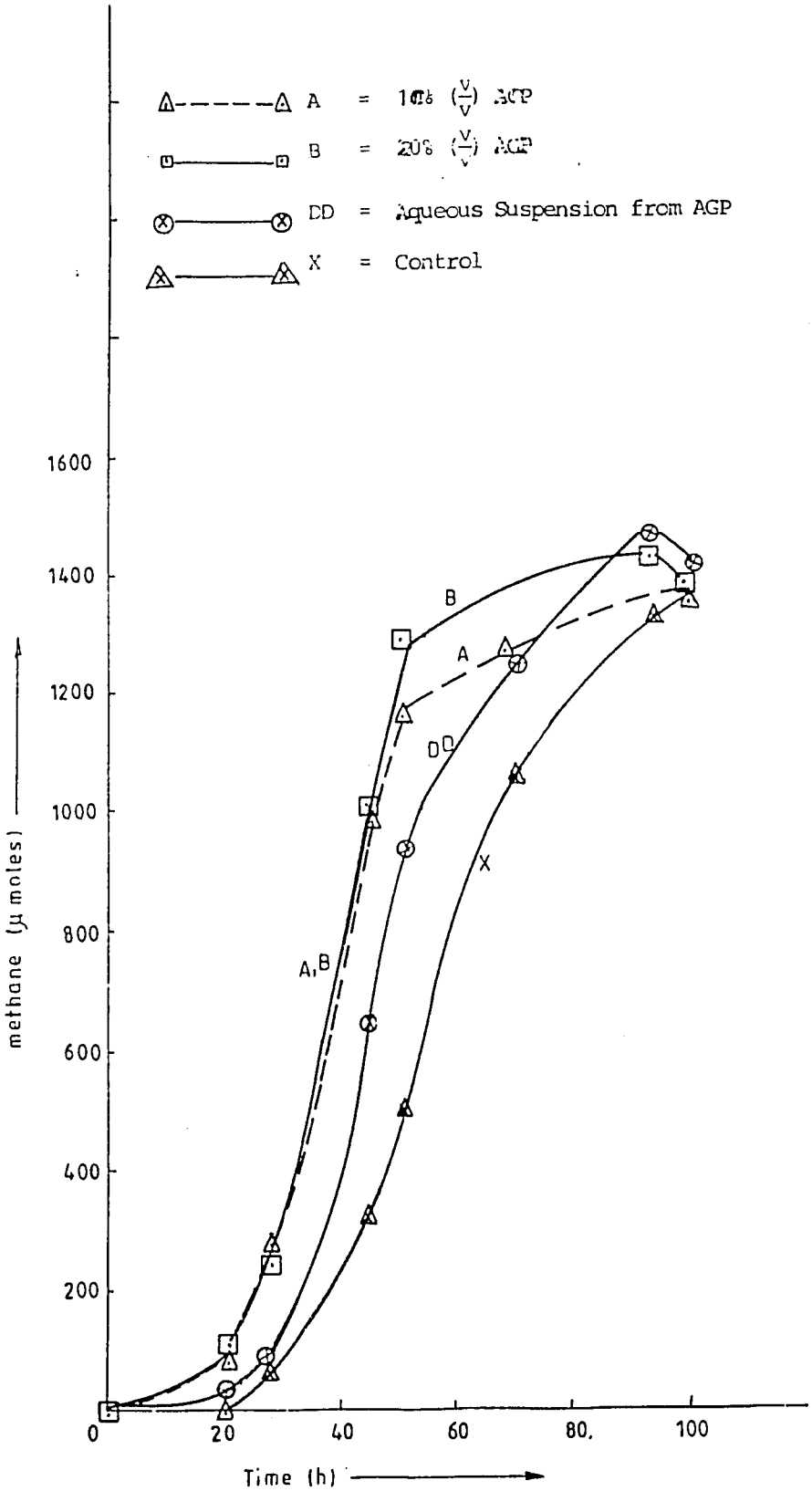

Fig. 1: Effect of AGP Conc. & Its Suspension

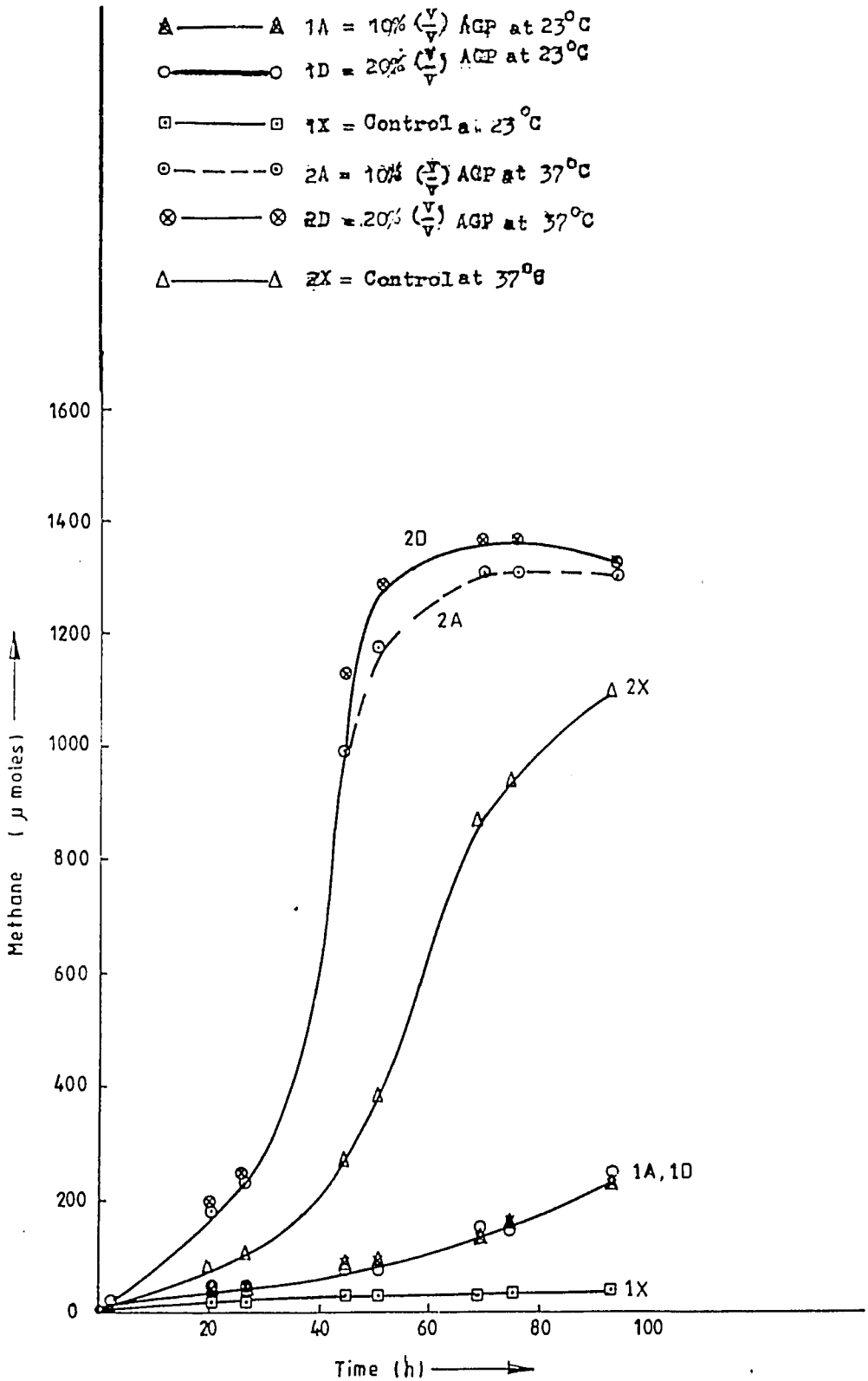

