ISOLATION, SCREENING AND FIELD APPLICATIONS OF CLOSTRIDIAL ISOLATES TO BE USED IN MICROBIAL ENHANCED OIL RECOVERY (MEOR)

By

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(MEOR)

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PREFACE

For over a decade, the United States and other western countries have faced increased oil prices because of an overall decrease in oil production and exploration in these countries and as a result of increased hostility towards the west by the third world that produces much of the crude utilized by said countries. Doscher (1981), however, says that the "overall recovery of crude oil from reservoirs in the United States, using proven conventional technology is estimated to be 148 billion barrels or 32% of the estimated 460 billion barrels originally in them". The 312 billion barrels that are left behind are the target of a renewed research effort to produce them in a cost effective manner in order to lower the dependence of the United States on foreign crude. The major factor in the life of an oilfield is economics, since oil is produced only as long as it is profitable to do so. Once a field is no longer able to turn a profit it is abandoned, with little regard as to how much oil is left underground. This oil can be from 25-50% of the original oil in place (OOIP).

At first natural sources of energy are available and cause oil to flow from a reservoir. This primary energy of

a well, involving either gas or water pressure or a combination of the two, is responsible for the movement of oil from the reservoir. Depending on a number of factors, the primary energy is capable of recovering from 5 to 75% the OOIP, with water serving as the greatest source of natural energy.

As the primary energy of a well is depleted, the rate of oil production decreases to a level where additional sources of energy are required to continue production. Most often "secondary" recovery involves flooding of the reservoir with water in order to mobilize the oil left within the pores. At the end of productive waterflooding, the oil remaining is still some 60% of the OOIP. The amount of this residual oil depends on three factors: oil in place at the start of waterflooding, reservoir sweep efficiency and microscopic displacement efficiency (Larsen, et al., 1982). This dead oil, after productive waterflooding is the target of tertiary oil recovery methods.

There are a number of tertiary processes capable of moving oil from the reservoir and thus extending the life of an oilfield. Here the only method that will be discussed is chemical flooding, where the basic mechanism of oil recovery is the introduction of chemical agents that act either by altering fractional flow relations, by lowering interfacial tension or raising viscosity, by changing phase

behavior by making oil partially soluble in an aqueous phas5 (solubilization) or by making it water soluble in an aqueous phase (swelling) (Larsen, et al., 1982). A number of substances are used in order to accomplish the above:

1. surfactants to lower the interfacial tension between the oil/water phase;
2. polymers that reduce fingering and therefore increase the sweep efficiency;
3. carbon dioxide flooding which will repressurize the reservoir.

In addition, depending on the minimum miscility pressure of the crude in question, carbon dioxide will combine with the lighter fractions to lower viscosity. Immiscible carbon dioxide flooding is also known to increase the movement of oil from a reservoir.

Any microbiologist could perceive that bacteria, when given the correct nutrient source, would be able to manufacture numerous end-products similar to those used in chemical enhanced oil recovery. In fact a number of investigators have come to the conclusion that bacteria may enhance oil recovery in a number of ways (Grula, et al., 1982; Hitzmann, 1982; Janskekar, 1984; Lazar, 1976; and McInerny, 1982). These ways include but are not necessarily limited to the following:

1. Production of low molecular weight acids such as acetic and butyric that would attack and dissolve the acid soluble portion of the reservoir rock. This would increase the effective permeability and add carbon dioxide to the free gas phase from carbonate rock dissolution.

2. Production of solvents such as acetone and butanol that can solubilize crude oil and water-wet reservoir rock.

3. Production of carbon dioxide that will move oil by either a miscible or immiscible action.

4. Production of biosurfactants that lower the interfacial tension between the oil and water phase.

5. Production of biopolymers that increase the areal sweep efficiency.

Although possible, it is unlikely that a bacterium or even a collection of bacteria could contribute significantly in all five categories. Therefore it would seem prudent for one to search for bacteria capable of contributing in as many ways as possible.

Most oil reservoirs are incapable of providing, even upon the addition of a nutritive support, ideal conditions for bacterial growth and hence optimal production of those compounds thought useful for enhanced oil recovery (EOR). Selection techniques must therefore be adopted that are tailored not only to the production of the necessary metabolic end-products, but the ability to do so under the environmental constraits found in the reservoir itself.

Some important constraints are salinity concentration, temperature (above and below optimums) and the low oxygen tension in a static reservoir. It would also be necessary to screen those bacteria thought useful for such a purpose for their ability to traverse from the wellbore into the reservoir. A survey of the literature dealing with this subject shows that while bacteria are quite capable of movement through permeable rocks (Jang, et al., 1983); Kalish, et al., 1964; Myers and McCready, 1966), reservoir damage by bacterial cells is quite possible (Crawford 1982; Raleigh and Flock, 1965). Through studies with cores, this damage has been determined to be the result of bacterial cells with a net negative charge coming in close proximity to minerals in the rock itself which bear an opposite net charge, as well as the permeability of the core itself (Jang, et al., 1983).

Then what would be the ideal bacterium for use in microbial enhanced oil recovery (MEOR)? In addition to producing the desired metabolic end-products in an inexpensive nutrient medium, it would seem necessary that the selected bacterium be able to grow in an essentially anaerobic environment, in the presence of sodium chloride and other ionic solutes, traverse the restricted network of res5rvoir rock and survive. Limitations must be set on temperature, pressure, solutes and their effect on

growth. In this light, the ideal bacterium as defined by McInerny (1982) will set the pH and salt limitations. "A thermophilic anaerobic bacterium that grows in 5% NaCl at neutral pH would be most suitable for MEOR processes." Since temperature is generally accepted to be primarily a function of the depth of the reservoir, as is pressure, a temperature limitation of 45 degrees celsius will also limit the pressure. An ideal bacterium must also be readily transported through porous rock. A spore forming bacterium would be ideal, since spores (no net charge) are transported through cores more efficiently than vegetative cells. A reduction is permeability does occur, yet it is not as great as that caused by vegetative cells (Jang, et al., 1983). Spores are also more resistant to high shear rates and pressures (Jang, et al., 1983) that are necessary to "drive" the spores or cells out from the bore hole into the reservoir itself. Sale, et al., (1970) report that spores are resistant to pressures of 1000-3000 atmospheres whether the pressure is held continously (compression) or discontinously (decompression). Ionic solutes such as NaCl and CaC_{12} were found to decrease the inactivation of spores. The percentages of surviving vegetative cells of Serratia marcescens, Brucella abortus, and Staphylococcus aureus when subjected to 118 atms of

pressure and rapid decompression are 69-41% for Serratia and 90-75% for Brucella and Staphylococcus (Foster, et al., 1962). One could argue that pressures of 118 atmospheres or 1734 psia (pounds per square inch atmosphere) would never be reached during well inoculation and rapid decompression would occur only during fracturing. Still the easier movement of spores through permeable formations and their increased resistance to pressure and shear rates are important parameters when one wishes to introduce the bio-producing agent into the formation. Since the ability to form spores is of such paramount importance in MEOR, the selection of the ideal bacterium is narrowed to members of the genera Bacillus and Clostridium. The choice may be further narrowed due to the anaerobic nature of the reservoir. As Zajics (1984) states "dissolved oxygen exists in both the connate water and the crude oil present in the reservoir, however, once the oxygen is consumed by microbial oxidative reactions one cannot expect replacement". This would mean that only facultative members of the genus Bacillus and the obligately anaerobic Clostridium species, capable of producing copious amounts of organic acids, alcohols, surfactant molecules, emulsifiers and gases in a low cost medium such as molasses, in the presence of 5% NaCl at an upper temperature limit of 45 degrees celsius

would be desirable.

Two further limitations on the use of bacteria are hydrogen sulfide production and possible pathogenicity. Saturation of an oil reservoir with pathogenic bacteria would not be prudent, for under normal oilfield maintenance a number of people could conceivably come in contact with these bacteria. Production of hydrogen sulfide is a minor inconvenience in the laboratory whereas in the field it would be a disaster as a result of its corrosive nature. Any bacterium, however, well suited for use in MEOR, if pathogenic or a producer of hydrogen sulfide, must be rejected at once.

This then gives us a complete definition of the ideal bacterium for MEOR. A non-pathogenic anaerobic or facultatively anaerobic sporeforming bacterium able to grow in the presence of 5% NaCl at an upper limit of 45 C, utilizing a low cost nutrient source to produce end-products such as organic acids, solvents, gases, etc., without producing hydrogen sulfide. Once a bacterium or combination of bacteria are obtained which meet this definition a study of the effects of these bacteria on the release of oil should be initiated, not only in the laboratory using sandpacks and cores, but in the field as well. The question is not whether bacteria are capable of producing compounds useful in EOR, but rather can a

bacterium isolated specifically for this purpose do so *in situ* and in sufficient quantities to cause oil mobilization and increased recovery.

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PART I

ISOLATION AND SCREENING

CHAPTER I

INTRODUCTION

There is no doubt that bacteria can and do in fact produce compounds that could be important in enhanced oil recovery. Two issues over the past few years have been hotly debated. They are: 1. Which are better mediators of enhanced oil recovery, anaerobic of aerobic bacteria? 2. Does one merely stimulate the indigenous microflora in a well or should various bacteria, capable of growth under reservoir conditions, be isolated and used to inoculate (provided suitable products can be and are produced in the connate water) the reservoir?

There can be little doubt that the greatest observable change in the chemical and physical properties of oil, whether in the field or laboratory occur under aerobic conditions (Westlake, 1982). This can be easily explained by the fact that the mono and dioxygenase enzymes required for bacterial degradation of hydrocarbons require free molecular oxygen. Still, the reservoir itself contains little dissolved oxygen, certainly not enough for sustained growth of an obligately aerobic bacterium. In fact, a

number of investigators (Hitzmann, 1982: McInerny, 1982; and Ramsey, et al., 1984) have suggested that anaerobic bacteria are the most useful for enhanced oil recovery (EOR) methods. Still others (Karashiewicz, 1975, and Lazar, 1976) use combinations of anerobic and anaerobic bacteria. In my opinion only facultatively anaerobic and obligately anaerobic bacteria would be able to grow under the anaerobic conditions in a static reservoir, without the addition of a free-oxygen releasing compound such as hydrogen peroxide.

The "indigenous bacteria versus selected organism" debate has supporters on both sides. Janskekar (1984) has suggested that the best source for inoculation would be the produced water, and Lazar (1976) as well as Karashiewicz (1975) used indigenous bacteria in field studies. It should be noted, however, that months of laboratory work were done by both Lazar and Karashiewicz to select the combination of indigenous bacteria that led to the most efficient release of oil from oil-saturated cores and sand-packs. Selection of the best oil movers from an indiginous population of bacteria from each well to be treated is time consuming. Chan and Yen (1984) found that Clostridium acetobutylicum was more effective at releasing oil from the column than indigenous bacteria present in the oil used to pack a sand column.

Therefore a decision was made to isolate, not just from oilfield sources, but any source, clostridia that would be useful for microbial enhanced oil recovery (MEOR). The preliminary screening would be based on the ability of isolates to grow in the presence of 5% NaCl. Secondary screening would include testing for production of copious amounts of solvents, acids, gases or emulsifiers known to be useful in EOR methods and the ability of said cultures to release oil from oil-impregnated sand and limestone rocks. The final screen should be to eliminate any potential pathogenic or hydrogen sulfide-producing cultures. The isolation procedures used were based on these principles.

The most difficult task was to isolate cultures capable of growing in the presence of 860 mM (5.0% w/v) NaCl. Aside from the fact that most bacteria (and indeed Cl. acetobutylicum) will not grow at sodium chloride levels above 520 mM (3.0% w/v), there is the renowned ability of the butanol forming bacteria to degenerate when frequently transferred without heat shocking the spore form. This degeneration asserted itself as cells that are unable to perform a solvent fermentation, (reminiscent of the butylic group) assumed dominance in a culture. (Kutzenok and Aschner, 1952; and Stephens, et al., 1985). Degeneration according to Kutzenok and Aschner (1952) can

be enhanced by the addition of 84 mM NaCl, ten times less than our primary isolation levels. This no doubt would have negative effects on any attempt to "train" cultures to grow in higher salt concentrations by continously transferring from lower to higher salt concentrations.

Data included in the first chapter of this dissertation on the effects of salt (NaCl) on growth, end-product production, and transport are not intended to enlighten the reader as to how sodium chloride causes these changes. Rather they pertain to the results of exposure of the cells to sodium chloride.

The intent was to isolate from nature, strains of Clostridium capable of growing in the presence of salt (as sodium chloride) at a level of 860 mM and produce metabolic end-products in amounts sufficient to cause an increased flow of oil from a reservoir. In order to accomplish this, selection schemes, based on principles outlined earlier, were used to isolate said strains from the environment.

CHAPTER II

MATERIALS AND METHODS

Initial Isolation Procedure

Cultures were originally isolated by placing a sample of water, soil, etc., from various sites such as salt flats, marshes originating from a number of states and Canada, in a tube containing one of the following media:

1. Tryptic Soy Broth (Difco) 1% glucose, 860 mM NaCl.
2. Tryptic Soy Broth (Difco) 1% sucrose, 860 mM NaCl.
3. 4% Molasses (v/v) 0.1% ammonium nitrate, 0.1% calcium carbonate, 860 mM NaCl.

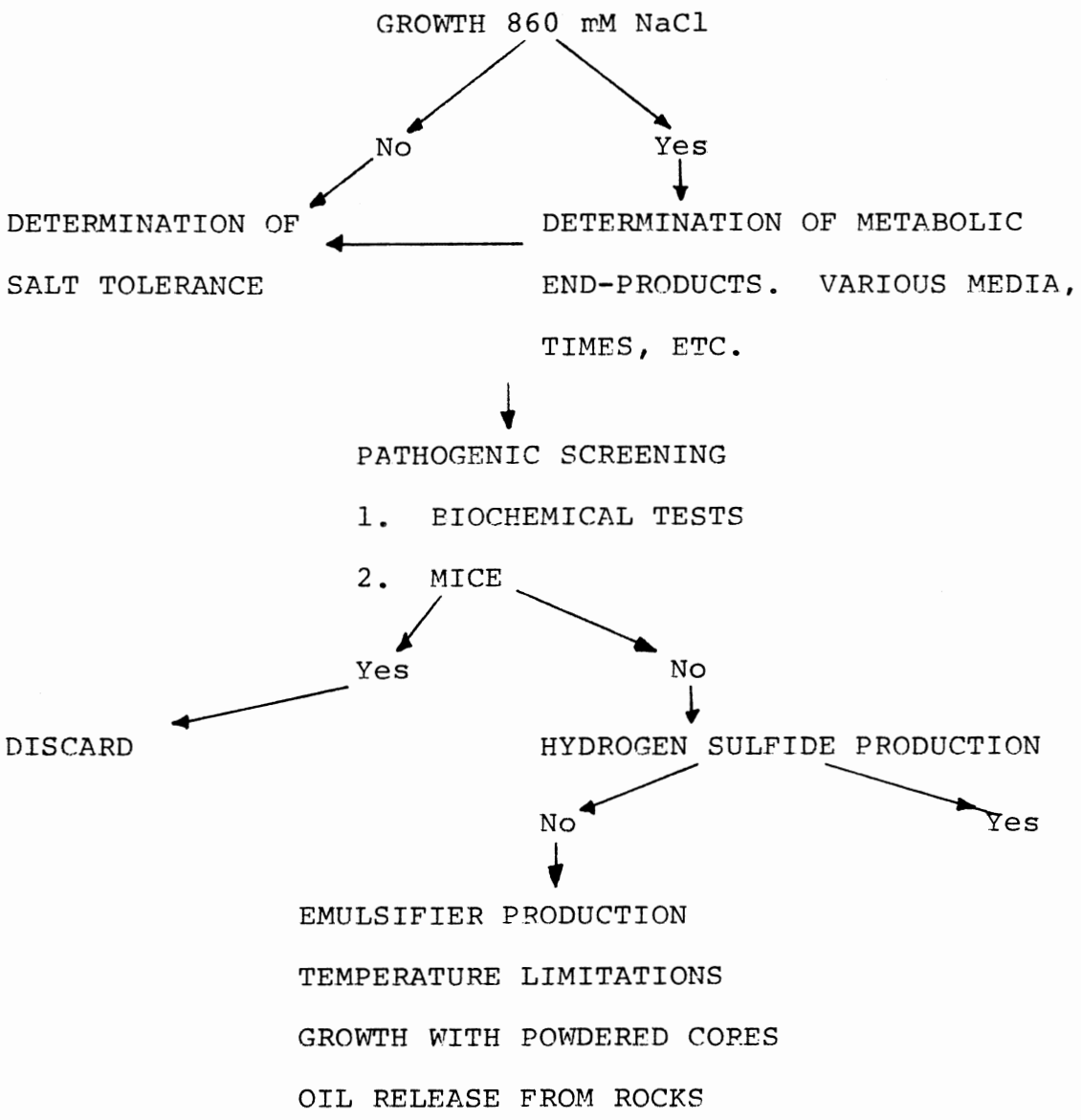
Vaspar was then layered on top of the medium and tubes were incubated (usually duplicates at 37 and 45 degrees C). Positive results were tubes that had become turbid due to growth or where gas production forced the vaspar up the tube.

Later, nitrogen flushed Hungate tubes with the appropriate medium added were used, omitting the vaspar step.

Secondary Screening Procedures

Figure 1 shows the schematic for secondary screening.

FIGURE 1. Secondary Screening Procedure for isolates of clostridia to be used in MEOR.


GROWTH 860 mM NaCl
No
Yes
DETERMINATION OF SALT TOLERANCE
DETERMINATION OF METABOLIC END-PRODUCTS. VARIOUS MEDIA, TIMES, ETC.
PATHOGENIC SCREENING
1. BIOCHEMICAL TESTS
2. MICE
Yes
No
DISCARD
HYDROGEN SULFIDE PRODUCTION
No
Yes
EMULSIFIER PRODUCTION
TEMPERATURE LIMITATIONS
GROWTH WITH POWDERED CORES
OIL RELEASE FROM ROCKS

Media

Media for identifying and quantitating acids, gases and solvents were:

1. Sucrose salts- 1% sucrose, 0.6% yeast extract, 0.1% tryptone, 0.1% ammonium nitrate, 0.01M combined phosphates, 20 mg/100 ml magnesium sulfate and Erwinia trace minerals (Grula, 1960).

2. Tryptic Soy Broth (Difco) with 1% sucrose.

3. Molasses medium- 4% molasses (v/v), 0.1% ammonium nitrate and 0.1% calcium carbonate.

Growth

Cultures were incubated for a specific interval at the desired temperature in nitrogen flushed Hungate tubes vaspar sealed tubes or in an anerobic jar (BBL). The interval of incubation was entirely dependent on the experimental design.

Transport (uptake) Studies

Cells were harvested in mid-log phase, spun at 8,000 x G for ten minutes, resuspended in phosphate buffer (0.01M) with 0.1% sodium thioglycollate purged with nitrogen and spun again at 8,000 x G. The washing procedure was repeated then cells were resuspended in a solution of 0.01M combined phosphates, Erwinia trace minerals (Grula, 1960) and aseptically added to a serum

bottle containing a nitrogen atmosphere. Uniformly labeled compounds (^{14}C - labeled glucose, sucrose or amino acids) were added and at one or three minute intervals, 1 ml removed, filtered through a 0.45 um filter (Gelman) then washed twice with cold 0.01M phosphate buffer (pH 7.00). Filter and cells were then removed, placed in a scintillation vial and counted in a Beckman LRS 7500 scinillation counter.

At the conclusion of the experiments, optial density readings were taken, converted to dry weight and using the appropriate dry weight curve micrograms of dry weight per culture per tube obtained.

Medium and Incubation

For uptake, cultures were grown in anaerobic gas packs (BBL) in Tryptic Soy Broth, plus 1% sucrose. All incubations and manipulations were performed at 37 degrees C.

Dry Weight Curves

Cells were grown overnight in Tryptic Soy Broth, washed twice with distilled water and resuspended in 10 ml of distilled water. Various aliquots were removed, diluted to 10 ml, and optical densities measured at 595 nms with Bausch and Lomb Spectrophotometer 100. After the optical density was determined, 5 mls of each dilution were removed and added to pre-weighed aluminum

pans that had dried for 48 hours in a 105 degree C oven. The pans were then placed in a 105 degree C oven. Twenty-four hours later, the pans were removed and weighed. After three consecutive weighings the pans were again placed in the oven for another 24 hours. At this time pans were removed every half-hour and weighed until three consecutive weights were determined that were the same. At this time, the average weight was determined.

Oil-Impregnation

Initial oil release studies were done using the apparatus described in Figure 2. No effort was made to lower contamination from the oil, rocks or air. Later the apparatus shown in Figure 3 was used. Rocks were sterilized by 48 to 72 hours incubation in a 105 degree C oven and oil was filtered through a 0.45 μm millipore filter. The apparatus was sterilized by autoclaving for 15 minutes.

In both cases, (apparatus from Figure 2 or 3) rocks were evacuated (hooked to in-house vacuum lines) before the addition of oil (to remove air trapped inside), and further evacuated for 30 minutes after the first contact of oil and rock. After 30 minutes under vacuum, the oil was poured off, and the rocks washed three times in distilled water to remove residual oil not trapped in

FIGURE 2. Apparatus used to place oil in rocks when no attempt was made to limit contamination.

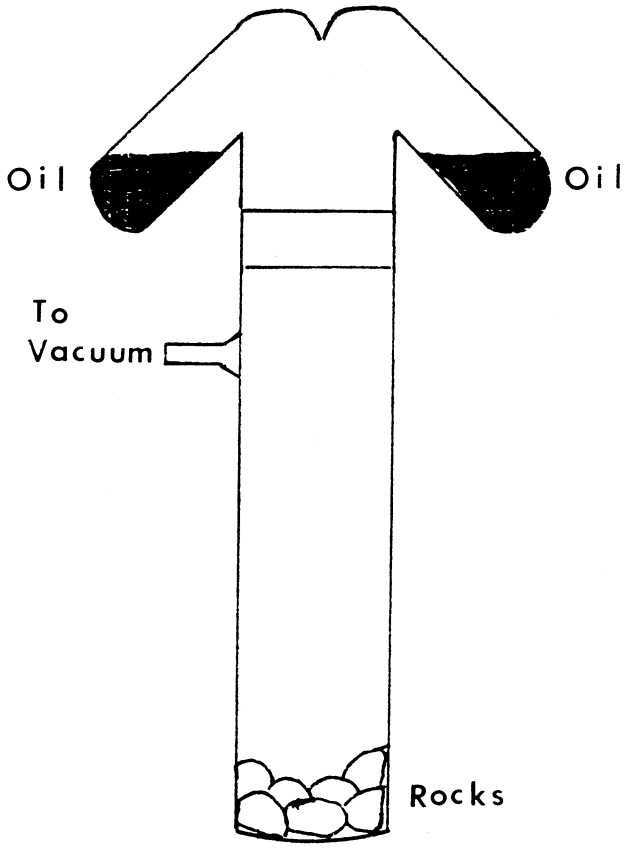
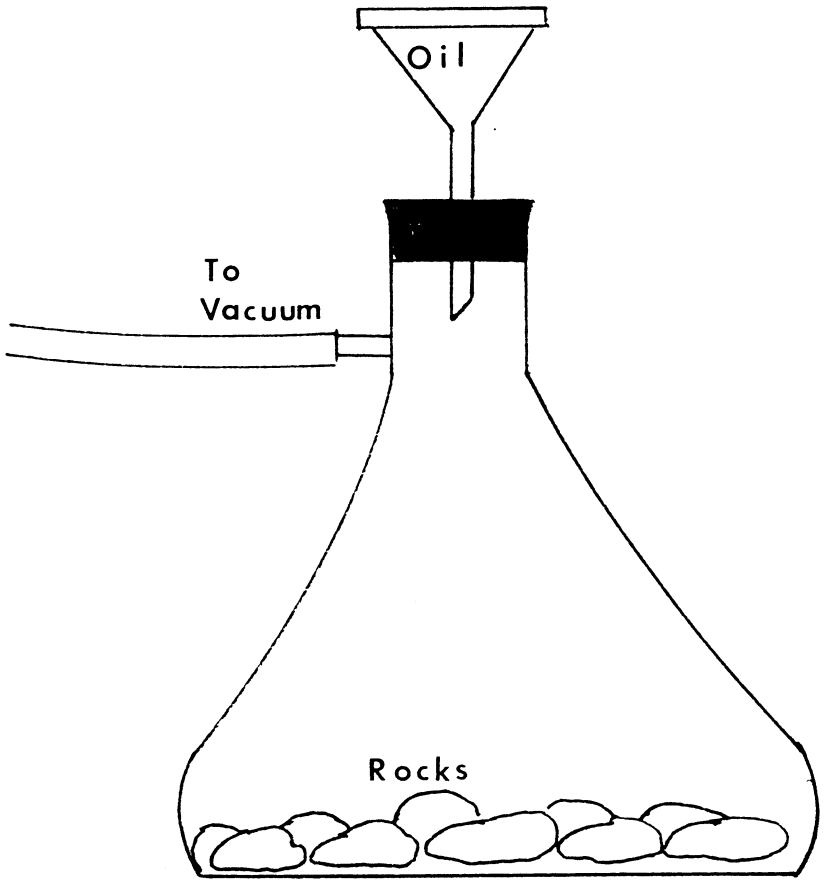

Oil
Oil
To
Vacuum
Rocks

FIGURE 3. Apparatus used to impregnate rocks with oil. All components were autoclavable and were treated thus to sterilize.


Oil
To
Vacuum
Rocks

pores. When the apparatus in Figure 3 was used, the distilled water was sterilized by autoclaving and added to the apparatus aseptically. Rocks were then placed on Whatman #1 filter paper to dry. Again, where aseptic measures were used, Whatman #1 filter paper was cut to fit in the bottom of a plastic petri dish and then sterilized by the method of Sanborn, et al., (1983) in a Sears Micro-oven. Rocks were added to sterilized 20 mm tubes with autoclaved media, using forceps that had been immersed in alcohol and flamed. Tubes were then layered with 2.5 mls of autoclaved vaspar. Attempts were made to ensure that all tubes received approximately the same amount of oil-laden rock and this was accomplished by weighing tubes before and after rock addition.

Oil Release

Oil release was determined by visually observing the amount of oil that rose to the surface of the medium as growth occurred. The oil film was scored according to its circumference (amount on top of the medium covered by oil) and volume on a scale of one to four. Initial controls consisted of uninoculated medium (run with inoculated tubes). Secondary controls were the inoculation of tubes with specific cultures such as *E. coli*.

Measurement of Gas

Gas was measured by the distance the plunger on a 5, 10 or 20 cc syringe was displaced by gas pressure as the needle penetrated the septum of a Hungate tube. Volumes were determined by the formula $\pi r^2 \times h$. Where π equals 3.142, r the radius of the syringe, and h the distance in mm the displaced distance of the plunger.

Determination of Acids and Solvents

Acid and solvent analysis were made using a Sigma 2 series, Perkin-Elmer gas chromatograph equipped with a flame inonization detector and a 6' glass column with 1/4" inside diameter. Peak areas were calculated using a Hewlitt-Packard calculating integrator.

Packing for analysis: 1-1820 carbopack
c/o 0.5% Sp-100.

For acid analysis: 60/80 carbopack
c/o .3% carbowax 20M 0.1%
phosphoric acid.

Programs

For solvent analysis:

Oven temperature: 200°C

Injection temperature: 150°C

Initial temperature: 80°C

Initial time: 2 minutes

Initial rate: 15°C/minute

Final temperature: 120°C

Final time: 5 minutes

Final rate: 25°C/minute

Post temperature: 145°C

Post time: 0.5 minutes

Carrier gas - nitrogen 20 ml/minute

For acid analysis:

Oven temperature: 200°C

Injection temperature: 150°C

Initial temperature: 120°C

Initial time: 2 minutes

Initial rate: 15°C/minute

Final temperature: 130°C

Final rate: 15°C/minute

Post temperature: 150°C

Post time: 1.0 minutes

Carrier gas - nitrogen 35 ml/minute

Sample Preperation

One tenth ml of 50% phosphoric acid was added to one ml of spent culture medium to acidify acid end-products. One microliter of this sample was added to the GC for analysis. Standard solvents and acids were: Methanol, ethanol, propanol, isopropanol, 2, 3 butanediol, methyl,

Analysis

Where necessary, samples were co-chromatographed (known solvents or acids mixed with the unknown) if a question arose as to peak identification. Internal standards (propionic acid or n-propanol) were also included if questions arose as to the validity of peak area.

CHAPTER III

RESULTS AND DISCUSSION

The first Clostridium sp. isolated that were capable of growth in 860 mM sodium chloride are listed in Table I. The isolation medium was Tryptic Soy Broth with 1% glucose. Culture designations (column one) were used to differentiate between cultures sensitive to salt (HS) and those capable of growth in 860 mM salt (HR). Two designations were necessary since cultures that could produce copious amounts of gas were not discarded. Sources of the material used in the isolation attempts were diverse and varied. In addition to soil and the feces of animals and birds, some isolates were obtained from connate water, estuaries, sea water and salt flats. Many attempts were made to first isolate clostridia from the environment and "train" them to grow in the presence of 860 mM sodium chloride. This was attempted by starting the cultures in a medium with low levels of sodium chloride, allowing them to grow to a visible optical density, then transferring to fresh medium with the salt concentration increased by 0.5 to 1.0%. Stepwise inoculations were done, attempting

TABLE I

FIRST CLOSTRIDIAL ISOLATES CAPABLE OF GROWTH IN 860mM SODIUM CHLORIDE

Culture	Type Culture	Source	Gas Production 86mM NaCl@24hrs.	Gas Production 86mM NaCl@48hr.
HR-4	Pure	Cow Manure	7 mm gas	Acid
HS-1	Mixed	Cow Manure	25 mm gas	N.G.
HR-3	Mixed	Soil	40 mm gas	25 mm gas
HR-9	Mixed	Cat feces	40 mm gas	19 mm gas
HR-13	Mixed	Dog feces	30 mm gas	20 mm gas
HS-4	Mixed	Cow Manure	55 mm gas	N.G.
HR-11	Mixed	Aquarium Water	18 mm gas	14 mm gas

Abbreviations Used: N.G.= no growth
Gas Production, distance in millimeters vaspar plug was pushed as a result of gas production.
Medium Tryptic Soy Broth with 1% sucrose.

to adapt isolated cultures to continously higher and higher levels of sodium chloride. All attempts to "train" isolates to grow in higher levels of sodium chloride were fruitless. While at times, cultures did appear to adapt to the higher levels, a second transfer in a medium of the same level of sodium chloride resulted in no growth on the part of the "adapted" strain.

The failure to attempt to "train" isolates to grow in the presence of salt can best be explained by the fact that degeneration in the butylic group of Clostridium occurs in 86 mM sodium chloride (Kutzenok and Aschner, 1952) after only a few transfers.

Cultures that could initially grow in 860 mM sodium chloride were designated HR and those that would not grow in 860 mM sodium chloride HS. Following the initial growth in 5% salt, cultures were subjected to purification, by standard microbiological techniques. This was accomplished by plating out on the appropriate medium, picking an isolated colony and repeating the procedure three times. After the purification, numerous letters would be added to particular cultures such that HR-9 would become cultures HR-9A, HR-9B and HR-9C. To those cultures that were able to produce a compound capable of forming a water external emulsification with crude oil, the letter E

would be added to the number. As a result of this procedure HR-69, HR-66 and HR-3 became HR-69E, HR-66E and HR-3E respectively. This was done in order to differentiate emulsifier producing cultures from those that did not.

On the average during an isolation attempt, only 10% of the tubes inoculated would show positive (turbidity and/or gas) and of that number perhaps 1% would be clostridia. Of the 1% remaining after an isolation attempt, some of those for some reason or another became unsatisfactory. At times it appeared that cultures simply lost the ability to grow in the presence of 860 mM sodium chloride, perhaps because of degeneration or the phase of the moon. Purification also appeared to affect the ability of cultures to grow in the presence of 860 mM NaCl; when a culture was purified, gas, solvent and acid production in the presence of salt were markedly reduced.

Still numerous cultures were isolated that retained the ability to grow in salt, even after purification on streak plates. Tables II and III list the solvents, acids and carbon dioxide produced by five isolates in Tryptic Soy Broth (TSB) or Molasses medium with NaCl levels of 86 and 860 mM, when incubated at 37^{o}C for 72 hours. A vast difference is seen not only between cultures grown in

TABLE II

SOLVENT PRODUCTION (mM) AND GAS PRODUCTION (mm)
BY VARIOUS CULTURES WITH 0.5 and 5.0% NaCl
IN EITHER MOLASSES OR TRYPTIC SOY BROTH
AFTER 72 HOURS INCUBATION AT 37°C

Culture	Medium	%NaCl	Ethanol	Isoprop.	n-Prop	n-But	Acetone
HS-4B	TSB	0.5	4.5	-0-	2.1	-0-	6.2
HS-4B	TSB	5.0	4.6	-0-	2.4	12.8	4.4
HS-4B	MOL	0.5	4.9	2.2	3.3	11.8	-0-
HS-4B	TSB	5.0	2.2	1.0	1.3	12.3	-0-
HR-94A	TSB	0.5	4.9	-0-	2.5	-0-	-0-
HR-94A	TSB	5.0	3.9	-0-	-0-	-0-	-0-
HR-94A	MOL	0.5	3.9	-0-	-0-	12.6	-0-
HR-94A	MOL	5.0	3.8	-0-	-0-	6.0	-0-
HR-3	TSB	0.5	4.2	-0-	3.8	12.3	6.9
HR-3	TSB	5.0	4.2	-0-	3.8	6.9	8.5
HR-3	MOL	0.5	3.6	-0-	-0-	13.2	-0-
HR-3	MOL	5.0	5.9	-0-	2.4	6.7	-0-
HR-72B	TSB	0.5	4.4	-0-	3.3	11.9	-0-
HR-72B	TSB	5.0	3.9	-0-	0.3	7.3	-0-
HR-72B	MOL	0.5	5.6	2.1	2.1	11.7	2.6
HR-72B	MOL	5.0	3.9	2.7	-0-	5.0	-0-
MX-1B	TSB	0.5	5.6	-0-	-0-	12.6	-0-
MX-1B	TSB	5.0	3.9	2.1	-0-	9.0	-0-
MX-1B	MOL	0.5	5.6	2.2	1.9	12.0	-0-
MX-1B	MOL	5.0	3.0	2.1	2.4	-0-	2.6

Abbreviations Used: Isoprop.= Isopropanol
n-Prop= n-Propanol
n-But= n-Butanol

TABLE III

ACID PRODUCTION (mM) AND GAS PRODUCTION (mm)
BY VARIOUS CULTURES WITH 0.5 AND 5.0% NaCl
IN EITHER MOLASSES OR TRYPTIC SOY BROTH
AFTER 72 HOURS INCUBATION AT 37°C

Culture	Medium	% NaCl	Gp Total	Acetic	Butyric	Isovaleric	Propionic
HS-4B	TSB	0.5	33.1	0.1	-0-	-0-	-0-
HS-4B	TAB	5.0	18.0	0.2	-0-	-0-	-0-
HS-4B	MOL	0.5	18.2	T	-0-	-0-	-0-
HS-4B	TSB	5.0	12.6	T	-0-	-0-	-0-
HR-94A	TSB	0.5	31.0	T	-0-	-0-	-0-
HR-94A	TSB	5.0	27.3	T	T	T	-0-
HR-94A	MOL	0.5	18.8	T	T	-0-	5.0
HR-94A	MOL	5.0	11.1	T	-0-	-0-	-0-
HR-3	TSB	0.5	36.5	1.5	-0-	-0-	-0-
HR-3	TSB	5.0	8.5	1.3	-0-	-0-	-0-
HR-3	MOL	0.5	17.6	-0-	-0-	-0-	-0-
HR-3	MOL	5.0	9.7	-0-	-0-	-0-	-0-
HR-72B	TSB	0.5	34.3	0.2	21.4	9.5	-0-
HR-72B	TSB	5.0	24.5	2.0	35.5	-0-	-0-
HR-72B	MOL	0.5	17.1	0.9	6.9	-0-	10.0
MX-1B	TSB	0.5	35.9	T	-0-	-0-	2.5
MX-1B	TSB	5.0	10.9	0.1	-0-	-0-	-0-
MX-1B	MOL	0.5	21.8	-0-	-0-	-0-	-0-
MX-1B	MOL	5.0	8.8	-0-	-0-	-0-	-0-

Abbreviations Used: Gp Total= Area in Millimeters Under Curve on Gas Chromatograph

the presence of salt, but cultures grown in TSB as compared to the molasses medium. Differences in end-product formation under various conditions (TSB vs Molasses and 86 mM NaCl vs 860 mM NaCl) are not surprising. In fact, a number of parameters are capable of changing the solvent/acid production ratio and therefore the gas production by a culture (Bahl and Gottschalk, 1984; Klu and Sundstrom, 1984; Montville, et al., 1985; and Turton, et al., 1983).

