CERTAIN NUTRITIONAL AND PHYSIOLOGICAL PROPERTIES OF AEROBIC BACTERIA ISOLATED FROM OIL RESERVOIRS AS RELATED TO THEIR REACTIONS WITH POLYACRYLAMIDES AND XANTHAN GUM

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Although the price of crude oil has levelled off recently, it remains high, and could very well rise again in the future, especially if demand increases, or oil supplies from other parts of the world are cut off for any reason. The probability of finding large new fields of oil in the continental United States is not high. Therefore, it is important to find new mwthods to recover as much oil as possible from known oil fields. This process is known as enhanced oil recovery. The final goal of enhanced oil recovery is to increase the flow rate of crude oil from the porous rocks of reservoirs to economic levels.

Oil has its origin from remains of dead organisms; most likely, algae, varoius marine microorganisms, and multicellular invertebrates. This organic debris accumulates in the shallow seas and then is entrapped in the silts and clays. After many millenia, these layers pile up on top of one another and finally sink to depth up to several thousand meters. As a result of this sinking, the layers are subjected to high temperatures and pressures, and thereby resulting in decomposition of organic materials anaerobically into hydrocarbons. Most of the oil is trapped in the bottom in dense salt or other impermeable material (18).

Oil is recovered from underground reservoirs via a series of operations. The release of internal pressure in the well causes the release of oil. When pressure is depleted, it is necessary to pump further oil

by mechanical means. It is the difference in the pressure between the fluids of the reservoir and the bore hole drilled into it, that causes oil to flow into the bore. But these methods recover only a limited amount of oil that is stored in the reservoir. Further enhancement of recovery can then be effected by secondary recovery. Water flooding is one of the methods used in secondary recovery. Water flooding is done by pumping water into the well which displaces part of the trapped oil from porous rocks. Water flooding has its own disadvantages, in terms of cost, etc. For instance, it will not recover all the oil that is left in the porous rocks.

The cost of treating water before injecting into the wells, and the cost of controlling the brine borne sulfate-reducing bacteria should be taken into consideration. These sulfate-reducing bacteria may lead into sulfide corrosion and to accumulation of organic masses on the reservoir sand face which reduce its permeability to water (18).

A number of methods have been developed in recent years to recover further quantities of oil from partially depleted reservoirs, by the use of mobility control agents. These agents enhance oil displacement from the porous rocks by increasing the viscosity of the displacing fluid. One of these processes include polymer flooding. Polymer flooding uses mainly two types of polymers. These are polyacrylamides, which are produced by a chemical polymerization, and biopolymers, which are produced by microorganisms. Both types of polymers are water soluble and have the property of forming very viscous solutions at low concentrations.

The most widely used biopolymer is Xanthan gum. It is produced by the microorganism Xanthomonas campestris which causes vascular disease

in plants like cabbage, cauliflower, rutabagas, etc. It is used in oil recovery because it possesses some unique properties which are advantageous for oil recovery use. For example, it is insensitive to high salt concentrations, does not precipitate or lose viscosity under normal conditions, is shear stable, is stable at high temperatures up to 200°F, and over a wide range of pH from 1.5 to 13. In addition to these, it is a good displacing agent. This gum is poorly adsorbed on the elements of the porous rock formations and it provides viscosities of a value to be useful in enhanced oil recovery. However, it can not withstand attack by "xanthanase." "Xanthanase" is a name given by Cadmus et al., (1) to a complex enzyme preparation that degrades Xanthan gum. Xanthan gum is biodegradable. The degree of biodegradability is not clear. Cadmus et al., (1) stated that, since solutions of Xanthan gum remains stable for a long time, and xanthanase-producing microorganisms are relatively uncommon, the polymer is relatively resistant to biodegradation (1). The diagrams of repeating units of Xanthan gum (1) and polyacrylamide are given in Figures 1 and 2 respectively.

Xanthan gum is an extracellular polysaccharide and is used in polymer flooding due to its high specific viscosity and pseudoplasticity (12). It is also widely used in food industries. The primary structure of the polymer consists of a main chain or a backbone made up of β (1→4) D-glucose residues linked as in cellulose. On every alternate glucose residue of the main chain, a side chain consisting of three sugars, is attached at C-(3) position (11). Each side chain consists of, in order, from terminal end, β-D-mannose, (1→4) β-D-glucuronic acid, (1→2) β-D-mannose (6-O-acetyl). About one-third of the terminal D-mannose residues bear a pyruvic acid acetal. The details of the structure are variable, depending on the strain. The number of pyruvyl groups can

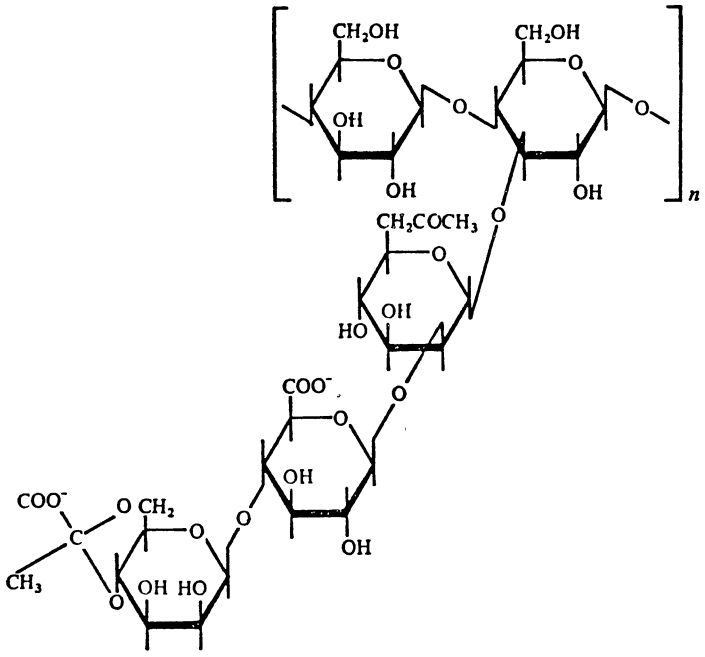

Figure 1. Repeating Unit of Xanthan Gum.

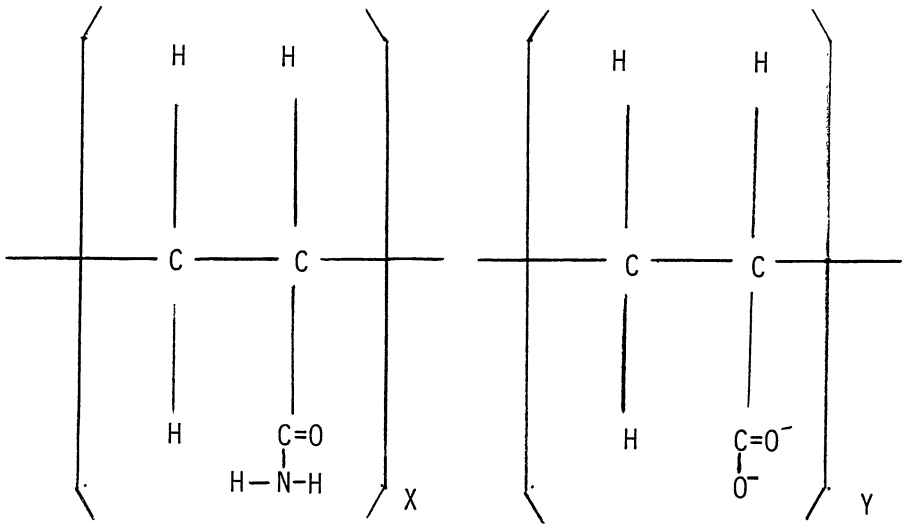

Figure 2. Structure of Polyacrylamide.
The ratio of X to Y is the degree of hydrolysis.

vary and this affects the properties of the polymer very greatly (4, 16). Because of the glucuronic acid and pyruvate groups, Xanthan gum exhibits many typical polyelectrolyte properties. The secondary and tertiary structures of Xanthan gum are still not known completely. The experiments of Holzworth et al., (9), used x-ray scattering from Xanthan gum fibers; this revealed a helix with five-fold symmetry and pitch 4.70 nm. The native polysaccharide consists of many subunits, 2 nm in diameter arranged in a right-handed double or triple helix. From the measurements of sedimentation coefficients and intrinsic viscosities, it is found that native Xanthan gum has a molecular weight of 15×10^6 daltons (10).

Xanthan gum is available in both powder and broth forms. Xanthan broth is obtained by growing Xanthomonas campestris on a well aerated medium containing glucose, organic nitrogen sources (amino acids), dipotassium hydrogen phosphate and trace elements. When fermentation is finished, the culture fluid will have a viscosity of about 17,000 centipoises (13). The solutions prepared from the broth possess better injectivity properties than the solutions prepared from the Xanthan Powder (5). Once the ordered conformation (multi-stranded helix) is destroyed, Xanthan gum exhibits a more random configuration and consequently higher viscosity (5).