Fig. 2a: Effect of Temperature

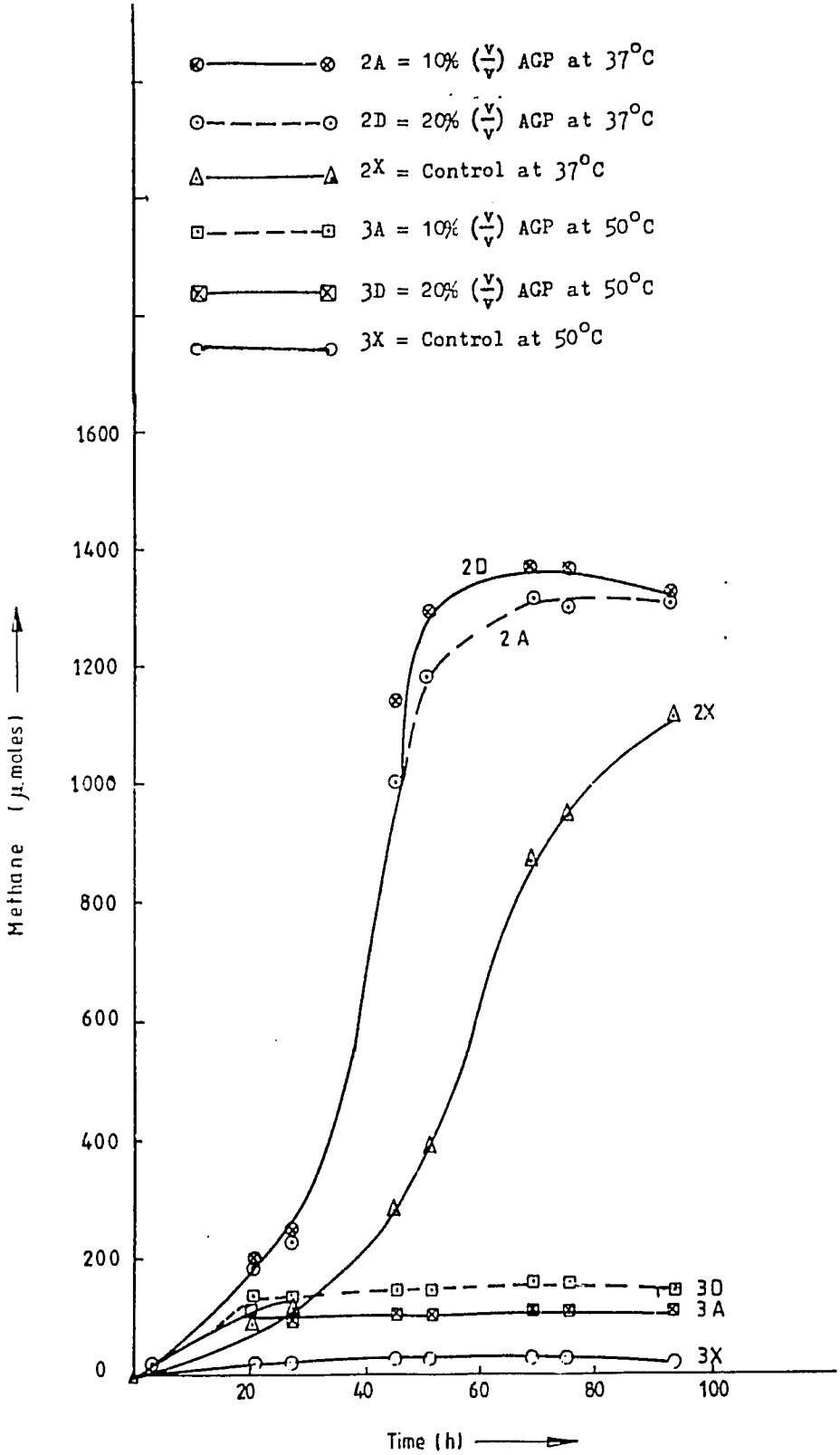

Fig. 2b : Effect of Temperature

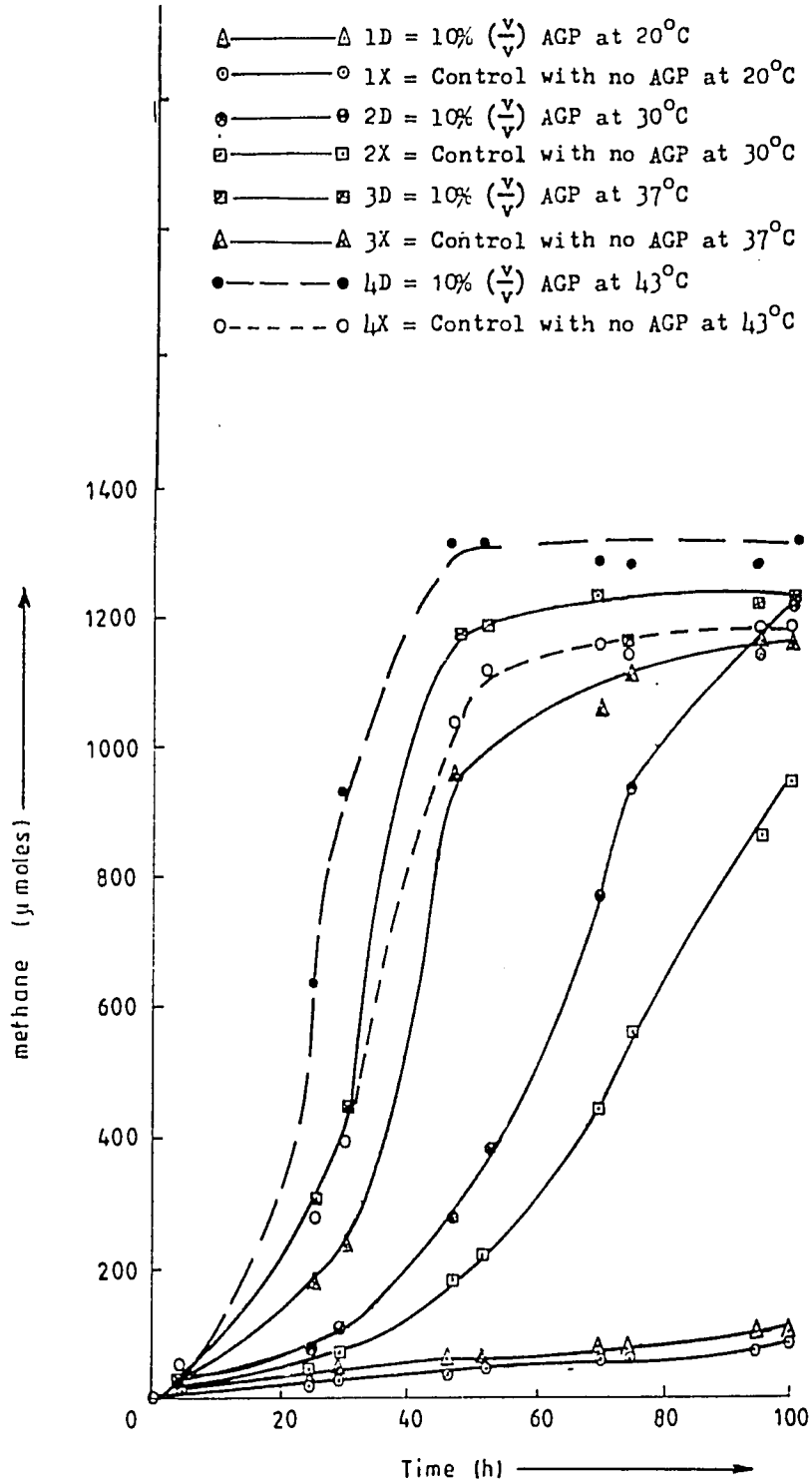