Of the cultures listed (Tables II and III), HR-72, MX-1B and HR-3 (now HR-3E) are still considered potential candidates for EMOR, but not just for the data presented here. Both tables show the contrast in production of acids, solvents and gases by cultures grown in two different salt concentrations (860 mM vs low salt 86mM) at 72 hours incubation. One of the first observations was that growth in 860 mM NaCl was less than growth at lower salt concentrations. Zalashko, et al., (1984) point out that the addition of 860 mM NaCl into the medium used to grow Rhodotorula glutinis caused a decrease in the amount of biomass formed, resulting from a decrease in the rate of growth, as well as having a considerable effect of salt on carbohydrate and lipid metabolism. Observations such as this have been made with Clostridium isolates, a decrease in not only the amounts, but kind of end-products formed with increasing levels of salt.

Figures 4 and 5 show growth curves of typical clostridial isolates in TSB plus 1% sucrose with and without 860 mM NaCl. Figure 1 is what can be termed "the best case" scenario, growth is delayed by only some 12 hours. While the 12 hour delay can at times be longer, it is not nearly as long as the delay shown by the isolate depicted in Figure 2. Here, the delay in growth is longer (at times as long as 72 hours) and maximum growth potential is never reached. Growth ceases at some point, usually 1/2 to 3/4 the maximum reached without salt. Consequently, if growth itself is delayed, gas, acid and solvent production must also be delayed although amounts equal to those produced in the same medium without salt are never reached.

Table IV contrasts the gas production (as measured by peak area with gas chromatography) of isolates inoculated and incubated at $37^{o}C$ for 48 and 96 hrs. with 86 mM NaCl and the same isolates incubated for 336 hours and 384 hours with 860 mM NaCl, in the sucrose-salt medium.

Gas production by several isolates with 860 mM NaCl mirrors that produced during the shorter incubation time of those in 86 mM NaCl. No isolate has produced more gas in 860 mM NaCl than lower concentrations, though there seems to be some that prefer 1 or 2% NaCl over lower concentrations.

Tables V and VI compare solvent productions of the

FIGURE 4. Growth of Culture HR-72 in Tryptic Soy Broth supplemented with 1% Sucrose. Open triangles show optical densities with 86 mM sodium chloride. Closed triangles 860 mM sodium chloride added. Incubation at 37 C.

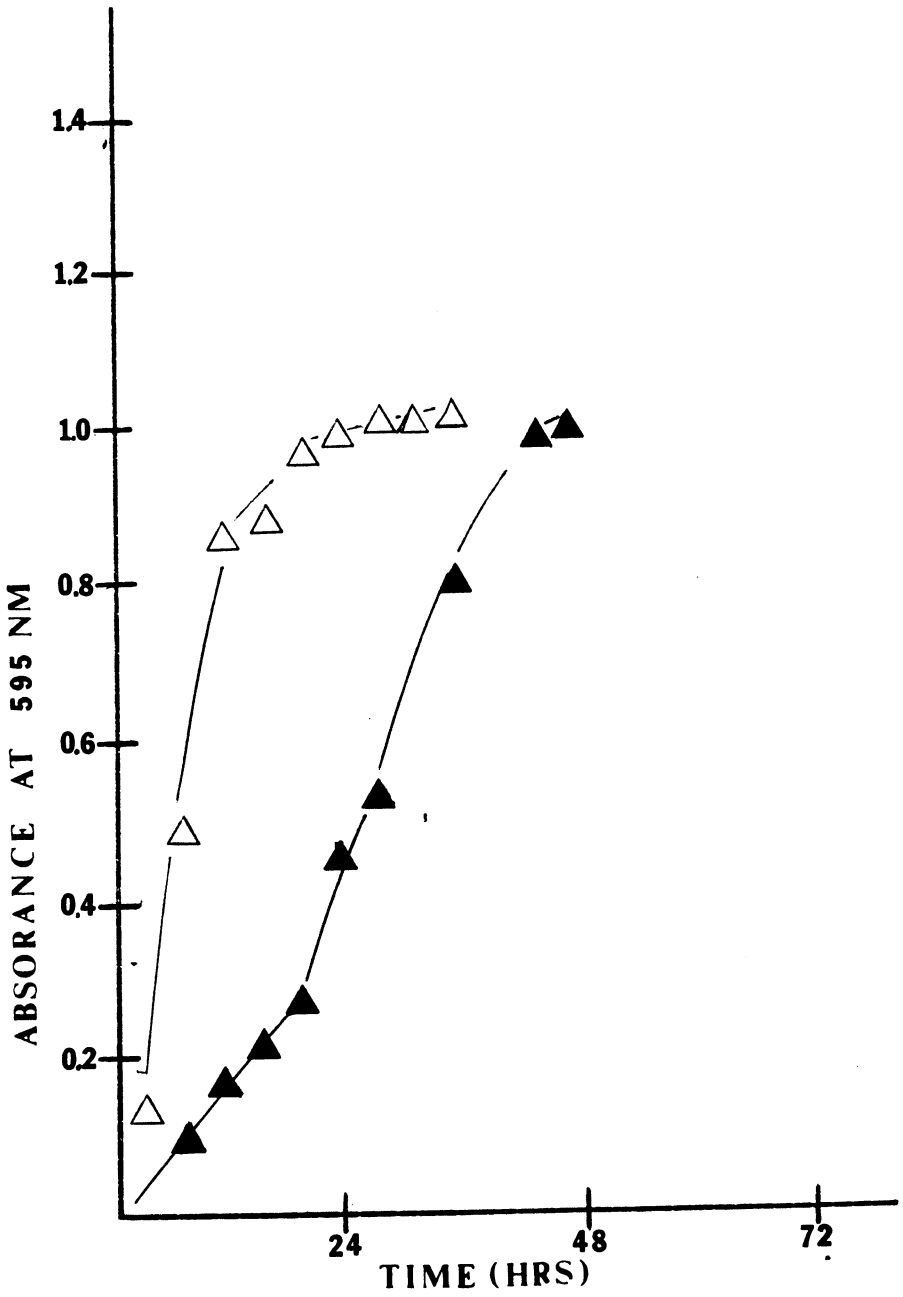

1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORANCE AT 595 NM
24
48
72
TIME (HRS)

FIGURE 5. Growth of Culture HR-94 in Tryptic Soy Broth supplemented with 1% Sucrose. Open triangles show optical densities with 86 mM sodium chloride. Closed triangles 860 mM sodium chloride added. Incubation at 37°C.

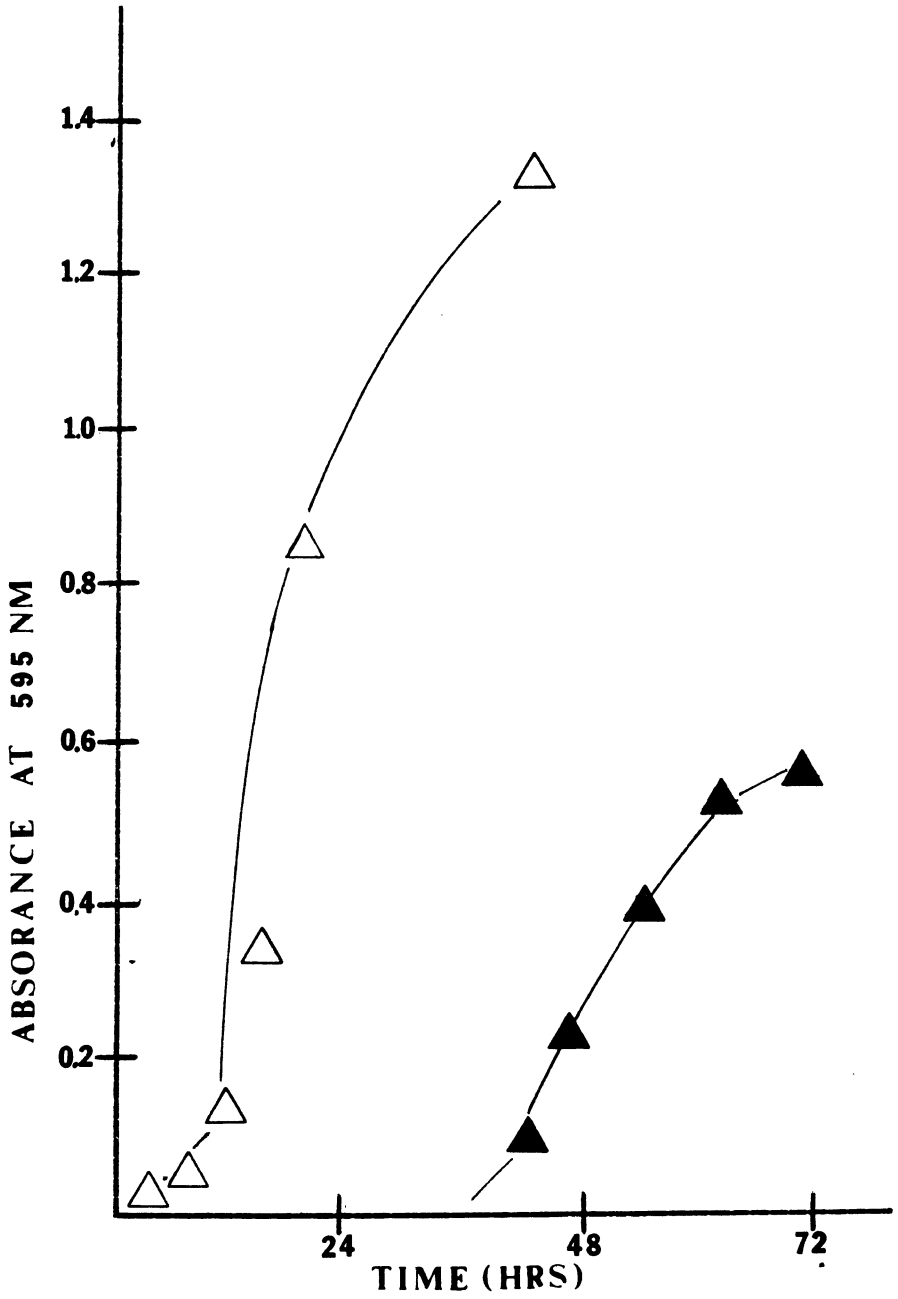

1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORANCE AT 595 NM
24
48
72
TIME (HRS)

TABLE IV

GAS PRODUCTION BY VARIOUS CULTURES IN THE SUCROSE SALTS MEDIUM AT 48 AND 96 HOURS IN THE PRESENCE AND ABSENCE OF 96 mM SODIUM CHLORIDE AND 336 AND 384 HOURS IN THE PRESENCE OF 860 mM SODIUM CHLORIDE

Culture	Gas Production MM x 10^4 86 mM NaCl 48 hours	96 hours	Gas Production MM x 10^4 860 mM NaCl 336 hours	384 hours
MX-1A	2.8	5.3	5.9	6.5
HR-5B	2.6	3.5	3.8	3.9
HR-32	4.0	5.0	3.8	3.6
HR-66	3.7	6.8	3.4	3.0
HR-3E	3.6	5.2	3.4	4.7
HR-69E	4.1	5.8	3.4	3.8
HR-51B	5.4	7.2	4.3	7.5

Values determined by peak area from gas chromatograph.

TABLE V

SOLVENTS (mM) PRODUCED BY VARIOUS CULTURES AT 48 HOURS IN 86mM SODIUM CHLORIDE AND 336 HOURS IN THE SUCROSE-SALTS MEDIUM

	Solvent Production 48 hrs. 86mM Sodium Chloride				Solvent Production 336 hrs. 860mM Sodium Chloride			
Culture	Etoh	Acetone	Isoprop.	But.	Etoh	Acetone	Isoprop.	But.
HR-5D	3.6	1.8	2.0	10.0	5.4	-0-	2.0	1.6
HR-32	3.9	-0-	2.1	-0-	4.9	1.9	1.0	-0-
HR-66E	3.9	1.8	2.1	-0-	2.6	1.9	1.5	-0-
HR-3E	3.9	-0-	1.8	-0-	4.2	2.1	1.5	-0-
MX-1A	2.8	-0-	1.8	-0-	3.1	-0-	2.0	-0-
HR-69E	4.2	-0-	2.1	-0-	2.5	1.8	1.5	-0-
HR-52	3.9	-0-	2.5	-0-	4.5	-0-	1.2	-0-
HR-51B	3.9	-0-	-0-	-0-	2.9	-0-	1.0	-0-

Abbreviations Used: Etoh= Ethanol Isoprop.= Isopropanol But.= n-Butanol

TABLE VI

SOLVENTS (mM) PRODUCED BY VARIOUS CULTURES AT 96 HOURS IN 86mM SODIUM CHLORIDE AND 384 HOURS IN 860mM SODIUM CHLORIDE IN THE SUCROSE-SALTS MEDIUM

	Solvent Production 96 hrs. 86mM Sodium Chloride				Solvent Production 384 hrs. 860mM Sodium Chloride			
Culture	Etoh	Acetone	Isoprop.	But.	Etoh	Acetone	Isoprop.	But.
HR-5B	4.2	1.8	2.2	10.0	5.4	-0-	2.2	1.9
HR-32	4.3	-0-	2.5	-0-	5.4	2.2	1.9	-0-
HR-66E	4.6	2.4	2.1	2.5	7.2	2.0	1.9	-0-
HR-3E	4.1	-0-	2.3	-0-	3.8	-0-	1.8	-0-
MX-1A	4.8	-0-	2.4	2.2	3.4	2.2	1.9	1.6
HR-69E	4.2	-0-	2.2	1.8	5.7	1.9	1.9	-0-
HR-52	5.3	-0-	2.9	-0-	6.0	-0-	1.9	0.2
HR-51B	3.8	-0-	1.8	-0-	6.7	1.8	1.0	-0-

Abbreviations Used: Etoh= Ethanol Isoprop.= Isopropanol But.= n-Butanol

same isolates in experiments where those grown in 860 mM sodium chloride are incubated for longer periods. While there is certainly an overall reduction in total numbers and total concentrations of solvents in salt, certain solvents are actually enhanced, especially in the case of acetone. Due to its ability to lower the surface tension of an aqueous solution and act as a polar solvent it is of great interest. The major focus of the search was on the ability of strains to produce acetone and butanol in high salt, not only because of the properties of these two compounds which would themselves quite possibly lead to an increased movement of oil from a reservoir, but also because of the concomitant production of acetic and butyric acids, (which go hand-in-hand) with acetone and butanol formation. The production of these acids and their action on carbonate rock would increase the amount of carbon dioxide in the free gas phase. This should not be taken to mean that cultures that produced only the lower or higher molecular weight (m.w.) alcohols in high salt concentrations were not welcome additions to the collection. Isopropanol and isoamyl or n-amyl alcohol are effective at washing oil from a reservoir in alcohol floods (Holm and Csaszar, 1962).

Table VII shows the end-product formation of two isolates HR-69E and HR-51B, that will be dealt with further in Part Three. The medium again is sucrose-salts with

TABLE VII

ACIDS AND SOLVENTS (mM) PRODUCED BY CULTURES HR-51B AND HR-69E AT VARIOUS TIMES AND SODIUM CHLORIDE CONCENTRATIONS SEPERATELY OR IN MIXED CULTURE IN THE SUCROSE-SALTS MEDIUM

Culture	Time (hrs.) %NaCl	Acids Acetic	Prop.	Buty.	Solvents Etoh	Acetone	Isoprop.	But.
HR-51B	24/0.5	0.1	1.8	7.0	2.5	1.8	-0-	1.4
HR-51B	48/0.5	0.3	2.5	7.0	3.1	1.8	2.8	1.3
HR-51B	96/5.0	0.1	-0-	-0-	2.7	-0-	-0-	-0-
HR-51B	192/5.0	0.2	-0-	-0-	3.5	-0-	-0-	-0-
HR-69E	24/0.5	0.1	1.9	7.0	2.6	1.8	1.8	-0-
HR-69E	48/0.5	0.2	2.0	10.3	2.8	-0-	2.4	-0-
HR-69E	96/5.0	0.1	-0-	-0-	2.9	1.8	-0-	-0-
HR-69E	192/5.0	0.3	2.0	-0-	4.4	1.9	-0-	-0-
69E/51B	96/5.0	0.1	-0-	-0-	3.1	-0-	-0-	-0-
69E/51B	192/5.0	0.6	2.1	2.1	3.2	2.0	-0-	-0-

Abbreviations Used: Prop.= Propionic Acid Buty.= Butyric Acid
Isoprop.= Isopropanol (2-propanol) But.= n-Butanol

incubation of 24 and 48 hours for tubes with 86 mM NaCl and 96 and 192 hours for tubes with 860 mM NaCl. Here it is evident that as a result of exposure to salt, a decreased production of acids and solvents occurs. Co-inoculation (using both cultures as inocula) provide an end-product pattern favorable for in situ use. Table IV shows that HR-51B produces more gas in 860 mM NaCl, while HR-69E (Tables II, III and VII) is able to produce more solvents and acids. The combination of the two does indeed enhance the total amount of acids formed, when compared to either separately. While the gas production of co-inoculated tubes is less than that when HR-51B is used alone it is possible that enhanced acid production and subsequent solvent production as the fermentation ran its course would offset the decreased gas production. This enhanced ability to produce suitable end-products in the presence of 860 mM sodium chloride also occurred when beet molasses was used as a nutrient source. Useful end-products were produced in sufficient quantaties to cause a release of oil from a reservoir.

Once isolates were obtained that could produce end-products thought to be useful for enhanced oil recovery in 860 mM NaCl, the next step was to determine the effects of high salt on not only the growth and end-product pattern, but the effects on transport and energetics of the cell.

Figure 6 shows the uptake of ^{14}C-glucose by isolate HR-66E, which is dramatically altered by the addition of NaCl. The uptake at 170 mM NaCl is less than half of the uptake when NaCl is not present, with further reduction as the salt concentrations increased. Though perhaps not quite as dramatic, the same pattern was observed for other isolates. Results of ^{14}C-glucose uptake by Cultures HR-32 and HR-5B (Figure 7) at 0 and 860 mM NaCl show that sucrose uptake is also affected by high salt concentrations, reducing the uptake of HR-32 to 50% and HR-5B to 25% of controls. Experiments were also done with cultures grown in salt, then subjected to uptake experiments in the presence or absence of salt. At times, it did seem that an adaptation had occurred, uptake of solutes transported by the PEP system appeared to remain at levels close to or equal to controls with no salt. This was thought to be an indication that perhaps an adaptation for growth in the presence of salt could occur. Initially though, the transport of solutes, such as glucose and sucrose is dramatically altered by high salt concentrations. Cultures that had not been grown in salt and as a result had not undergone said adaptation, never transported solutes at a rate in the presence of salt comparable to those of controls. Whether this is because the mechanism of transport is somehow damaged by high levels of salt, resulting in a lowered

FIGURE 6. Uptake by Culture HR-66E of uniformly labeled carbon-14 sucrose in concentrations of sodium chloride from 0-1400 mM sodium chloride. Open circles refer to uicromoles per mg dry weight of sucrose transported into the cells after 3 minutes incubation. Open triangles 6 minutes incubation. Open squares 9 minutes incubation.

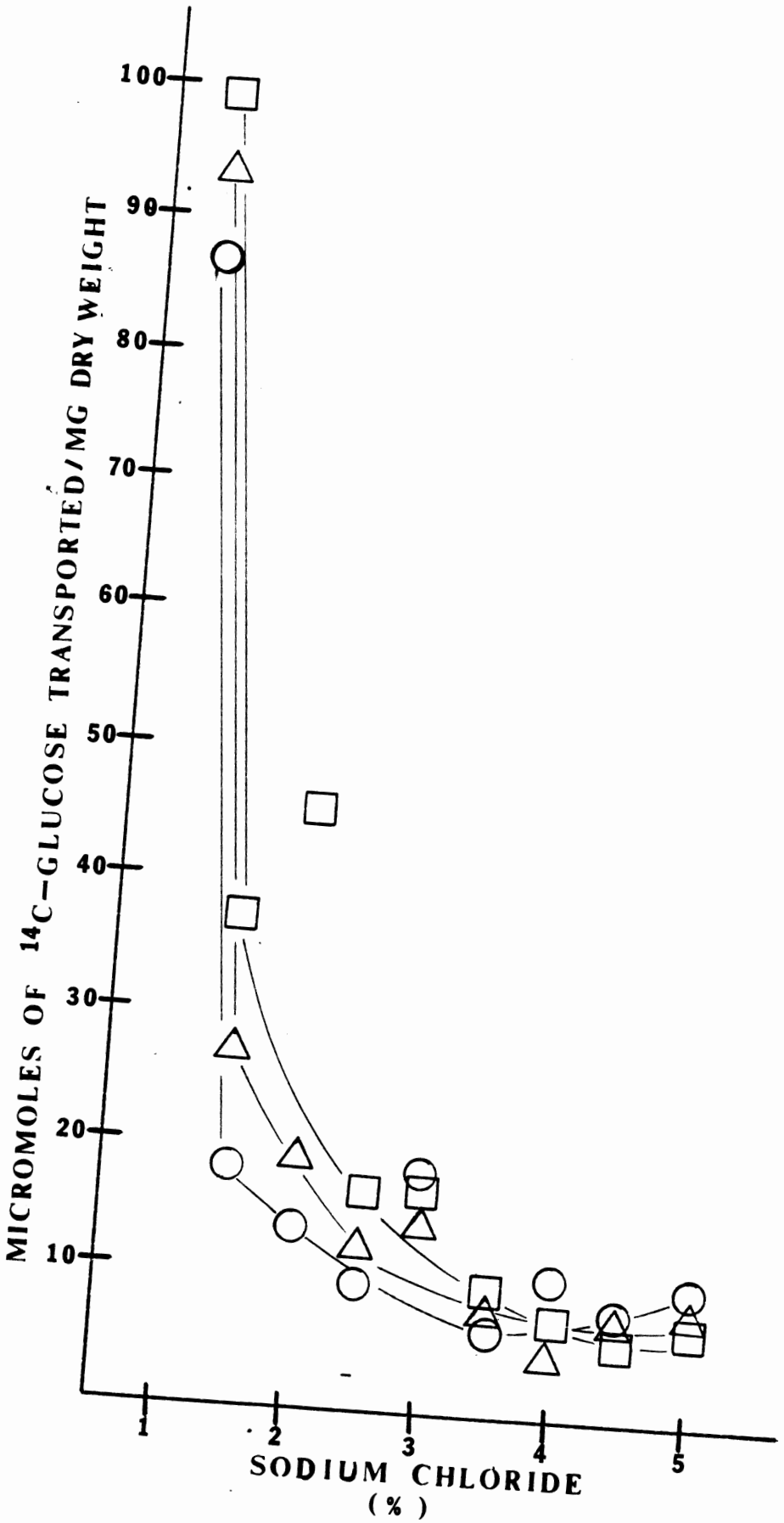
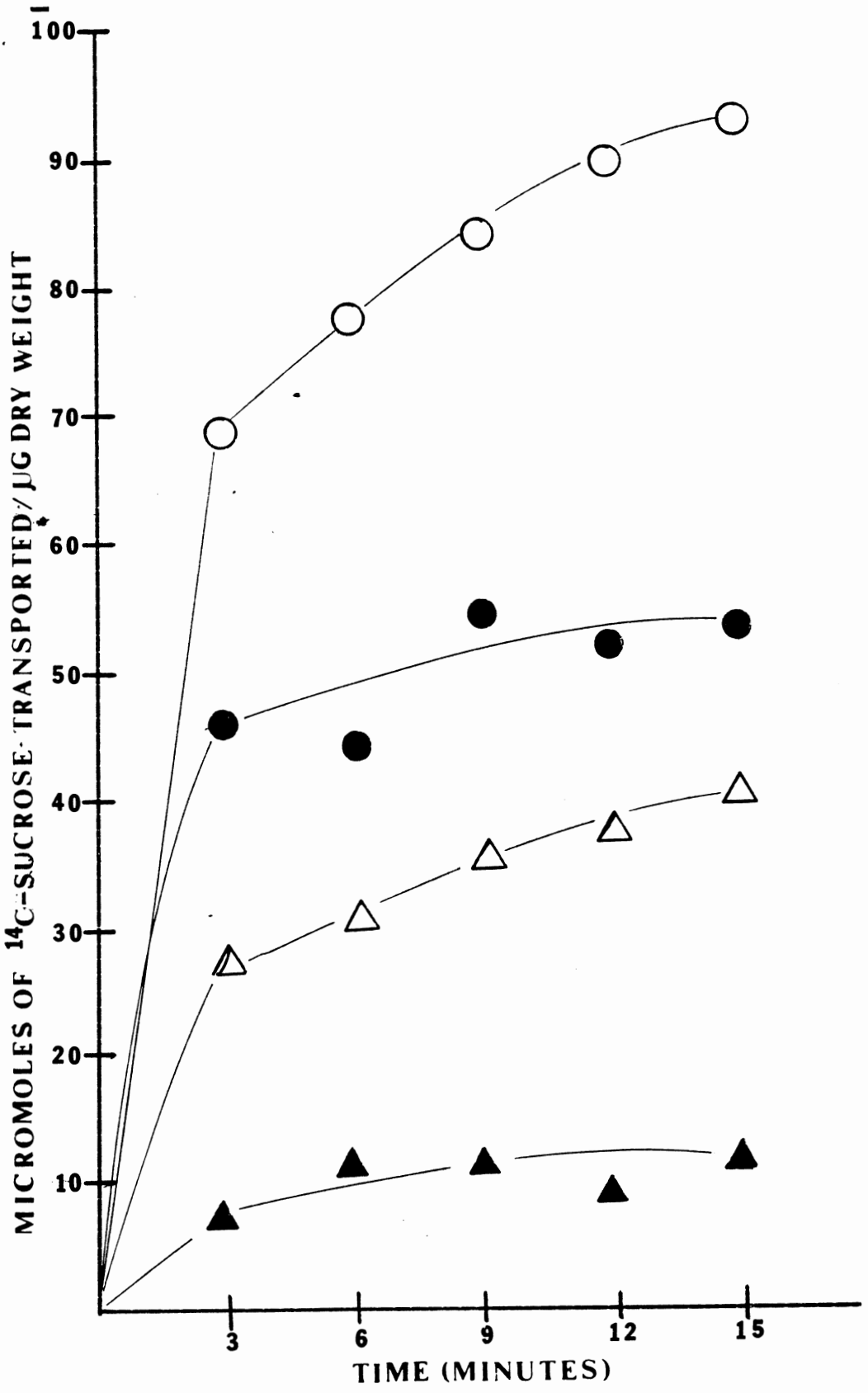

MICROMOLES OF ^{14}C–GLUCOSE TRANSPORTED / MG DRY WEIGHT
100
90
80
70
60
50
40
30
20
10
1
2
3
4
5
SODIUM CHLORIDE
(%)

FIGURE 7. Uptake of ^{14}C labeled sucrose by cultures HR-5 (triangles) and HR-32 (circles). Open symbols refer to controls, transport in 86mM sodium chloride. Closed symbols transport in 860mM sodium chloride.

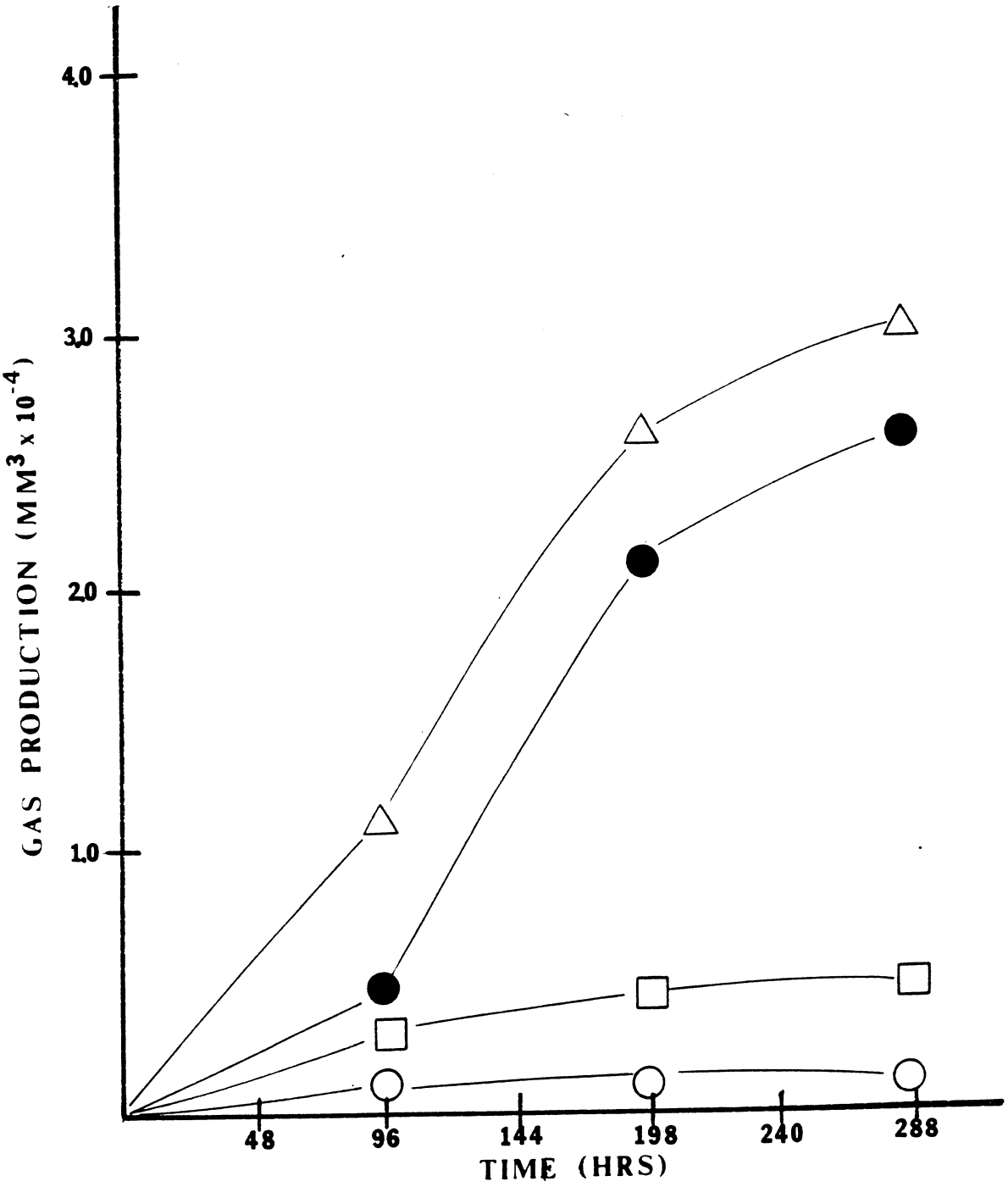

MICROMOLES OF ^{14}C-SUCROSE TRANSPORTED/ µG DRY WEIGHT
100
90
80
70
60
50
40
30
20
10
3
6
9
12
15
TIME (MINUTES)

specificity or rate of transport, or if it is because of an irreversible or reversible damage to cells when they are put in contact with high levels of salt, was not addressed in these experiments. Plate counts of cells transferred from a growth medium with low salt, to one with high concentrations 860 mM salt do show a reduction in the population of viable cells.

From the uptake experiments it would appear that NaCl can indeed affect the uptake of glucose, sucrose, and acids (data not shown). This lowered rate of solute transport could therefore explain the lag period and differences in growth pattern observed when isolates are grown in the presence of high levels of salt.

The growth medium has an effect on an isolates ability to resist salt. The original isolates were obtained in TSB with 1% glucose and most were unable to survive the switch to molasses (some perhaps due to an inability to utilize sucrose). Even the type of molasses can have a dramatic effect on growth of a culture. Figure 8 shows the gas production (MM by the syringe method) of isolate HR-51B in different types of molasses at 860 mM NaCl. With respect to various types of molasses a vast difference in gas production occurs. Two kinds of molasses failed to support growth at all in 860 mM NaCl. Since molasses is favored for downhole use as a feedstock (due to its low cost) and

FIGURE 8. Growth of HR-51B in various kinds of molasses at 860mM sodium chloride. Open triangle beet molasses. Closed circle TK#1. Open square cane molasses. Open circle cattle molasses.


4.0
3.0
2.0
1.0
GAS PRODUCTION (MM^3 x 10^{-4})
48
96
144
198
240
288
TIME (HRS)

after initial screens, beet molasses specifically showed the best potential as a growth medium, the isolation medium was changed. New isolates were obtained, utilizing 4% v/v beet molasses, 0.1% calcium carbonate and 860 mM NaCl. This medium rendered a number of cultures superior to the intitial isolates as to their ability to grow in the higher concentrations of NaCl. Transport studies were not attempted with these isolates.

To illustrate the approach used in relating laboratory studies to field tests, Figure 9 shows the gas production of four cultures in the beet molasses medium made with connate water (700 mM NaCl) from a well under consideration for a field test using one of the test strains. For this well, HR-32 and MX-1A would be prime candidates for well inoculation on the basis of gas production. Acid and solvent data would also be gathered and combinations of organisms used for inoculation in an attempt to maximize the total amounts of solvents, acids and gases produced in the connate water, and a decision made as to which culture or cultures would be used.

In addition to screening for acids, gases and solvents, attempts were made to screen isolates for their ability to release oil from sandstone and limestone saturated with oil. Initial experiments (Table VII) showed that under conditions where no attempts were made to limit contami-

FIGURE 9. Growth of various cultures in beet molasses medium with connate water containing approximately 700mM sodium chloride. Open square culture MX-1A. Closed circle culture HR-32. Open triangle culture HR-69E. Open circle culture HR-51B.

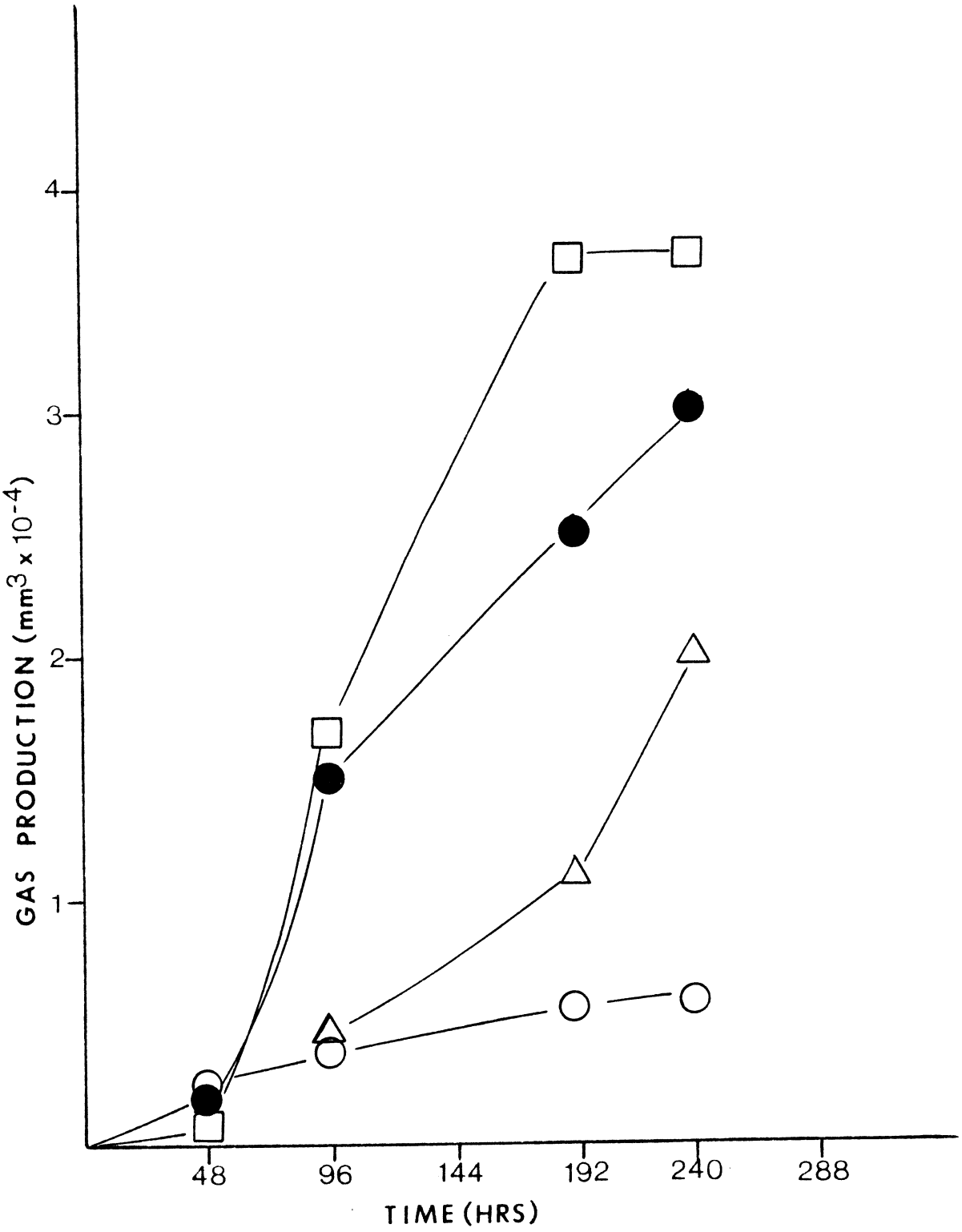

GAS PRODUCTION ($mm^3 \times 10^{-4}$)
4
3
2
1
48
96
144
192
240
288
TIME (HRS)

TABLE VIII

OIL RELEASE BY VARIOUS CULTURES IN EITHER THE SUCROSE SALTS MEDIUM OR BEET MOLASSES MEDIUM

Culture	Type Rock	Medium	Oil Release
HS-4	Sand	Sucrose	4+
HS-4	Sand	Molasses	4+
HS-4	Limestone	Sucrose	4+
HR-3	Sand	Sucrose	3+
HR-3	Limestone	Sucrose	4+
HR-3	Limestone	Molasses	4+
HR-3	Sand	Molasses	4+
HR-46	Sand	Sucrose	3+
HR-46	Limestone	Sucrose	3+
HR-46	Sand	Molasses	3+
HR-46	Limestone	Molasses	3+
HR-27	Sand	Sucrose	0
HR-27	Limestone	Sucrose	1+
HR-27	Sand	Molasses	0
HR-27	Limestone	Molasses	1+
HR-50	Sand	Sucrose	1+
HR-50	Limestone	Sucrose	1+
HR-50	Sand	Molasses	1+
HR-50	Limestone	Molasses	1+

nation, certain cultures had the ability to release greater amounts of oil than others and though no quantitation of gas production determined, this ability seemed to be related to the amount of gas produced by a culture. Later experiments (Table IX) where gas production was recorded, and aseptic techniques used, there was indeed a correlation between oil release and gas production. This correlation occurred when bacteria other than test strain were used, (Enterobacter, E. coli, etc.). Oil release was determined to be related to gas production.