Oil reservoirs often have trouble with sulfate-reducing bacteria, especially after polymer flood. When an oil reservoir has problems with the sulfate-reducing bacteria, which results in sulfide corrosion and accumulation of organic masses in the reservoir, it is said to be turned "sour." In my study, samples of oil and water were obtained from reservoirs that had turned sour. Large numbers of sulfate-reducing bacteria were found in these samples. Along with the sulfate-reducing bacteria, some aerobic, pigmented, isolates were found.

Because of their pigmentation, it is sometimes convenient to refer to these organisms by the colloquialism "pink". There appears to be a close association --possibly a form of mutualism--between these microorganisms and sulfate-reducing bacteria. I have reason to believe that pink organisms play an important role in situ. Therefore, it is important to learn something of their properties and capabilities as far as the polymer degradation goes.

Oil companies are interested in the fate of Xanthan gum once it is injected into the reservoir. The current trend seems to be away from its use, because of a common perception of its high biodegradablity.

The experience of Cadmus et al., (1), who state their belief that Xanthan-degrading organisms are uncommon in natural environments, would seem to contradict this. It is possible that its seemingly high degradability may be related to the high impurity of the broth products. It is important to settle the question of biodegradability, because the oil industry will not use the product, in spite of its many excellent qualities, if it believes the polymer to be biodegradable. Hence, in addition to studying the relationships of the aerobic pink organisms with sulfate-reducing bacteria, it is important to study their properties and biodegradation abilities independent of sulfate-reducing bacteria. I have, therefore, undertaken this study to learn something of the nutritional and physiological characteristics of these organisms, and of their interactions with Xanthan gum. Because of similar considerations, I have also done tests for the ability of these microorganisms to degrade certain polyacrylamides.

CHAPTER II

MATERIALS AND METHODS

Test Organism

In the course of this project, a large number of samples of produced water and crude oil were obtained from reservoirs that had turned sour. From these samples, along with the sulfate-reducing bacteria, some aerobic, pink pigmented organisms were also found. At least eight strains of these "pink organisms" were isolated from the reservoir samples. Identification of these organisms is under investigation in the Department of Microbiology, Oklahoma State University, Stillwater, Oklahoma. I have chosen two of these strains, designated as C and 2E to conduct our studies.

These organisms are gram negative, motile rods, whose size is approximately 0.5 x 2 um. These organisms form pigmented colonies on tryptic soy agar (TSA) plates. They are able to grow both at 25°C and 37°C, showing better growth at 37°C. Both strains produce shiny capsular substance and, depending on the medium they are growing in, they produce an insoluble pink pigment. Neither strain can grow on nutrient agar. Each possesses a single polar flagellum; this is compatible with the genus Pseudomonas.

Stock cultures were maintained on TSA slants stored at room temperature. The stock cultures were transferred every 7-10 days. The colonial morphology of these strains is given in Table I.

TABLE I

COLONIAL MORPHOLOGY

Strain	Morphology *
C	The colonies are medium sized, circular, raised, with entire margin, butyrous in consistency and pinkish tan colored on tryptic soy agar plates.
2E	The colonies are medium sized, circular, convex, with entire margin, viscid and pink colored on tryptic soy agar plates.

* Time of observation and temperature of incubation is 24 hours and 34°C respectively.

Media and Growth of Cultures

Composition of the chemically defined basal medium is given in Table II. In most experiments, sodium lactate, added in the form of 60% syrup was used as sole carbon and energy source. Media were adjusted to pH 7.0 $\pm$ 0.2, with 0.1 N sodium hydroxide or hydrochloric acid,using an Orion Research digital pH millivoltmeter 611.

In some experiments, Postgate's Medium C was used. This medium was devised for growing sulfate-reducing bacteria, without a heavy black precipitate of ferrous sulfide. Composition of this medium is given in Table III. It was used in our studies of Xanthan gum degradation.

An enrichment broth containing salt (Table IV) used by Cadmus et al., (1), was used in attempting to test for Xanthan gum degradation by these bacteria.

Postgate's Medium B was used in a few experiments,to study the growth characteristics of these organisms. The composition of Medium B is given in Table V.

The inoculum for all the experiments was prepared by washing cells off an 18 to 24 hour TSA slant culture with 5 ml of sterile saline (0.85% NaCl). The cells were centrifuged and resuspended twice. The concentration of cells in the final suspension was adjusted to an absorbance of 1.0, before inoculation. The inoculum size was kept constant throughout all experiments. Sterile Pasteur pipettes were used to deliver the drops of inoculum. One drop of inoculum per 5 ml of medium was used. Erlenmeyer flasks (250 ml) containing 75 ml of the medium were inoculated with standardized inoculum and incubated at 37°C, usually with shaking on a rotory shaker. The degree of growth was determined by measuring the absorbance of the bacterial suspensions at 540 nm with a

TABLE II

COMPOSITION OF BASAL MEDIUM

Chemicals	Amount/100 ml
Carbon-Energy source*	
NH_4Cl	400 mg
$MgSO_4.7H_2O$	3 mg
KH_2PO_4	136 mg
K_2HPO_4	174 mg
Trace minerals:	
H_3Bo_3	0.5 ug
$CaCo_3$	10.0 ug
$CuSO_4.5H_2O$	1.0 ug
$FeSO_4(NH_4)_2SO_4.6H_2O$	50.0 ug
KI	2.0 ug
$MnSO_4.H_2O$	2.0 ug
MoO_3	1.0 ug
$ZnSO_4.7H_2O$	5.0 ug

*This was usually lactate (sodium salt), the source which promotes growth of most strains. When other compounds were used, it is indicated in the text in connection with the specific experiment.

TABLE III

COMPOSITION OF POSTGATE'S MEDIUM C

Chemical	Grams/Liter
KH_2PO_4	0.5
NH_4Cl	1.0
Na_2SO_4	4.5
$CaCl_2.2H_2O$	0.06
$MgSO_4.7H_2O$	0.06
Sodium lactate	6.0
Yeast extract	1.0
$FeSO_4.7H_2O$	0.004
Sodium Citrate.$2H_2O$	0.3
Sodium Chloride	25.0

TABLE IV

*MEDIUM FOR ISOLATION OF XANTHAN GUM-DECOMPOSING MICROORGANISMS

Substance	Amount/Liter
Xanthan gum	3.0 gms
$(NH_4)_2SO_4$	0.5 gms
Yeast extract	0.8 gms
Tryptone	0.4 gms
KH_2PO_4	1.5 gms
K_2HPO_4	0.7 gms
NaCl	40.0 gms
Salt solution B	2.5 mls

*Reference: Cadmus et al., Applied and Environmental Microbiology. 44, p. 5-11. (1982).

TABLE V

COMPOSITION OF POSTGATE'S MEDIUM B

Chemical	Grams/liter
KH_2PO_4	0.5
NH_4Cl	1.0
$CaSO_4$	1.0
$MgSO_4.7H_2O$	2.0
Sodium lactate	3.5
Yeast extract	1.0
Ascorbic acid	0.1
Thioglycollic acid	0.1
$FeSO_4.7H_2O$	0.5

Bausch and Lomb Spectronic Model 88. The cuvettes used have a diameter of 10mm. Deionized distilled water was used as the blank in those experiments with the chemically defined medium, since it is devoid of any color.

In order to determine whether Xanthan gum was degraded in cultures in which a decrease in viscosity occurred, I have followed the methods of Schweiger (17) and Haidel et al., (8). I analyzed the culture supernatants for the monomers, D-glucose, D-glucuronic acid, D-mannose, pyruvate and acetate. When a reduction in viscosity was observed, the cultures were centrifuged and the supernatant was dialyzed, using a 12,000 MW cutoff dialysis bag, against 0.01 M phosphate buffer, at 4°C. After 24-48 hours, the dialysis bag was removed, and discarded along with the components inside. The buffer (possibly containing any smaller components of Xanthan gum) was concentrated using a freeze dryer. One hundred ml of buffer were concentrated to 1 ml (concentrated 100 times). This concentrated liquid was used for further analysis by gas chromatography and thin layer chromatography to detect the free sugars and acetic acid.

Chemicals

The biopolymer Xanthan gum, a polysaccharide produced by Xanthomonas campestris, was used in two forms. One, given the trade name Flocon, is a product of Pfizer, Inc. It is a chemically impure product, intended for use in enhanced oil recovery. It is essentially the fermentation liquor with 2000-3000 ppm formaldehyde added. I used this material in only few tests. The other form was a purified powder, obtained from Sigma. The molecular weight of Xanthan gum ranges from 2 to 50 x 10^6

daltons.