Fig. 3: Effect of Temperature

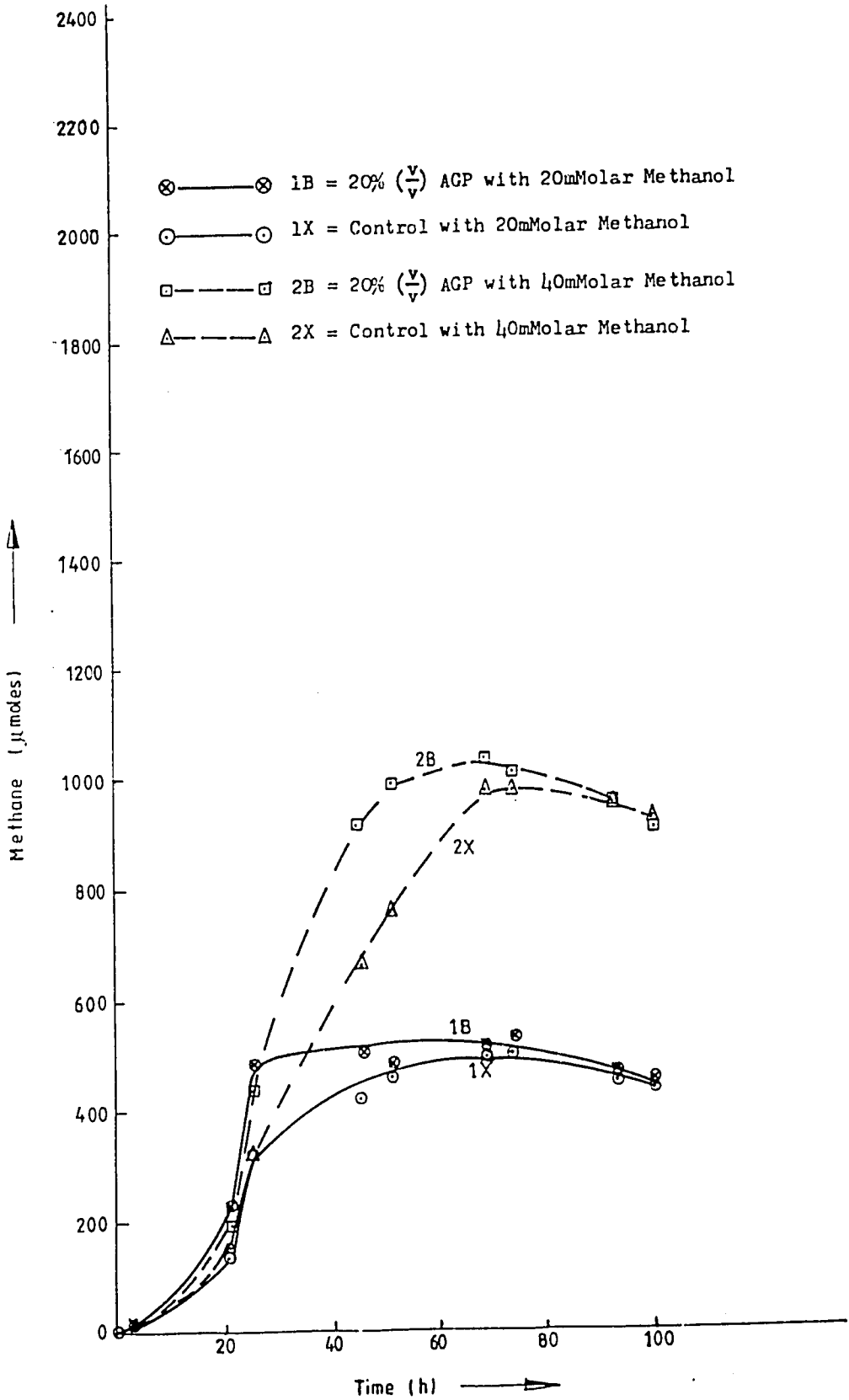

Fig. 4a : Effect of Methanol Substrate Conc.