The final screening consisted of tests to eliminate any potential pathogenic or hydrogen sulfide producing cultures. The reasons for eliminating any pathogenic cultures should be obvious. Releasing large numbers of a potentially pathogenic bacterium into the environment would not be prudent. Hydrogen sulfide production by bacteria already present in oil reservoirs (sulfate-reducing bacteria) is costly to the oil industry each year, because of the corrosive nature of the gas.

Cultures were screened for the production of hydrogen sulfide by exposing tubes in which growth was in mid or late log phase to strips of Whatman paper saturated with lead acetate. Cultures positive for hydrogen sulfide production would turn the paper brown. All cultures that appeared to be potential candidates for MEOR application

TABLE IX

GAS PRODUCTION AND OIL RELEASE FROM SANDSTONE ROCKS USING ASEPTIC OIL ROCKS WITH THE SUCROSE SALTS MEDIUM. INCUBATED FOR 96 HOURS AT 37°C

Culture	Gas Production mm	Oil Release
MX-1B	18,395	3+
HR-3E	19,709	4+
HR-69E	20,506	4+
HR-4	13,171	1-2+
HR-51B	15,669	2+
HR-66E	18,167	3-4
Enterobacter	21,687	4+
Salmonella	-0-	-0-
E. coli	19,303	2-4+
Serratia	-0-	-0-
Control	-0-	-0-

were tested in all media as well as Sulfate-Indone-Motility Medium (SIM) and Kligler's Iron Agar. Any culture that produced hydrogen sulfide in detectable amounts was discarded.

Based on biochemical information given in the 8th edition of Bergey's Manual (Buchanan and Gibbons, 1974), five simple biochemical tests can be used to screen clostridia for possible pathogenicity:

1. Digestion of meat broth
2. Hydrolysis of casein
3. Beta Haemolysis on blood agar plates
4. Hydrogen sulfide production
5. Non-utilization of sucrose

Since most pathogenic members of the genus Clostridium are proteolytic, positive reactions in all five categories would mean a presumptive positive as to a pathogenic nature. All pathogenic or presumed pathogenic cultures were immediately discarded.

Summary

A number of strains were isolated that met all or most of the screening criteria. Sensitivity to NaCl varied but in general as the salt concentration increases, growth rate decreases and finally at a certain maximum

(characteristic of a given strain) growth ceases altogether. Before the maximal salt level is reached, growth is delayed, total acid, gas and solvent production are decreased and spore formation is affected. The magnitude of this salt effect is dependent on the isolate, in that while some are dramatically affected at low concentrations of NaCl others are not. Co-inoculation using certain cultures can to a certain extent overcome or at least modify this effect.

The effects of high levels of NaCl on processes such as growth, fermentation pathways and solute transport, again vary with the culture, but are always inhibited or delayed as the level of NaCl is increased. Variations in extent occur among strains; the ability of one culture to transport sucrose may be lowered by 50% in 860 mM NaCl, and others ability to transport sucrose may be lowered by 75% at the same concentration.

A number of clostridia were isolated capable of producing metabolic end-products in sufficient amounts in a medium that contains 860 mM NaCl to mobilize oil from an oilwell depleted of its primary energy and at the productive end of waterflooding. These cultures were isolated from a variety of sources specifically for use in an in situ fermentation under conditions set by the

reservoir itself (as to saline content, toxic ions, temperature, etc.). Potential candidates were to be screened for their ability to produce the necessary end-products either separately or together in a medium exactly the same as that to be used for injection. This was accomplished by obtaining produced water from the wells (or the water to be used for medium make-up) and using same to make a similar medium in the laboratory. Since the injection medium practically cannot be sterilized before introduction into the reservoir, this screen would have to be done in an unsterilized medium. As controls, uninoculated tubes were incubated with inoculated tubes. This procedure served two purposes; controls for our screen and bacteria indigenous to the well (or water used for the medium) could be added to the collection of MEOR isolates.

Those isolates able to produce the desired products in the least amount of time, in the greatest amounts, would be used to inoculate the test wells. Produced waters as a result of inherent properties selected various isolates as the best candidates for well or reservoir inoculation. Only a select few of the hundreds of cultures screened were able to grow in a large majority of the test waters. Some 15 to 20 cultures

appeared as the best candidates for use in MEOR and of these, three to five were able to achieve a four star rating. It was these cultures that we hoped to employ in various reservoirs.

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PART II

ASPECTS OF *IN SITU* FERMENTATIONS

CHAPTER I

INTRODUCTION

The basis of Microbial Enhanced Oil Recovery (MEOR) is bacterial growth and concomitant production of metabolic end-products within the porous media (rock matrix) of an oil-producing reservoir. Viable bacteria, and a suitable nutrient source are introduced into the reservoir and a fermentation allowed to occur. The metabolic end-products, being produced at the oil-water interface within the reservoir directly affect the viscosity, contact angle, and displacement efficiency, leading to an increased production of crude oil.

Bacterial growth within the harsh confines of a reservoir requires the addition of or the presence in the medium of micronutrients. Elements such as magnesium, calcium, iron and various trace elements are needed to assure maximum growth and hence maximum production of metabolic end-products. A buffering system, to offset the large amounts of acid produced at the beginning of clostridial fermentations, is required for prolonged growth with maximum use of the substrate. While it is true that most connate or produced waters (if used to make up the

medium) will provide the bulk of the trace elements required and also contain carbonates that will aid in buffering the system, they will not necessarily contain enough for optimum growth and metabolite production. Any addition of micronutrients to the inoculation medium increases the cost of the procedure, and in order to make MEOR economically viable, should be avoided. Ideally, nothing more than a inexpensive readily fermentable carbon and energy source should be added to the water to be used for well inoculation. This assumes that a nitrogen source is already available in the medium or is supplied as an inherent part of the carbon and energy source, or in the connate water or make-up water as usable nitrate. This means that all inorganic elements and buffering capacity not provided as part of the injection medium or connate water must come from the reservoir rock itself. Acidic products of metabolic activity must react with basic components of the reservoir rock and become neutralized so that the fermentation proceeds as planned with maximum production of desired non-acid metabolic end-products.

Reservoirs consisting of dolomite or calcite would quite readily lend themselves to neutralizing low molecular weight (m.w.) acids produced by bacteria, since they consist of magnesium and calcium carbonate, which are known to be excellent buffers. Sandstone reservoirs, being

intermixed with clays and calcium carbonate cement would also add some buffering capacity. What effects the levels of acid soluble portions of a particular core would have on the fermentation ability of a bacterium is a question that to date has not been answered.

In the case of Clostridium, there are a number of parameters that can quite drastically alter the fermentation scheme. These include phosphate limitations (Bahl and Gottschalk, 1984), carbon dioxide pressure (Klu and Sundstrom, 1984), pH (Hicky and Johnson, 1981; Montville, et al., 1985; Stephens, et al., 1985) cell density (Gottschal and Morris, 1982), and other inorganic ions (Monot, et al., 1982). All of these parameters could be influenced directly by the chemical composition of the reservoir rock in which the fermentation takes place. The pH of the medium would certainly be dependent on the acid neutralization capacity of the core; this would no doubt influence not only the cell density, but also gas production and therefore gas pressure.

Interest in the clays (intermixed mostly in sandstone cores, but sometimes in carbonate reservoirs) is mainly concerned with their ion exchange capacity. Are they also capable of supplying buffering capacity? Can the clays bind the amino acids required for spore germination so efficiently that they are unavilable for the germination

process? This latter is very important because the use of spore inocula for injecting wells has many advantages, but would necessitate good conditions for spore germination.

The culture chosen for use in core studies was isolate SEG-3, a saccharolytic Clostridium isolated from produced water obtained from a well in Texas. SEG-3 will grow in a simple chemically defined medium: one of several carbohydrates, an ammonium salt, and inorganic nutrients. Germination of spores on the other hand requires one or more of several amino acids (from the aspartic or glutamic acid family). The major end products of sucrose fermentation are the gases carbon dioxide and hydrogen, acetic and butyric acids and solvents such as ethanol, acetone, isopropyl alcohol, and n-butanol. The relative amounts of end-products depend on conditions and length of the fermentation period.

The ability to grow when core minerals provide the majority of inorganic nutrients and buffering capacity is not unique to SEG-3. Several other Clostridium spp. have been isolated which are capable of growth under similar conditions in the presence of cores. Many Gram positive and Gram negative bacteria were unable to grow under these conditions, unless the core medium was supplemented with phosphate.

CHAPTER II

MATERIALS AND METHODS

Culture

The culture used for the core studies were strain SEG-3, a Gram-positive obligately anaerobic spore-forming rod of the genus Clostridum. Major metabolic end-products were: Acetone, butanol, ethanol, acetic and butyric acid, carbon dioxide and hydrogen. Minor end-products were isopropanol and isobutyric acid.

The culture is saccharolytic, requiring one of several sugar or sugar alcohols for growth. No known growth factors were required other than sugar and a nitrogen source, but growth was enhanced by a number of amino acids, vitamins and inorganic ions (iron, etc.). Results of carbohydrate tests are given in Table X.

Measurement of Gas

Gas was measured by the distance the plunger on a 5, 12, and 20 cc syringe was displaced by gas pressure as the needle penetrated the septum on a Hungate tube. Volumes were determined by the formula $\pi r^2 \times h$. Where π equals 3.142, r= the radius of the syringe, and h=

TABLE X

RESULTS OF CARBOHYDRATE UTILIZATION
BY ISOLATE SEG-3

Carbohydrate	Reaction
Melizitose	Acid + Gas
Ribose	Acid + Gas
Inulin	Acid + Gas
Adonitol	Negative
Melibiose	Acid + Gas
Sorbose	Acid + Gas
Mannose	Acid + Gas
Raffinose	Negative
Dulcitol	Negative
Cellobiose	Acid + Gas
Galactose	Acid + Gas
Inositol	Negative
Mannitol	Acid + Gas
Sorbitol	Acid + Gas
Amygadlin	Acid
Erythritol	Negative
Dextrin	Acid + Gas
Glycogen	Acid + Gas

the displaced distance of the plunger.

Determination of Acids and Solvents

Acid and solvent analysis were made using a Sigma 2 series, Perkin-Elmer gas chromatography equipped with a flame ionization detector and a 6' glass column with 1/4" inside diameter. Peak areas were calculated using a Hewlitt-Packard calculating integrator.

Packing for solvent analysis: 1-1820 carbopack c/o 0.5% Sp-100

For acid analysis: 60/80 carbopack c/o 0.3% carbowax 20M/0.1% phosphoric acid

Programs

For solvent analysis:

Oven temperature: 200°C

Injection temperature: 150°C

Initial temperature: 80°C

Initial time: 2 minutes

Initial rate: 15°C/minute

Final temperature: 120°C

Final time: 5 minutes

Final rate: 25°C/minute

Post temperature: 145°C

Post time: 0.5 minutes

Carrier gas - nitrogen 20 ml/minute

For acid analysis:

Oven temperature: 200°C

Injection temperature: 150°C

Initial time: 2 minutes

Initial rate: 15°C/minute

Final temperature: 130°C

Final rate: 15°C/minute

Post temperature: 150°C

Post time: 1.0 minutes

Carrier gas - nitrogen 35 ml/minute

Sample Preparation

One-tenth ml of 50% phosphoric acid was added to one ml of spent culture medium to acidify acid end-products. One microliter of this sample was injected in the GC for analysis.

Standard Curve

Standard curves were obtained by injecting known concentrations of the appropriate end-products and determining the peak areas. Plots of peak area versus concentration (mM) were obtained for all metabolic end-products. Unknown concentrations were then determined by linear regression using suitable standard curves.

Analysis

Where necessary, samples were co-chromatographed with known solvents or acids if a question arose as to peak identification. Internal standards (propionic acid or n-propanol) were also included if questions arose as to the validity of peak area.

Core Analysis

Cores were pulverized to a fine powder by the use of a mortar and pestle, then throughly mixed and analyzed by x-ray diffraction.

Phosphate Analysis

Total phosphate in powdered cores and yeast extract was determined by atomic absorption. Samples were hydrolyzed with nitric acid, then analyzed on a Hewlitt-Packard atomic absorption spectrometer and total phosphate determined by comparison with a standard curve.

Medium

The medium for core experiments (initial fermentation) was 1% sucrose, 0.1% yeast extract, 0.1% ammonium nitrate. In experiments to determine the effect of yeast extract on growth and gas production, this addition was omitted when cells in the log phase of growth were used. Nitrogen sources other than ammonium nitrate were also tested for their ability to support growth.

When tubes were refed, at times seven different media were used to refeed cultures:

1. 1% sucrose
2. 1% sucrose, 0.1% ammonium nitrate
3. 1% sucrose, 0.1% yeast extract
4. 1% sucrose, 0.1% ammonium nitrate, 0.1% yeast extract
5. 1% sucrose, 0.05% yeast extract
6. 1% sucrose, 0.05% ammonium nitrate
7. 1% sucrose, 0.05% yeast extract, 0.05% ammonium nitrate

At no time during a refeed would exogenous phosphate (above that already present in yeast extract) or trace elements be added back.

Incubations were at 30^{o}C.

Refeeding Procedure

The initial refeed procedure was to invert the tube and remove 2 mls of spent medium from the tube with a 5 ml syringe. This method was later amended, and the 2 mls removed by inserting an alcohol flamed 3 inch 21 gauge needle into the medium and removing 2 mls without inverting the tube and thus not disturbing the medium. This increased gas production and decreased incubation time to reach maximum gas production in those tubes

refed only 1% sucrose by lowering the washout rate. Removal by this method also reduced the amount of small particles of core material removed via the syringe.

Two mls of fresh medium were then aseptically added.

CHAPTER III

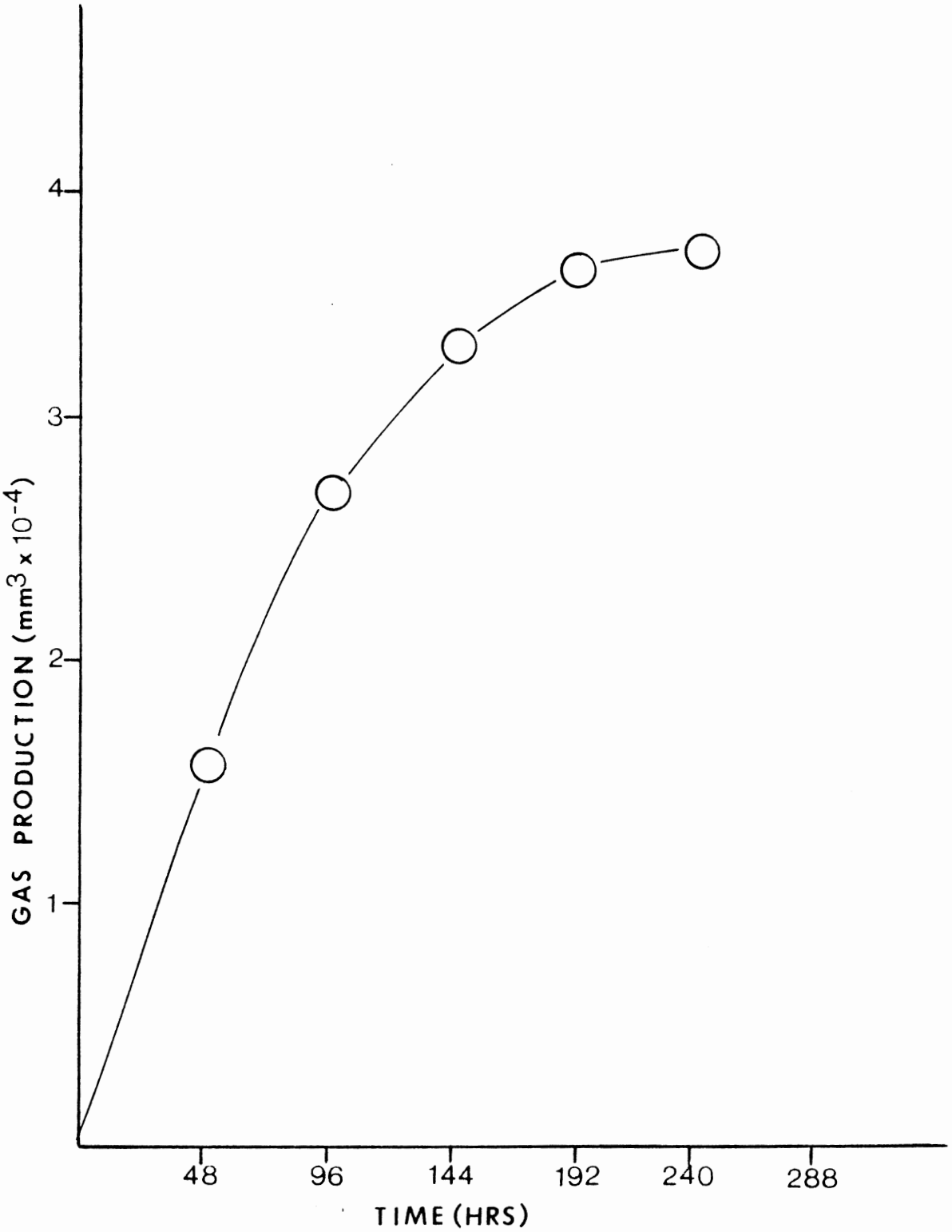
RESULTS AND DISCUSSION

Total gas production (as well as solvent and acid production) by the butylic group of clostridia is dependent on a number of complex interactions and the availability of certain necessary co-factors.

First, in order to determine the total amount of gas one might expect from the core medium (1% sucrose, 0.1% yeast extract, 0.1% ammonium nitrate), Erwinia trace elements were added as well as 20 mg/100 mls magnesium sulfate and once a vigorous fermentation had begun, 0.01M combined phosphate buffer are added to the medium every 48 hours. The addition of the phosphate buffer every 48 hours serves two purposes; allowing the fermentation to continue until all of the sucrose is utilized, without respect to pH and end-product inhibition (dilution by the phosphate buffer). Figure 10 shows the total gas that one might expect from the core medium in a non-phosphate-limited experiment. Total gas production under these conditions (N=16) would be 37,400 mm.

Atomic absorption analysis of yeast extract shows that the total phosphate concentration is 3.6 mg/100mg. It

FIGURE 10. Total gas produced by SEG-3 at 30 C in a medium consisting of 1% ammonium nitrate, 0.1% yeast extract, 20mg/100ml magnesium sulfate, 0.01M combined phosphates and 1% sucrose. Every 48 hours, 0.01M combined phosphates are added.


GAS PRODUCTION ($mm^3 \times 10^{-4}$)
4
3
2
1
48
96
144
192
240
288
TIME (HRS)

follows then that at the concentration of yeast-extract in the core medium there is 0.36 mg/tube of phosphate. One of the cores (to be discussed later), the Austin Chalk, contains phosphate levels of 0.2 mg/100 mg.

Figure 11 is a representation of an experiment whereby the yeast extract and ammonium nitrate of the core medium were kept at 0.1% and various levels of sucrose were used. This experiment was designed to determine the exact amount of gas attributable to each component to the core basal medium. In this experiment, two buffering systems are used:

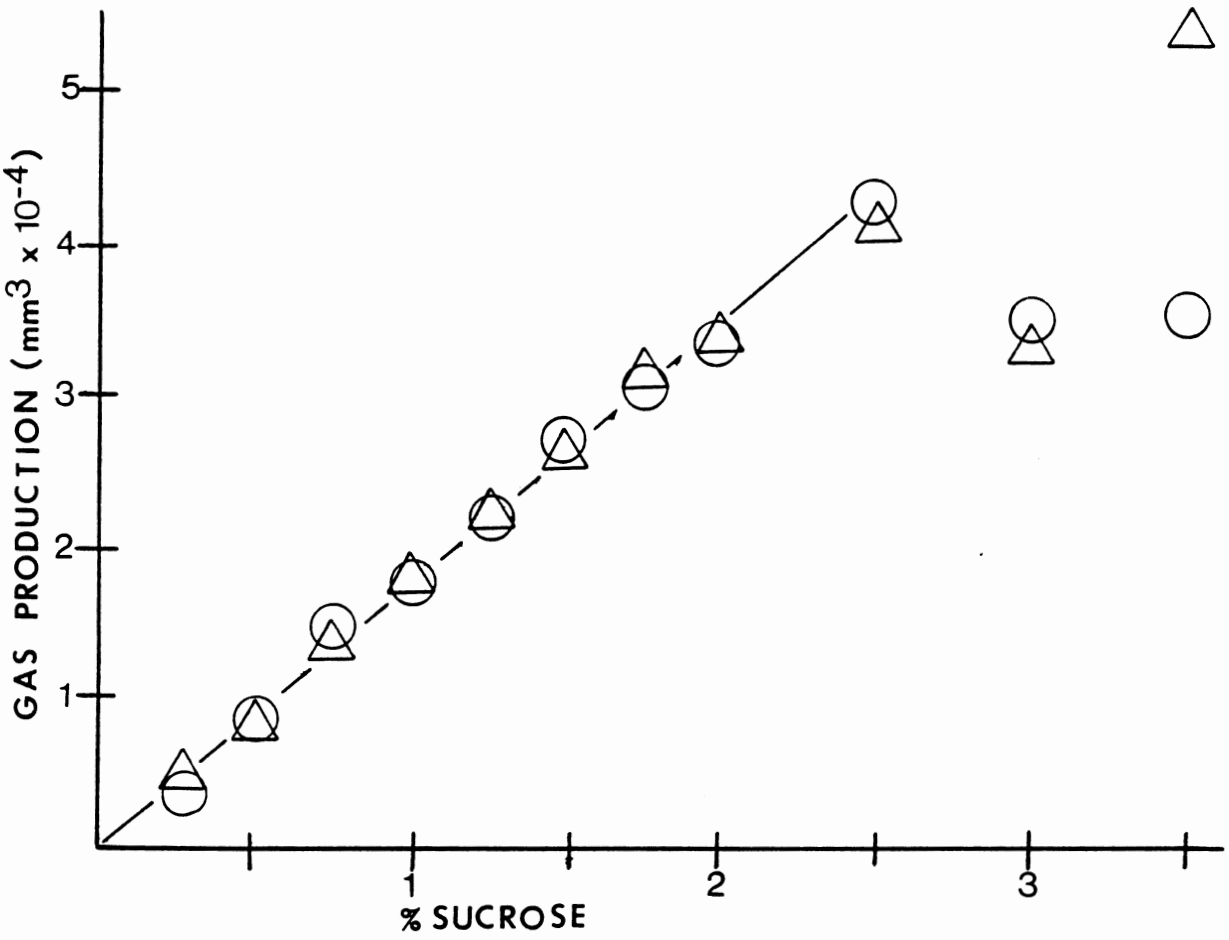
1. After a vigorous fermentation has begun, 0.01M combined phosphate buffer is added to the tube every 48 hours. Since no spent medium is removed, toxic end-products are diluted and acidic end-products neutralized, but total nutrients are unaffected.

2. 0.1 gm of Austin Chalk was added per tube (final phosphate concentration= .56 mg/10 mls phosphate)

Gas production from both methods of adding phosphate are nearly identical (Figure 11). Gas production, is dependent on the amount of sucrose in each tube and independent of the concentration of yeast extract and ammonium nitrate at 0.1% concentrations and phosphate down to 0.4 mM (concentration when grown in the presence of the Austin Chalk).

Other experiments, utilizing buffers other than phos-

FIGURE 11. Gas production by culture SEG-3 in the core basal medium with various levels of sucrose where buffering capacity is supplied by: A. (open circles) by the addition every 48 hours of 10mM combined phosphate. B. (open triangles) 0.1gm/tube of the Austin Chalk. All incubations 432 hours.


GAS PRODUCTION ($mm^3 \times 10^{-4}$)
5
4
3
2
1
1
2
3
% SUCROSE

phate as a nutrient of this bacterium is low. This in itself is not surprising, primarily because of the fermentation scheme of the butylic group of Clostridia. There is a production of an excessive amount of ATP through the Embden-Meyerhof-Parnas pathway and acetate production, but little ATP is utilized for biomass production and none is hydrolyzed for the production of solvents or butyrate (Papoutsakis, 1984). All of the above, coupled with the fact that sandstone cores contain only a trace of phosphate per 100 mg, suggest that under the conditions used phosphate concentration does not become limiting to gas production.

Preliminary Core Experiments

Table XI shows the core composition of the first cores utilized in these experiments. There is a wide range of composition and the acid soluble constituents of the cores are quite variable from one core to another. Core number five, having the highest percentage of dolomite would in effect contribute the best buffering capacity, the best menstruum for growth and as a result allow the most growth and gas production.

The buffering capacity of a core might also be determined by comparing the relative amounts of butanol produced by SEG-3 during the fermentation in the presence

TABLE XI

ANALYSIS BY X-RAY DIFFRACTION OF CORES
1, 2, 4, 5, 6 AND 7

Constituent	% Per Core by Number					
	1	2	4	5	6	7
Quartz	65-80	65-80	80-100	2-5	40-100	45-55
Feldspar	2-5	5-10	2-5	-0-	10-40	10-15
Calcite	0.5-2	5-10	0.5-2	T	3-10	T
Dolomite	-0-	-0-	2-5	40-50	-0-	-0-
Kaolinite	2-5	5-10	5-10	-0-	1-3	2-5
Illite	2-5	2-5	2-5	-0-	1-10	5-10
Mixed Layer	2-5	2-5	-0-	-0-	3-20	15-25
Chlorite	2-5	205	-0-	-0-	0.1-1	2-5
Pyrite	-0-	0.5-2	-0-	-0-	-0-	-0-
Anhydrite	-0-	-0-	-0-	45-55	-0-	-0-

of each core. While a number of factors, such as concentration of acidic end-products, cellular internal pH, nutrient limitation, or temperature and oxygen tension will to an extent act as triggering mechanisms for solventogenesis, the external pH has long been regarded as a key factor in determing the outcome of the acetone butanol fermentation (Jones and Woods, 1986). Most strains of Clostridium acetobutylicum do not produce solvents unless the pH falls below 5.00. The considered optimum pH for solvent production is 4.5.

The results shown in Figure 12, using log cells for inoculation and cores at 0.5 gm/tube, did not confirm this hypothesis. All but one of the sandstone cores containing a much lower level of acid-soluble constituents provided almost as good a menstruum for growth (based on gas production) as core number five, the core containing the highest acid-soluble components (and hence buffering capacity). In fact, core number seven without dolomite or calcite allowed SEG-3 to produce the same amount of gas as core number five. If butanol production is taken as an indication of buffering capacity, core number seven, Table XII, would seem to contribute the most buffering capacity. The lower butanol production could be a result of core number seven keeping the medium above the breakpoint for butanol formation.

FIGURE 12. Total gas production by SEG-3 in the core medium in the presence of various cores. Closed square-core number 1. Open square-core number 2. Closed circle-core number 4. Closed triangle-core number 5. Open triangle-core number 6 and Open circle-core number 7.

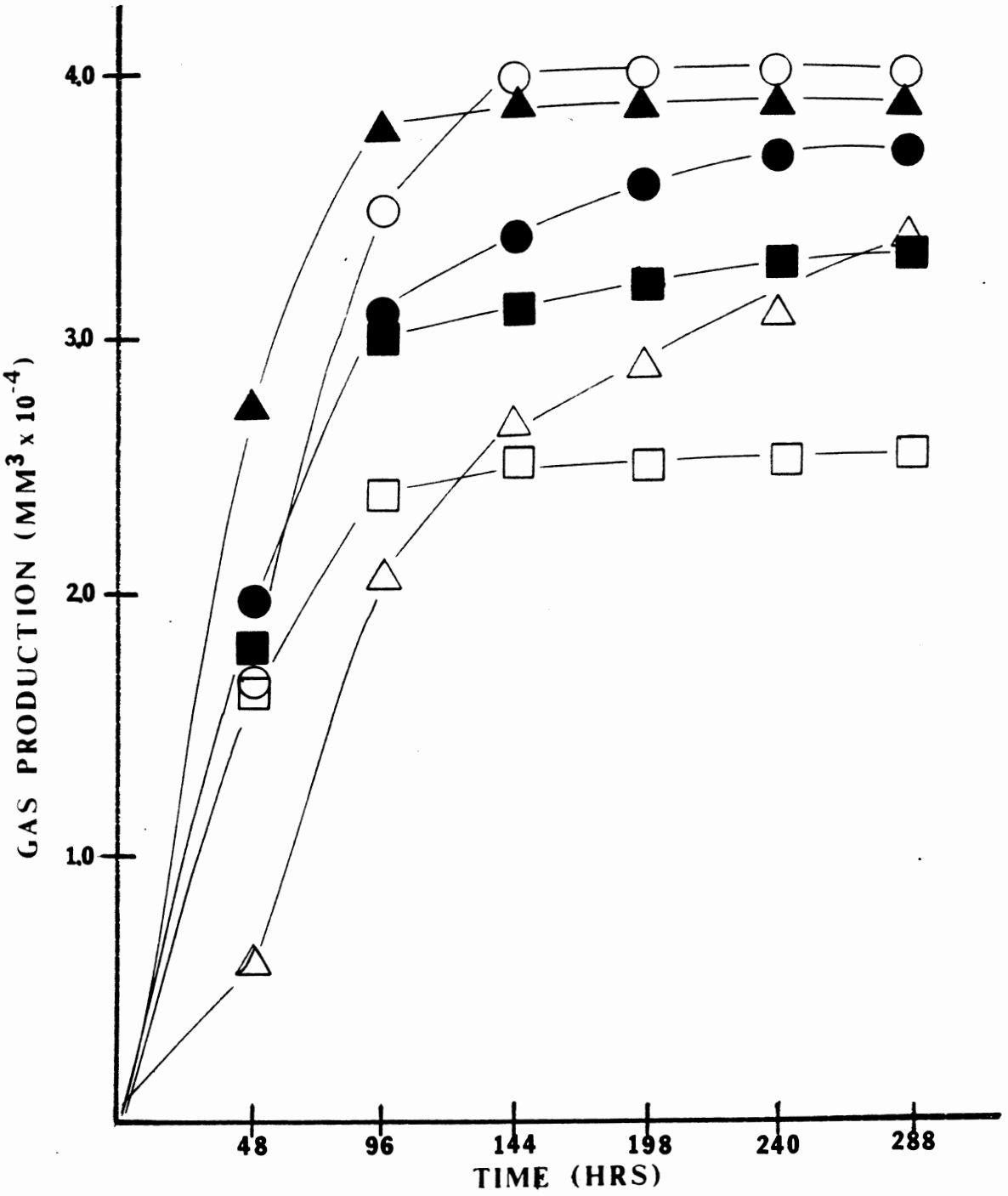

4.0
3.0
2.0
1.0
GAS PRODUCTION ($MM^3 \times 10^{-4}$)
48
96
144
198
240
288
TIME (HRS)

TABLE XII

ACID AND SOLVENT PRODUCTION (mM) BY SEG-3 IN THE CORE MEDIUM IN THE PRESENCE OF VARIOUS CORES AT 0.5 GM/TUBE

Core	(N=5) Ethanol	Acetone	Butanol	Acetic	Butyric
1	3.9 ± 0.6	2.6 ± 0.3	10.8 ± 2.5	0.1 ± 0.1	13.6 ± 5.9
2	5.6 ± 2.6	-0-	9.1 ± 4.8	0.3 ± 0.1	18.6 ± 3.6
4	4.7 ± 2.5	1.8 ± 0.5	9.9 ± 2.4	0.4 ± 0.2	18.3 ± 7.8
5	6.3 ± 3.3	1.9 ± 0.1	5.1 ± 3.0	0.3 ± 0.1	21.8 ± 1.5
6	6.4 ± 4.6	3.2 ± 0.6	10.3 ± 2.1	0.3 ± 0.1	19.7 ± 3.5
7	3.9 ± 0.6	-0-	4.0 ± 0.7	0.4 ± 0.2	22.1 ± 5.5

It is evident from Table XII that all cores contain constituents capable of buffering the core medium, if the relative amount of butanol produced is a direct indication of the pH of the medium. As related earlier though, a number of factors can be responsible for this shift and the relative amount of buffering capacity may be only one component. In the case of Clostridium butylicum, George and Chen (1983) suggest that the onset of butanol formation is due to several related parameters: acidic pH, high weak acid concentration and high cell densities. In the case of these cores, high cell density would not seem to be a factor since the relative numbers of viable cells of SEG-3 are constant from core to core.

Initially, it was hoped that a standard curve could be established plotting core weight (grams) versus total gas production in 0.1 gm increments from 0.1 to 1 gm. Inoculation of SEG-3 into the core basal medium with Erwinia trace minerals added, but no buffering source, the gas production was approximately 5,000 mm. Gas production in the core medium without trace minerals or a buffering source was slight, although a small amount of growth was observed. If a sandstone core had only slight buffering capacity, then the ten fold difference between 0.1 and 1.0 gm should manifest itself with an increased amount of gas production as well as lowered amounts of butyric acid

converted to butanol. Core number one was the only core that gave any indication of a significant difference between the 0.1 gm/tube and the 1 gm/tube fermentation. A shortage of the powdered cores used in Figure 12 and Table XI forced the abandonment of experiments utilizing these cores.

Core Studies

There were sufficient quantities of six other cores to perform a number of experiments to determine the effects of these cores on growth, gas production and fermentation ratios. The compositions of these cores are shown in Table XIII. Two, the Kansas City Lime and Austin Chalk were limestone, and four were sandstone cores, the Dutcher, Bryden, Douglas and Redden Sands. There is a vast range of calcite (acid soluble carbonate) concentrations, from a high in the two limestone cores to a low concentration in the Redden Sand, to essentially zero in the remaining three sandstone cores. The Redden Sand contains (one the average) little clay, while the other sandstone cores contain no detectable dolomite or calcite, but a wide variety of clays. Determination of how each of these variables affects the fermentation would lead to a better understanding of <u>in situ</u> growth of clostridia.