Polyacrylamides were also used in some of the experiments. They are copolymers of acrylic acid and acrylamide with molecular weights ranging from 3×10^6 to $9\text{-}10 \times 10^6$ daltons. The ratio of carboxy groups to the total number of side groups on the polymer is called the degree of hydrolysis. Most chemicals were obtained from commercial sources in the purest form available. Certain chemicals were obtained as samples from various companies. These are given in Table VI.

In some early experiments, the solutions of Xanthan gum were prepared by slowly sprinkling the exact amount of dry Xanthan gum powder in to the basal medium to get a final (W/V) desired concentration. The flasks containing the medium, were gently swirled by hand while adding the polymer. Once, all the dry powder was added, a magnetically driven stirrer was used to obtain a thoroughly mixed, uniform polymer solution. The media were autoclaved after the polymer was added. Since the concentrations used were 0.1% and 0.2% (W/V), no undissolved particles (microgels) were found during the preparation of media. Polyacrylamide solutions were prepared in the same way.

After certain experimentation, it was found that autoclaving of Xanthan gum in basal medium, especially in the absense of added lactate, resulted in a significant decrease of viscosity. Since, viscosity is the key property that I was interested in, it was undesirable to have this kind of decrease in viscosity during autoclaving. As an alternative, the Xanthan gum solutions were sterilized separately, and added aseptically to the autoclaved medium. A 0.5% (W/V) stock solution of Xanthan gum was freshly prepared for every experiment. Because of the high concentration, it was necessary to remove undissolved particles

TABLE VI

CHEMICALS

Chemical	Lot #	Physical state	Source
DBNPA *	MM810220	Yellow liquid	Dow Chemical co.
Glutaraldehyde	91F-0448	Yellow liquid	Sigma
Kathon	-	Yellow liquid	Rohm and Haas
Flocon	G82002	Viscous solution	Pfizer
Polyacrylamide J279	-	Dry, amorphous, powder	Dowell Division, Dow Chemical
Polyacrylamide J333	-	Dry,amorphous, powder	Dowell Division, Dow Chemical

*2, 2 dibromo-3-nitrilopropionamide (a broad spectrum, low persisting biocide)

(microgels) of the polymer by filtration through a copper mesh plate[1] (2). After removing microgels, the polymer solution was autoclaved at 16 PSI pressure for 15 minutes. After cooling, the solution was added to the sterile medium to get final concentrations of 0.1% or 0.2%. Autoclaving Xanthan gum in a pure aqueous solution produced very little or no decrease in viscosity.

Since Flocon is already in solution (3%), it only required dilution in the medium and did not form any microgels. Flocon (3% broth) was provided by AMOCO. It contains 2000 to 3000 ppm formaldehyde. This liquid was stored at $4^{0}C$ at all times. Before using, it was warmed to room temperature and diluted in deionized distilled water to get a final concentration of 1%. This 1% solution was autoclaved and diluted further in sterile medium to get the desired concentration. It was observed that solutions prepared from Flocon of a given concentration had lower viscosities than solutions of same concentration of xanthan powder.

Solutions of biocides were prepared using an automatic pipette with sterile tips. Accurate amounts of biocides were delivered into sterile media, and diluted serially to obtain the desired concentrations.

Methods of Analysis

Measurement of Viscosity

The usefulness of a polymer solution in enhanced oil recovery depends in its viscosity. In the case of biopolymers, absolute viscosity, measured in centipoises, is the most valid criterion, whereas with polyacrylamides, screen factor (related to, but not absolutely equivalent to

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[1]In oil recovery applications, it is necessary to use the polymer solution free of microgels, since they could cause the plugging of reservoir rocks.

true viscosity) is a better index of its ability to release oil. Usually for polyacrylamide solutions, screen factor rather than absolute viscosity is determined because it is simpler and easier to do so. If a polymer solutions loses its viscosity over a period of time, it would fail in the oil reservoir. A decrease in viscosity or screen factor generally means that some form of depolymerization or degradation of the molecule has occured. Polyacrylamide molecules are especially susceptible to mechanical stress caused by shear forces; violent agitation of a solution, as in a blender, for as little as five minutes can markedly reduce screen factor or viscosity.

Screen factor (14) is simply the ratio obtained by dividing the time to run a polymer solution through a standardized bulb with time to run the same volume of water through the bulb (14). A screen viscometer[2] was used to measure the screen factor of polyacrylamides. A diagram of cross section of a screen viscometer is given in Figure 3. In passing solutions through the viscometer, a ten inch water pressure vacuum was used to avoid the degradation of polymer by pulling it through the screen too rapidly. The temperature was kept constant for each experiment. The screen factor is given by the following equation:

$$F_s = \frac{t_p}{t_s} F_t$$

$$F_t = 1.00 + 0.017\ (T-25)$$

$$\text{At } 25\ C, F_s = \frac{t_p}{t_s}$$

F_s = Screen factor

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[2] Courtesy of Dowell Division, Dow Chemical Company.

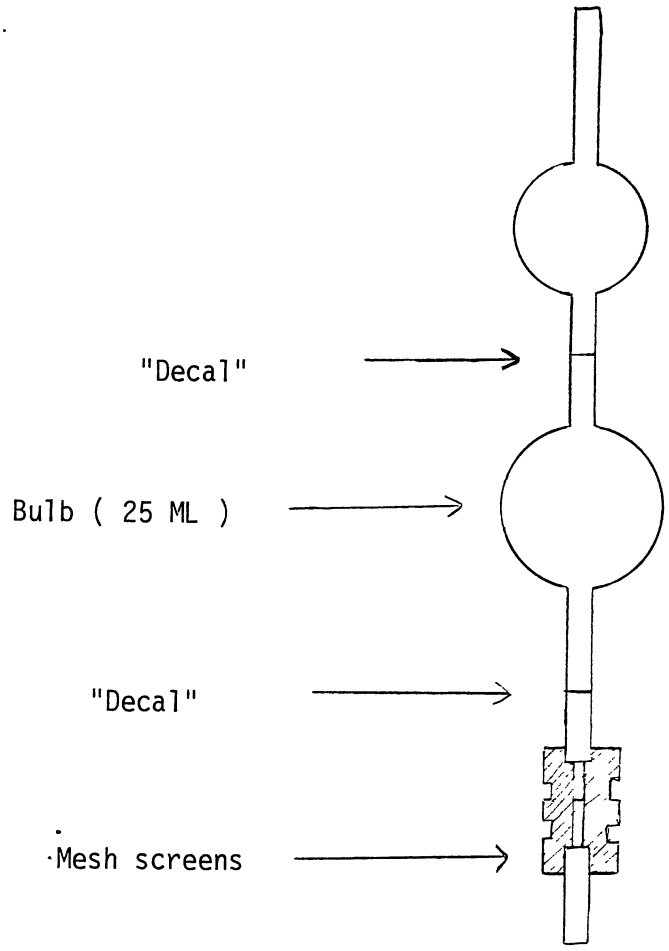

Figure 3. Screen viscometer.

F_t = Temperature correction factor

t_p = Time to run polymer solution through the given standardized volumetric bulb (seconds)

t_s = Time to run solvent (which is distilled water) through the given standardized volumetric bulb (seconds)

Measuring the True Viscosity of Xanthan Gum Solution

It is preferable to measure true viscosity for Xanthan gum solutions. The concentrations of Xanthan gum used in our studies were 0.1% and 0.2% (W/V). At a given W/V concentration, Xanthan gum in solution has a much higher viscosity than polyacrylamide. The viscosity of Xanthan gum was measured using a Brookfield viscometer. A UL adapter was used to measure the viscosity for smaller volumes of solutions. Viscosities were always measured at 6 RPM, in centipoises. Since viscosity of a liquid is a function of the temperature, it was necessary to keep the temperature during measurements constant as far as possible, by using a constant temperature water bath. However, I found in my studies that two or three degrees difference in temperature would not measurably alter the viscosity of Xanthan gum solution. A diagram of the Brookfield viscometer is given in Figure 4. While measuring viscosities, care was taken to prevent any particulate matter from getting in the UL adapter. Solutions or media containing Xanthan gum and test organisms were centrifuged at 6000 RPM for 15 minutes using an SS-34 rotor, to remove cells from the medium. A solution of 16.5 ml is needed to fill the UL adapter. Units given in tables are in centipioses.