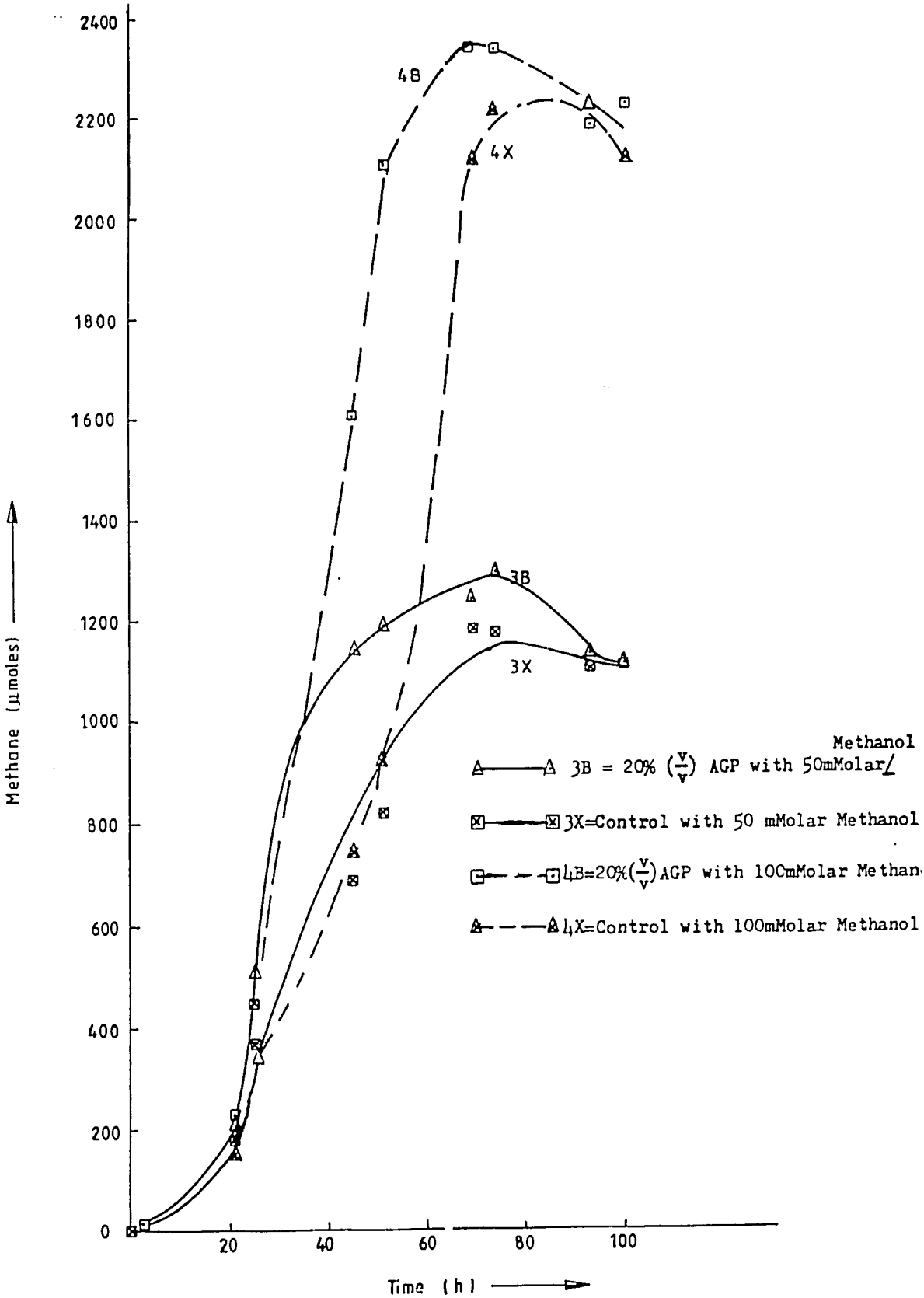

Fig. 4b: Effect of Methanol Substrate Conc.