Experiments using these cores in amounts from 0.1 to 1 gm (0.1 gm increments) did not result in a uniformly

TABLE XIII

CORE ANALYSIS. NUMBERS REFER TO PERCENT OF EACH CONSTITUENT AS DETERMINED BY X-RAY ANALYSIS

Constituent	Kansas City Lime	Dutcher Sand	Bryden Sand	Austin Chalk	Douglas Sand	Redden Sand
Quartz	5-10	30-40	40-50	5-10	40-50	50-100
Feldspar	-0-	2-5	15-25	2-5	15-20	-0-
Calcite	80-100	T	T	50-65	T	0.5-2
Dolomite	5-10	-0-	-0-	-0-	-0-	2-5
Kaolinite	-0-	5-10	5-10	2-5	5-10	2-5
Illite	-0-	5-10	-0-	2-5	5-10	-0-
Mixed Layer	-0-	20-30	5-10	-0-	5-10	0.5-2
Chlorite	-0-	5-10	5-10	0.5-2	5-10	-0-
Pyrite	-0-	0.5-2	0.5-2	-0-	0.5-2	-0-
Anhydrite	-0-	-0-	-0-	-0-	-0-	0-1
Siderite	-0-	2-5	0.5-2	-0-	0.5-2	-0-
Smectite	-0-	-0-	-0-	10-50	-0-	-0-
Mica	-0-	-0-	5-10	-0-	-0-	-0-

graded response. Figure 13 shows the gas produced with various weights of the Austin Chalk and Bryden Sand. There is no difference in gas production between 0.1 gm of core/tube or 1 gm/tube in the case of either core. For this reason, experiments to standardize gas production of cores by weight were abandoned.

Since the weight of core (from 0.1 to 1 gm/tube) did not appreciably change the amount of gas produced, a level of 0.5 gm/tube powdered core/tube was arbitrarily chosen. This would allow not only sufficient core material for a complete fermentation, but conserve on the amount of core used per experiment.

Experiments were done to determine the total amount of gas produced in the presence of 0.5 gm of various cores in the core basal medium. Figures 14 thru 19 show the results of spore inocula (closed symbols) as compared to log cell inocula (open symbols). Delays occurring with all cores when spore preparations were compared to log cell inocula are an inherent property of using spores for inoculation and represents the time required for germination and outgrowth of the spores.

Table XIV compares the final pH in the core medium after the initial fermentation (288 hrs. post-inoculation). The cores containing more than trace amounts of calcite have a final pH well above 5.00, while the sandstone cores

FIGURE 13. Total gas by SEG-3 at 288 hours post-inoculation versus core (weight/grams). Open circles-Bryden Sand. Open triangles-Austin Chalk.

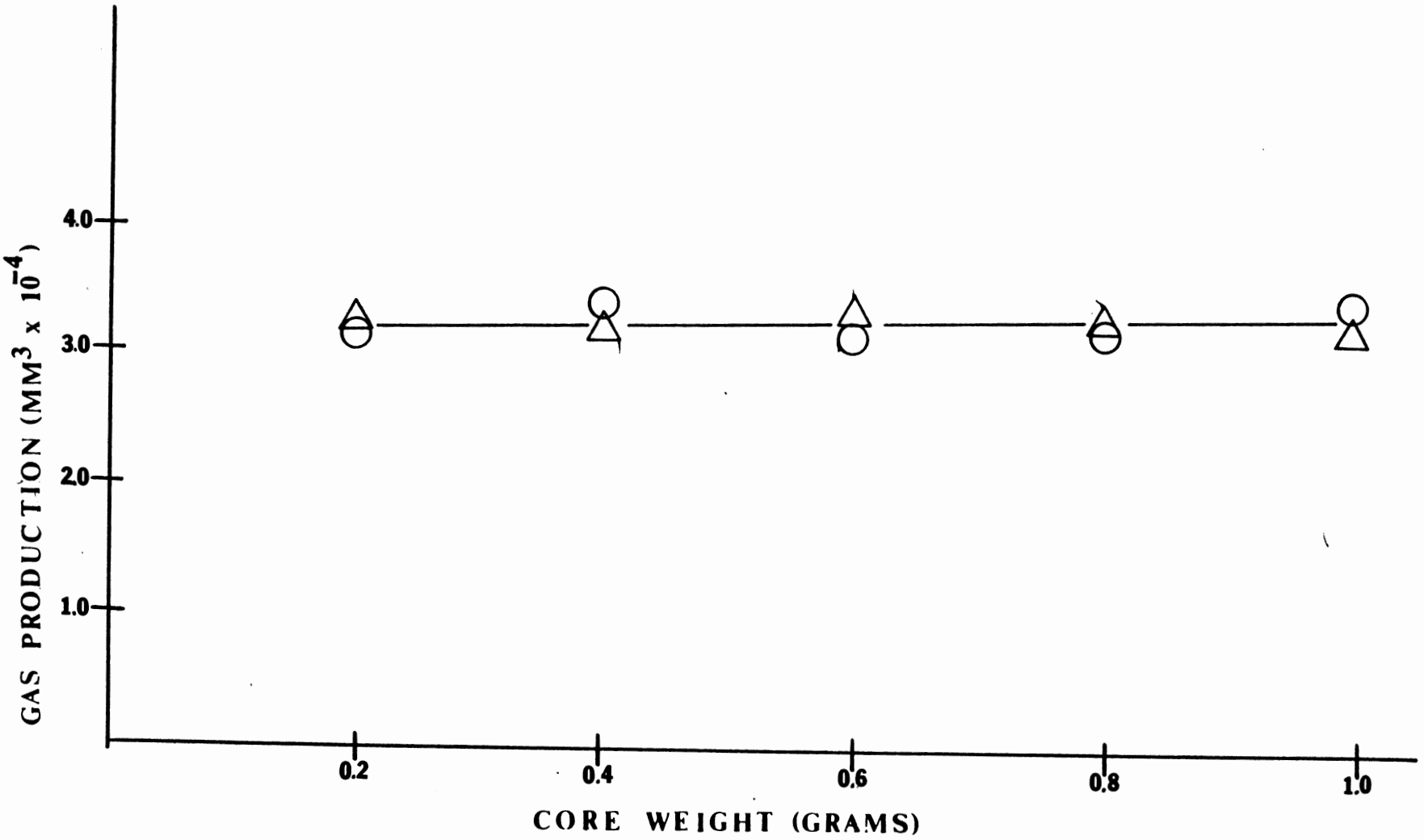

GAS PRODUCTION ($MM^3 \times 10^{-4}$)
4.0
3.0
2.0
1.0
0.2
0.4
0.6
0.8
1.0
CORE WEIGHT (GRAMS)

FIGURE 14. Total gas (mm) produced by SEG-3 in the core medium in the presence of the Austin Chalk. Open triangles-log cell inoculation (N=20). Closed triangles-spores used as inocula (N=43).

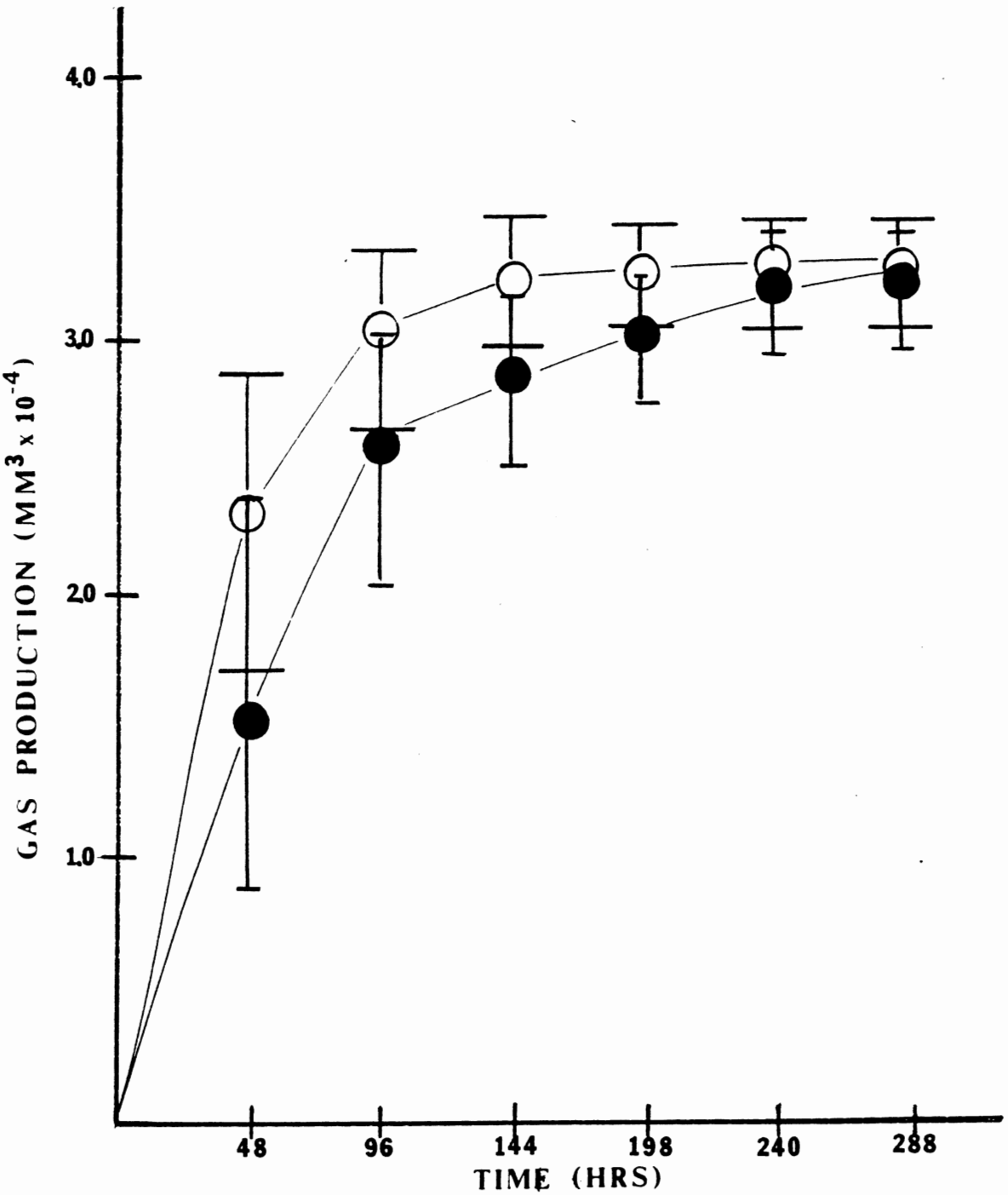

4.0
3.0
2.0
1.0
GAS PRODUCTION ($MM^3 \times 10^{-4}$)
48
96
144
198
240
288
TIME (HRS)

FIGURE 15. Total gas (mm) produced by SEG-3 in the core medium in the presence of the Kansas City Lime. Open triangles-log cell inoculation (N=15). Closed triangles-spores used to inoculate (N=22).

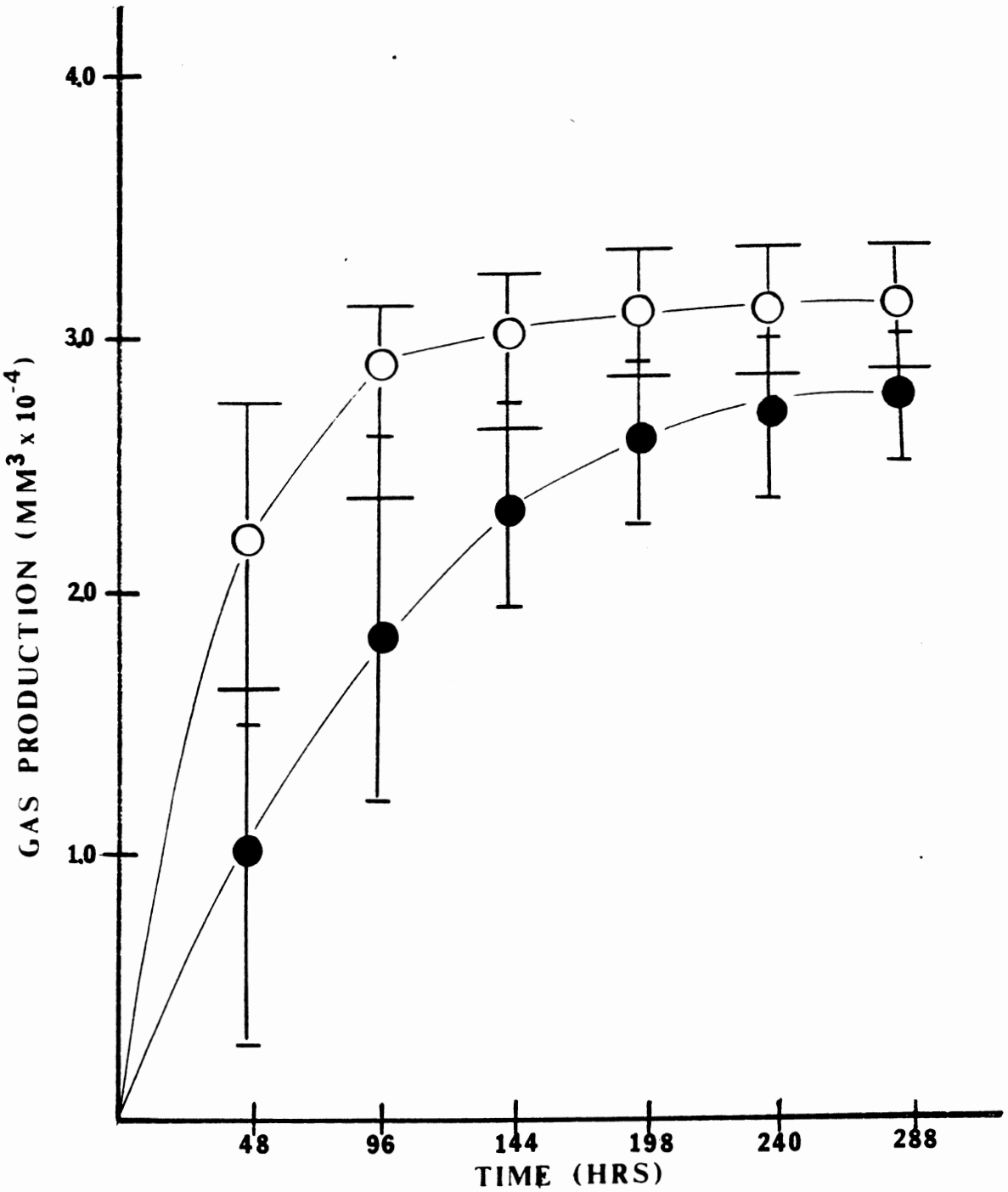

4.0
3.0
2.0
1.0
GAS PRODUCTION ($MM^3 \times 10^{-4}$)
48
96
144
198
240
288
TIME (HRS)

FIGURE 16. Total gas (mm) produced by SEG-3 in the core medium in the presence of the Redden Sand. Open triangles-log cell inoculation (N=28). Closed triangles-spores used to inoculate (N=27).

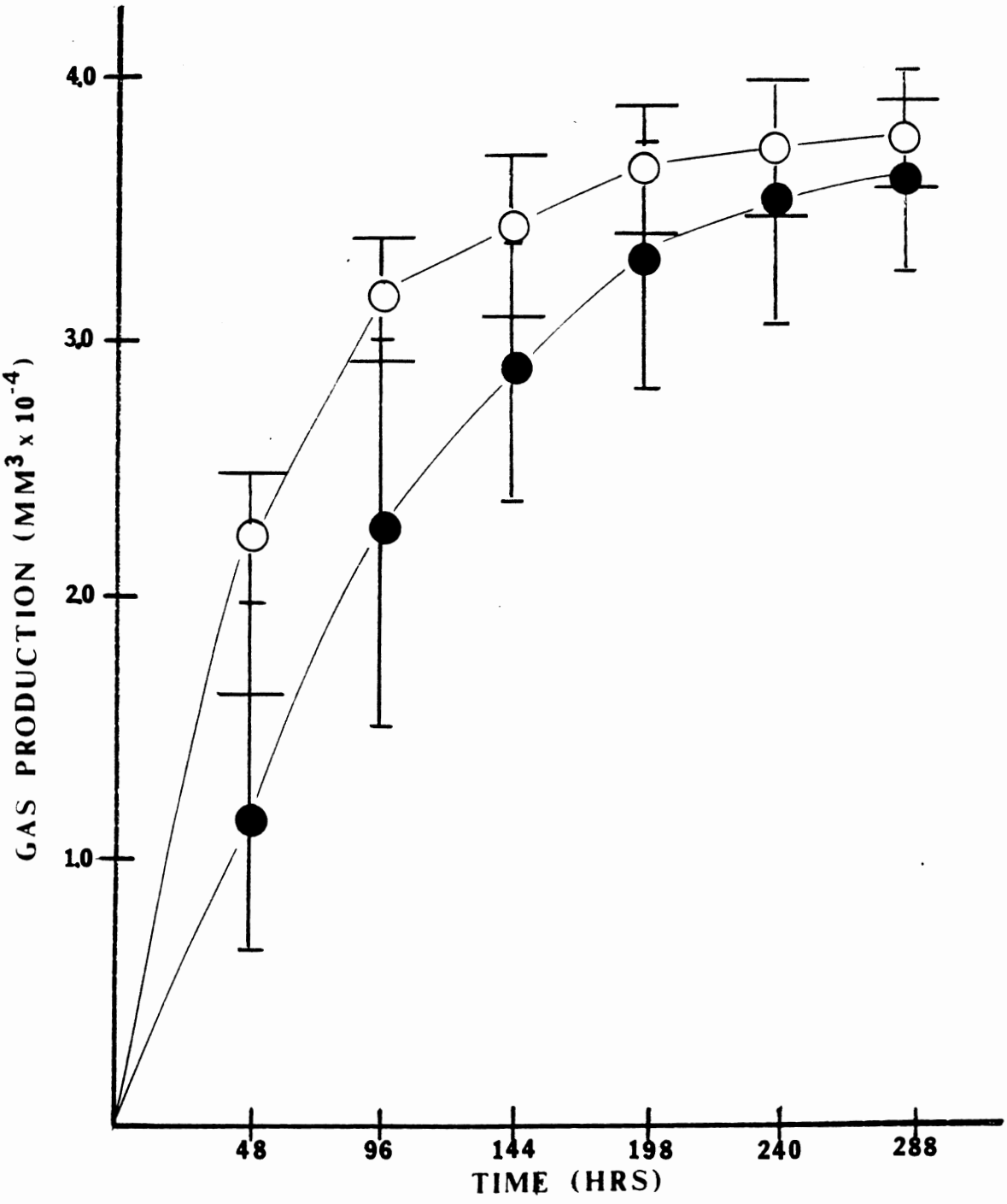

4.0
3.0
2.0
1.0
GAS PRODUCTION ($MM^3 \times 10^{-4}$)
48
96
144
198
240
288
TIME (HRS)

FIGURE 17. Total gas (mm) produced by SEG-3 in the core medium in the presence of the Dutcher Sand. Open triangles-log cell inoculation (N=30). Closed triangles-spores used to inoculate (N=44).

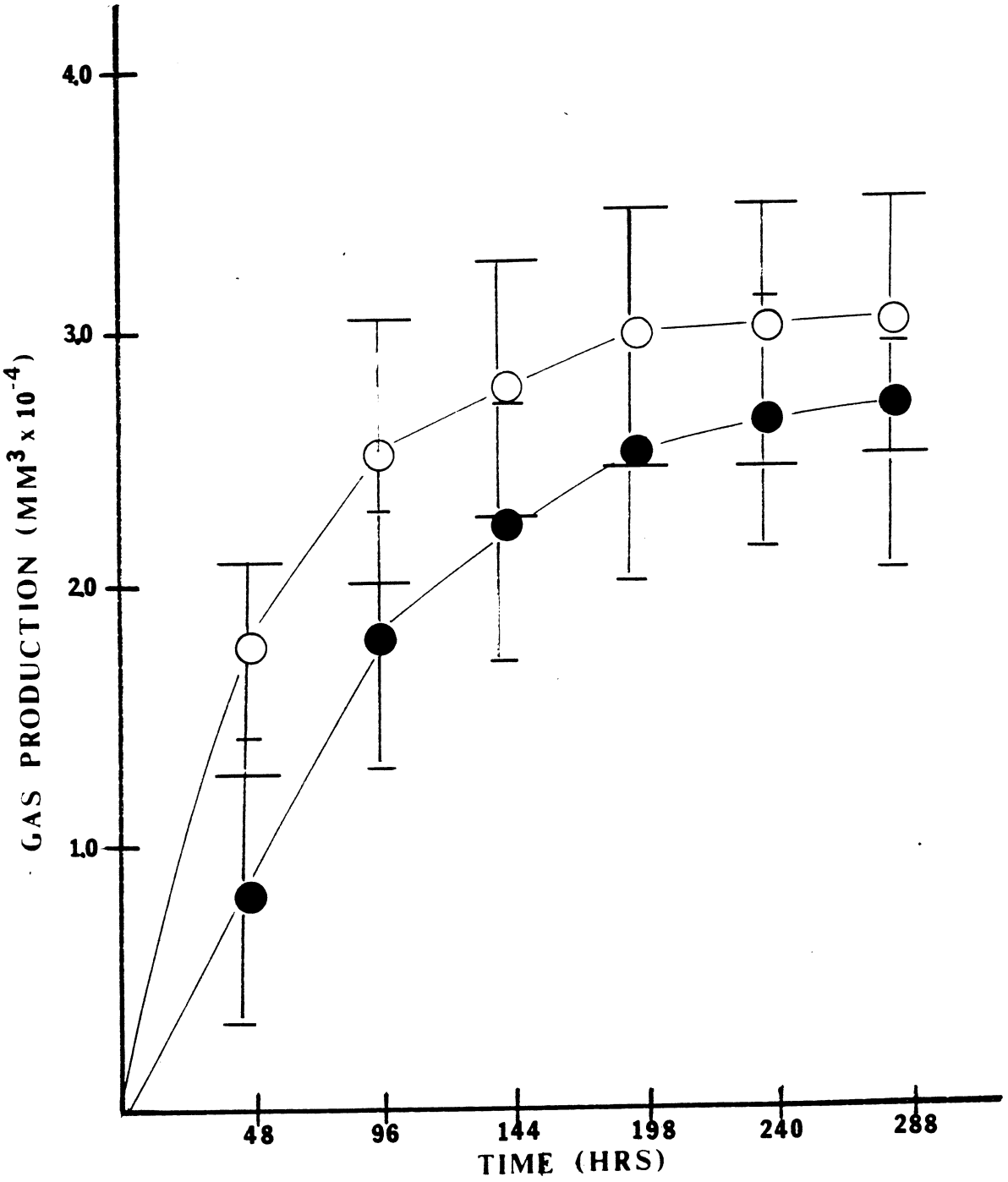

4.0
3.0
2.0
1.0
GAS PRODUCTION ($MM^3 \times 10^{-4}$)
48
96
144
198
240
288
TIME (HRS)

FIGURE 18. Total gas (mm) produced by SEG-3 in the core medium in the presence of the Bryden Sand. Open triangles-log cell inoculation (N=28). Closed triangles-spores used to inoculate (N=43).

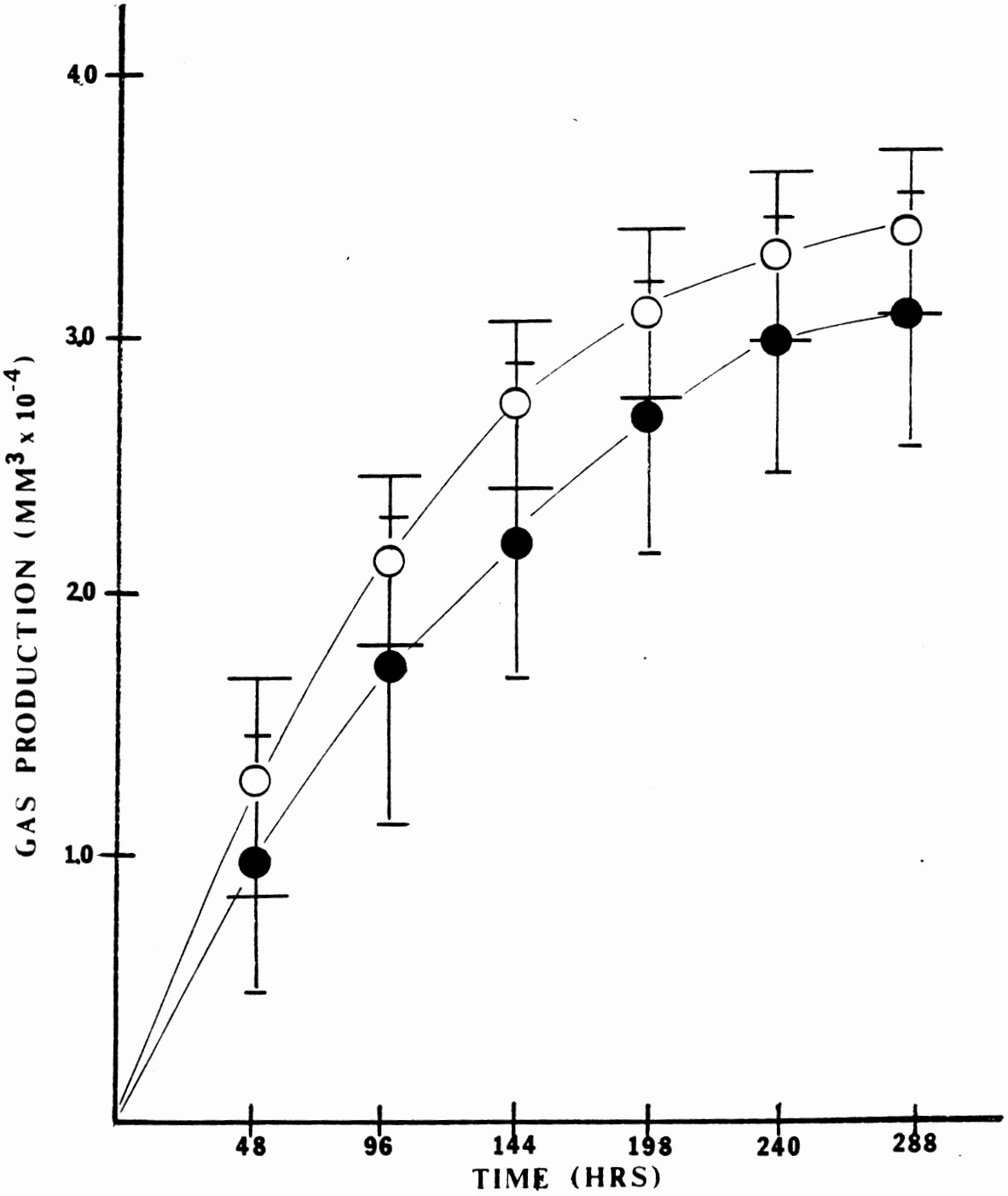

4.0
3.0
2.0
1.0
GAS PRODUCTION ($MM^3 \times 10^{-4}$)
48
96
144
198
240
288
TIME (HRS)

FIGURE 19. Total gas (mm) produced by SEG-3 in the core medium in the presence of the Douglas Sand. Open triangles-log cell inoculation (N=26). Closed triangles-spores used to inoculate (N=42).

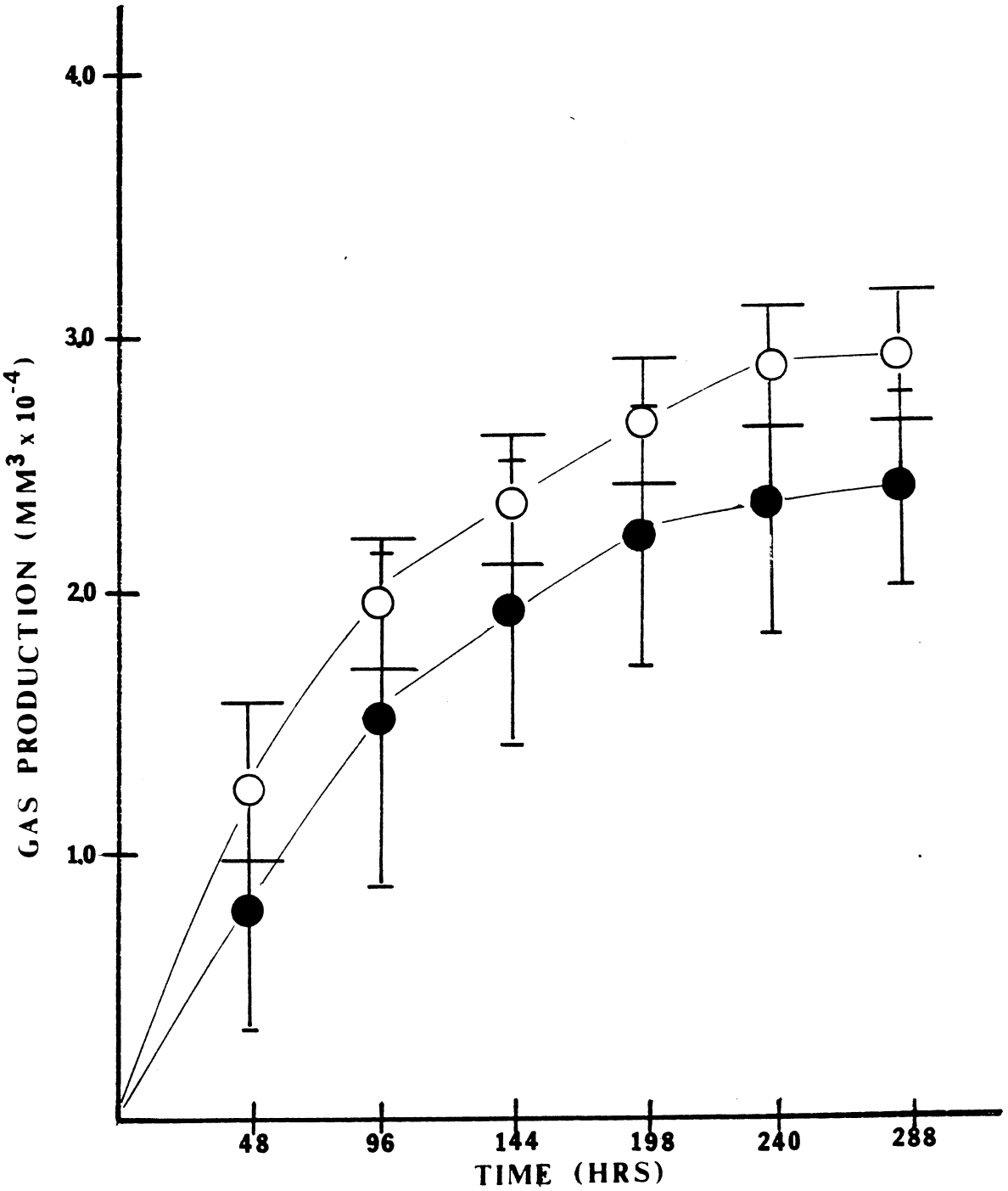

4.0
3.0
2.0
1.0
GAS PRODUCTION ($MM^3 \times 10^{-4}$)
48
96
144
198
240
288
TIME (HRS)

TABLE XIV

FINAL pH SPENT MEDIUM FROM VARIOUS CORES
288 HOURS POST-INOCULATION
INITIAL FERMENTATION

Core	N (replicates)	Final pH
Austin Chalk	31	5.81 ± 0.18
Kansas City Lime	22	5.79 ± 0.18
Redden Sand	21	5.37 ± 0.16
Dutcher Sand	24	5.01 ± 0.08
Bryden Sand	37	4.87 ± 0.10
Douglas Sand	32	4.63 ± 0.20

are at or below 5.00.

Table XV compares and contrasts the solvent and acid production by SEG-3 with each core supplying the buffering capacity, after the initial inoculation. This is clear evidence that there are differences between fermentations in the various cores. The most striking of these differences is the butanol production. Assuming that butanol production is indeed a function of the pH of the growth medium then those tubes with the highest concentration of butanol would be the least buffered. This would lead to the conclusion that the Austin Chalk, Kansas City Lime and Redden Sand are excessively buffered (low butanol production) while the strict sandstone cores are not.

A second way to determine the total buffering capacity as well as the effects of cores on a fermentation is to refeed the cultures. This in effect means to remove 2 ml of spent medium and add back 2 ml of medium that is five times as concentrated as the original fresh medium. The growth response of the bacterium should in theory be the same as the initial response, if conditions for growth are such that a response is possible. The response of more gas production when the culture is refed as above, in the presence of a core that is supplying certain microelements and buffering capacity, is a measure of the "refeedability"

TABLE XV

INITIAL SOLVENT AND ACID (mM) PRODUCTION BY CULTURE SEG-3 IN 10 MILLILITERS OF THE CORE MEDIUM, IN THE PRESENCE OF VARIOUS CORES AT 0.5 GM/TUBE

Core	M	Ethanol	Acetone	Butanol	Acetic	Butyric
AC	16	1.8 ± 1.2	7.1 ± 2.5	0.5 ± 1.0	0.1 ± 0.1	17.6 ± 2.2
KC	10	7.5 ± 2.4	7.5 ± 1.9	-0-	0.1 ± 0.1	20.0 ± 1.9
R	8	0.7 ± 1.3	7.3 ± 1.7	0.9 ± 1.8	0.1 ± 0.1	20.0 ± 1.9
DT	16	3.4 ± 1.6	5.6 ± 3.0	18.8 ± 5.1	0.1 ± 0.1	18.2 ± 1.9
B	16	3.1 ± 0.9	7.7 ± 1.4	16.6 ± 5.7	0.1 ± 0.1	15.1 ± 3.2
DG	16	1.8 ± 1.6	8.3 ± 2.4	23.3 ± 5.3	0.1 ± 0.1	11.3 ± 2.0

Abbreviations Used: AC= Austin Chalk KC-= Kansas City Lime R= Redden Sand
DT= Dutcher Sand B= Bryden Sand DG= Douglas Sand

of the system. Refeeding as described above can be carried out a number of times depending on the core. The ability of a core material to support gas production upon several refeedings is a property of that core with considerable significance for sustained growth by a microbial culture in situ. Cores in this research differ significantly in their refeedability. There is little if any difference in the first refeed of the Austin Chalk, Kansas City Lime or Redden Sand. After a subsequent refeed, the Redden Sand quickly losses its ability to support gas production. The Kansas City Lime does not allow as much gas production as does the Austin Chalk under similar conditions.

Table XVI gives the results of long term experiments in tubes using the Austin Chalk at 0.5 gm/tube, some of which have been actively growing for close to one year after being refed. The one tube shown in this table that is no longer actively growing represents a special case and will be discussed in detail on following pages. From this table, the Austin Chalk is capable of providing enough buffering capacity for active growth over an extended period of time. No limits have been found, as yet, for the Austin Chalk or Kansas City Lime, but the total gas production under these conditions was not quite as good in the Kansas City Lime.

The limit for the Redden Sand appears to be the third

TABLE XVI

GAS PRODUCTION, pH AND PERCENT NUTRIENTS UTILIZED BY LONG TERM INCUBATIONS IN THE PRESENCE OF THE AUSTIN CHALK AT 0.5 GMS/TUBE

Tube #	Inoculation Date	Final pH/Date	Sucrose	Yeast Extract	NH_4Cl	Gp Total
15	7-19-84	5.95/5-22-85	14.0	0.65	0.60	4.62
25	7-19-84	4.71/5-22-85	14.0	0.75	0.60	2.20
35	7-19-84	5.79/6-22-85	14.0	0.45	0.60	5.80
45	7-19-85	5.65/6-22-85	13.0	0.55	0.70	4.02
55	7-19-84	5.95/6-22-85	14.0	0.45	0.50	4.92
65	7-19-84	5.99/6-22-85	14.0	0.75	0.50	4.65
45	8-29-84	5.37/1/25/85	12.0	0.30	0.40	5.04
7	1- 5-85	5.21/6- 4-85	10.0	0.25	0.20	4.45
8	2-16-85	5.30/6- 4-85	9.0	0.25	0.20	4.00
32	2-16-85	5.30/6-16-85	7.0	0.10	0.40	2.87
33	2-16-85	5.21/6-10-85	7.0	0.10	0.40	2.74
34	2-16-85	5.20/6-10-85	8.0	0.45	0.40	2.26

All Incubations at 30 Degrees Celsius. Gp Total represents total gas production per tube in cubic millimeters x 10^5.

refeed, a limit that will allow us to utilize the resevoir itself a number of times. As to the other sandstone cores, the Bryden Sand, because of gas production on refeed ranks fourth on refeedability. No differences occur between the Dutcher and Douglas Sands as no response is seen on the first refeed. One would be hardpressed however to discern a compositional difference between the Bryden, Douglas and Dutcher Sands. It is therefore quite likely that the differences observed i.e. growth and gas production on refeed in the Bryden Sand are the result of the availability of clays (notably siderite) to provide the necessary buffering capacity.