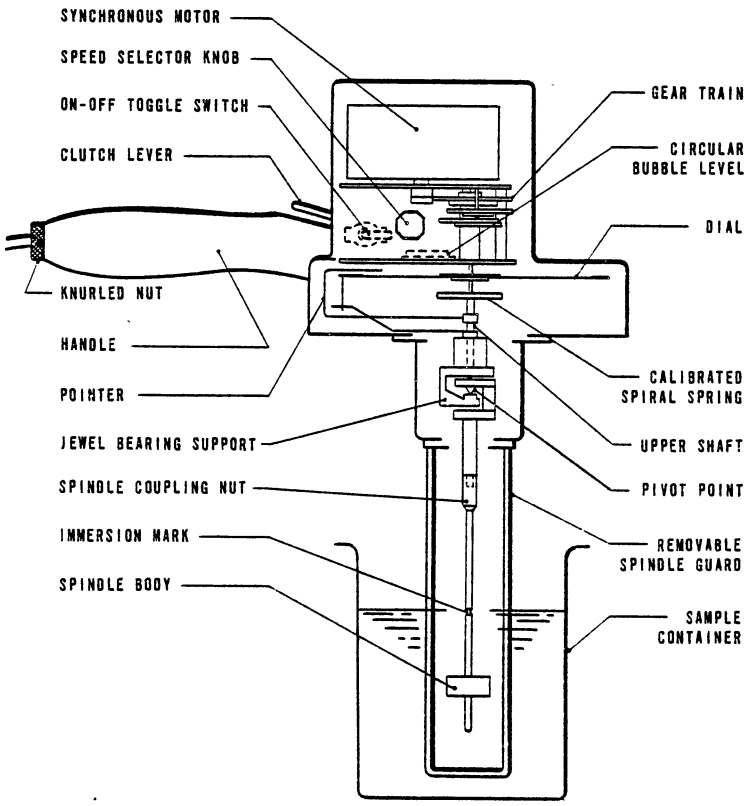

Figure 4. Diagram of Brookfield viscometer.

Source: Instrument Manual supplied by Brookfield Engineering Laboratories, Inc.

Thin Layer Chromatography (TLC)

Thin layer chromatography was used in our experiments to detect the free sugars and sugar acids that may have been produced in the media containing Xanthan gum. The plates for our assay purposes, were prepared in the lab, using cellulose.[3] The solvent system used was pyridine-ethylacetate-acetic acid-water (5:5:1:3). p-Anisidine-phthalic acid reagent was used to spray the TLC plates. This produces a colored derivative with sugars or uronic acids.

Gas Chromatography

Acetic acid content present in the samples was estimated by using gas chromatography. A carbopack glass column supplied by Supelco Inc., was used in the analysis. All samples were acidified with small amounts of H_3Po_4 to assure that the acid is protonated. A detailed description of the procedure used was described by Dicorica and his co-workers (3).

Analysis for Pyruvic Acid

The method of Haidle and Knight (8) was used to test for the presence of pyruvic acid. This was used to detect pyruvate as an end product of loss of viscosity of Xanthan gum. This is a colorimetric test for carbonyl-containing compounds.

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[3] Cellulosepulver MN 300 was used to prepare the plates for TLC. The method was given on the container's label.

CHAPTER III

EXPERIMENTAL

Effects of Antimicrobial Compounds on Growth of Strains C and 2E

Since this work was sponsored by AMOCO Production Company, a portion of our work consisted of testing the effectiveness of certain antimicrobial agents (biocides) used by the oil industry, against these bacteria, all of which had been recently isolated from oil reservoirs. The biocides tested were Kathon[1], 2, 2 dibromo-3-nitrilo-propionamide (DBNPA--manufactured by the Dow Chemical Company) and glutaraldehyde.

In all the experiments, the biocide was added carefully to sterile medium by using an automatic "Fin Pipet." I assumed these to be self-sterilizing and hence they were not autoclaved[2]. Serial dilutions were prepared from the highest concentrated stock or original solution. The inoculum size was controlled by adding a certain volume of a washed cell-suspension of known density. The experiments were done in test tubes (18 mm diameter) with 10 ml volumes and were incubated at 34-36°C with shaking on a reciprocal shaker.

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[1] This is a trade name. Product is manufactured by Rohm and Haas; chemical composition is unknown.

[2] Uninoculated controls, in which the highest concentration of biocide was added, were always prepared, and did not show any growth. In addition, randomly chosen cultures, which showed growth were streaked out to determine possible presence of contamination.

All these biocides, as we received them, were concentrated liquids and yellow in color. They are most commonly used by the oil industry to control the microbial flora in various phases of their operation. Microorganisms in oil reservoirs are thought to bring about degradation of polymers, may cause corrosion of metals underground, and in general, are undesirable; hence the interest of the oil industry in their control.

The antimicrobial agents tested were found to be effective in inhibiting growth of the aerobic pigmented organisms, especially in defined basal medium. Data on growth of strains C and 2E in the presence of varying concentrations of Kathon in the chemically defined basal medium (Strain 2E), in the chemically defined medium plus 1% (W/V) peptone (Strain C)[3], and in the tryptic soy broth (TSB) are given in Tables VII, VIII and IX respectively.

In the minimal (basal) medium, as low a concentration as 2 ppm of Kathon inhibited growth of Strain 2E for as long as 9 days. Strain C was inhibited for 72 hr by 2 ppm Kathon (Table VIII), even though the medium was supplemented with 1% peptone. No growth of strain C was observed even after 72 hrs. This is somewhat noteworthy because in our experience with biocide testing, a rich medium will lower the effectiveness of chemical antimicrobial agents. This effect was noted in the test of Kathon against both strains C and 2E in TSB (Table IX). In this medium, a fivefold higher concentration (10 ppm) was insufficient to prevent good growth in 24 hr. At 20 ppm, strain 2E was inhibited for 45 hr; strain C grew in 45 hours in the presence of 20 ppm Kathon.

The other two antimicrobial agents, DBNPA and glutaraldehyde, were

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[3] Peptone used with strain C because it will grow only very slowly in defined medium plus lactate, without a supplement.

TABLE VII

INHIBITION OF GROWTH OF STRAIN 2E BY
KATHON IN BASAL MEDIUM

Strain	Concentration of Kathon (ppm)	Absorbance, 540nm*		
		24 hrs.	48 hrs.	9 days
None	None	0.00	0.00	0.00
2E	None	0.59	0.90	0.55
2E	50	0.02	0.02	0.02
2E	10	0.03	0.02	0.04
2E	8	0.02	0.02	0.04
2E	5	0.03	0.03	0.04
2E	2	0.03	0.03	0.04

* Zero time absorbance was 0.02 units in the inoculated controls.

TABLE VIII

INHIBITION OF GROWTH OF STRAIN C BY KATHON IN BASAL MEDIUM + 1% PEPTONE

Strain	Concentration of Kathon (ppm)	Absorbance, 540 nm* 24 hrs.	48 hrs.	72 hrs.
None	None	0.00	0.00	0.00
C	None	1.00	1.45	1.50
C	50	0.04	0.02	0.02
C	10	0.01	0.01	0.01
C	5	0.02	0.00	0.01
C	2	0.02	0.03	0.01

* Zero time absorbance was 0.02 units in the inoculated controls.

TABLE IX

INHIBITION OF GROWTH OF STRAINS C AND 2E
BY KATHON IN TRYPTIC SOY BROTH

Strain	Concentration of Kathon (ppm)	Absorbance, 540 nm* 24 hrs.	45 hrs.
None	None	0.00	0.00
C	None	1.70	1.70
C	50	0.02	0.02
C	40	0.01	0.01
C	30	0.01	0.01
C	20	0.01	1.64
C	10	1.80	1.65
2E	None	1.95	1.70
2E	50	0.02	0.02
2E	40	0.02	0.02
2E	30	0.01	0.01
2E	20	0.02	0.02
2E	10	1.85	1.65

* Zero time absorbance was 0.01 units in the inoculated controls.

also tested against these two strains. The effect of DBNPA on growth of strain 2E in defined basal medium is given in Table X. No growth was observed for strain 2E in presence of DBNPA. The profound influence of using a rich medium on the effectiveness of given biocide is shown very clearly in Table XI using TSB. It was observed that a concentration of 300 ppm DBNPA was required to prevent growth of strain 2E for 96 hr whereas with strain C, 350 ppm was needed. It is possible, using these figures, to get an approximation of the minimum inhibitory concentration of DBNPA for strains C and 2E in TSB. On the basis of 96 hr incubation, a value between 250 and 350 ppm could be given for strain 2E, and between 300 and 350 ppm for strain C. These values for a minimum inhibitory concentration are at least 100 times greater than the corresponding values would be in the chemically defined medium.

The results obtained using glutaraldehyde are given in Tables XII and XIII. As shown in Table XII, glutaraldehyde inhibits the growth of both strains in chemically defined basal medium at all the concentrations tested except at 2 ppm. Both strains are able to grow in the presence of glutaraldehyde, in TSB, at all concentrations tested.