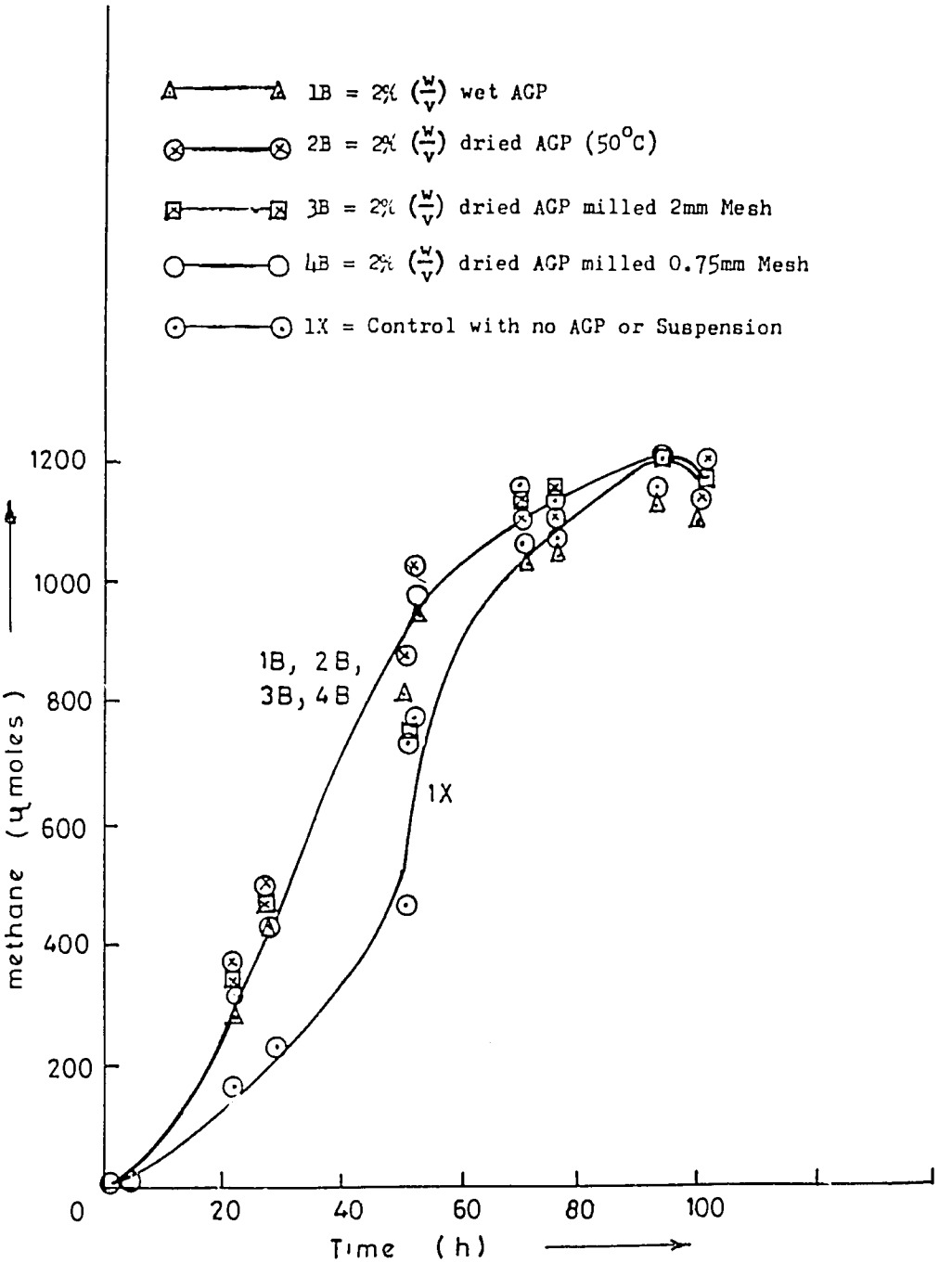

Fig. 5a : Effect of milling & filtering

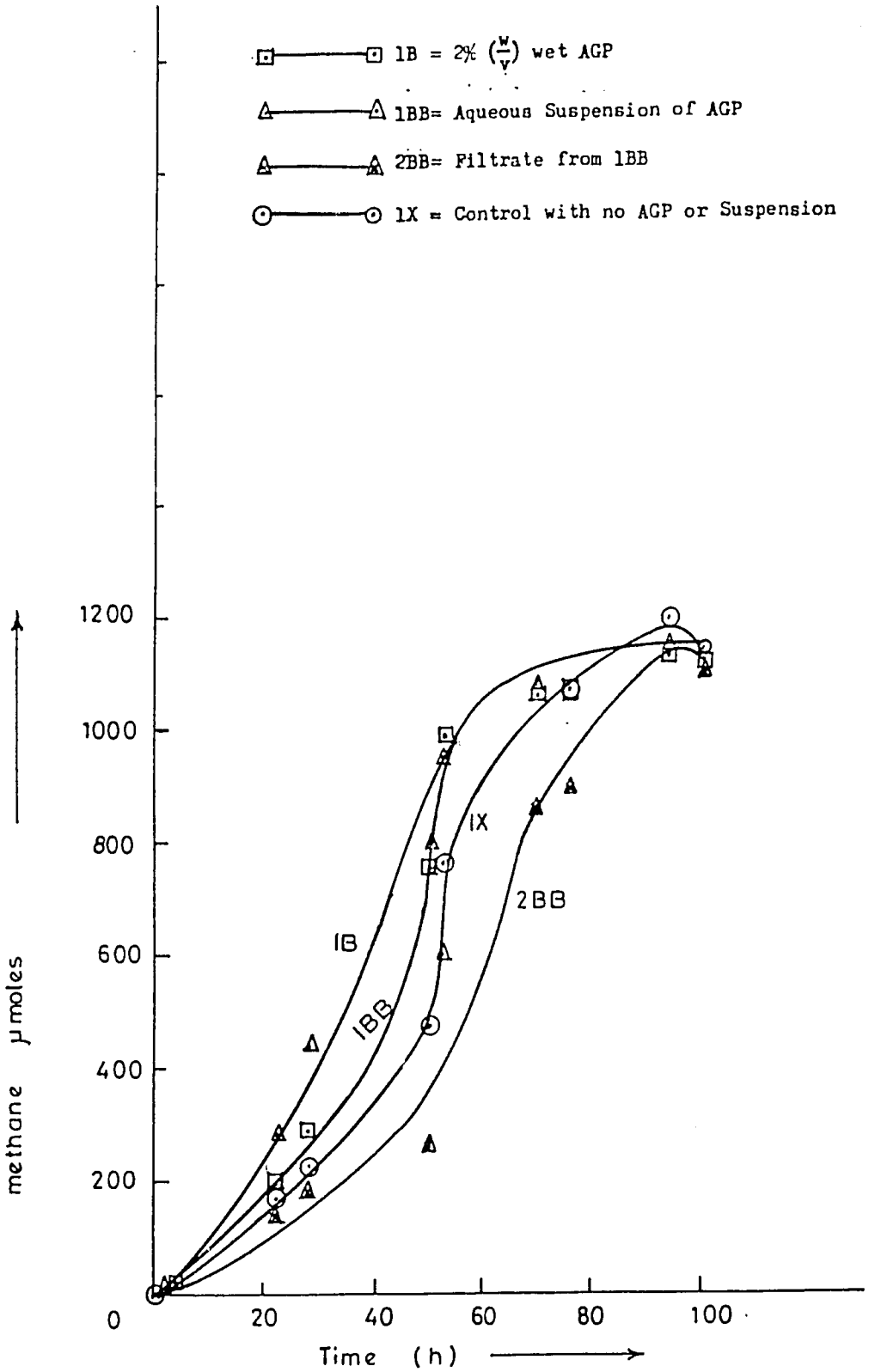

Fig. 5b: Effect of milling & filtering.