Table XVII compares the solvent and acid production 288 hours after the first refeed with the addition of either 0.1% ammonium nitrate or yeast extract. Major differences occur in the production of isopropanol and isobutyric acid, which appear to result from the presence of yeast extract and ammonium nitrate added to the refeed medium. Yeast extract in the case of the limestone cores and Redden Sand causes an early shift to isopropanol production. This same shift will occur later if only sucrose is used to refeed. Yeast extract also seems to have an effect on the amount of butanol formed. In the case of the Austin Chalk and Redden Sand, adding yeast extract to the refeed medium dramatically increase the production of

TABLE XVII

RESULTS OF ACID AND SOLVENT (mM) DATA FROM SEG-3 288 HOURS AFTER FIRST REFEED WHEN REFED WITH 1% SUCROSE SUPPLEMENTED WITH EITHER 0.1% AMMONIUM NITRATE OR 0.1% YEAST EXTRACT. ALL INCUBATIONS AT 30 DEGREES CELSIUS (N=5)

Core	Supplement	Ethanol	Acetone	Isoprop.	Butanol	Acetic	Butyric	Isobut.
AC	YE	3.2±0.6	4.9±0.4	3.7±0.3	14.4±4.9	0.2±.1	39.2±3.0	-0-
AC	AM	1.2±1.7	5.9-0.5	-0-	4.6±3.5	0.3±.5	44.6±4.2	0.6±.2
KC	YE	3.1±0.5	8.0±0.9	6.5±1.0	4.3±1.0	0.2±.1	20.9±0.8	-0-
KC	AM	2.9±-0-	5.3±1.6	-0-	2.7±0.3	0.2±.1	31.8±3.9	1.5±.7
R	YE	3.5±0.2	7.1±2.6	2.8±0.5	22.6±8.1	0.2±.1	39.2±0.9	-0-
R	AM	2.7±-0-	-0-	-0-	9.2±0.4	0.2±.1	42.5±2.1	5.3±.5
DT	YE	3.1±0.1	8.9±0.1	-0-	19.7±2.8	0.3±.1	25.5±0.7	-0-
DT	AM	3.2±-0-	6.3±0.8	-0-	16.5±2.2	0.3±.1	31.5±7.7	0.8±.2
B	YE	3.1±.1	7.6±1.2	-0-	17.3±1.2	0.2±.1	30.0±1.6	-0-
B	AM	3.2±-0-	6.7±0.3	-0-	21.2±8.3	0.2±.1	31.8±7.1	1.5±.5
DG	YE	2.9±-0-	6.9±0.5	-0-	17.7±0.4	0.3±.1	20.8±6.2	-0-
DG	AM	2.9±0.5	7.1±0.8	-0-	17.5±0.9	0.3±.1	35.1±10	0.8±.2

Abbreviations Used: AC= Austin Chalk KC= Kansas City Lime R- Redden Sand
DT= Dutcher Sand B= Bryden Sand DG= Douglas Sand
Isoprop.+ Isopropanol Isobut.+ Isobutyric Acid
YE= Yeast Extract AM= Ammonium Nitrate

butanol. This suggests that while the pH of the medium and hence buffering capacity provided by a core may be an important parameter for the switch in fermentation from acidogenic to solventogenic it is not the only factor. Numerous articles (Allcock, et al., 1981: Bahl and Gottschalk, 1984; Griffith, et al., 1985; Montville, et al., 1985; Rosenfield and Simon, 1950; Ulmer, et al., 1983 and Turton, et al., 1982) suggest that a pH near the breakpoint is only one of the factors responsible for the shift from acid to solvent fermentation. Addition of ammonium nitrate shifts the fermentation scheme in favor of isobutyric acid production. Fermentation patterns of cultures in those cores which lend themselves to refeeds, show a common characteristic as to kinds and amounts of products formed. In general, as refeeding progresses, the fermentation schemes occurring in the acid soluble cores begins to resemble that of the lower acid-soluble cores as acetone and butanol formation increases.

It should be noted, that a weight of 0.5 gm/tube of powdered core is less on a weight/volume basis than one would encounter in the reservoir. Experiments were attempted with the amount of core one would encounter, assuming a 15% porosity. Gas production from these experiments mirrored those of the same core where only 0.5 gm/tube was used, but sampling difficulties occurred in respect to pH

and gas chromatography analysis.

During refeed experiments, certain tubes have produced upon refeed more gas than normal. The first of these was tube 45 in the Austin chalk inoculated August 29, 1984, shown in Figure 20. The numbers refer to the number of refeeds with 1% sucrose only. For comparison, Figure 21 shows the average gas production per refeed of six tubes (inoculated 7/19/84) refed with 1% sucrose. It must be noted that these tubes were refed using the old procedure of inverting the Hungate tube to remove 2 mls of spent medium. Using this method of removal, the washout rate was high and minute pieces of core were also sucked into the syringe as the tubes were inverted, thus removing a small portion of the core at each refeed. In tubes refed with 1% sucrose (no nitrogen) cultures were unable to divide and therefore regenerate those cells lost by washout. A characteristic of the end of the solvent phase of the acetone-butanol fermentation is the loss of motility of the cells. As a result cells fall to the bottom of the tube and a general clearing of the upper portions of the medium occurs. For this reason, the refeed procedure was amended and a flamed 3 inch 21 gauge needle was injected into the medium without inverting the tube. As a result, the 2 mls removed from the top of the medium contained cells, thus lowering the washout rate of viable bacteria.

FIGURE 20. Total gas (mm) produced by SEG-3 in the core medium where trace elements and buffering capacity are supplied by 0.5gms of the Austin Chalk. This figure depicts tube 45 in the Austin Chalk. Gas production was above average in this tube on refeed. Open triangles-gas production during the initial inoculation. Numbers refer to the times refed, therefore, at the end of the first refeed, the culture has been subjected to 2% total sucrose, then on second refeed 3%, etc.

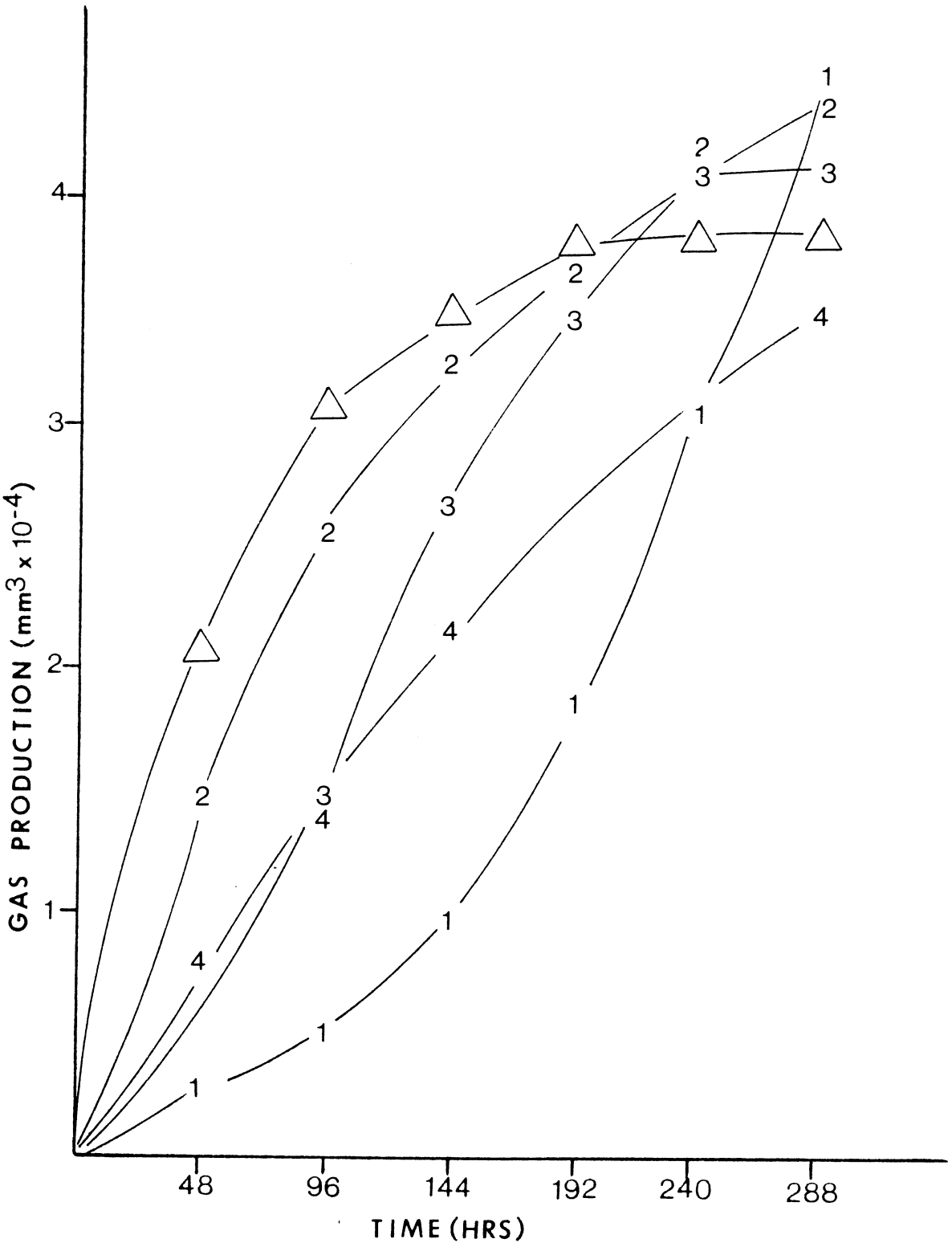

GAS PRODUCTION ($mm^3 \times 10^{-4}$)
TIME (HRS)
1
2
3
4
48
96
144
192
240
288

FIGURE 21. Average total gas (mm) produced in five tubes inoculated with SEG-3 in the core medium where trace element and sole buffering capacity are supplied by 0.5 gms/tube of the Austin Chalk. Tubes were refed with the first refeed method and are representative of Austin Chalk tubes after refeed using this procedure. Open triangles-gas production during the initial incubation. Numbers refer to the refeeds. Therefore, 1 equals the first addition of fresh medium to the culture.

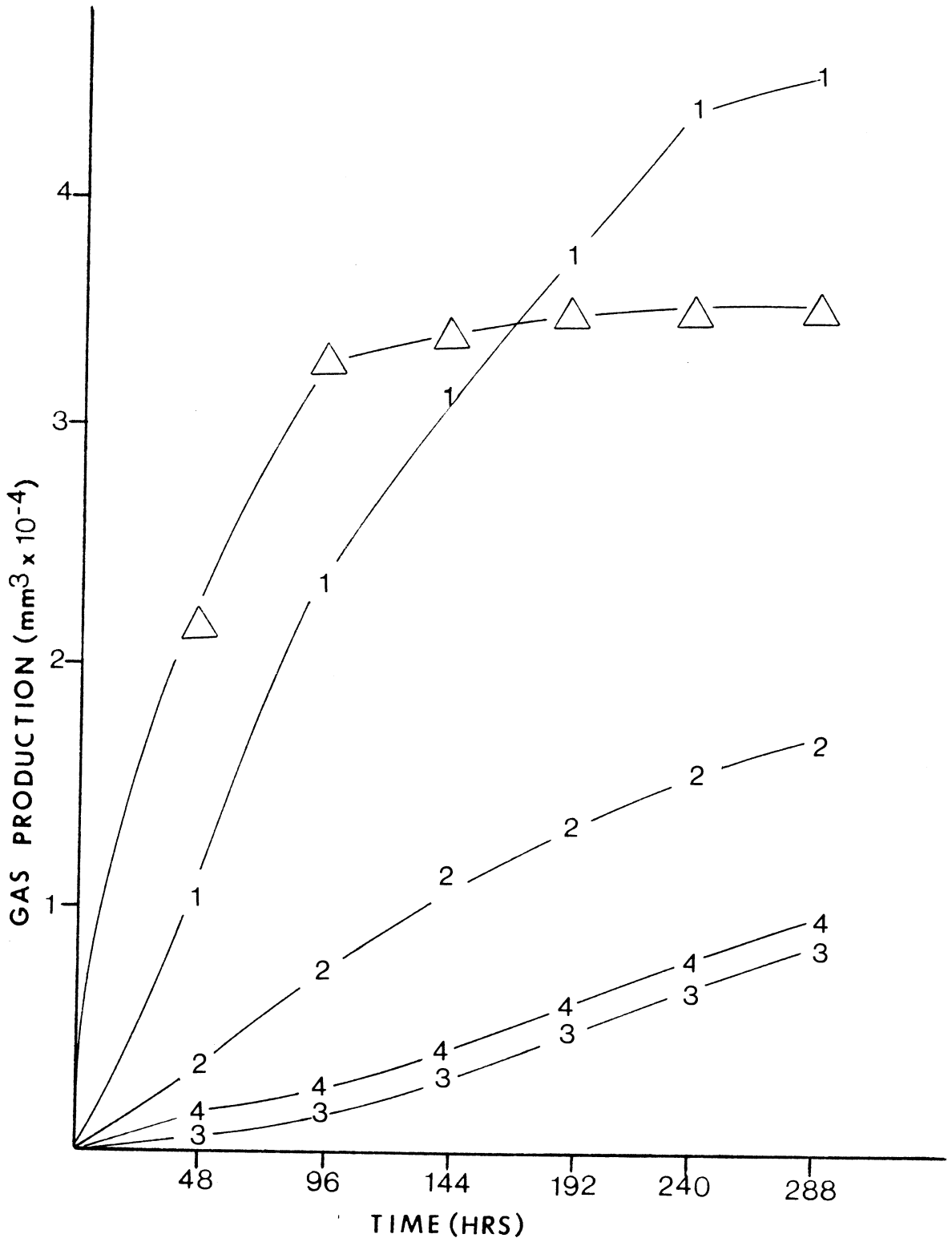
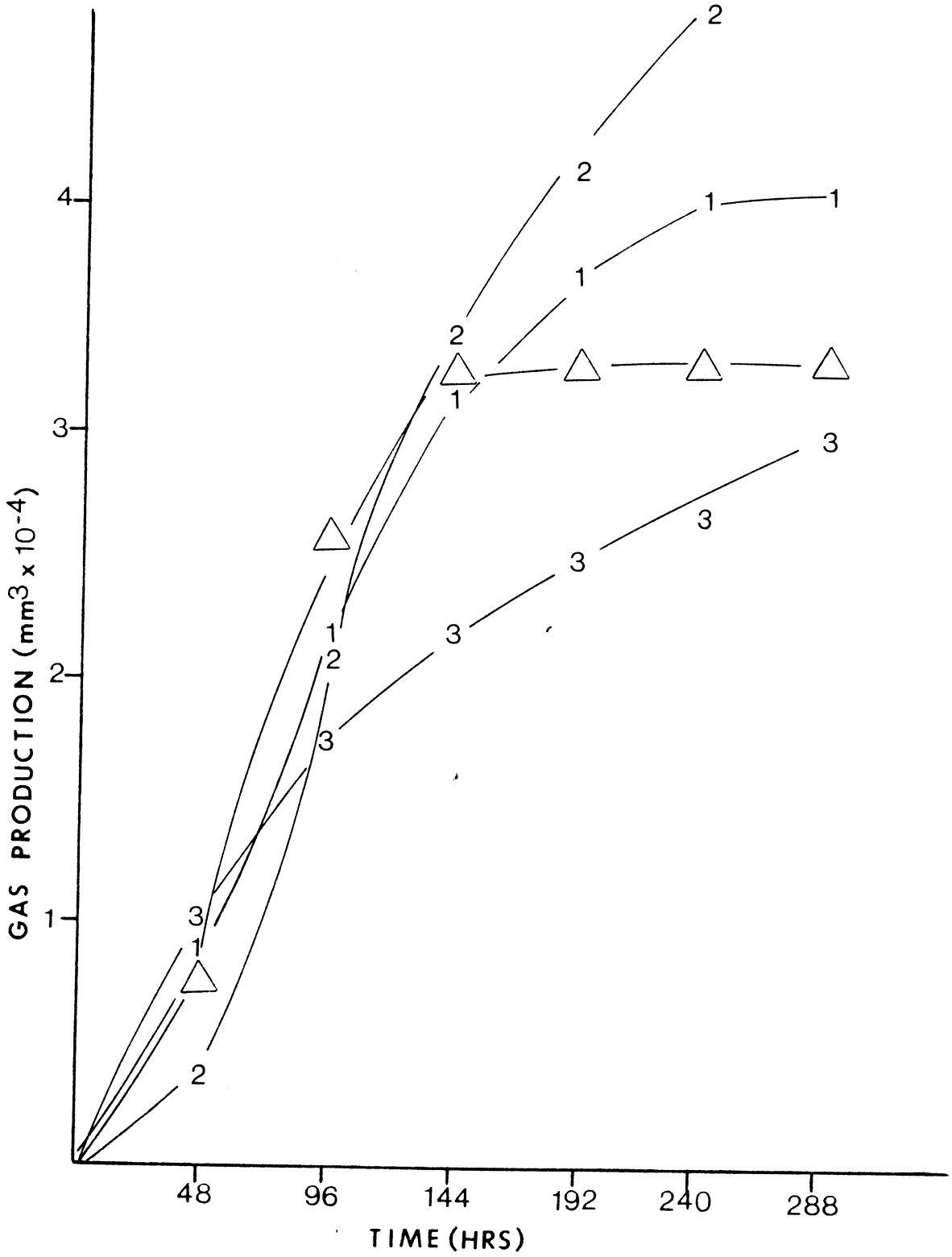

GAS PRODUCTION ($mm^3 \times 10^{-4}$)
TIME (HRS)
1
2
3
4
48
96
144
192
240
288

Figure 22 shows the results when two tubes upon refeed using the new procedure with 1% sucrose. Although there clearly is a difference between these tubes and the ones in Figure 12 and from Figure 11, it is still evident that Tube 45, using the old refeed procedure was still very unusual. Gas production rates and total production was higher in Tube 45.

Attempts were made to subculture from Tube 45 and determine if this ability to produce increased amounts of gas could be seen in the presence of other cores. This was accomplished by using the 2 mls of spent medium removed from Tube 45 to inoculate other tubes with the Austin Chalk and other cores and Tryptic Soy Broth (TSB). Results of these experiments and subsequent inoculations with the culture from Tube 45 which was designated HSEG-3 proved of little value. If anything, the culture seemed to be somewhat less capable of growth in cores other than the Austin Chalk when compared to the parent strain. After heatshocking, the strain became identical to the parent in regards to growth in the Austin Chalk, although there was a somewhat enhanced gas production upon refeed. Other tubes with enhanced gas production were detected during the course of refeeds, assuring initial inoculation, or even during experiments when cores were not used (with media such as 1% sucrose, combined phosphates or molasses).

FIGURE 22. Average total gas (mm) produced in two tubes inoculated with SEG-3 in the core medium where trace element and sole buffering capacity are supplied by 0.5gms/ tube of the Austin Chalk. Tubes were refed with the new refeed method and are representative of Austin Chalk tubes after refeed using this procedure. Open triangles-gas production during the initial incubation. Numbers refer to the refeeds. Therefore, 1 equals the first addition of fresh medium to the culture.


GAS PRODUCTION ($mm^3 \times 10^{-4}$)
TIME (HRS)
1
2
3
4
48
96
144
192
240
288

These cultures had two things in common, an increased ability to produce gas under a given set of conditions and the rapid loss of that ability upon reculturing. It was not until an experiment began on January 5, 1985, with all six cores (24 replicates each) being refed every 288 hours that two tubes, one in the Bryden and one in the Redden Sand, produced gas for a greater number of refeeds than in earlier trials.

Figure 23 illustrates the gas production by Tube B7 containing the Bryden Sand (replicate number 7) at 0.5 gm/tube. The initial gas production was not impressive and neither was the first refeed (about normal for a tube with the Bryden Sand). On the second refeed, gas production increased to levels equal to the initial and the third refeed as well was high. This had never occurred before with Bryden Sand in numerous refeed attempts.

Figure 24 shows the gas production by Tube R7 (Redden Sand, replicate number 7), with the first refeed being about normal for the Redden core. The second refeed, though, was much higher than the initial production or first refeed. A normal second refeed in the Redden Sand would normally result in less gas production than either the initial gas production or first refeed.

Cells from both tubes (separate inoculations) were used to inoculate other tubes containing various cores.

FIGURE 23. Total gas from tube B-7 during the initial incubation in the core medium (open triangle) the first (1), second (2) and third (3) refeed (no gas production detected).

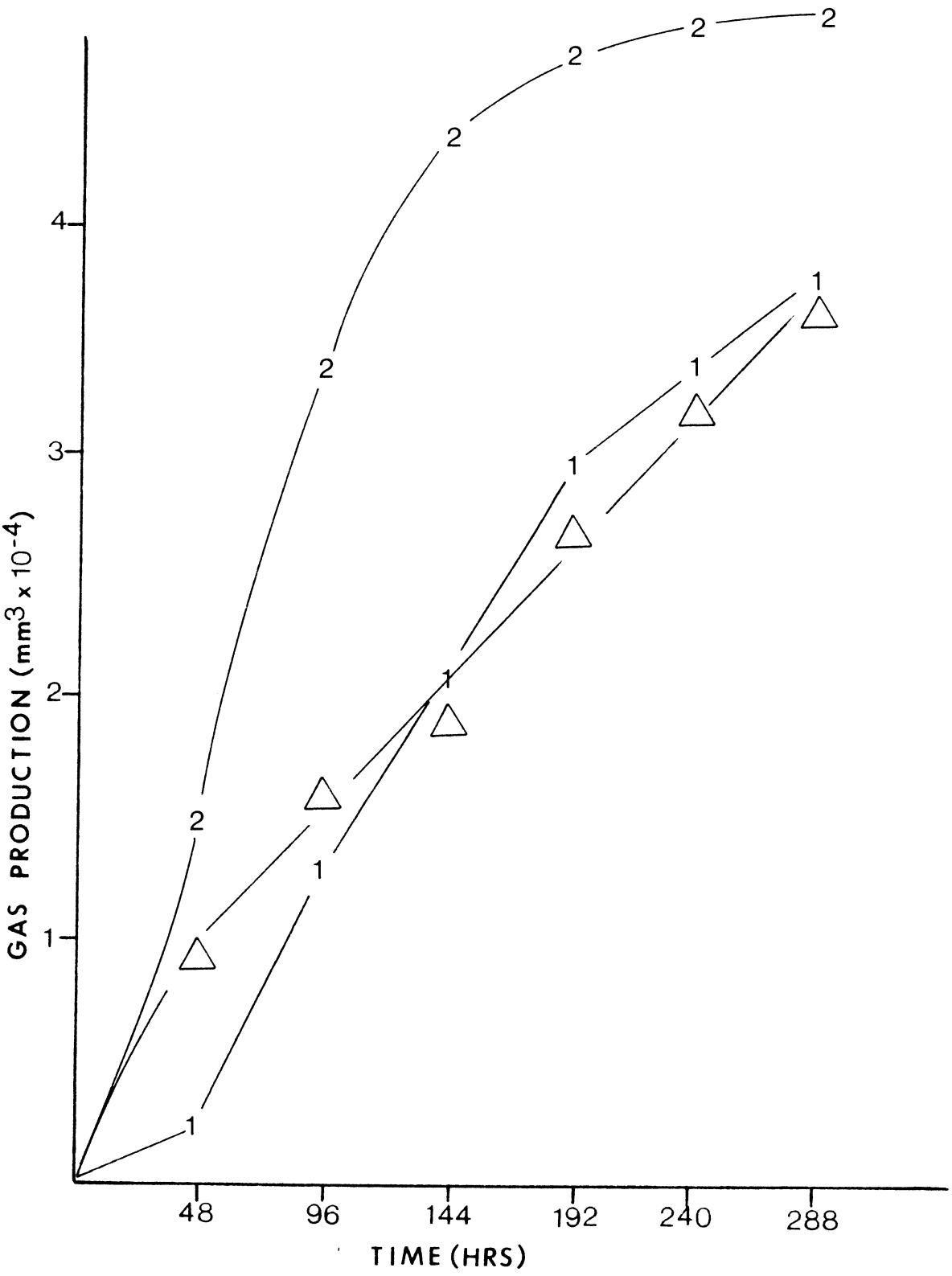
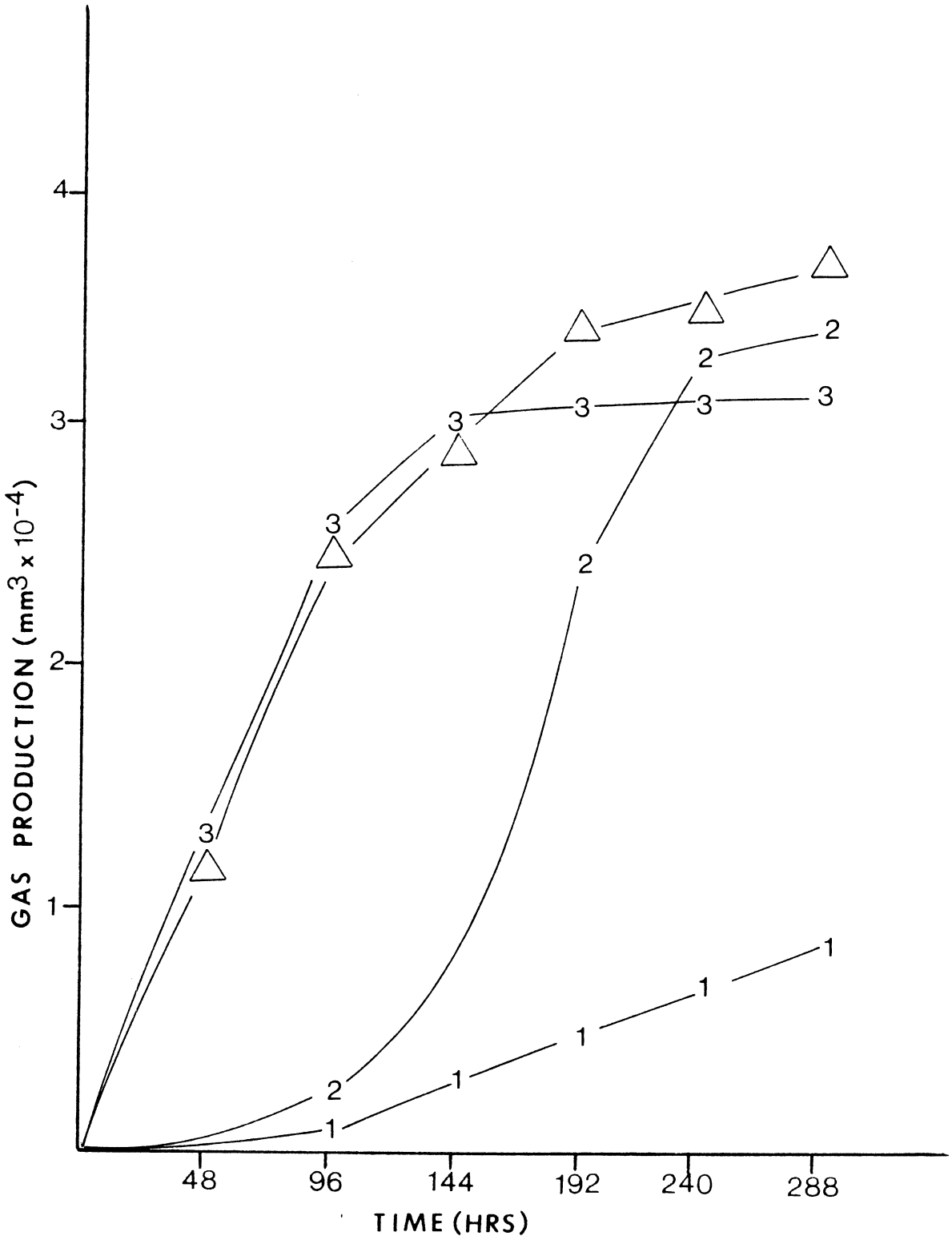

GAS PRODUCTION ($mm^3 \times 10^{-4}$)
TIME (HRS)
1
2
3
4
48
96
144
192
240
288

FIGURE 24. Total gas from tube R-7 during the initial incubation in the core medium (open triangle) the first (1), second (2) and third (3) refeed.


GAS PRODUCTION ($mm^3 \times 10^{-4}$)
4
3
2
1
48
96
144
192
240
288
TIME (HRS)

The results were similar to those seen in tubes R7 and B7, that is refeeding of these tubes resulted in an increased ability to produce gas. Cultures of both strains, now designated B7 and R7 were made using TSB with 1% sucrose and the spores then transferred to sterile soil. Subsequently both cultures were used in core experiments. The ability to produce large amounts of gas upon refeed in all cores (including the Douglas Sand) was not lost even upon reculturing and heat shock.

Table XVIII contrasts the gas production between the core derived strains, B7 and R7 and the progenitor SEG-3, in a 2% sucrose, 0.1% yeast extract, 0.1% ammonium nitrate medium with various cores at 0.5 gm/tube. From this table and other data, it appears that R7 lies between the parent strain and B7 in the ability to produce gas in the presence of cores. Gas production by B7 in the presence of the Douglas Sand is twice that of SEG-3, and as one would expect, almost identical in the Kansas City Lime and Austin Chalk.

In order to discern differences between the parent strain and B7, all cores were inoculated with cells in the log phase of growth of the respective cultures and refed.

Figure 25 depicts the initial gas production of both strains in the Bryden Sand. Upon initial inoculation, it is possible that the difference between the parent and the

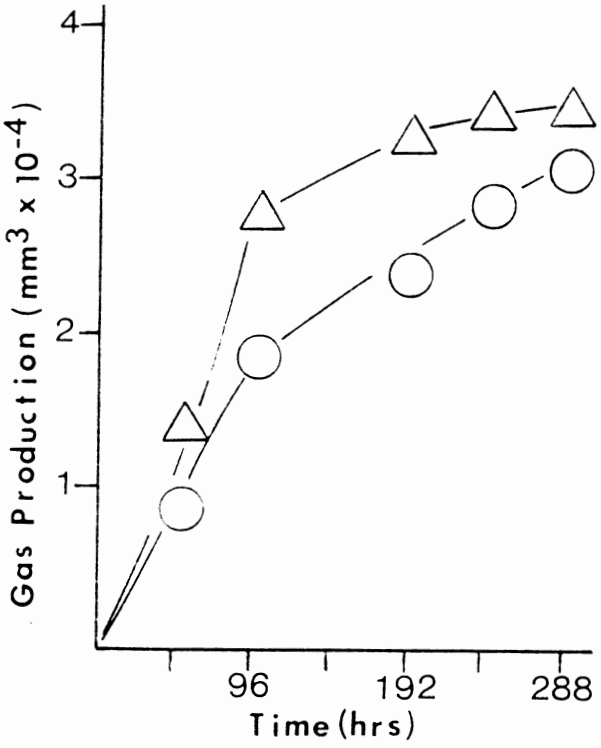
TABLE XVIII

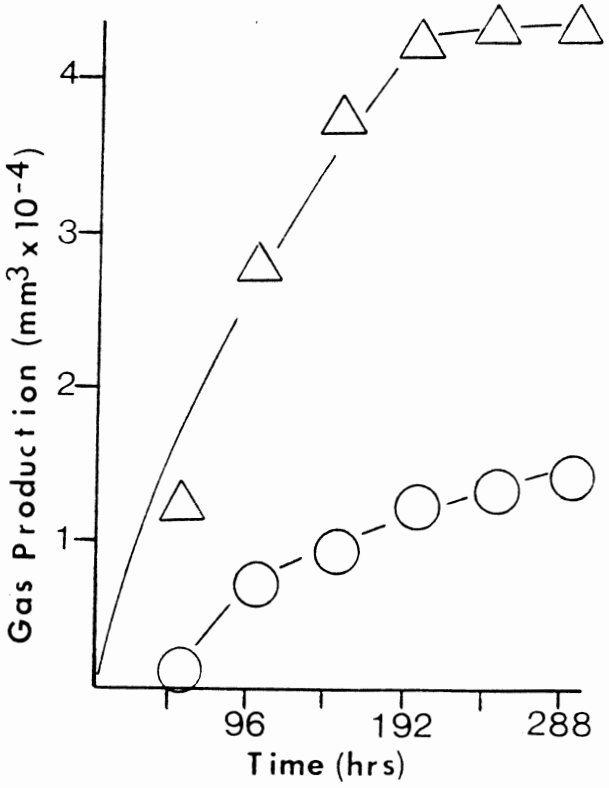
TOTAL GAS PRODUCED BY SEG-3 AND THE CORE DERIVED STRAINS B7 AND R7 WHERE THE CORE BASAL MEDIUM CONTAINS 2% SUCROSE

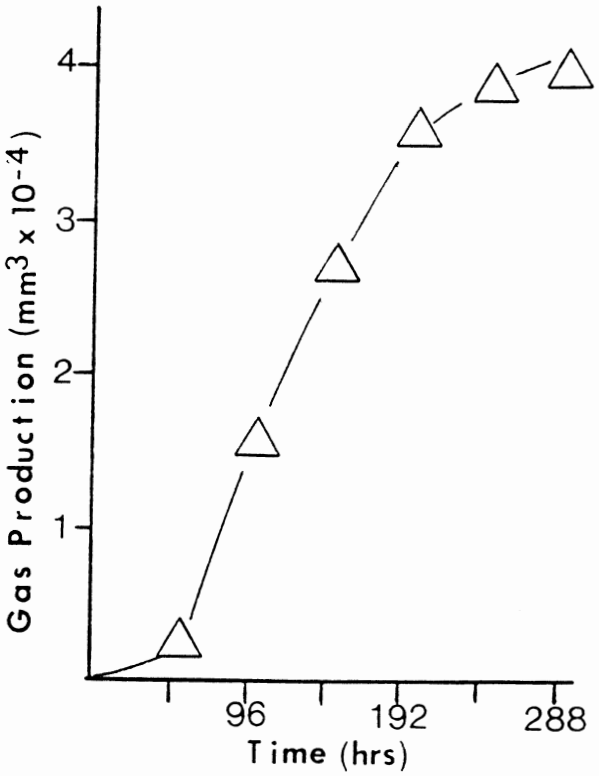
Core	Culture	Gp Total
Austin Chalk	B7	6.9
Austin Chalk	R7	6.6
Austin Chalk	SEG-3	7.0
Kansas City Lime	B7	8.1
Kansas City Lime	R7	5.6
Kansas City Lime	SEG-3	7.3
Redden Sand	B7	8.0
Redden Sand	R7	7.5
Redden Sand	SEG-3	6.7
Dutcher Sand	B7	7.3
Dutcher Sand	R7	5.2
Dutcher Sand	SEG-3	4.6
Bryden Sand	B7	7.0
Bryden Sand	R7	5.0
Bryden Sand	SEG-3	4.8
Douglas Sand	B7	6.9
Douglas Sand	R7	4.5
Douglas Sand	SEG-3	3.4

Gp Total equals total gas produced at 576 hours post-inoculation cubic millimeters x 10^4

FIGURE 25. Comparisons of culture B-7 (open triangle) and SEG-3 (open circle) in the presence of the Bryden Sand. Center-initial inoculation. Lower left-first refeed. Lower right-third refeed total 3% sucrose. (At third refeed SEG-3 produced no detectable gas) N for each culture = 5.


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

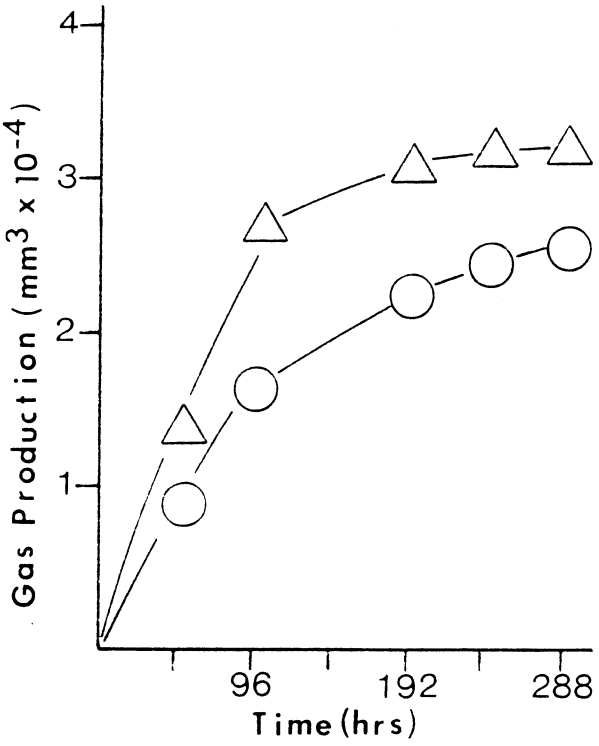
core-derived strain is merely the latter's enhanced ability to grow, under conditions set forth by the medium and the core. Upon the first and second refeed a vast difference is seen between the two. Gas production by B7 does not decrease with two refeeds, although the parent strain rapidly loses the ability to produce measureable gas.