The approximate minimum concentrations of the three biocides to inhibit the growth of strains C and 2E in the two media used are given in Table XIV. The effect of the growth medium upon the minimum concentration necessary to inhibit growth is very striking. In the case of Kathon, this concentration is approximately 10 times higher in TSB than in the chemically defined medium, with both strains C and 2E. In the case of DBNPA it is approximately 100 times greater in TSB for strain 2E. We do not have the minimum inhibitory concentration of DBNPA for strain C in the chemically defined medium since it does not grow or grows

TABLE X

INHIBITION OF GROWTH OF STRAIN 2E
BY DBNPA IN BASAL MEDIUM

Strain	Concentration of DBNPA (ppm)	Absorbance at 540 nm*		
		24 hrs.	48 hrs.	96 hrs.
None	None	0.00	0.00	0.00
2E	None	0.52	1.40	0.75
2E	50	0.01	0.02	0.02
2E	25	0.02	0.02	0.02
2E	10	0.02	0.02	0.02
2E	5	0.02	0.03	0.03
2E	2	0.02	0.03	0.02

* Zero time absorbance was 0.01 units in the inoculated controls.

TABLE XI

MINIMUM INHIBITORY CONCENTRATION OF DBNPA WITH RESPECT GROWTH OF STRAINS C & 2E IN TRYPTIC SOY BROTH

Strain	Concentration of DBNPA (ppm)	Absorbance at 540 nm*		
		24 hrs.	48 hrs.	96 hrs.
None	0.00	0.00	0.00	0.00
2E	0.00	>2.0	>2.0	2.00
2E	300	0.03	0.03	0.03
2E	250	0.02	0.47	2.00
2E	200	>2.0	>2.0	2.00
2E	150	>2.0	>2.0	2.00
C	0.00	>2.0	>2.0	2.00
C	400	0.01	0.01	0.01
C	350	0.00	0.00	0.00
C	300	0.02	0.02	1.40
C	250	0.03	0.03	2.00
C	200	>2.0	>2.0	2.00
C	100	>2.0	>2.0	2.00

* Zero time absorbance was 0.02 units in the inoculated controls.

TABLE XII

EFFECT OF GLUTARALDEHYDE ON GROWTH OF STRAINS C AND 2E IN BASAL MEDIUM

Strain	Concentration of Glutaraldehyde (ppm)	Absorbance at 540 nm*		
		20 hrs.	90 hrs.	6 days
C	0	0.02	0.75	0.82
C	100	0.01	0.01	0.01
C	50	0.02	0.01	0.02
C	10	0.01	0.01	0.04
C	2	0.01	0.85	0.89
2E	0	0.32	0.82	0.71
2E	100	0.02	0.02	0.03
2E	50	0.02	0.02	0.02
2E	10	0.02	0.02	0.05
2E	2	0.02	1.13	1.22

* Zero time absorbance was 0.01 units in the inoculated controls.

TABLE XIII

EFFECT OF GLUTARALDEHYDE ON GROWTH OF STRAINS C AND 2E IN TRYPTIC SOY BROTH

Strain	Concentration of Glutaraldehyde (ppm)	Absorbance, 540* 20 hrs.
None	0	0.00
2E	0	1.86
2E	100	1.99
2E	50	>2.0
2E	10	>2.0
2E	2	1.88
C	0	1.90
C	100	1.97
C	50	1.88
C	10	1.83
C	2	1.92

* Zero time absorbance was 0.02 units in the inoculated controls.

TABLE XIV

EFFECT OF MICROBIOCIDES ON GROWTH OF STRAINS C AND 2E IN DIFFERENT MEDIA

Strain	Medium	Microbiocide	Minimum inhibitory Concentration (ppm)
2E	Tryptic soy broth	Kathon	20 - 30
2E	Basal medium	Kathon	<2
2E	Tryptic soy broth	DBNPA	300 - 400
2E	Basal medium	DBNPA	<2
2E	Basal medium	Glutaraldehyde	>2 -<10
C	Tryptic soy broth	Kathon	10 - 20
C	Basal medium + 1% peptone	Kathon	2
C	Tryptic soy broth	DBNPA	300 - 350
C	Basal medium	Glutaraldehyde	>2 -<10

slowly in this medium; therefore one percent peptone is usually added. There is no reason to believe that the use of TSB as a medium to test the MIC of DBNPA for strain C would not result in a large increase in MIC.

With 2 ppm glutaraldehyde, these strains are able to grow in defined basal medium. It is generally recognized that a higher concentration of an antimicrobial substance is required to inhibit growth in the presence of large amounts of organic matter, or in a rich medium (e.g., TSB) than in low concentrations of organic matter (minimal medium). However, the reason for the difference in biocide concentration ratio (ca. 10-fold with Kathon, and ca. 100-fold with DBNPA and glutaraldehyde) is unknown. It seems reasonable to suggest to users of the biocides, however, that if not all the characteristics of underground environment were known, Kathon would be a safer choice to maintain effectiveness in the presence of organic matter.

A Nutritional Study

The first part of this work was a study of some of the nutritional properties of the strains C and 2E. A knowledge of their nutritional requirements should enable control measures to be more quickly and rationally developed. Our nutritional study included the effects of various media, pH and complex materials such as casamino acids, groups of individual amino acids, and vitamins on growth of these organisms.

Growth in Various Media

These strains were tested for their ability to grow in the chemically defined basal medium, using glucose as sole carbon and energy

source and NH_4Cl as sole nitrogen source. I found no growth, however. Apparently these strains can not use glucose as sole carbon and energy source. However, supplementation of the defined basal medium with Bacto-peptone allowed good growth (Table XV). Growth as a function of peptone concentration is given in Figure 5.

From results in Table XV, it is seen that peptone is markedly stimulatory for both strains, greatly shortening the lag phase and increasing amount of growth (biomass) at 19 hours as indicated by the absorbance of cultures.

Effect of Yeast Extract

Abundant growth on Tryptic soy media, together with little or no growth on nutrient agar or broth, indicates that these organisms require a rich medium for growth. Accordingly, we tested the effect of yeast extract on growth of strain C, in the basal chemically defined medium, with no glucose but with 0.1% peptone added. This level of peptone is just suffucient to support minimal growth. If yeast extract at low concentration should markedly increase growth, this would indicate that it was supplying one or more needed vitamins for growth. If a high concentration of yeast extract were required for maximum growth, the indication of the nature of the requirement would be much less specific. Strain C, at two temperatures, 25°C and 37°C, was tested over a range of yeast extract concentrations varying from zero to 0.5%. Results at 24 hours are given in Figure 6. There is not an all or nothing effect of yeast extract, but rather that amount of growth is a function of yeast extract concentration over a range up to 0.2%. This is most likely because a number of factors, rather than just one, are responsible for

TABLE XV

EFFECT OF 1% PEPTONE ON GROWTH OF STRAINS C AND 2E IN DEFINED BASAL MEDIUM

Strain	Medium	Absorbance at 540 nm*	
		8 hrs.	19 hrs.
C	Basal + Glucose	0.00	0.00
C	Basal + Glucose + Peptone	0.07	>2.0
C	Basal + Lactate	0.03	0.05
C	Basal + Lactate + Peptone	1.50	>2.0
2E	Basal + Glucose	0.02	0.02
2E	Basal + Glucose + Peptone	1.30	>2.0
2E	Basal + Lactate	0.03	0.57
2E	Basal + Lactate + Peptone	1.85	>2.0

* Zero time absorbance was 0.01 units in the inoculated controls.