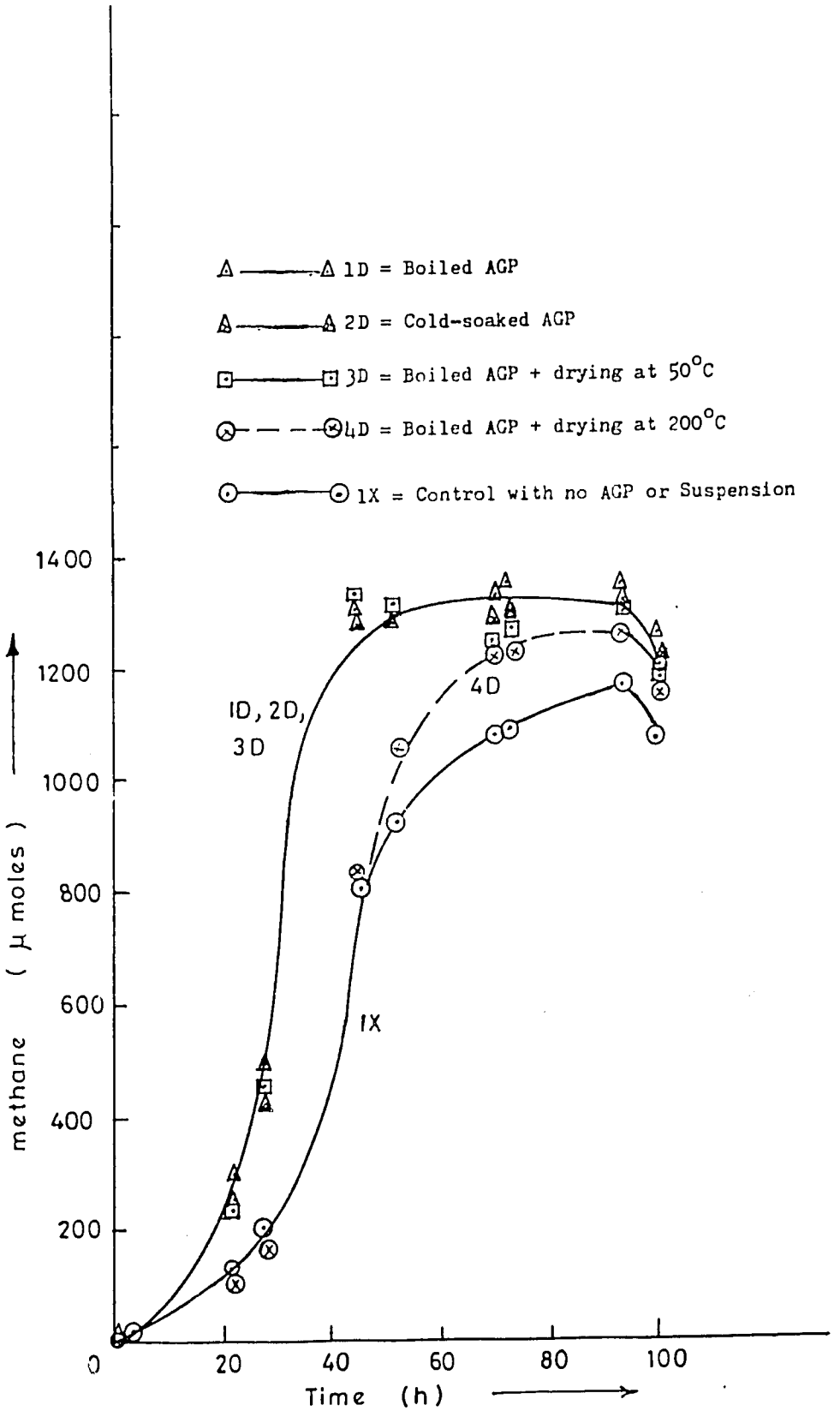

Fig. 6a : Effect of heating and filtration of AGP.

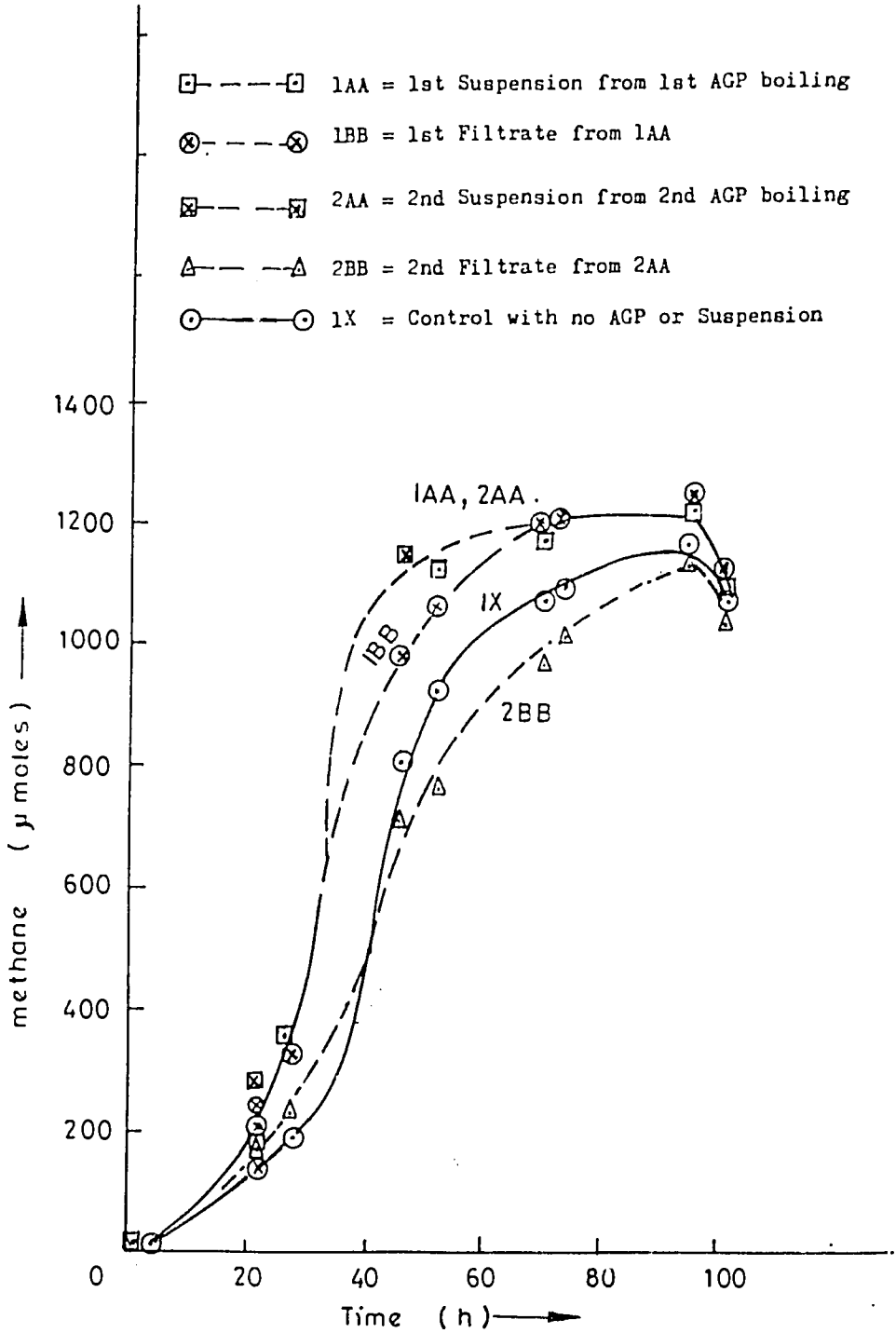

Fig. 6b : Effect of heating and filtration of AGP.