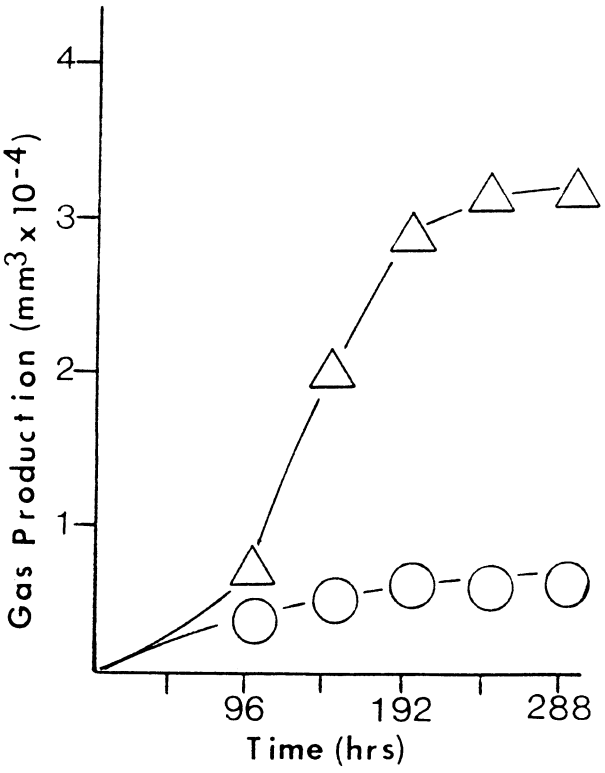
Figure 26 relates the differences between the strains as to their ability to produce gas in the Douglas Sand inititally and on refeeds. There appears to be a difference between the two cultures upon initial inoculation. At the first and second refeed, B7 continues to produce gas, while SEG-3 did not. The ability of B7 to produce gas not only in this core, but the Dutcher Sand (Figure 27) is the best demonstration that B7 has an increased ability to produce gas in the presence of cores with a lower acid solubility.

The ability of B7 to outgrow (based on total gas production) SEG-3 doesn't manifest itself when cores with excellent refeedability are used (Figures 28, 29 and 30) either with initial inoculation or with the first refeed. This no doubt is a function of the core and not the cultures, since in the case of the Redden Sand after the second refeed there exists a difference between strains.

The foregoing experiment demonstrates that the

FIGURE 26. Comparisons of culture B-7 (open triangle) and SEG-3 (open circle) in the presence of the Douglas Sand. Center-initial inoculation. Lower left-first refeed. Lower right-third refeed total 3% sucrose. (SEG-3 did not produce gas at third refeed). N for each culture = 5.


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

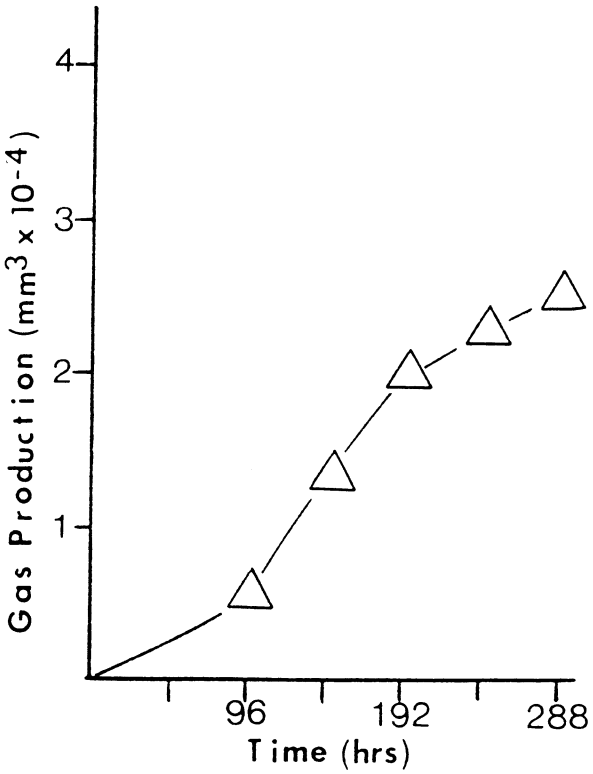
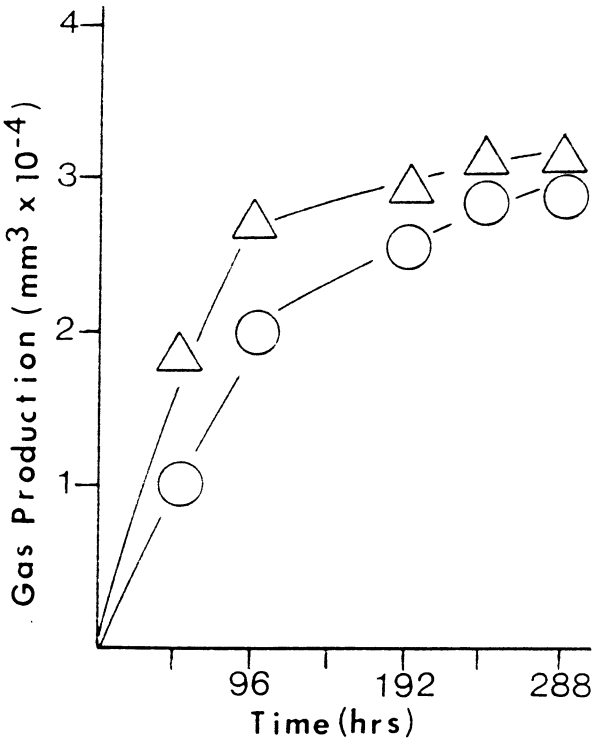
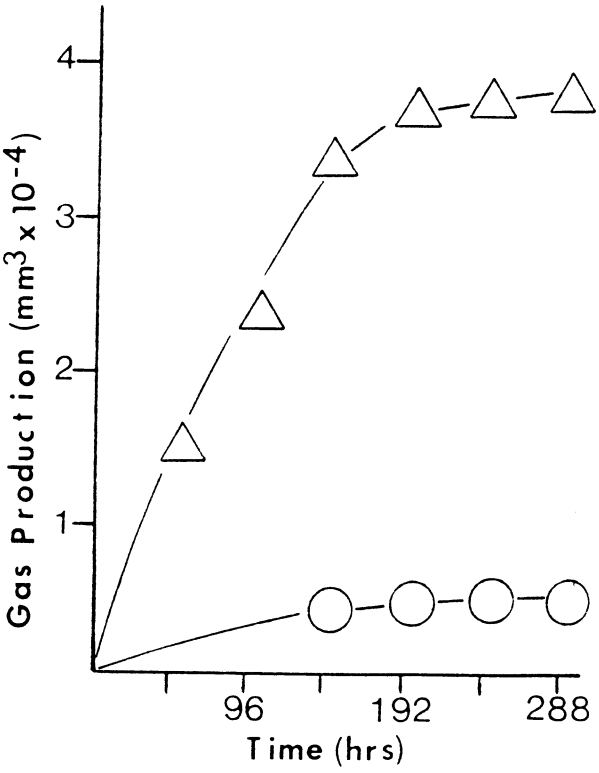

Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

FIGURE 27. Comparisons of culture B-7 (open triangle) and SEG-3 (open circle) in the presence of the Dutcher Sand. Center-initial inoculation. Lower left-first refeed. Lower right-third refeed total 3% sucrose. (SEG-3 did not produce gas at third refeed). N for each culture = 5.


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

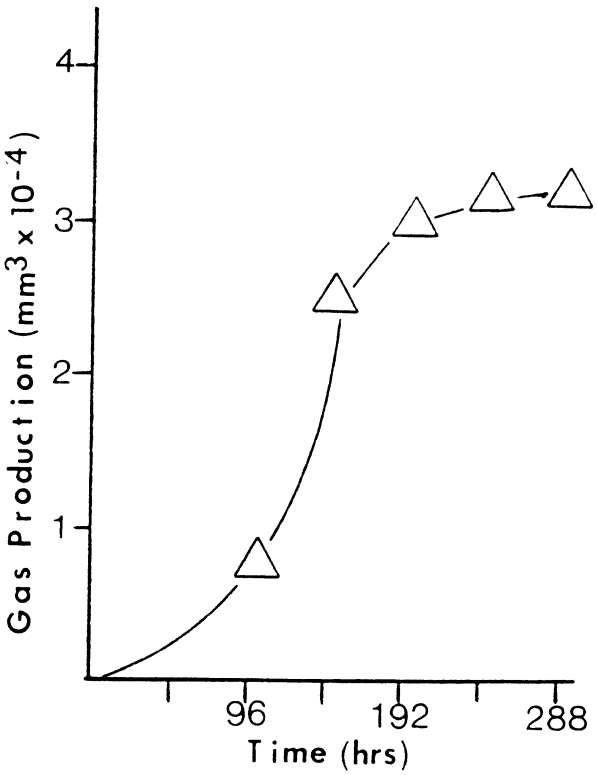
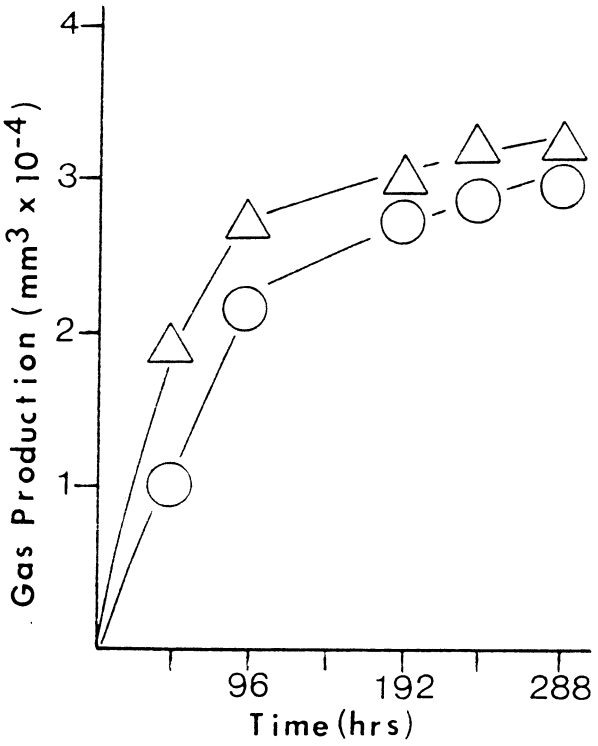
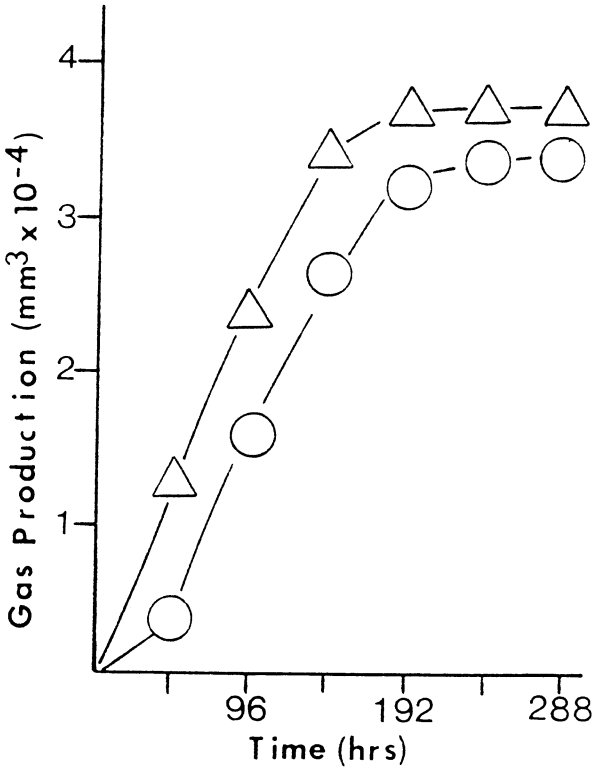

Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

FIGURE 28. Comparisons of culture B-7 (open triangle) and SEG-3 (open circle) in the presence of the Austin Chalk. Center-initial inoculation. Lower left-first refeed. Lower right-third refeed total 3% sucrose. N for each culture = 5.


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

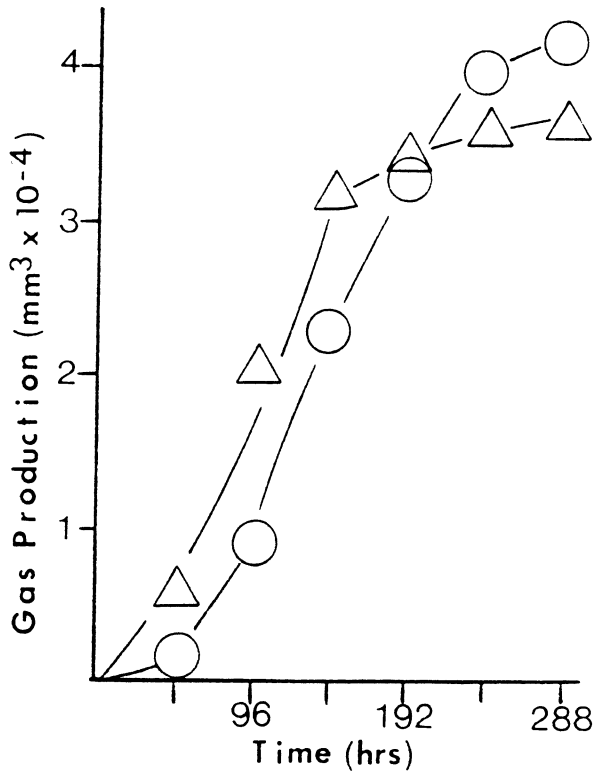
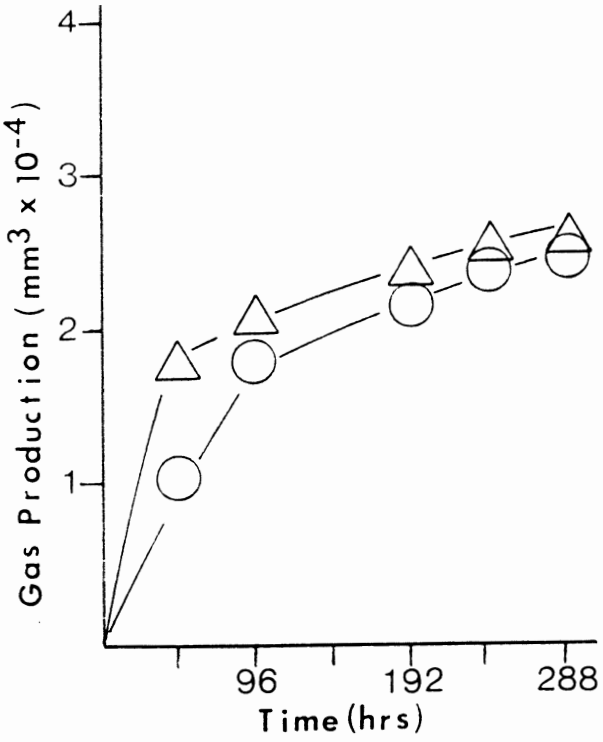
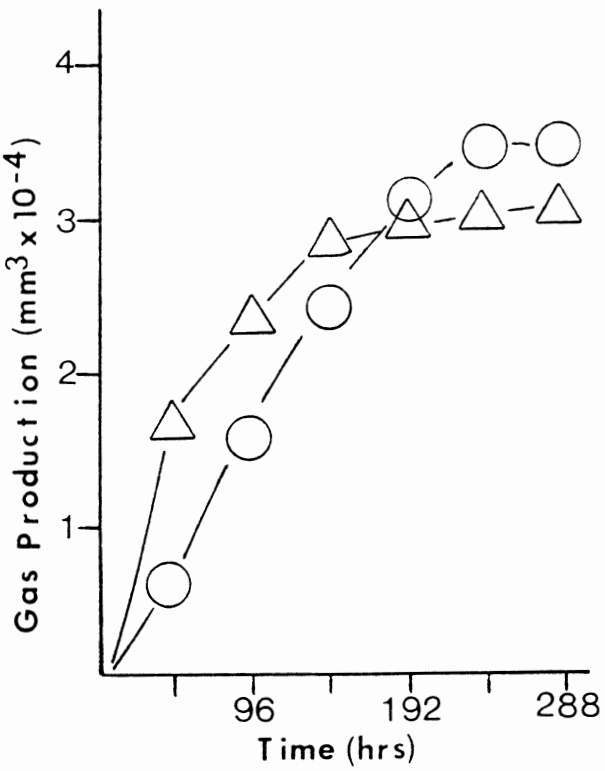

Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

FIGURE 29. Comparisons of culture B-7 (open triangle) and SEG-3 (open circle) in the presence of the Kansas City Lime. Center-initial inoculation. Lower left-first refeed. Lower right-third refeed total 3% sucrose. N for each culture = 5.


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

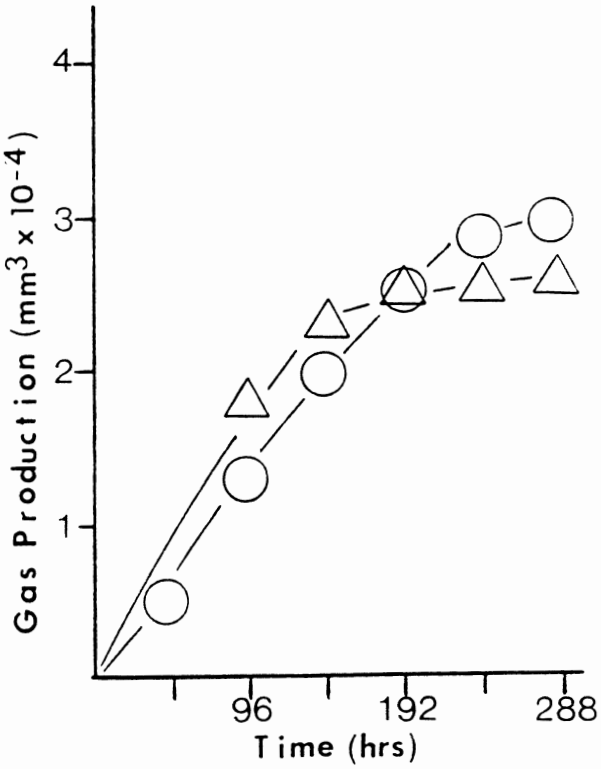
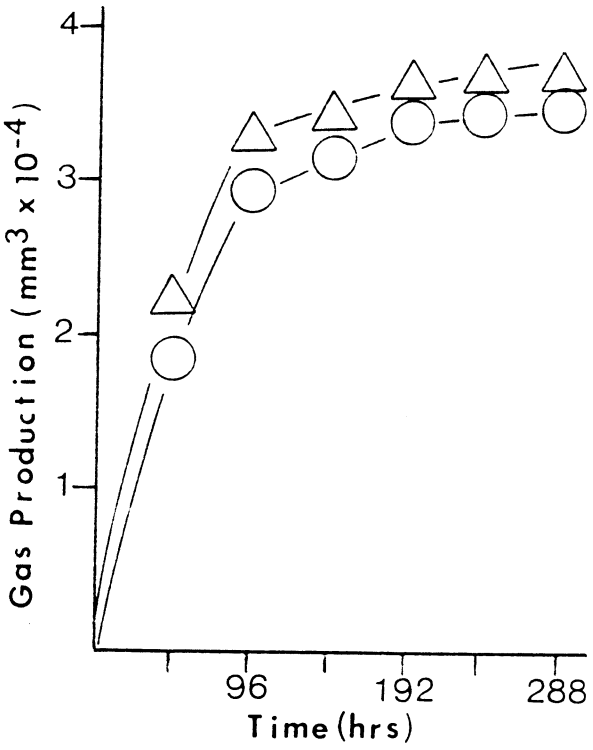
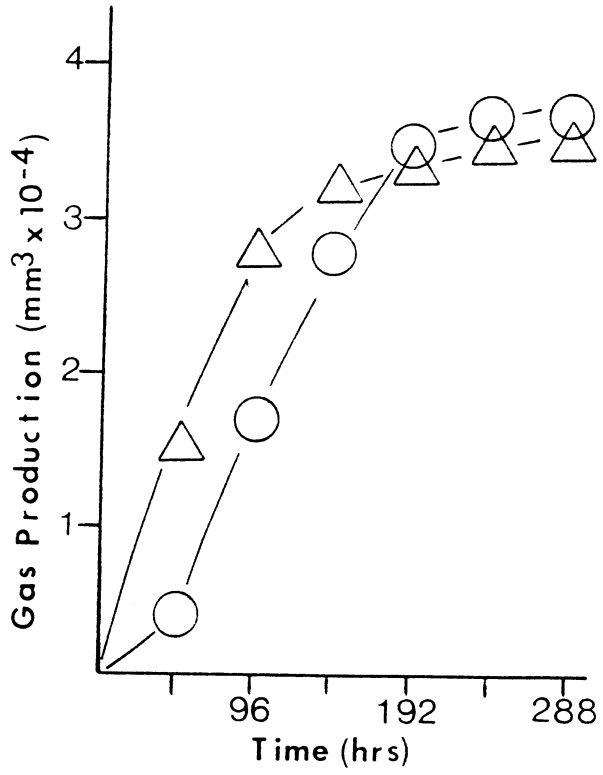
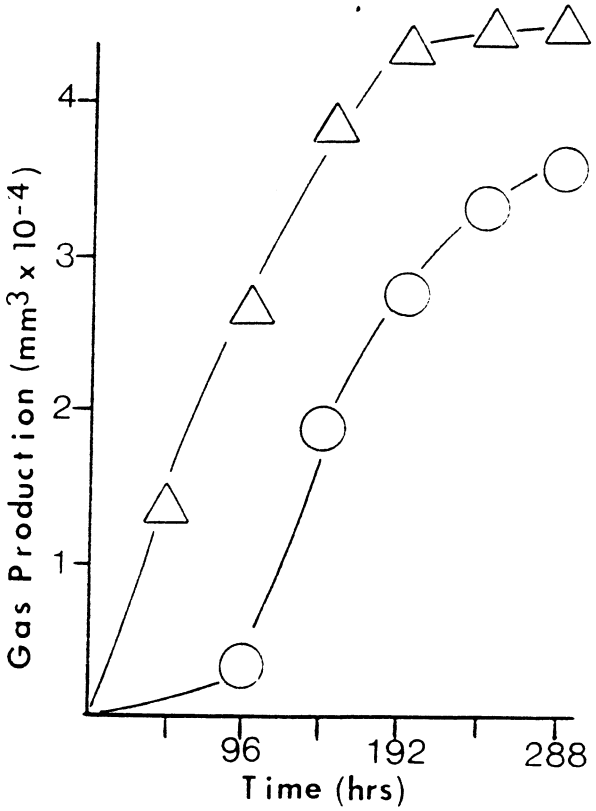

Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

FIGURE 30. Comparisons of culture B-7 (open triangle) and SEG-3 (open circle) in the presence of the Redden Sand. Center-initial inoculation. Lower left-first refeed. Lower right-third refeed total 3% sucrose. N for each culture = 5.


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)


Gas Production ($mm^3 \times 10^{-4}$)
4
3
2
1
96
192
288
Time (hrs)

core-derived strain B7 is distinct in its ability to produce gas in comparison to the parent strain, especially in the presence of cores with low refeedability. Table XIX shows the acid and solvent production by the core derived strain B7, amounts which are impressive in relation to those produced by SFG-3 (data in Table XVII).

Additional information would be needed before any assertions could be made as to the nature of the increased ability of B7 to grow on refeed in certain cores. Indications are that when the parent strain is grown with the Austin Chalk or Kansas City Lime at 0.5 gm/tube and upon refeed, the 1% sucrose is supplemented with 0.1% ammonium nitrate or yeast extract after the second or third refeed, gas production ceases. When only sucrose is used at the first few refeeds and ammonium nitrate or yeast extract added only at the sixth or seventh refeed or if the level of yeast extract or ammonium nitrate is lowered to 0.05%, growth is not affected. From Table XVII we see that the addition of ammonium nitrate to the medium at the 0.1% level, causes a shift to isobutyric formation. If 0.1% yeast extract is added, the cores with good refeedability begin to produce isopropanol (reduction of acetone). Both shifts occur when cultures are refed only sucrose, (in the case of cores allowing the process to continue) only these shifts occur at later refeeds. This and the fact that the

TABLE XIX

ACID AND SOLVENT (mM) PRODUCTION BY CULTURE B7 AT THE SECOND OR THIRD REFEED, 288 HOURS AFTER THE FRESH MEDIUM HAS BEEN ADDED. (N=5)

Core	Ethanol	Acetone	Butanol	Acetic	Butyric	Isobutyric	Refeed
DT	3.7 ± 1.3	17.0 ± 3.0	29.3 ± 3.4	0.1 ± 0.1	62.6 ± 5.6	11.7 ± 3.4	2nd
DT	4.6 ± 1.2	30.0 ± 5.7	49.1 ± 4.5	0.1 ± 0.1	63.3 ± 6.7	11.7 ± 3.5	3rd
B	3.9 ± 0.9	14.1 ± 4.2	23.0 ± 5.6	0.1 ± 0.1	56.9 ± 4.8	11.7 ± 2.3	2nd
B	3.7 ± 0.5	13.5 ± 3.4	20.3 ± 9.8	0.1 ± 0.1	66.8 ± 5.4	11.7 ± 3.2	3rd
R	4.3 ± 2.3	14.3 ± 5.0	7.3 ± 1.2	0.1 ± 0.1	68.6 ± 7.6	11.7 ± 5.6	2nd
R	4.2 ± 3.4	5.6 ± 3.0	12.3 ± 1.0	0.1 ± 0.1	75.6 ± 4.3	11.9 ± 3.8	3rd

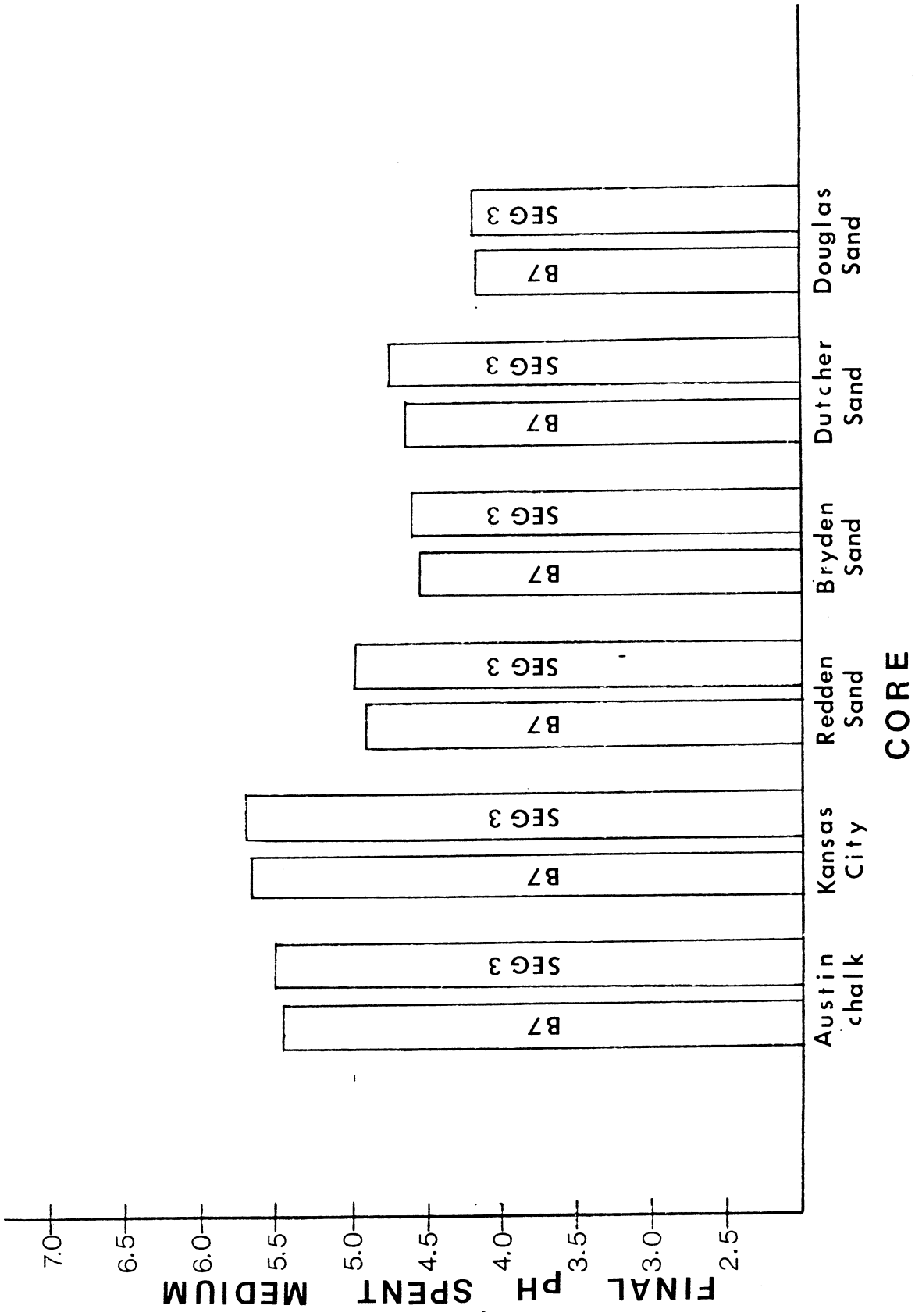
Abbreviations Used: DT= Dutcher Sand B= Bryden Sand R= Redden Sand

final pH values from tubes with SEG-3 or B7 inoculations are for the most part equal (at each refeed) then it would seem that B7 quite possibly has an increased resistance to some end-product of metabolism or is able to grow at a lower pH. Figure 31 is a bar graph showing the differences in the final pH between SEG-3 and B7. Even though the pH final from B7 is lower, refeeding the culture causes renewed fermentation, but not in the case of SEG-3. Indeed there is a report in the literature of a mutation in a strain of Clostridium acetobutylicum that decreases its sensitivity to butanol (Westhuizen, et al., 1982). Table VIII shows that the production of isobutyric acid by SEG-3 results in degeneration of gas production, while in the case of B7 there is no loss of gas production.

The core work has answered a number of questions. From the data presented, no additional trace elements or a buffering agent need to be added to the inoculation medium as these can be readily supplied by the reservoir rock itself. In all experiments done to date, the ratio of core to medium has been considerably less than would occur in situ during fermentation. Experiments attempting to remedy this situation (assuming a 15% porosity) have not been successful in that raw data could not be obtained.

The type of core will be expected to have an effect on the balance between an acid and a solvent fermentation.

FIGURE 31. Bar graph comparing the final pH of spent medium where various cores provide trace element and buffering capacity from cultures B-7 and SEG-3 (N=5).


FINAL pH SPENT MEDIUM
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
CORE
Austin chalk
B7
SEG 3
Kansas City
B7
SEG 3
Redden Sand
B7
SEG 3
Bryden Sand
B7
SEG 3
Dutcher Sand
B7
SEG 3
Douglas Sand
B7
SEG 3

The relationship between gas production, buffering capacity and refeedability of a given core is not clear. Cores with the highest amount of acid soluble constituents do not as a rule produce the most gas on the initial inoculation. Subsequent refeeds of cultures grown in the presence of high refeedability cores will lead to an increase in total gas production per core over the sandstone cores. A number of factors no doubt contribute to the amount of buffering capacity attributable to a core. No doubt the higher percentage of calcite of the Kansas City Lime, Austin Chalk and Redden Sand is partially responsible for the increased refeedability of these cores. Clays though perhaps functioning in an ion exchange capacity or providing an insoluble matrix for cell attachment or various end-products must also play a role in increased gas production, simply because the Kansas City Lime, which contains no such matrix did not generally yield gas production as high as those with such a matrix. It would therefore seem that refeedability of a core or initial gas production from a core is based not on calcite or dolomite alone, but interaction with clays as well.

The difference in the refeedability of the Bryden Sand over the Douglas and Dutcher Sand cannot be attributed clearly to clay content or any other constituent. In the case of these three cores, the differences must no doubt

lie in the availability of the clays in the Bryden Sand to function as an ion exchange bed. For some reason, in the rock matrix, the clays in the Dutcher and Douglas Sands are masked and unavailable to neutralize metabolic acids.

Clays do not bind the amino acids required for germination tightly enough to stop the process. This is evident since spore germination occurred in all cores.

The emergence of cultures B7 and R7 point to the possible use of cores as a screening or selection technique. Whatever mutation responsible for the increased ability of B7 and R7 to grow in the presence of cores, it is apparently quite stable and extends to other media as well. It would therefore be quite feasible to culture bacteria better suited to a particular reservoir, by growth cultivation not only in the water to be used for injection, but also in a medium where reservoir rock (or cores with similar composition) supplies trace element and buffering capacity above that supplied by the medium.

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PART III

FIELD TESTING

CHAPTER I

INTRODUCTION

Microbial enhanced oil recovery (MEOR) is an enhanced oil recovery (EOR) technique that involves injecting viable bacteria and a suitable nutrient source into a producing oil reservoir. As a result of growth and metabolic activities of the bacteria, various metabolites are produced that can cause the release of oil and thus increase the production. This becomes important when a well or entire oilfield reaches a production level low enough to be uneconomical.

In the case of Clostridium, the useful metabolic end-products are low molecular weight alcohols, such as ethanol and n-butanol, polar solvents such as acetone, acids such as acetic and butyric, carbon dioxide, and emulsifiers. Each of these compounds in turn has an effect on the physical/chemical properties of the oil. The low molecular weight alcohols and solvents will effectively wash oil from the pore spaces, by affecting the contact angle of oil, and by lowering interfacial tensions between the oil and water. Acetone and butanol

are also capable of water-wetting the formation. Carbon dioxide, can effect either a miscible or immiscible displacement depending on the pressure required for miscibility, which is a function of the oil itself. Acids react with carbonate in the reservoir to add carbon dioxide to the free gas phase and act as acidizing agents. Emulsifiers act to keep minute droplets of oil in the free-flowing water phase.

MEOR can be divided into two basic categories, single well stimulation and microbial flooding. In the case of single well stimulation, the area immediately adjacent to the wellbore of a single producing well is treated; in the case of microbial flooding a percentage of the swept reservoir is treated, using in place flooding patterns, such that the bacteria move some distance away from the point of injection, and other wells in the area will be affected. This may involve turning a producing well into an injection well.

In the case of single well stimulation, Donaldson (1984) has theorized that the major contribution of Clostridium in EOR is the production of desired metabolic end-products in situ which accomplish a very effective cleaning of the rock pores from the inside. Acids, produced by the bacteria react with various minerals, loosening the clay particles and other inorganic fine

materials that may have clogged pores. Solvents dissolve the precipitated organic deposits, allowing them to regain a mobile condition. The pressure exerted by the carbon dioxide furnishes the energy to blow the mineral fines and sludge into the wellbore, effectively increasing the pore size of the formation around the wellbore. This change in pore size, from small to large at the wellbore will establish a new capillary pressure-saturation relationship and oil production "begins again at an increased rate that can be as much as 50-100% of the original flow depending on the viscosity of the oil and conditions before and after cleanout" (Donaldson, 1984).

The effect of carbon dioxide produced in situ is probably quite different from that of carbon dioxide flooding (injecting carbon dioxide from the surface). Carbon dioxide (Holm, 1976) achieves miscible displacement at pressures of 1100-3000 psi when the carbon dioxide has a purity of 90% or greater. It has also been shown that the amount of carbon dioxide required to recover an additional barrel of crude oil was 30,000 standard cubic feet (Doscher, 1981). It is improbable that a bacterium could be isolated capable of producing enough carbon dioxide to release a detectable amount of oil if the mechanism of action of gas produced in situ by bacteria were not radically different from that of injected carbon

dioxide. However, the experience of a number of investigators has convinced them that in situ production of carbon dioxide does result in enhanced production of oil (Janshekar, 1985).

There are few documented reports in the literature of field tests. The most thoroughly documented information has come from Eastern Europe, from work done by Karashiewicz (1974), Lazar (1976) and Lazar and Constantnence (1984). Between the years 1961 and 1971, Karashiewicz performed a number of experiments in oil deposits in Romania using bacterial cultures isolated from soil samples and deposit water. Bacterial inoculations were mixtures of Arthrobacter, Clostridium, Mycobacterium, Peptococcus and Pseudomonas using a molasses based medium. According to Karshiewicz (1974) metabolic action of these bacteria caused the decomposition of the heavier fraction of petroleum crude, with an observable decrease in the viscosity and specific gravity. It is doubtful that any strain of hydrocarbon-oxidizing bacteria would cause the decomposition of heavier crudes until the preferential lighter fractions had been utilized at the expense of the molecular oxygen required for growth. Still the by-products of the degradation of the lighter fraction of crude which are by their own right surface active compounds, could cause the loss of viscosity and lower

specific gravity observed.

The microbial activation of crude occurred primarily under favorable geological conditions (zones of higher permeability) in the formation. Microbial injection produced surpluses of crude from wells in the immediate vicinity which reached on the average, 200% or production before injection. This rate was maintained for time periods ranging from two to eight years.