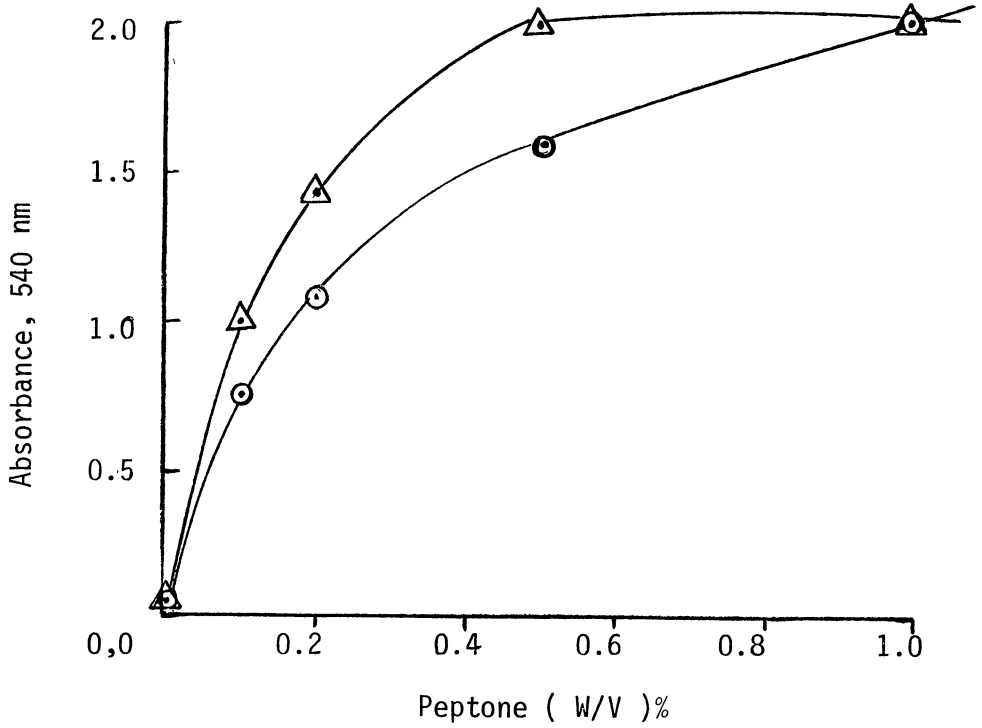

Figure 5. Effect of concentration of peptone on growth of strains C and 2E in basal medium plus glucose. Time of incubation is 15 hrs. △ Strain C ⊙ Strain 2E

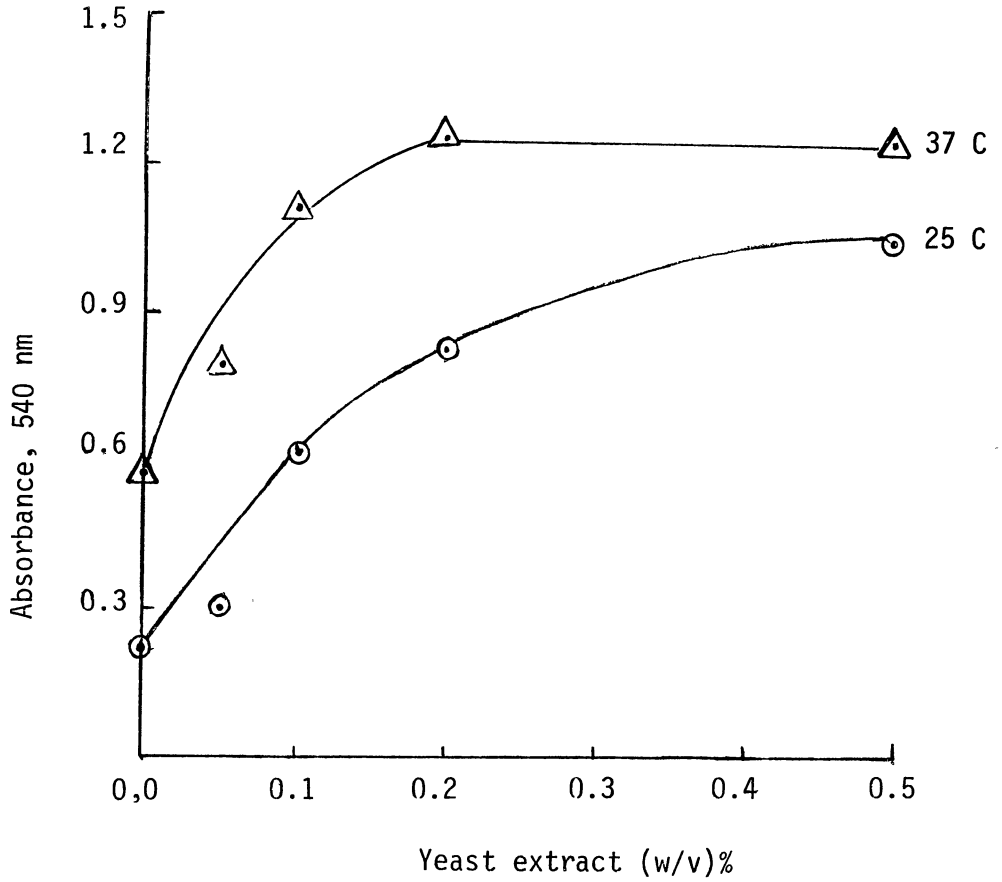

Figure 6. Effect of concentration of yeast extract on growth of strain C. (Basal medium with 0.1% peptone) Time of incubation is 24 hrs.

stimulation by yeast extract. At 37°C, amount of growth at a given time and yeast extract concentration is greater than that at 25°C; ultimate extent of growth attained is also higher at 37°C than 25°C.

Effect of Lactate as Carbon and Energy Source

Other strains of aerobic organisms, isolated from oil reservoirs, and possessing an intracellular pink or orange pigment, had been shown to utilize lactate as a carbon-energy source in either Postgate's medium B or medium C (15). I therefore set up tests to determine whether lactate could serve as a sole carbon-energy source in the chemically defined medium. Since strains C and 2E do not utilize glucose in this medium without the addition of a complex material such as peptone or yeast extract, it was also thought to be of interest to determine whether glucose would either stimulate or inhibit growth under conditions that would allow growth in the basal defined medium. Therefore I added glucose, or lactate, or glucose plus lactate as carbon-energy source. Results are given in Table XVI. This experiment was done using test tubes and with shaking. Here, the chemically defined basal medium contained 1% (W/V) Bacto-peptone in some controls. It can be seen that lactate allowed very good growth of both strains. Under the conditions of the experiment, glucose could not be shown to be stimulatory for either strain. It may have been somewhat inhibitory for strain C. Lactate is utilized very readily by strain 2E and rather slowly (72-96 hr) by strain C, when used as sole source of carbon and energy. Since lactate is utilized readily by both strains, even under relatively stringent nutritional conditions, it was the carbon-energy source of choice for many subsequent experiments in this work.

TABLE XVI

GROWTH OF STRAINS C AND 2E IN BASAL MEDIUM AMENDED WITH PEPTONE IN THE PRESENCE OF GLUCOSE, LACTATE, OR GLUCOSE PLUS LACTATE

Strain	Medium	Absorbance at 540 nm* 18 hrs.**
C	Basal + Glucose + Peptone	0.82 x 2
C	Basal + Lactate + Peptone	0.91 x 2
C	Basal + Glucose + Lactate + Peptone	0.85 x 2
C	Basal + Lactate (no peptone)	0.00 x 2
2E	Basal + Glucose + Peptone	0.90 x 2
2E	Basal + Lactate + Peptone	1.10 x 2
2E	Basal + Glucose + Lactate + Peptone	1.10 x 2
2E	Basal + Lactate (no peptone)	0.24 x 2

* Two fold dilution was made to obtain absorbance reading.
** Zero time absorbance was 0.02 in the inoculated controls.

Effect of Sodium Chloride on Growth of Strains C and 2E

Since these strains were obtained from the Wilmington field (Long Beach, California), which is near the ocean, they might well be of marine origin. Therefore, it is important to test their tolerance of sodium chloride. The aqueous phase in many oil reservoirs may contain a concentration of NaCl up to 10 percent. Preliminary observations had shown that various strains of these organisms were actually stimulated by low concentrations of salt, but they could not grow in the presence of 10% NaCl. It was therefore thought to be of interest to confirm and extend these findings on the influence of NaCl on their growth. Using the basal medium (strain 2E) and basal medium plus 1% peptone (strain C), we tested salt concentrations of 2.5%, 5.0% and 10.0%. Results are given in Table XVII. Strain C readily tolerated, and appeared to be slightly stimulated by 5 % NaCl in basal medium containing peptone. Strain 2E, on the other hand, grew very poorly, if at all, at a concentration of 2.5%. Neither strains grew significantly at 10% NaCl.

Effect of Initial pH on Growth of Strains C and 2E

A series of tests were done in order to more rigorously specify the effects of various parameters on the growth of strains C and 2E. I believed it was important to optimize growth as fully as possible under relatively well-defined conditions before testing the effect of Xanthan gum on growth.