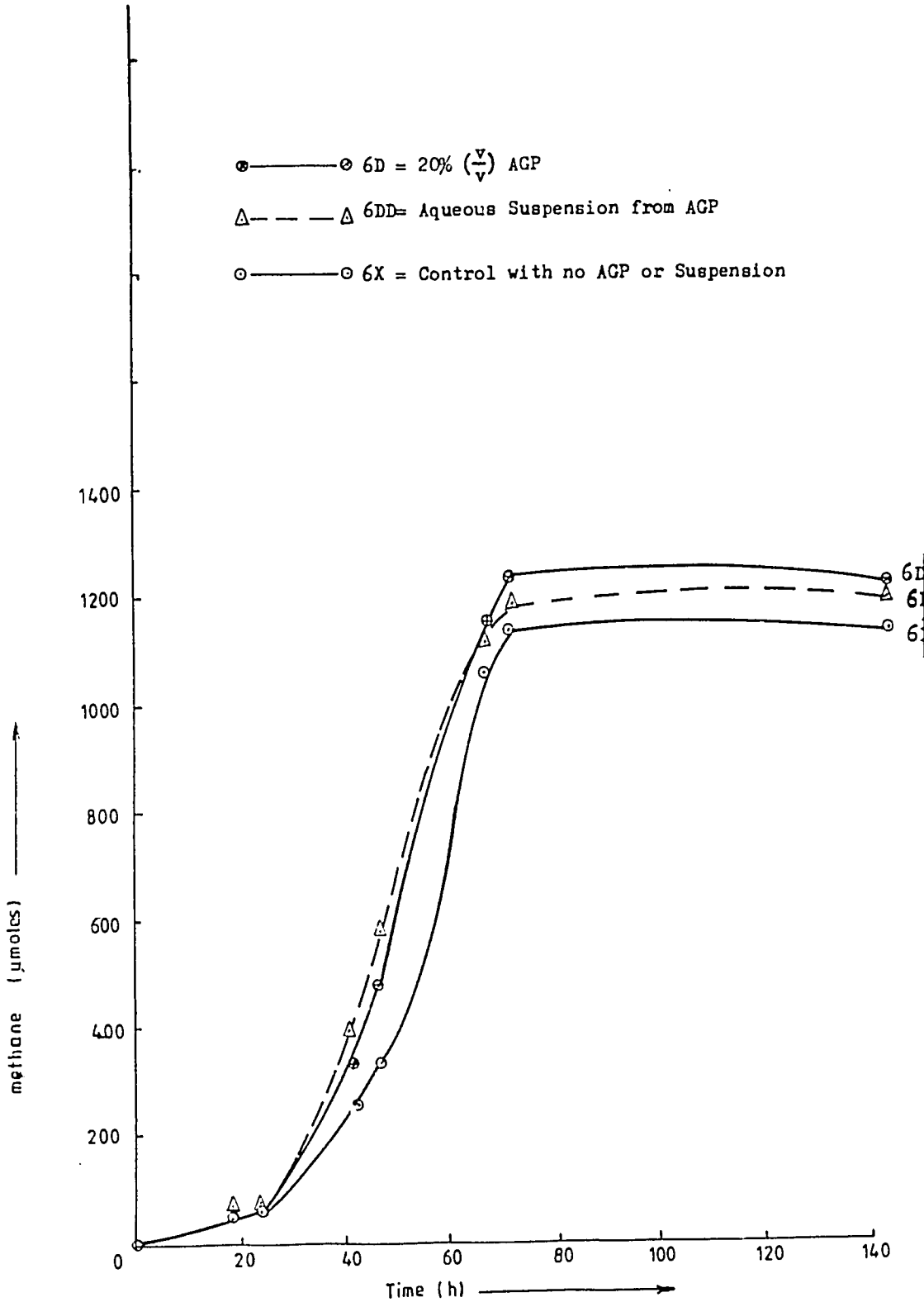

Fig. 7: Effect of Agribyeprod and its suspension on AGP pregrown Culture fermentation

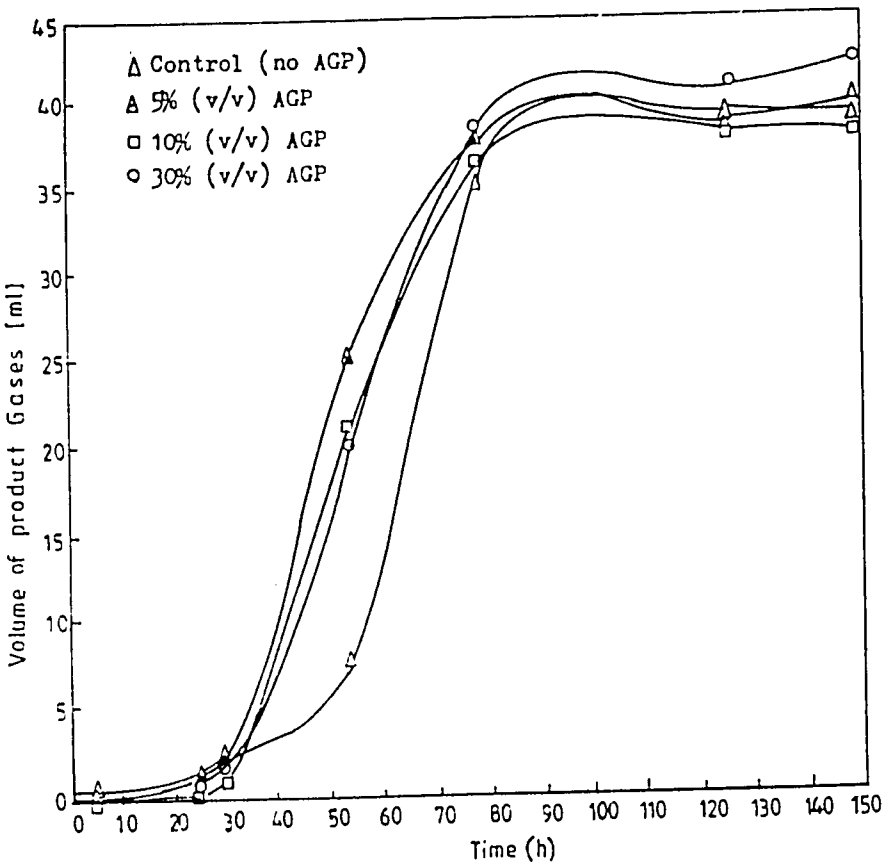

Fig. 8a: Growth of M. barkeri on methanol (with agitation)