Lazar and Constantnence (1984) between the years 1975 and 1981 subjected seven reservoirs to microbial treatment using an inoculum consisting of a mixture of the genera Pseudomonas, Clostridium, Mycobacterium, Bacillus, Peptococcus and the family Enteobacteriaceae, which were recovered from a number of sources.

An increase in crude oil production was obtained from these seven reservoirs for periods of 7 months to five years. Alterations in crude oil viscosity, density and volatilization were also reported.

In the United States the most documented field test using bacteria is the often-cited test carried out by Yarbrough, Updegraff and Coty in 1954, as described by Yarbrough and Coty (1982) in the Upper Cretaceous Nacotoch formation, Union County, Arkansas, using the bacterium Clostridium acetobutylicum.

The field test was begun by injecting fresh water into

the reservoir. This is a procedure that if done in a reservoir with a "clean" sand (no swelling or moving clays) could quite conceivably eliminate or partially alleviate salt problems associated with MEOR. Two months later, beet molasses was added to the injection water at a final concentration of 2% v/v. Over a six month period 18 separate 220 gallon inocula of Clostridum acetobutylicum were added to the inoculation medium.

Analysis of gas, water and oil samples from the test well showed the production of large amounts of carbon dioxide, short-chain fatty acids (formic through caproic and caprylic) with small amounts of ethanol, n-butanol and acetone. The fatty acids are not products one would expect if Clostridium acetobutylicum were solely responsible for the fermentation. The authors postulated that perhaps Clostridium kluyveri had also participated in the fermentation and was responsible for the production of the longer chain fatty acids. Clostridium kluyveri produces butyrate, caproate and hydrogen from ethanol and acetate.

Seventy days post-injection, fresh water breakthrough occurred and in 80-90 days fermentation products and sugar appeared in the produced water. One well was assumed to have been favorably affected by the bacterial fermentation. The average oil production from November, 1954, to May, 1955, was 2.1 bbl/day, 3.5 times the production rate

before inoculation.

The field data presented above and in other reports (unpublished) lead one to believe that addition of bacteria and a nutritive support to an oil well can in fact bring about an enhanced production of oil. Published reports do, however, attribute oil recovery to a specific bacterium or group of bacteria. One goal was to determine if the clostridia we isolated would be able to produce the desired metabolic and end-products downwell. Ramsey, et al., (1984) have stated: "It is well known that bacteria produce these compounds (acids, alcohols, surfactants, gases and emulsifiers), but it is not known whether they will do so under reservoir conditions of anaerobiosis, temperature, salinity and pressure".

The major problem faced when doing field tests is the lack of absolute control of a field or well that would enable controlled testing of the selectively isolated cultures. The dependence upon wells controlled by others has necessitated the utilization of reservoirs that were not optimum for one reason or another for the early experiments although it is doubtful that conditions matching the rigor or excellence of the laboratory could ever be found in the field.

At the end of the field tests, the following questions were to have been answered:

1. Can clostridial spores survive the injection process?

2. Once spores are introduced into the formation will they successfully germinate?

3. Can a viable fermentation occur in the reservoir?

4. Will injected bacteria outcompete indigenous microflora and microflora present in the injection medium as contaminants?

5. Will a process such as MEOR damage producing wells?

6. What effect will the growth of bacteria in the confines of a producing well have on oil production?

CHAPTER II

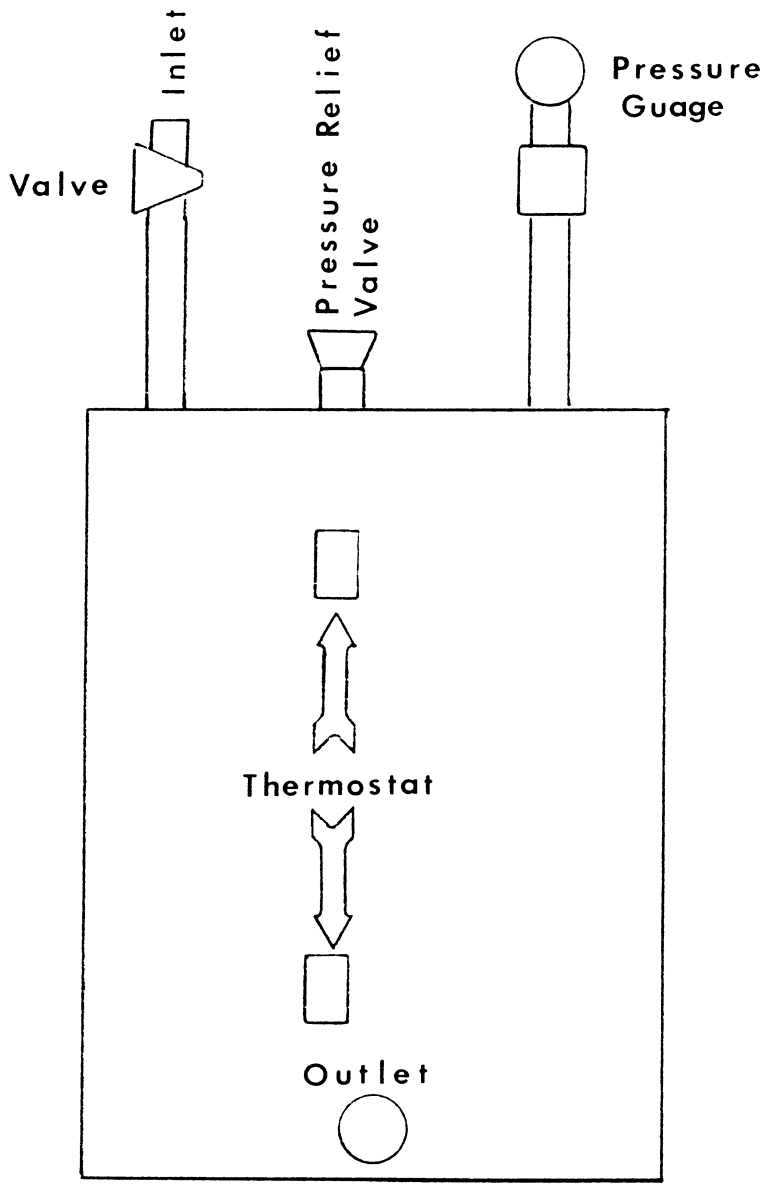
MATERIALS AND METHODS

Preparation of Spores

Spores used for well inoculation were prepared by converting electric water heaters into low-budget fermenters (Figure 32). Sterilization was accomplished by heating the enclosed medium to 80°C for one hour at which time the pressure within the jacket reached 20 psi. Initially the medium was allowed to cool overnight; later a small pump was attached to the inlet and outlets of the tank and the medium circulated, resulting in a substantial savings in time required for the medium to cool. Inoculation to scale-up were at all times stepwise and consisted of the addition of 10% v/v mid to late log cells to fresh medium. Cells were initially grown in Tryptic Soy Broth with 1% sucrose, then used (at 10% v/v) to inoculate the fermenters which contained the following: 1% sucrose, 0.1% milk solids, 0.1% ammonium nitrate and 0.05% sodium carbonate using filtered tap water. The tap water was filtered using a "water pik charcoal" (Teledyne Corporation) filter cartridge to remove chlorine and organic debris.

The temperature was controlled at 37-42°C via a

FIGURE 32. Schematic of fermentor.


Inlet
Valve
Pressure Relief Valve
Pressure Guage
Thermostat
Outlet

thermostate.

The progress of the fermentation was followed by slide observations of medium drawn from both the top and bottom of the fermenter. The fermentation was complete only when the ratio of sporulating to vegetative cells from the bottom portion of the medium was approximately 1:1, and a few or no cells from the top of the medium were visible when a loopful of medium was spread on a slide and observed microscopically. This meant that the cells which had sporulated (about 90%) had largely settled to the bottom of the tank. The time required for a complete fermentation varied, but by eleven days most were complete.

Spores were harvested, in the cases of the Bixby and Chanute Wells, after the fermentation was complete and spores had settled to the bottom of the tank by siphoning the spent medium from the top, harvesting the residual 10-15 gallons left in the tank, then washing the bottom of the tank once using filtered water. The resultant 25-30 gallons were allowed to stand until a thick layer of cells/spores appeared at the bottom. The supernatant was removed by siphoning and spore/water mixture was stored at ambient temperature. Little loss of viability was observed over a two-week period utilizing this method. Since spores were rarely kept for more than one week, loss

of viability was not a problem.

Numbers of viable spores/ml used to inoculate per well were determined by collecting a sample at the well before inoculation, returning to the laboratory and subjecting same to 80°C for twenty minutes to kill all vegetative cells. Numbers were determined by plate counts. Spore counts were determined under the assumption that only viable spores survived the heat shock.

Spores were transported to the wells in 55 gallon barrels or five gallon plastic carboys depending on the size of the inoculum. This was possible since no precautions were needed as to atmosphere around cells, temperature, etc.

Wells and Fields for Test

Bixby, Oklahoma

The well, drilled in 1981 to a depth of 1750 feet in the Dutcher formation with an initial production of 10 bbl/day had fallen to less than 1 bbl/day before the initial injection. It is a gas driven well, with no water production. Known head pressure of 40 psi was determined after a two week shut-in period. Reservoir temperature was determined to be about 37°C.

Chanute, Kansas

Field was drilled into the Bartlesville Sand at a dept of about 1000 feet. BHST (bottom hole static

temperature) of the wells was determined to be 15°C. This field had never been subjected to waterflood, but was in decline at the time of injection. After a one-week shut-in period before injection, producing wells were on a vacuum and a valid gas control sample could not be obtained.

Covington Field, Jack County, Texas

The field was producing from the Gunsight Sand at a depth of between 405 and 481 feet. At time of injection, the field was under extensive waterflood. Gas pressure within the reservoir was not detectable.

Inoculation of Well

Bixby, Oklahoma

For the initial field test, the pump on a water transport truck was used. The pressure to enter the formation efficiently could not be generated using this method and for the second inoculation a fracturing truck capable of generating pressures up to 2000 psi was employed.

Covington, Texas, Field

A portable Cat (Caterpillar) centrifugal pump was used to inoculate Covington Well Number 22. A cement truck (HT-400) was required to generate the pressure necessary to inject into Wells No. 18 and 24.

In all cases, the spores were placed in the wells before the medium in order to assure that the front of the medium contained viable spores and as such the spores would reach further into the formation than they would if mixed with the nutrient medium before injection. This meant we would always have viable spores ahead of, or at the front of the injected medium in order to assure that the entire formation filled with medium contained viable spores.

Medium for Injection

Bixby, Oklahoma, Well

Medium for the initial injection contained 8.6% cane molasses, 0.1% ammonium nitrate, 0.1% milk solids, and 0.05% sodium carbonate. The molasses had been stored for a number of years and was quite acidic, therefore, the sodium carbonate was added to maintain the pH in the usable range. Pond water was used to prepare the injection medium.

Second injection - 4% beet molasses, 0.1% ammonium nitrate and 0.1% milk solids. Produced water from an adjoining lease was mixed 1:1 with fresh water to prepare the injection medium.

Chanute, Kansas

A 4% v/v solution of a 1:1 mixture of corn syrup and cane molasses mixed with produced water from the field

and 0.1% ammonium nitrate.

Covington, Texas, Field

A 1.5% sucrose, 0.1% milk solids, 0.1% ammonium nitrate and 0.5% beet molasses was mixed with produced water for injection.

Analysis of Samples

Samples were collected at specific intervals and analyzed for the presence of metabolic end-products. This interval was generally on the order of one week, since the wells were at some distance from the laboratory. Bacterial counts were done by standard microbiological methods, using a medium of Tryptic Soy Broth plus 1% sucrose. Counts were made both aerobically and anaerobically. Sugar content was determined using anthrone.

Characterization of Bacteria Present in Oilfield Waters

Before injection a number of bacteria were isolated, subjected to biochemical tests and characterizations from the Chanute and Covington Fields. Virtually no bacteria were present in the Bixby Well. When a well (or wells) that had been treated was put back into production, colonies from the lowest countable dilution plate were again subjected to characterization to determine the origin of the major population growing as a result of nutrient addition.

Isolation of Bacteria Present In Wells Before Injection

In an effort to isolate only those bacteria associated with the wells themselves, a five gallon bucket was placed under the relief valve from the tubing. The wells were pumped until the bucket was full, and then a water sample was aseptically taken with a syringe and added to a sterile tube, and transported to the laboratory. Asepsis under the conditions described is open to debate as a result of the open environment. Counts were done by standard procedures as to viable aerobic and anaerobic colony forming units/ml. Isolated colonies were picked and streaked three times on a nutrient agar plus 1% glucose medium to purify. Purified cultures were then given a designation and subjected to various biochemical tests.

Characterization of Nutrient Contaminants

During the Covington Field Test, bacteria present in the nutrient used for well injection were also subjected to the same tests as those already present in the reservoir. This was done to determine the origin of each bacterium participating in the downwell fermentation.

Determination of Acids and Solvents

Acid and solvent analysis were made using a Sigma 2

given a designation and subjected to various biochemical tests.

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Acid and solvent analysis were made using a Sigma 2 series, Perkin-Elmer gas chromatograph equipped with a flame ionization detector and a 6' glass column with 1/4" inside diameter. Peak areas were calculated using a Hewlitt-Packard calculating integrater.

Packing for solvent analysis: 1-1820 carbopack c/o 0.5% Sp-100

For acid analysis: 60/80 carbopack c/o .3% carbowax 20M/0.1% phosphoric acid

Programs

For solvent analysis:

Oven temperature: 200°C

Injection temperature: 150°C

Initial temperature: 80°C

Initial time: 2 minutes

Initial rate: 15°C/minute

Final temperature: 120°C

Final time: 5 minutes

Final rate: 25°C/minute

Post temperature: 145°C

Post time: 0.5 minutes

Carrier gas - nitrogen 20 ml/minute

For acid analysis:

Oven temperature: 200°C

Injection temperature: 150°C

Initial temperature: 120°C

Initial time: 2 minutes

Initial rate: 15°C/minute

Final temperature: 130°C

Final rate: 15°C/minute

Post temperature: 150°C

Post time: 1.0 minutes

Carrier gas - nitrogen 35 ml/minute

Sample Preperation

To one ml of spent culture medium 0.1 ml of 50% phosphoric acid was added to acidify acid end-products. One Microliter of this sample was injected into the GC for analysis.

Standard Curve

Standard curves were obtained by injecting known

concentrations of the appropriate end-products and determining the peak areas. Plots of peak area versus concentration (mM) were obtained for all metabolic end-products. Unknown concentrations were then determined by linear regression using suitable standard curves.

Analysis

Where necessary, samples were co-chromatographed (known solvents or acids run with unkown) if a question arose as to peak identification. Internal standards (propionic acid or n-propanol) were also included if questions arose as to the validity of peak area.

CHAPTER III

RESULTS AND DISCUSSION

Bixby, Oklahoma, Well

The initial experiment, done with the 8.6% cane molasses mixture, involved pumping 80 bbls of the medium into the well followed by a thirty day shut-in period. During this period, the gas pressure at the head never rose above 10 psi.

Upon opening the well, analysis of water samples revealed the presence of solvents such as ethanol, acetone and n-butanol (data not shown) in concentrations indicating a fermentation had occurred but not sufficient to cause any migration of oil from the reservoir. Salinity measurements revealed that the sodium chloride level in the returned water to be on the order of 5%; unfortunately this development had not been anticipated (since the pond water was fresh). The quality of the cane molasses and the presence of 5% sodium chloride would cloud any results that might have been obtained from this experiment. It was also determined from the low head pressure (10 pounds) and the lack of production from the well that the fresh water had essentially shut the reservoir off.

Analysis of core material (obtained two weeks after the initial injection, from a new well drilled in the field), show that 20-30% of the reservoir rock contained a mixed layer clay (a swelling clay) with lesser amounts of chlorite, illite and kaolinte which are known to migrate as fresh water removes or dilutes the cationic cloud from these clays. This fact might also explain the presence of sodium chloride in the returned water.

A second experiment was therefore planned using 4% beet molasses, 0.1% ammonium nitrate and 0.1% milk solids. From previous experiments (Part One) it was known that beet molasses provided a better base for growth in high salt than cane molasses. Water from a nearby waterdriven well, diluted 1 to 4 with fresh water, (resultant salinity of 3% as sodium chloride) was used to prepare the medium, in order to introduce some salt into the formation. As a result, there should be no migration or swelling of clays.

Two cultures, HR-51B and HR-69E were used in the inoculation. They were chosen for their ability to produce large amounts of carbon dioxide, high solvent production and the ability of HR-69E to produce a bioemulsifier. Both were also capable of growing at a NaCl concentration of 860 mM. Table XX contrasts the solvent and acid production of HR-69E and 51B in the medium designed for the

test with HR-69E alone, HR-66E (another emulsified producing culture) and Control (uninoculated medium). It is apparent that the co-inoculation of these two cultures provided the best ratio of fermentation products capable of enhancing oil production. The control growth has since been attributed to a Clostridum, most likely present as spores in the beet molasses itself.

Twenty liters of a spore preparation containing 2.6 x 10^{12} spores per ml (as determined by plate counts) were injected into the casing followed by 40 liters of medium. One hundred sixty additional barrels of medium were then injected into the casing as depicted in Table XXI. The well was then shut-in and gas pressure was monitored for thirty days at the head with a liquid filled gauge. When possible liquid samples were removed from the tubing and analyzed. This was done to follow the course of the fermentation, although any fermentation in the tubing would be quite different from that in the formation proper, but it was the only measurement possible.

Table XXII shows the gas pressure as measured at the head and the percentage of carbon dioxide and methane at each sampling. Initial gas composition before the first inoculation was 98% methane with a trace of carbon dioxide. From the percentage of carbon dioxide and increased pressure at the head, it is quite evident that biogenic gas

TABLE XX

SOLVENT AND ACID PRODUCTION (mM) BY CULTURES IN 4% BEET MOLASSES, 0.1% AMMONIUM NITRATE, 0.1% MILK SOLIDS WITH DUTCHER SALT WATER DILUTED 1/4 WITH FRESH WATER

Culture	Ethanol	Acetone	Butanol	Acetate
69E & 51B	6.4	3.3	20.8	1.4
69E	2.6	-0-	1.9	0.2
66E	2.6	-0-	2.3	0.1
Control	2.8	-0-	4.8	0.4

TABLE XXI

PRESSURES USED AND RATES OF MEDIUM INJECTION AT A WELL NEAR BIXBY, OKLAHOMA

Medium Added	Flow Rate (bbl/min)	Pressure (psi)
8 bbls	3.0	0
18 bbls	1.5	50
160 bbls	1.5	400

TABLE XXII

RESERVOIR GAS PRESSURE MEASURED AT THE HEAD AND COMPOSITION OF GASES FROM THE INOCULATED WELL NEAR BIXBY, OKLAHOMA

Post-Inoculation (Days)	Pressure (psi)	Gases (%) CO	CH
0	40	T	98
7	55	2.0	44
14	100	4.0	37
21	15	5.0	22
30	80	T	34
32	0	T	28

either contributed to the free gas phase of the well or microbial action had opened the near wellbore, by removing fines (minute grains of clay or sand) and/or paraffinic deposits that had accumulated in the pore throats. Increased gas pressure (2 1/2 times) and a higher percentage of carbon dioxide in the free gas phase when compared to samples taken before injection suggest both did occur.

Two weeks post injection, a liquid sample was removed from the tubing and analyzed. The pH of the medium had fallen from 6.5 to 5.5, and the presence of ethanol and butanol was demonstrated. This confirmed that bacterial action was indeed occurring in the casing and not just in the wellbore.

Table XXIII shows the various solvents present in liquid samples taken on opening day (30 days post-injection) with pH and plate counts. Determination of the acids present in fluid samples, by gas chromatography show that acetic and butyric acids were produced. From this Table it is apparent that bacterial growth has occurred with the production of a number of low molecular weight alcohols and solvents. Acid production consequently lowered the pH of the produced fluid to 4, a value we know from laboratory data causes a cessation of growth.

When first put back into production, the well produced only spent medium, with a light skin of oil. Two days post-

TABLE XXIII

SOLVENTS DETECTED (mM), BACTERIA/ml AND pH OF SPENT MEDIUM SAMPLES FROM THE BIXBY WELL ON OPENING DAY COLLECTED AT VARIOUS PUMPING TIMES

Time Post-Opening (hrs:min)	pH	Ethanol	Acetone	Isoprop.	Prop.	But.	Bacteria per ml.
0:00	4.00	19.8	23.1	2.5	3.0	4.1	1.86×10^8
1:46	4.00	16.2	-0-	2.4	2.4	2.4	1.78×10^7
2:20	4.00	16.5	2.0	-0-	-0-	1.8	1.67×10^7
4:45	4.00	14.2	-0-	-0-	-0-	1.8	8.90×10^4

Abbreviations used: Isoprop.= Isopropanol (2-propanol) Prop.= n-Propanol
But.= n-Butanol

opening, the oil/water ratio began to increase and within a 20 minute time frame some 4 bbls of oil/spent medium were literally blown into the holding tank. Samples taken on this day had a pH of 4, with anaerobic bacterial counts of 1×10^7 cfu per ml and good solvent production.

Liquid samples were taken and analyzed for counts of viable bacteria, pH, solvent and acid production as well as residual sugar at 2 and 3 weeks post-opening (Table XXIV). These samples came from the formation (near wellbore) since the well had at numberous times been pumped off (all liquid pumped from the bore hole). Anthrone assays of returned injection medium showed residual sugar at 3000 µg/ml, some 2% of the original concentration of fermentable sugar.

Unfortunately in early December, the lease was sold and for a variety of reasons the field was not kept in production for a number of months. During the period of opening and final shut-down the well produced two barrels of oil per day for the approximately 35 days that the well remained in production, a rate of two times the production before microbial treatment. Interestingly, after three weeks of production, very little molasses was returned, in fact it became impossible to collect aqueous samples. Efforts to remove water from the oil by gasoline or kerosene extraction were futile.

TABLE XXIV

SOLVENTS (mM) AND BACTERIA/ml FROM THE BIXBY WELL AT ONE AND TWO WEEKS POST-OPENING

	Ethanol	n-Propanol	Isobutanol	Butanol	Bacterial Counts
One Week Post-Opening	12.3	3.8	-0-	8.6	8.9×10^6
Two Weeks Post-Opening	14.3	2.7	8.1	2.5	1.6×10^7

As to whether or not the production from this well was enhanced, a clear lack of data makes any definite conclusion almost impossible. Since thirty days of production (at previous rate of 1 bbl/day) were missed, it is reasonable to assume that 30 bbls are "made-up" oil, and do not represent enhanced recovery. Of the remaining 40 barrels produced over a thirty-five day period, 35 would have been produced anyway and the remaining 5 are the result of enhancement. It must be stressed though, that the question of "made-up" oil clouds the issue and we have no way to determine that the well would have indeed "made-up" the oil that would have been produced during the shut-in period. The answer of how well the treatment worked from the standpoint of oil production could only have been answered by continued monitoring of the production.

From this test we were able to learn a number of things. Viable spores can be injected into a reservoir and these spores upon germination and outgrowth can produce metabolic end-products within the formation itself. The proportions of solvents present are not explainable by laboratory data or any known pathway, and it is not known how pressure or the growth of bacteria in the confines of a formation affect solvent production. Attempts to "wash" solvents or acids from oil produced by this well prior to inoculation were unsuccessful, and therefore, the detected

acids and solvents were no doubt biogenic in nature.

Bacteria re-isolated from this well were anaerobic in nature, and as one would expect, bacteria other than those injected participated in the fermentation. The majority of the population (based on biochemical tests, colony morphology and cell morphology) was thought to be strain HR-69E and HR-51B as bacteria resembling these two cultures were readily isolated from the last dilution plates. Other notable bacteria, were a Gram-positive facultative coccus, a Bacillus and at least one Clostridium.

The large increase in gas pressure at the head is probably not due solely to biogenic gas production, since the pressure appeared to level off at 80 psi (after reaching a peak of 115 psi then dropping suddenly) and quite possibly could be related to the clean-out of the wellbore itself, or merely repressurization of the near wellbore. Removal of fines, paraffin or other deleterious entities from the near wellbore and the subsequent opening thereof, could account for some increased pressure. Bacterial growth in and around the wellbore and resultant metabolic end-products could conceivably produce such an effect. Removal of fines and paraffinic deposits that have accumulated in the pore throats over years of production would increase the effective permeability and as a

result change the capillary pressure and increase the production of oil.

It is also quite possible that this thirty day shut-in period allowed sufficient time for the reservoir to repressurize the wellbore area. The initial shut-in period (two weeks) may not have been of sufficient time to allow equilibration between the reservoir and wellbore.

Still there is evidence that a large amount of gas was produced by the fermentation. Five percent of the gas was carbon dioxide (control sample 0%). Several liquid samples were placed in evacuated serum bottles and vortexed for an extended period of time (to enduce release of the gas from the liquid) and the headspace analyzed. Gas evolved from these samples (oil/spent molasses and spent molasses) was entirely carbon dioxide. This gas, undoubtedly biogenic in nature, by forming a gas in oil emulsion would indeed facilitate the movement of oil from the reservoir.

We were unable to measure hydrogen production because the gas chromatograph was equipped with a flame ionization detector. Therefore no comments can be made as to the presence or absence of this gas.

Well near Chanute, Kansas

A field near Chanute, Kansas, was made available for

a MEOR test. The field was drilled into the Bartlesville Sand at a depth of about 1000 feet. The BHST (bottom hole static temperature) of the well was approximately 15°C, which was far below the lowest temperature the mesophilic isolates could be expected to face in a reservoir. Still the salinity was low (3% as NaCl) and a few of our isolates were able to produce gas, acids and solvents in a corn syrup-cane molasses medium when incubated at 15°C. Corn syrup-cane molasses was used because it was available locally and the differences between fermentation in beet molasses and corn syrup-cane molasses in the produced water at 15°C were minimal.

Figure 33 is a map of the field. Well No. 3 was chosen for inoculation, with the hope that the effects of the fermentation as reported in Eastern Europe might be seen at the surrounding wells (Karajweiz, 1972).

Plate counts taken from Wells 2, 3, 7 and 8 before inoculation are shown in Table XXV. A number of bacteria were isolated and characterized from the well destined for injection, and from surrounding wells. Acids and solvents were not detected in liquid samples taken from Wells 2, 3, 6 and 8 before inoculation.

On June 2, 1984, Well No. 3 was inoculated with 20 liters of a 3.83×10^{14} spores/ml, followed by the introduction of 160 short/bbls of a 4% corn syrup-cane molasses

and 0.1% ammonium nitrate medium. During the injection of medium the pressure at the head never exceeded 100 psi with a rate of 4 bbls/min. A sample of molasses medium was taken, added to a nitrogen-flushed Hungate tube, inoculated with the spore preparation, transported to the laboratory and incubated at 15°C for three weeks. Results of this incubation appear in Table XXVI. After three weeks of incubation there was little conversion of acids to solvents, and no real shift from acidogenic to solventogenic fermentation. This lack of conversion from an acid type fermentation to solvent was thought to be a result of the low temperature.

On July 10, 1984, the well was circulated for twenty-four hours by pumping from the casing. Samples were then removed and subjected to analysis (Table XXVII). Plate counts showed viable anaerobic bacteria at 1.7×10^7 cfu per ml and residual sugar at 333 µg/ml. Counts were also performed on samples from surrounding wells (Table XXVIII). These samples were also tested for sugar as well as solvents and acids. No detectable sugar, acids or solvents were present in the samples from surrounding wells. The increased numbers of cells (from the surrounding wells) are most likely explained by the increase in ambient temperature between the first counts and those taken later. Still it is quite evident that fermentation was occurring in

FIGURE 33. Contour map of the Kansas field.

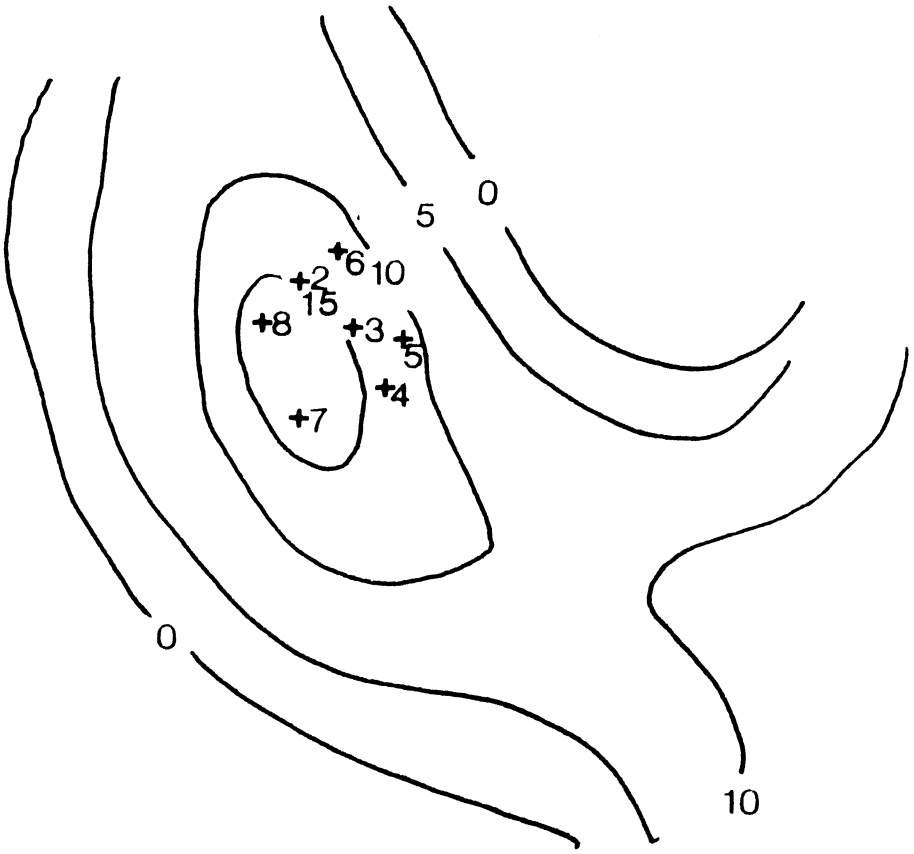

0
5
+6
10
+2
15
+8
+3
5
+4
+7
0
10

TABLE XXV

AEROBIC COUNTS AS CFU PER ml OF VARIOUS WELLS IN THE KANSAS FIELD DONE NOVEMBER 28, 1983 BEFORE INOCULATION

Well Number	Bacteria (ml)
2	1.6×10^2
3	1.4×10^2
8	1.2×10^2
7	0.9×10^2

TABLE XXVI

FERMENTATION RESULTS ACHIEVED IN THE LABORATORY USING THE INJECTION MEDIUM AND INOCULATION BACTERIA. INCUBATED 3 WEEKS AT 15°C IN NITROGEN FLUSHED HUNGATE TUBES

Acid		Solvent	
Acetic-	2.7 mM	Ethanol-	18.0 mM
Proprionic-	2.1 mM	Isopropanol-	1.9 mM
Isobutyric-	0.7 mM	Isobutanol-	1.5 mM
n-Butyric-	4.9 mM	n-Butanol-	Trace
Gas-	20,000 mM		

TABLE XXVII

ACID AND SOLVENT DATA COLLECTED FROM THE KANSAS WELL JULY 10, 1984

Acid (mM)		Solvent (mM)	
Acetic-	1.9 mM	Ethanol-	11.8 mM
Proprionic-	0.8 mM	Isopropanol-	4.5 mM
Isobutyric-	0.8 mM	Isobutanol-	1.5 mM
n-Butyric-	1.5 mM	n-Butanol-	2.9 mM
		Acetone-	2.6 mM

TABLE XXVIII

COUNTS FROM WELLS SURROUNDING THE INJECTION WELL IN THE KANSAS FIELD TAKEN JULY 10, 1984

Well Number	Bacterial/ml
2	1.8×10^4
4	1.6×10^4
7	1.5×10^4
8	2.4×10^4
5	10

Well No. 3 and that it mirrored the fermentation by the injection bacterium HR-3 in the laboratory.

Gas pressure at the head never rose above five pounds during the initial inoculation period (46 days), although the percentage of carbon dioxide in gas samples was high (19%). Before inoculation the formation was so devoid of pressure as to be on a continous vacuum, and the collection of a valid control sample was impossible; therefore an increase of 5 pounds pressure was thought to be substantial.

On July 18, 1984, samples were taken from the surrounding wells and Well No. 3 was refed an additional 160 bbls of 4% corn syrup-cane molasses. Table XXIX shows the increase of gas pressure with time after refeeding. The percentage of carbon dioxide remained high, the methane remained low, indicating that the gas pressure at the head was due solely to biogenic gas production. Although a higher pressure at the head would have been desirable (perhaps the lower temperature of the formation contributed to a lower production of gas), it still takes quite a bit of gas to raise the head pressure around a well five or ten pounds. Solvent data from surrounding wells (Table XXX) were encouraging and it was thought that perhaps breakthrough had been achieved. However no samples taken from the surrounding wells after one week after refeeding showed detectable amounts of solvents or acids.

TABLE XXIX

GAS PRESSURE MEASURED AT THE HEAD OF THE INJECTED WELL NEAR CHANUTE, KANSAS AFTER REFEEDING

Date	Pressure (psi)
August 3, 1984	4.0
August 5, 1984	6.5
August 7, 1984	7.5
August 22, 1984	10.0

TABLE XXX

SOLVENTS (mM) DETECTED AT WELLS SURROUNDING THE INJECTED WELL IN THE KANSAS FIELD

Well No.	Date	Ethanol	n-Butanol	n-Amyl
6	7-18-84	2.4	1.5	2.0
	7-25-84	Trace	Trace	Trace
7	7-18-84	2.2	-0-	-0-
	7-25-84	Trace	-0-	-0-
4	7-18-84	2.3	-0-	-0-
	7-25-84	-0-	-0-	-0-
8	7-18-84	2.3	-0-	-0-
	7-25-84	-0-	-0-	-0-

On August 23, 1984, Well No. 3 was opened and placed in production. Solvent and acid data from liquid samples were similar to those found earlier and plate counts ranged from 7.8×10^6 to 1.08×10^7 cfu per ml.

From the data presented, this experiment can be termed a technical success. Fermentations below ground occurred as they had in the laboratory under similar conditions of temperature and medium used. Culture HR-3 was reisolated in large numbers from Well No. 3 (as well as a Bacillus that had previously been isolated and characterized). HR-3 was not, however, isolated from any surrounding wells. The increased cell counts in surrounding wells after inoculation were attributed to the two Bacillus spp. isolated and characterized from Well No. 2 before injection. There is no evidence that increased numbers of this bacterium resulted from movement of injection medium from Well No. 3.

There was no damage to the well due to microbial action or plugging as a result of injecting large numbers of spores. The face of the well-bore was not affected by plugging, as indicated by the low pressures required to inject the refeed medium and no loss of production when the well was returned on-line. Unfortunately no increase in oil production was detected in Well No. 3 or in the surrounding wells. It must be stressed though that no product or procedure to increase oil production in a well

is 100% effective. Had the formation temperature of this well been 20°C higher, the increased production of metabolic end-products might have led to an increased migration of oil from the reservoir if sufficient oil remained in the ground to be released. In retrospect, from the standpoint of field testing, this well should not have been chosen for experimentation because of the low formation temperature detected during the initial screening.

Covington, Texas, Field

A final field test was carried out in a field in northern Texas, drilled into the Gunsight sand formation at a depth of between 405 and 481 feet. Pertinent well information is shown in Tables XXXI and XXXII. All wells were one to two years old cased completions, without packers and under extensive waterflood.