The effect of initial pH on the growth of these organisms in a chemically defined minimal medium (only NH_4Cl as nitrogen source) was

TABLE XVII

EFFECT OF NaCl ON GROWTH OF STRAINS C AND 2E

Strain	Medium*	Concentration of NaCl (w/v)%	Absorbance at 540 nm** 20 hrs.	72 hrs.
2E	Basal (w/o peptone)	None	0.49	0.99
2E	Basal (w/o peptone)	2.5	0.41	1.45
2E	Basal (w/o peptone)	5.0	0.05	0.05
2E	Basal (w/o peptone)	10.0	0.05	0.04
C	Basal + peptone	None	1.10	1.50
C	Basal + peptone	2.5	1.20	1.45
C	Basal + peptone	5.0	1.25	1.70
C	Basal + peptone	10.0	0.05	0.05

* The medium contained lactate as carbon and energy source
** Zero time absorbance was 0.02 units in the inoculated controls.

determined. Lactate was the carbon-energy source. The medium was prepared in 10 ml volumes in 18 mm test tubes; incubation was with shaking on a reciprocal shaker (medium speed), at a temperature of 34°C. The initial pH values of the media were adjusted using an Orion research digital pH meter, before autoclaving. Table XVIII summarizes the results of this experiment. Although the rate and extent of growth were far below optimum, it may be seen that the response of the two strains to initial pH is significantly different. An initial pH of 6.2 enhances the growth (48 hr) of strain 2E whereas it inhibits strain C.

Effect of Casamino Acids on Growth of Strain 2E

Since growth in the chemically defined medium is delayed in initiation, and does not become extremely heavy even after 48 hours incubation, it was believed of interest to determine effects of peptone and of hydrolyzed casein, on length of lag phase (as reflected in time of appearance of visible growth) and on ultimate growth attained. The materials tested were acid-hydrolyzed casein (acid hydrolyzed Casamino acids, Difco), and enzyme hydrolyzed casein (enzyme hydrolyzed Casamino acids, Difco). Both of these materials are primarily sources of amino acids and their effects on growth might give us a clue as to whether required factors were amino acids or vitamins.

Table XIX shows that acid hydrolyzed casein and enzyme hydrolyzed casein strongly stimulated growth of strain 2E in the chemically defined basal medium.

In order to determine to what extent hydrolyzed casein could serve as a sole source of carbon, of nitrogen, and of carbon and nitrogen combined, a test using strain 2E only, was done in: (1) the defined basal

TABLE XVIII

EFFECT OF INITIAL pH ON GROWTH OF STRAINS C & 2E IN BASAL MEDIUM (W/O PEPTONE)

Strain	Initial pH	Absorbance at 540 nm *	
		24 hrs.	48 hrs.
C	6.2	0.01	0.06
C	7.5	0.03	0.15
C	8.0	0.03	0.10
2E	6.2	0.16	0.45
2E	7.5	0.20	0.27
2E	8.0	0.06	0.14

* Zero time absorbance was 0.01 units in the inoculated controls.

TABLE XIX

EFFECT OF HYDROLYZED CASEIN ON GROWTH
OF STRAIN 2E IN BASAL MEDIUM*

Strain	Medium	Absorbance at 540 nm**	
		24 hrs.	42 hrs.
None	Basal + Acid hydrolyzed Casein	0.00	0.00
2E	Basal (w/o hydrolyzed Casein)	0.20	0.56
2E	Basal + Acid hydrolyzed Casein	1.50	1.30
2E	Basal + Enzyme hydrolyzed Casein	1.40	1.10

Concentration of hydrolyzed Casein is 0.1% (w/v)

* Lactate was used as carbon-energy source.

** Zero time absorbance was 0.02 units in the inoculated controls.

medium (complete) supplemented with acid hydrolyzed Casamino acids; (2) the defined medium with NH_4Cl omitted;(3) this medium with lactate omitted; and (4) with both lactate and NH_4Cl omitted. Results are given in Table XX.

It is seen that acid hydrolyzed Casamino acids can serve very well as a source of nitrogen, since omission of NH_4Cl does not interfere with growth at all. However, as a source of carbon-energy, they do not do well unless NH_4Cl is also omitted. This suggests that the deamination of amino acids might be somehow inhibited (feed back inhibition ?) by the presence of ammonia, thus rendering carbon skeletons (e.g., pyruvate) a limiting factor in growth-effectively depriving the organism of carbon, energy, or both.

Specific Nutritional Requirements of Strains C and 2E

Effect of Amino Acids on Growth of Strains C and 2E

It appeared to be of interest to pursue the nutritional requirements of the strains in a little more detail. I had noted on many occasions that they grow more rapidly and abundantly in very rich medium; for example, growth is abundant on TSA, but scanty or non-existent on nutrient agar. Nethier will grow in the chemically defined medium with glucose as carbon-energy source. With lactate, strain 2E grows moderately well and strain C slowly. Since the stimulatory factors present in casein hydrolysate, yeast extract, or peptone were probably organic in nature, I did screening tests on ablity to stimulate growth in the basal medium (with lactate), of five groups of amino acids.

The first group, designated as A, contained all the aliphatic amino

TABLE XX

EFFECT OF ACID HYDROLYZED CASEIN ON GROWTH
OF STRAIN 2E AS SOLE NITROGEN SOURCE *

Strain	Medium	Absorbance at 540 nm** 24 hrs.
None	Basal Medium	0.00
2E	Basal + Hydrolyzed Casein	0.73
2E	Basal + Hydrolyzed Casein (w/o lactate)	0.18
2E	Basal + Hydrolyzed Casein (w/o NH_4Cl)	0.74
2E	Basal + Hydrolyzed Casein (w/o lactate, NH_4Cl)	0.61

* Concentration of acid hydrolyzed Casein is 0.1% (w/v)
** Zero time absorbance was 0.02 units in inoculated controls

acids (alanine, valine, leucine and isoleucine). The second group, A_2, contained the aromatic amino acids and proline (phenylalanine, tyrosine, tryptophan and proline). Group A_3 contained the acidic amino acids and their amides (aspartic acid, aspargine, glutamic acid and glutamine). Group A_4 contained the basic amino acids (lysine, arginine and histidine). The last group A_5 contained the hydroxy- and sulfur-containing amino acids (serine, threonine, cysteine, and methionine), and glycine. Group designated as A_0 contained no amino acids.

Amino acids were prepared in solutions in groups, at a final concentration (total amino acid content) of 10 mg per 100 ml of medium. Stock solutions of amino acids were filter sterilized and then added aseptically to the medium. The experiment was done in 18 mm test tubes with 10 ml volume in each. Each tube was inoculated with 2 drops of (0.5 O.D) cell suspension. Incubation was at 34°C with shaking on a reciprocal shaker (90 oscillations per minute). Absorbance of tubes was read after incubation time of 96 hrs. The results are presented in Figure 7.

The stimulation by groups of amino acids is not surprising. Bacteria capable of growth on a minimal medium will grow much faster if they do not have to synthesize their amino acids. In this experiment, strain 2E showed much lower growth than usual in the control (basal medium without amino acids). It is possible that the density of the inoculum was lower than usual, resulting in more delayed growth in the control. It is interesting that two of the groups (A_2 and A_3) showed little or no stimulation, whereas the other three groups were very stimulatory.

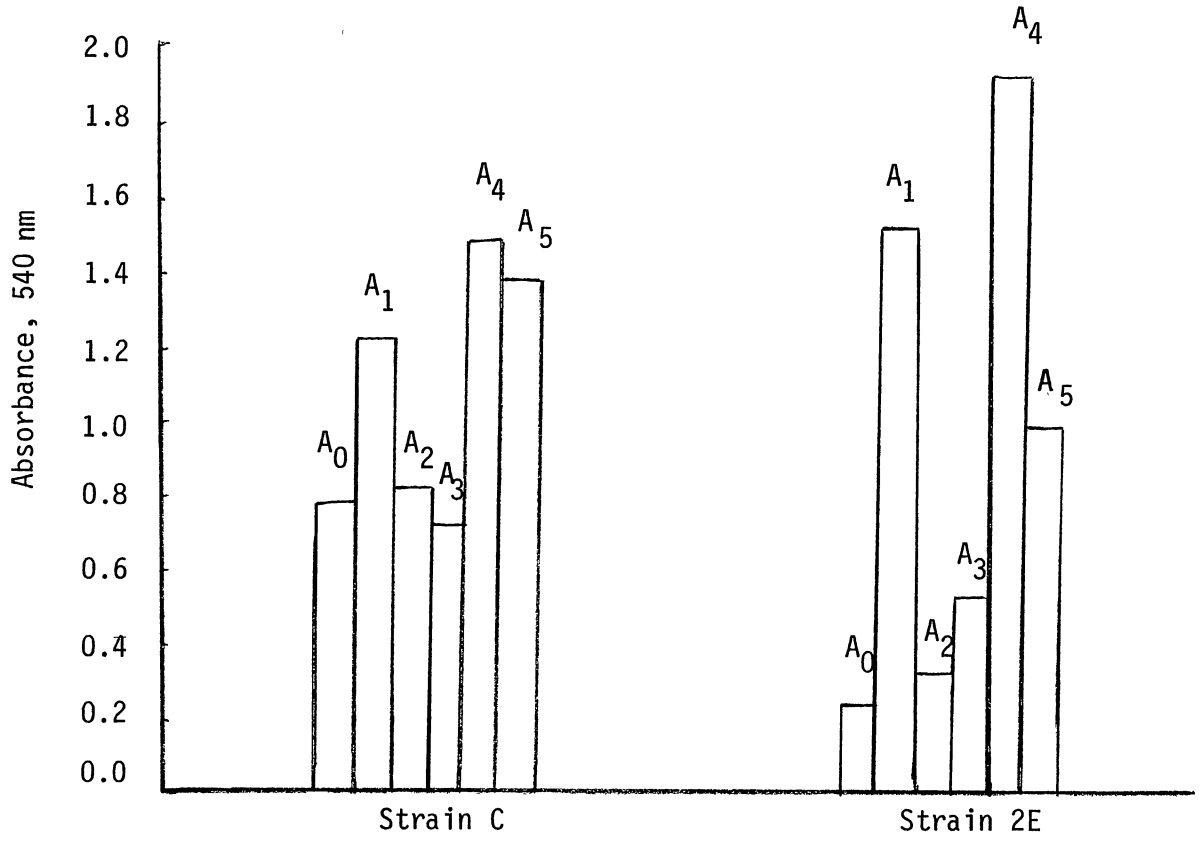

Figure 7. Growth of strains C and 2E in presence of groups of amino acids. Time of observation is 96 hrs.