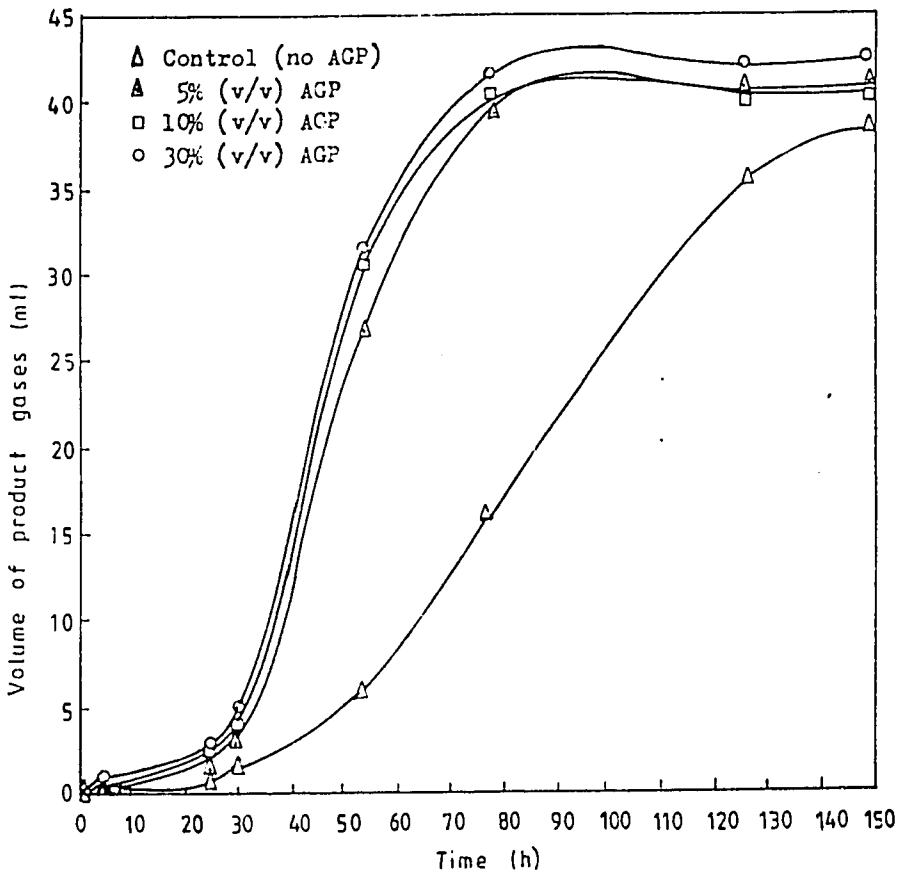

Fig. 8b: Growth of M. barkeri on methanol (w/o agitation)

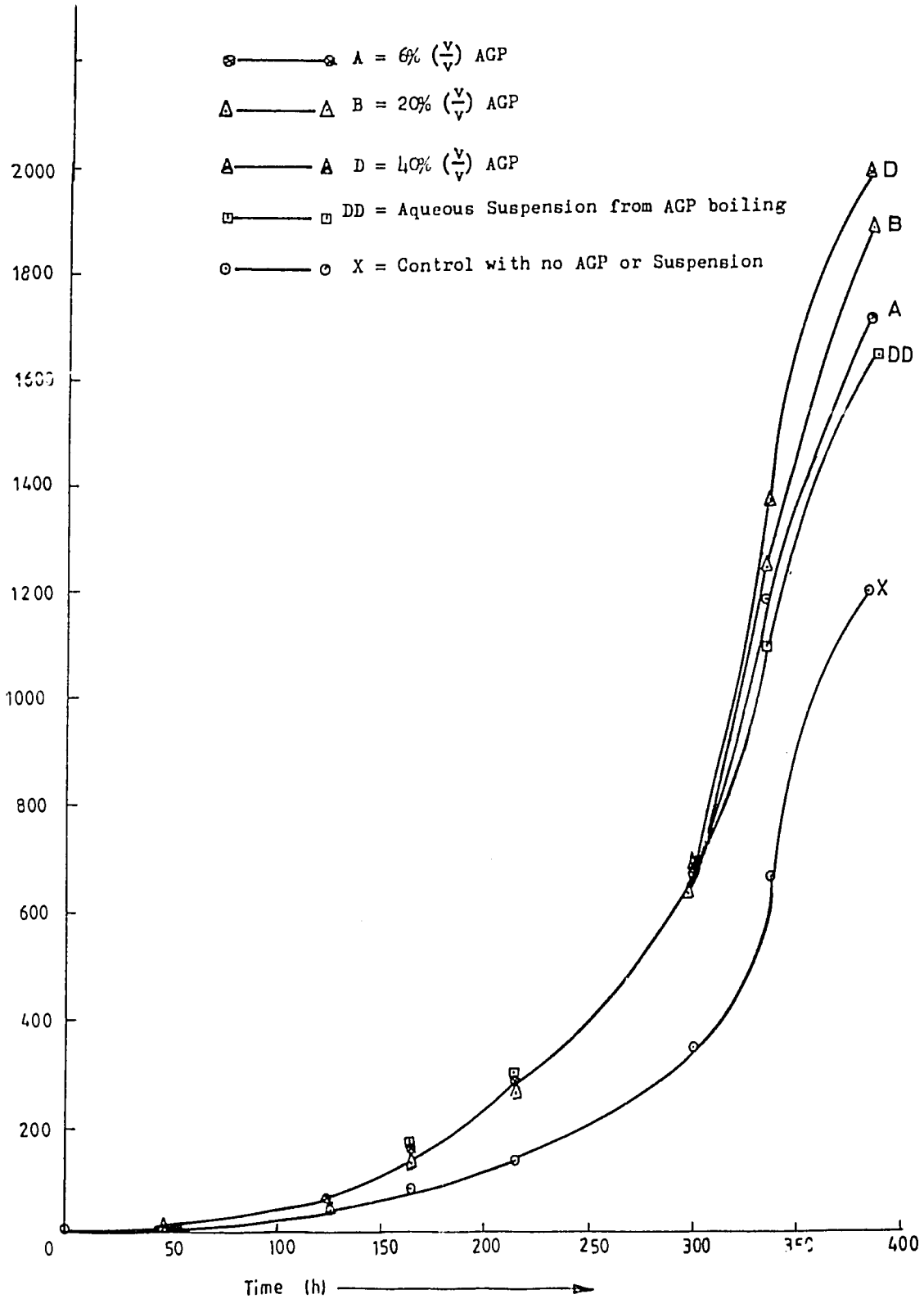

Fig. 9: Effect of Acetate Substrate on AGP Containing Culture

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- Miss Leonor Santos (Technical)
- Miss Paula Morgado (Informatics)
- Mr E. Costa-Ferreira, Miss Irene and Mrs Ana Viegas for general project assistance.

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