The initial protocol of the test is summarized in Table XXIII, utilizing three wells on an adjacent lease into the same formation as control wells. The known fracture pressure of the reservoir (350 lbs.) would not be used during injection of either nutrients or spores. The three Covington wells were to be inoculated with spores and nutrients, and the three Ellison wells were used as controls. For controls, one well (Ellison One) would be injected with nutrients only, to determine how the

TABLE XXXI

WELL DATA OF THE THREE COVINGTON WELLS TO BE INOCULATED

Well No.	Depth (ft)	Perforated Zone (ft)	Pay Zone (ft)
18	442	6.5	18
22	405	5.5	16
24	481	9.0	12

TABLE XXXII

WATER ANALYSIS OF BOTH PRODUCED AND INJECTION WATER FROM COVINGTON WELLS

Injection Water Analysis

Specific Gravity	=	0.990	
pH	=	8.3	
Resistivity	=	0.003	ohms/m /m
Temperature	=	24°C	

Ion	ppm
HCO	529
Cl	1200
SO	219
Ca	109
Mg	265
Fe	10
Na	475
Total Dissolved Solids	2820

Produced Water Analysis

Specific Gravity	=	0.992	
pH	=	8.2	
Resistivity	=	0.002	ohms/m /m
Temperature	=	24°C	

Ion	ppm
HCO	783
Cl	2200
Ca	100
Mg	133
Fe	15
Na	1360
Total Dissolved Solids	4610

TABLE XXXIII

INITIAL PROTOCOL OF FIELD TEST IN COVINGTON FIELD

Well	Treatment
Covington 18	Injected spores and nutrients
Covington 22	Injected spores and nutrients
Covington 24	Injected spores and nutrients
Ellison 1	Injected nutrients
Ellison 2	Control Well
Ellison 4	Control Well

indigenous bacteria or bacteria introduced with the nutrients would affect oil movement from a reservoir as compared to the injection of the Clostridium species we planned to use. The other control wells (Ellison 2 and 4) were to be shut-in, but not treated, to determine how the shut-in period would affect oil production. We would then have, while not a perfect control, one suitable for addressing the "made-up" oil portion of the enhanced oil equation. It was thought that the use of this protocol would answer a number of questions:

1. How would the indigenous bacteria of the reservoir react upon nutrient injection.

2. Would they indeed upon nutrient stimulation grow with a concomitant release of oil.

3. What effect would shutting in wells, for an extended period of time have on the oil production, once they were put back in production? These would be controls for made-up oil.

By staying below the fracture pressure of the formation, all changes in oil production could be regarded as a response to microbial action.

All wells were to have been previously fractured (unfortunately this proved not to be the case).

The Clostridium chosen for this test was isolate SEG-3, detailed in Section II (core work). The low bottom hole static temperature of the wells (20° C) although within the

growth range or SEG-3 was lower than the optimum of 30-37° C. A treatment procedure was then designed to heat the nutrient medium upon injection to a temperature of about 30°C. While there was no doubt that the temperature of the medium would soon equal that of the reservoir, it was thought that for that time period before equilibration the culture would gain somewhat of an advantage over an unheated injection.

Prior to treatment samples were collected and bacterial counts obtained. Table XXXIV shows the counts from the three wells to be inoculated. Bacteria for the most part were similar to the pink-pigmented facultative methyltrophs described by MacManus, et al., (1983).

On March 7, 1985, the field test began. Figure 34 shows the basic schematic which was to be used for well inoculation. Field data suggested that a pressure of 350 psi would fracture the formation, and therefore it was felt that pressures should never exceed this value, and since operator indicated that the wells had already been fractured there would be no need to exceed working pressures of 400-500 psi. To this end a cat pump capable of a working pressure of 750 psi with the adjustable pressure bypass set at 300 lbs. was to be used for well inoculation. This meant that if a pressure over 300 lbs. was reached, the

TABLE XXXIV

BACTERIAL COUNTS FROM COVINGTON WELLS
BEFORE INJECTION

Well	Aerobic Bacteria/ml	Aerobic Bacteria/ml
Covington 18	5.9×10^4	4.5×10^4
Covington 22	1.3×10^4	2.0×10^4
Covington 24	3.0×10^4	5.3×10^4

TABLE XXXV

SPORE PREPARATIONS USED TO INOCULATE COVINGTON WELLS

Well	Volume Injected	Spores/ml	Date Prepared
Covington 18	210 liters	3.1×10^{14}	2-28 / 3- 4-1985
Covington 22	210 liters	2.3×10^{15}	2-22 / 2-26-1985
Covington 24	157 liters	1.0×10^{16}	3- 4 / 3- 7-1985

medium was by-passed from the tubing connected to the well, back to the medium storage tank. Between the pump and mixing tanks (Figure 34) and the wells, schedule 80 PVC (plastic pipe rated to 600 psi) pipe was laid with which to inject the medium into the wells.

Table XXXV gives the pertinent data on the inoculum used for each well. The first well to be inoculated was Covington 22. The treatment log is shown in Table XXXVI. Temperature of the medium entering at the head was approximately 32^{o} C. Counts taken from the medium tank gave aerobic numbers of 2.35×10^{6} bacteria per ml. Samples taken after the medium traversed both the filter and pump gave aerobic counts of 4.5×10^{4} and anaerobic counts of 1.0×10^{4}.

Treatment of Covington Well No. 18 was begun on March 9, 1985 using the treatments as described in Table XXXV. Unfortunately repeated failures of the schedule 80 pipe (normally where collars were used to join two pieces of pipe) dictated that 2" steel pipe be laid to Covington 18 and a HT-400 ceement truck holding the maximum pressure at 600 pounds be used for injection. Table XXXVII shows the treatment log for this well.

Immediately after treatment of Well No. 18, the treatment of Covington Well No. 24 was begun, using the HT-400 truck. At this time we were informed that in fact

TABLE XXXVI

TREATMENT LOG COVINGTON 22

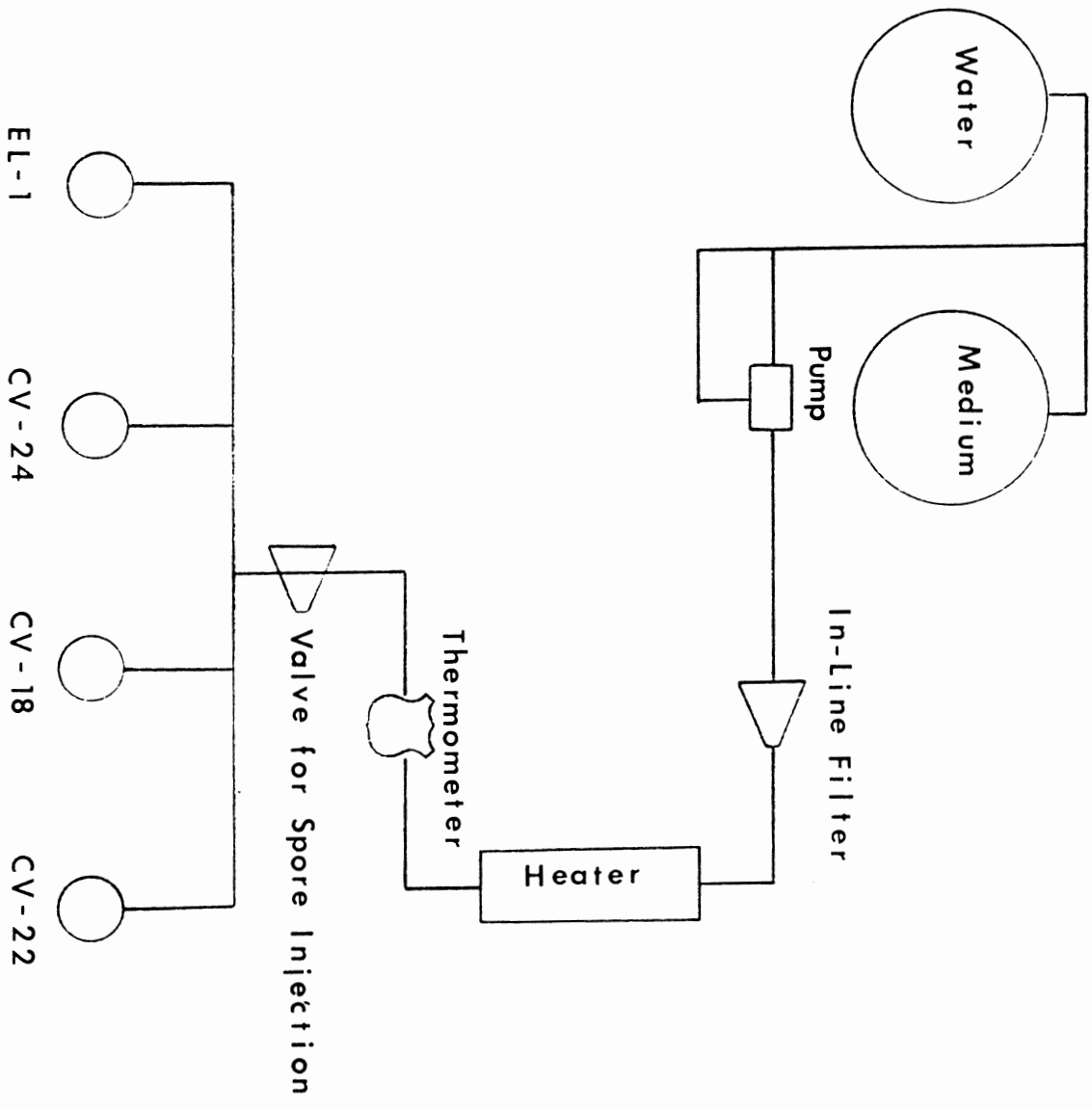
Time (hr:min)	Rate (gpm)	Pressure (psi)	Total Vol. Injected (gal)
Begin H_2O Spacer			
:00	12.1	14	60
:05	12	17.5	180
:10	12	27.5	238
:15	11.7	62.5	238
:20	10.9	185	292
:25	10.0	205	342
:30	10.1	240	392
:40	10.5	250	497
Begin Injecting Bacterial Spores			
1:00	10.4	250	706
1:10	10.6	248	812
1:30	10.6	248	1024
1:50	10.8	248	1240
2:20	10.7	245	1561
Begin Nutrients			
2:30	10.2	245	1663
3:00	11.3	250	2002
3:30	11.6	253	2350
4:30	12.2	255	3082
4:55	12.3	255	3390
End of Spores			
6:00	12.4	253	4196
8:10	12.0	250	5756
8:20	12.0	225	5876
8:30	12.2	250	5998
9:00	12.2	258	6358
10:55	12.2	155	7761
End of Spacer			

TABLE XXXVII

TREATMENT LOG COVINGTON WELL NO. 18

Time (hr:min)	Rate (gpm)	Pressure (psi)	Total Vol. Injected (gal)
Begin H O Spacer			
0	12.5	15	62.5
5	12.5	42.5	188
10	12.5	70	188
12	12.5	92	212
40	12.5	92	562
Start Bacterial Spores			
45	.86	330	566
100	.86	335	579
PVC Pipe Failures-Used 2" tubing and began pumping w/HR-400 psi at 600			
1:00	5	600	
1:10	10	600	679
1:15	12	600	739
1:25	15	600	889
Begin 7 bbls of spore suspension followed by H O Spacer			
1:30	20	600	989
1:35	32	600	1149
1:40	42	600	1359
1:50	52	600	1879
1:55	55	600	2154
Begin Nutrients			
2:00	80	600	2429
2:05	84	600	2704
:05	90	600	6004
End of Nutrients - Begin H O Spacer			
3:08	10	25	6034
3:10	0.9	350	6036
3:15	2.3	3	6048
3:20	2.7	3	6062
3:25	2.7	390	6075
3:30	3.1	405	6091
3:35	3.1	400	6107
3:40	3.3	396	6124
3:45	3.4	390	6141

FIGURE 34. Schematic and outlay of equipment used to treat Covington wells.


Water
Medium
Pump
In-Line Filter
Heater
Thermometer
Valve for Spore Injection
EL-1
CV-24
CV-18
CV-22

Covington Well No. 24 had not been fractured. Subsequent injection pressures of 1200 psi required to enter the formation confirmed this fact. Table XXXVIII gives the treatment log for Well No. 24.

Use of the HT-400 truck to enter Covington Wells 18 and 24 meant that the apparatus used to heat the medium before injection could not be used. Therefore, the temperature of the medium upon injection was approximately 25^{o}C.

Plans were made on March 10, 1985, to inject medium into Ellison Well No. 1 using the original equipment design (Figure 34). No spores were to be used in this well. This was to be the medium control well to determine how the indigenous bacteria or bacteria introduced into the formation via the medium would affect oil production. Attempts to introduce medium into Ellison No. 1 using the CAT pump were futile and after repeated attempts to do so were discontinued. No doubt this was the result of the well never having been fractured even though the operator had assured us that all wells had been previously fractured. The medium designed for this well (at 32^{o}C) was therefore instead diverted to Covington Well No. 24. Therefore, the protocol was changed somewhat in that we had no medium, only control well, and one of the three test wells (No. 24) received twice the medium as the other two test

TABLE XXXVIII

TREATMENT LOG COVINGTON WELL NO. 24

Time (hr:min)	Rate (gpm)	Pressure (psi)	Total Vol. Injected (gal)
Begin H O Spacer			
0:00	40	100	
0:03	8	700	129
0:05	5	990	141
0:07	7	1000	153
0:09	8	1200	166
0:23	50	800	280
Start Bacterial Spores			
0:30	53	650	630
Begin Nutrients			
0:33	84	650	880
0:45	84	590	1830
0:49	84	580	2130
0:54	84	560	2532
Begin H O Overdisplacement			
0:59	84	450	2932

wells. It was also decided, that Covington Well No. 24 would be used to monitor the fermentation in all three wells and therefore no spacer was used to chase the medium from the casing.

Table XXXIX relates the plate counts from Covington Well No. 24 approximately two months after the injection. The well was pumped over a six hour period. Samples were taken for counting numbers and types of bacteria present. This was an attempt to determine just where any active bacteria (other than the culture injected) might have arisen since we had previously isolated and partially characterized bacteria from the wells themselves, injection medium etc. The progression of different types of cells as the well was being pumped from the first hour to the sixth was quite impressive (Table XXXIX).

A microscopic examination revealed that as time progressed, more and more Clostridium cell types and fewer of the various other morphological groups appeared. Of the other four major groups of cultured bacteria, the yellow pigmented Gram-positive rod and the Gram-negative rod, were present in the medium used for injection. The white Gram-positive bacterium (Table XXXIX) was isolated from some of the wells prior to inoculation. As to the unknown Clostridium, there is no direct evidence as to it's origin. It's lavender pigment made differentiation from

TABLE XXIX

BACTERIAL COUNTS FROM TEST WELL NO. 24

Time Pumping (hrs)	Bacteria/ml x 10^6	Description
1	0.2	SEG-3
	0.5	Gram positive (yellow) facultative anaerobe
	2.0	Gram negative facultative anaerobe
2	0.01	SEG-3
	0.04	Gram positive facultative anaerobe (white)
		Gram negative facultative anaerobe
4	0.5	SEG-3
	0.03	Gram positive facultative anaerobe (white)
6	20.0	SEG-3
	0.02	Clostridium unknown
	0.8	Gram positive facultative anaerobe (white)

SEG-3 quite easy, but it had not been detected previously in the injection medium or reservoir.

Due to the extreme low pressure in the reservoir, no pre-injection gas samples for controls were available. A slight increase in pressure was detected on the Ellison control wells. Data from the test wells are shown in Table XL.

Table XLI shows a representative gas analysis from the wells after treatment. The high percentage of carbon dioxide and hydrogen are good indicators of biogenic gas production (gas analysis done courtesy of Halliburton Services).

Acid and solvent data in Table XLII reveals the fermentation pattern of Covington Wells No. 22 and 24. No data (acid, solvent and cell counts) were obtained from Covington Well No. 18 which was a result of mechanical failure of the pump unrelated to microbial injection. The pump on the well should have been pulled before treatment, and was to have been pulled as soon as the wells were put back into production.

On June 7, 1985, the Covington wells were put back into production. Through a misunderstanding of some sort the Ellison wells had been placed back on production in mid-May. The Ellison wells were to have been shut-in until it was decided to bring the Covington wells on-line.

TABLE XL

MEOR PRODUCED GAS PRESSURES (PSI)

Well No.	18	22	24
Date			
4- 3-85	Vacuum	Vacuum	34
4-11-85	Vacuum	Vacuum	6
4-17-85	Vacuum	Vacuum	7
5-20-85	0.5	3	8
6- 3-85	0.5	3	8
Wells Retruned to Production			
6- 7-85	-0-	-0-	3
6-25-85	-0-	-0-	3

TABLE XLI

REPRESENTATIVE GAS ANALYSIS OF BIOLOGICALLY PRODUCED GAS FROM THE COVINGTON FIELD

Gas	% By Volume
Propane	0.12
Isobutane	0.42
Butane	0.86
Isopentane	1.06
Carbon Dioxide	57.94
Ethane	10.28
Methane	1.59
Hydrogen	24.00

TABLE XLII

REPRESENTATIVE ACID AND SOLVENT DATA (mM) FROM THE COVINGTON FIELD

Well No.	Acetone	Ethanol	Butanol	Acetic	Propionic	Butyric
24	1.3	1.4	1.2	43.2	43.9	38.9
22	0.3	10.8	2.1	44.8	27.7	33.1

Figures 35 and 36 give the total production (bbls/day) from both the Covington and Ellison leases. As expected, both leases show an initial jump in production after opening. A narrower, but taller peak occurred with the Covington lease. These data show total fluid produced per day, not bbls of oil, oil cuts (WOR ratio) were taken from each of the wells seperately.

Figure 37 shows the oil production from the inoculated wells, Covington No. 22 and 24 (production data from 18 were essentially useless, because of mechanical problems that some parties failed to remedy) and the shut-in control Ellison Well No. 2. From Covington Well No. 22 there was a gradual increase in oil cut (percentage of oil produced compared to water) in the first few days of production, but a decrease in Covington Well No. 24 and Ellison Well No. 2 over the same period.

Figure 38 shows the oil cut from the two treated wells (Covington 22 and 24) and two control wells, Ellison 2 and 4. Again a positive response from Covington Well No. 22 and a positive initial slope (albeit small) from Covington Well No. 24. Neither control well had a positive slope with respect to production. One would be hard pressed to attribute a major effect on oil production as a result of microbial treatment. The treated wells do however show a positive slope with respect to oil cut and production

FIGURE 35. Total fluid production (bbls/day) Covington lease. Experiment began day 60. Base line production day 0-day 60. Wells reopened day 150.

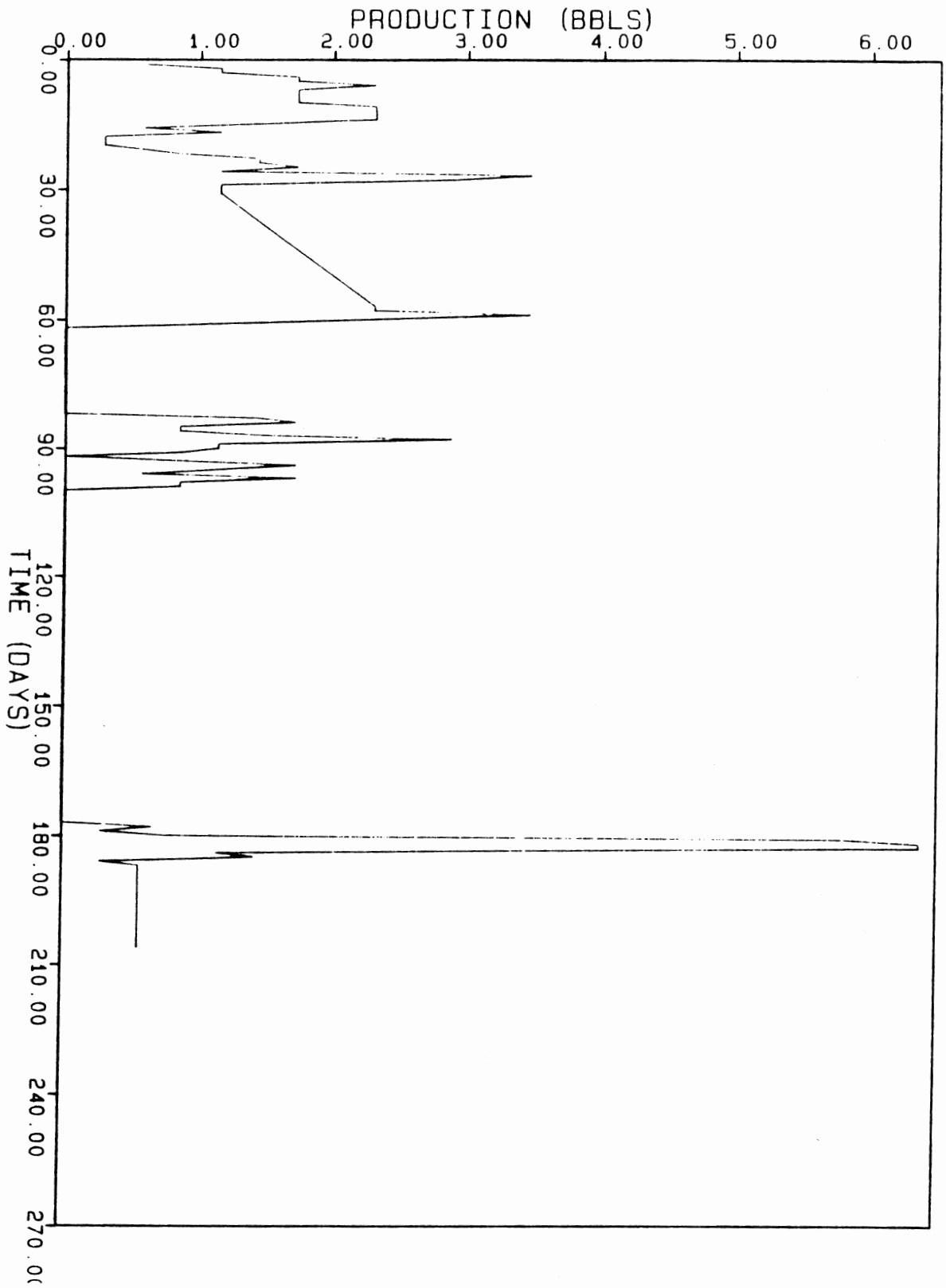

PRODUCTION (BBLS)
0.00
1.00
2.00
3.00
4.00
5.00
6.00
TIME (DAYS)
0.00
30.00
60.00
90.00
120.00
150.00
180.00
210.00
240.00
270.00

FIGURE 36. Total fluid production (bbls/day) Ellison lease. Experiment began day 60. Base line production day 0-day 60. Wells mistakenly reopened around day 90.

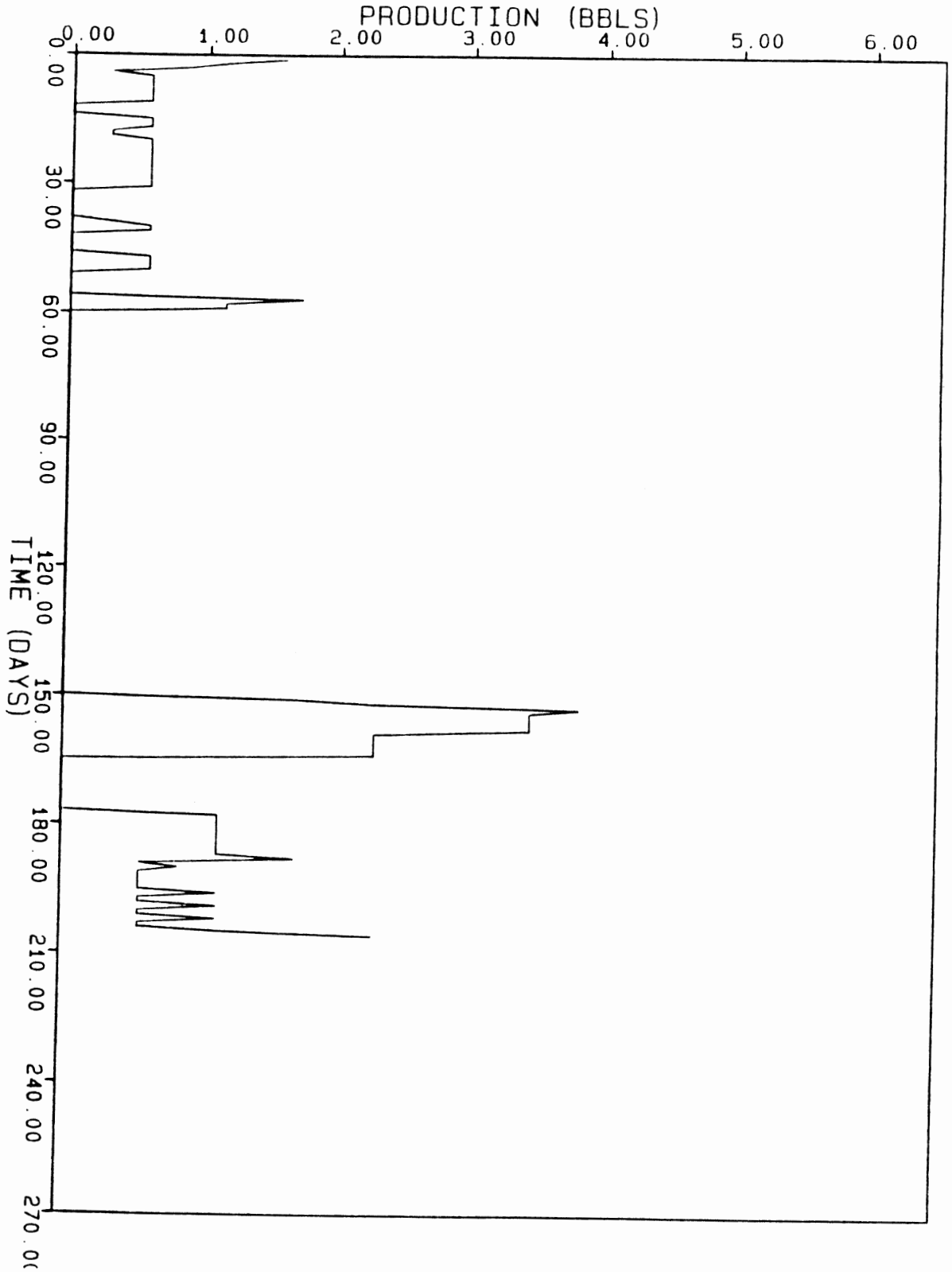

PRODUCTION (BBLS)
0.00
1.00
2.00
3.00
4.00
5.00
6.00
TIME (DAYS)
0.00
30.00
60.00
90.00
120.00
150.00
180.00
210.00
240.00
270.00

FIGURE 37. Oil production (gallons/day) treated (Covington wells) and control (Ellison wells) wells after inoculation. Open Triangles - Ellison 2. Closed Triangles - Ellison 4. Open Circles - Covington 22. Open Squares - Covington 24.

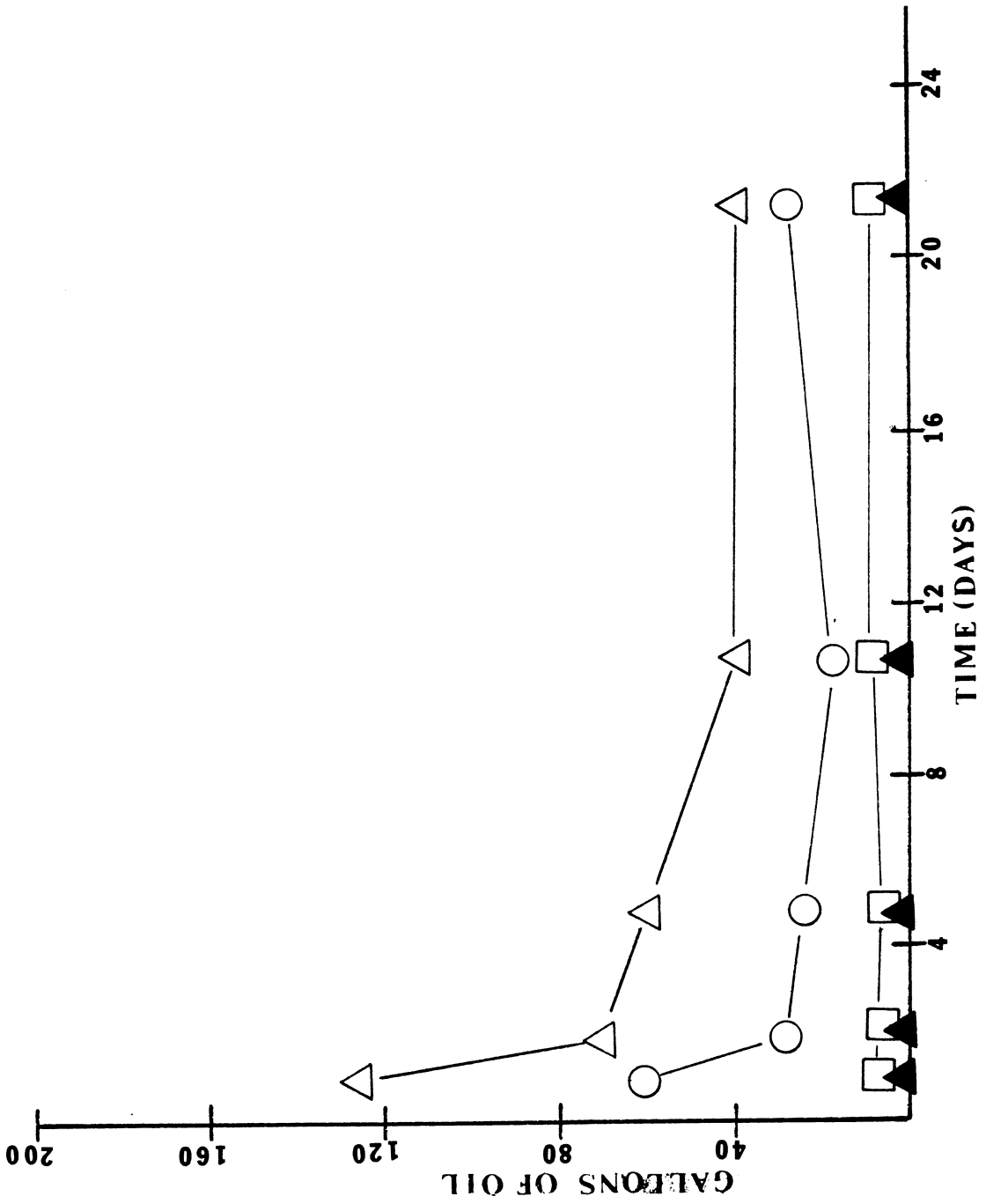
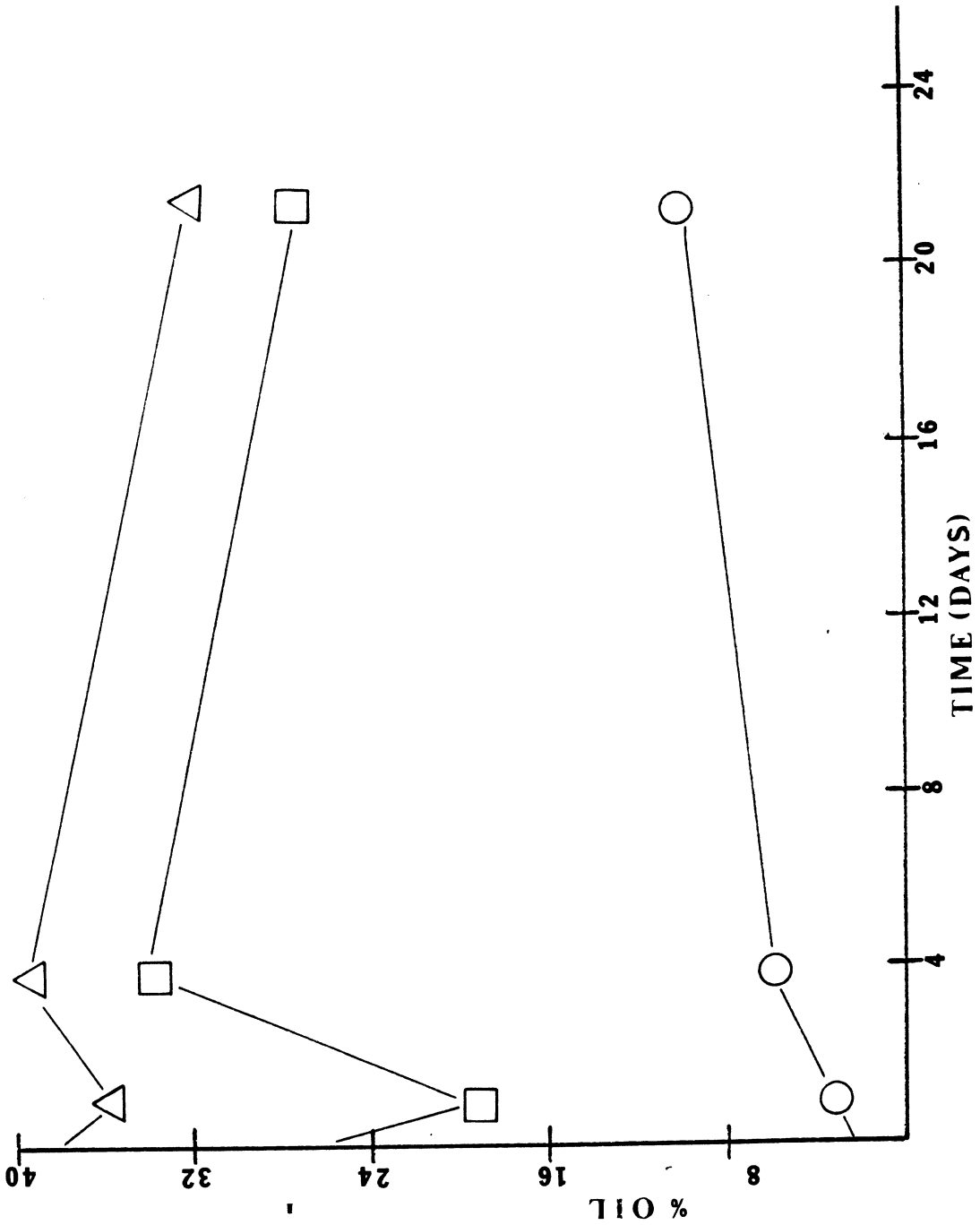

GALLONS OF OIL
40
80
120
160
200
TIME (DAYS)
4
8
12
16
20
24

FIGURE 38. Oil cut (% oil) produced fluid) treated (Covington wells) and control (Ellison wells) wells after inoculation. Open Triangles - Ellison 2. Closed Triangles - Ellison 4. Open Circles- Covington 22. Open Squares - Covington 24.


TIME (DAYS)
4
8
12
16
20
24
% OIL
8
16
24
32
40

while the control wells do not.

Returning to Figure 37 and comparing with Figure 38 we are able to come to the following conclusions:

1. There are some indications that a positive effect on oil production has occurred; these are the positive slope with respect to oil-cut (Figure 6) and total production (Figure 7) from the test wells, when compared to control wells. Ellison Well No. 4, even after being shut-in for an extended time does not appear capable of making up any oil. In fact it would appear that Ellison Well No. 4 produces only water, even after an extended period of non-production.

2. The inoculation treatment did not damage the wells. Any mechanical problems that arose during the treatment process were not the result of microbial injection or growth.

Again though we were victims of circumstance, mechanical problems with Covington Well No. 18, and at least one such problem with Covington Well No. 24 hampered our data retrieval. It must also be noted that at the time of well inoculation, the waterflood was discontinued. For various reasons (out of our control) the waterflood was never resumed. One can only speculate as to the beneficial effect resumption of the waterflooding process would have had on oil production from both the Covington and Ellison fields.

The primary success of the test is from a microbial standpoint because of the large production of biogenic gases, solvents and acids. However this field was not the

candidate for the cultures isolated, being some 10°C below the optimum for this organism. This obstacle was partially overcome through the use of the heated injection medium.

Microbial counts from both Covington wells at various times during post-injection production ran from X x 10^7 to X 10^8. For the most part the bacteria present were essentially identical to isolate SEG-3, being Gram-positive obligately anaerobic spore forming rods.

Summary

The same conclusions from all three field tests can be drawn. Clostridial spores could be injected along with a nutrient mixture into a formation at various pressures (up to 1200 psi). Once in the reservoir those same cultures were able to outgrow competing indigenous and nutrient-contaminating bacteria and perform a viable fermentation. The nature of the fermentation products suggest that indigenous or transient bacteria apparently play only a minor role in the fermentation.

Few statements can be made as to the enhancement of oil production. The loss of two fields (one because it was sold, and the other simply abandoned) in the middle of the test prevented adequate testing. All three fields were recently drilled, none completed before 1981 and by the time of inoculation were extremely marginal, suggesting that perhaps most of the oil that these wells were

capable of producing was already out of the formation. All three fields were available (for treatment) because they were on the verge of economic death. As of this date, two of the fields are no longer in operation and the third has been sold. In most cases we went into the wells blind, since we lacked well logs and were mislead as to their history. Still, these were the only wells available and in most cases the operator had quite a bit of "out of the pocket" expense. Data has however been presented that suggests MEOR has some potential.

For more meaningful data, it would seem logical that the following procedure be used:

1. A reservoir temperature of about 37°C.
2. Use of a well or wells that have been in production for a number of years and only recently have been at the lower end of the decline curve.
3. The crude oil should be of low gravity, since any physiochemical changes in heavy oil would be more apparent. One can also be quite sure that there is sufficient heavy oil left in a reservoir after primary and secondary recovery.
4. Control of the wells should certainly remain in the hands of the investigator for the duration of the test.

It is my belief that all four criteria can be met. Whether or not MEOR can stand on its own or fall, can be determined if someone is willing to pay for meaningful field data.

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Doctor of Philosophy

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