Effect of Vitamins on Growth of Strains C and 2E

A similar experiment was done with various vitamins, except that they were tested individually at concentrations of 10 ug/100 ml, for their ability to stimulate growth of strains C and 2E. Basal medium with lactate as sole carbon-energy was used in this experiment. Inoculum preparation and incubations were the same as in the amino acid experiment. The results are presented in Figure 8. As shown in the graph, all the vitamins tested are stimulatory for both strains C and 2E.

Growth Characteristics of Strains C and 2E in Postgate's Media B and C

Postgate's media B and C are commonly used for growth of sulfate-reducing bacteria. I was interested in determining whether the pigmented aerobic ("pink") strains show any special characteristics, particularly in regard to production of pigment, production of extracellular polysaccharide, and possible reduction of sulfate, when they grow in Postgate's media. Cells of strains C and 2E were grown in 125 ml Erlenmeyer flasks with 25 ml medium (either B or C) in each. The flasks were incubated at 34°C with shaking on a rotory shaker, run at medium speed. Strain 2E showed dark pinkish growth--a similar degree of pigmentation--in both media B and C. Strain 2E did not cause blackening of medium B[4]. Strain C also showed dark pinkish growth, but in medium C only. The degree of pigmentation in medium C is much higher

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[4] Medium C is designed not to show any blackening; a change which results from the production of $S^{=}$ or HS^{-} in the presence of Fe^{++}, thus precipitating black FeS.

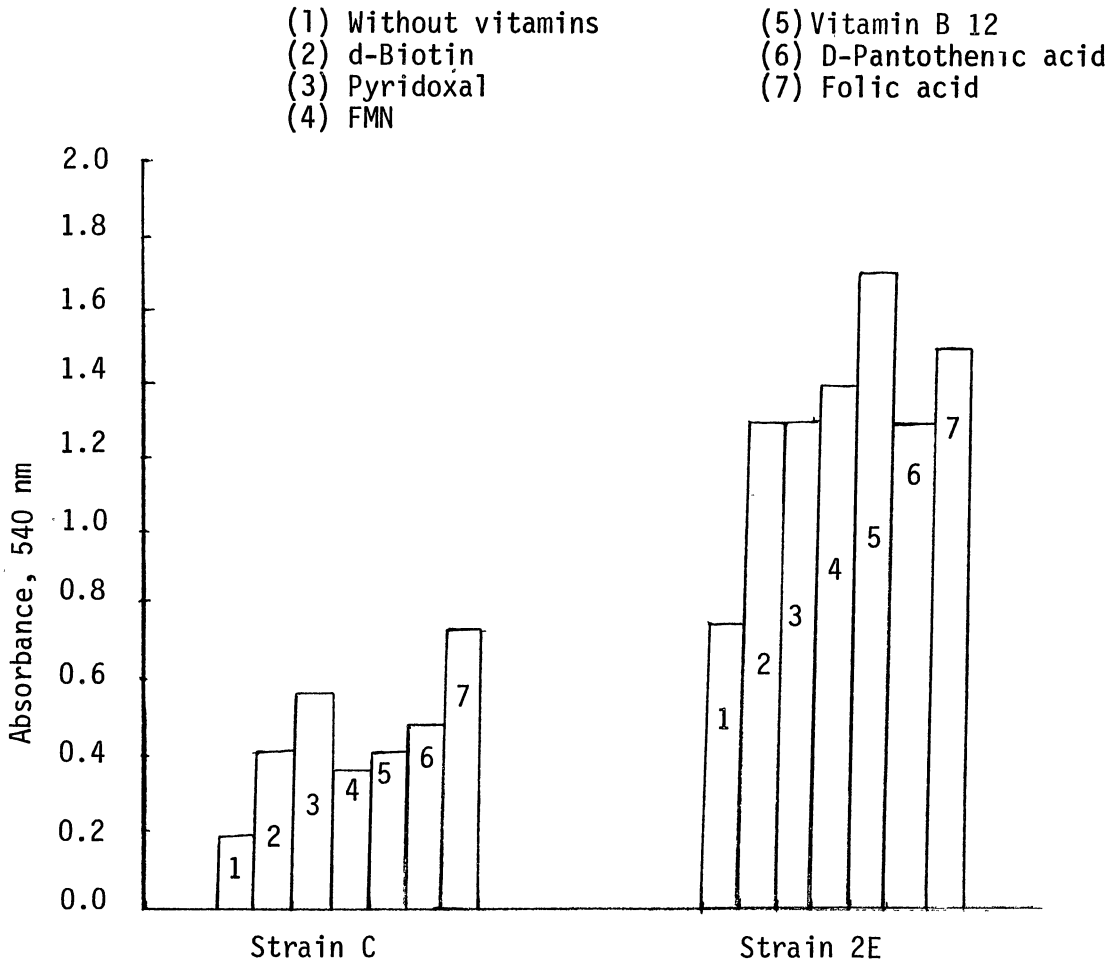

Figure 8. Growth of strains C and 2E in presence of vitamins. Time of observation is 40 hrs.

than that of strain 2E. Strain C caused complete blackening of medium B within a week. This blackening apparently arises through production of HS^- from thioglycollate rather than $SO_4^=$ reduction, since subsequent studies showed that these strains do not carry out respiratory sulfate reduction.

Both strains when grown in medium C and kept on the desk top without shaking, will form a viscous mat on the bottom of the container. This is due to the production of an extracellular slime or polysaccharide. The largest amount appears to be produced by strain C when grown in Postgate's medium C. Both media, and also medium F (Postgate) support excellent growth of strains C and 2E.

Effect of Polyacrylamides on Growth of Strains C and 2E

People in oil industry who have done field work with polyacrylamides believe that they somehow stimulate or enhance growth of microorganisms, which in some cases appear to be assosciated with a degradation of the polymer molecule. Since our strains were isolated from oil reservoir samples (crude oil or produced water), and since they show some kind of a mutualistic relationship with sulfate-reducing bacteria, we felt that some tests with polyacrylamides were in order. This is true primarily because growth of sulfate-reducing bacteria (SRB's) in the presence of polyacrylamide has been shown to bring about a degradation of the polymer molecule (7). I felt it was important to determine what effect(s) polyacrylamide might have on the pigmented aerobic strains, and also what effect(s), if any, the bacteria might have on the polymer.

The first polyacrylamide we chose to work with had a molecular

weight of 6-7X 10^6 daltons [5], was 25%-35% hydrolyzed (refers to hydrolysis of NH_2 group from amide side chain, not to a breaking of covalent bonds in the backbone of the molecule[6]).

I first tested whether these strains were stimulated in growth by polymer J333. The medium used was the defined medium with lactate as carbon-energy source, and 0.1% peptone. Two concentrations of polymer, 0.05% and 0.1%, were tested. The fifty milliliters samples were inoculated with 10 drops of cell suspension. The inoculum had an O.D. of 1.5. Incubation was at 34^{o} C with shaking on a rotory shaker. The results in Table XXI show that this polyacrylamide at 0.1% concentration does not inhibit the growth of these strains, but in regard to stimulation the results are inconclusive because of the very heavy and rapid growth in all cultures, including controls.

Since microbial growth in the presence of polyacrylamide has been associated with a loss of viscosity of the solution, I determined viscosities of these cultures after 72 hours of growth. The cells were removed by centrifugation prior to the measurement of viscosity. There was no significant decrease or increase in the viscosity of the polyacrylamide solutions as compared with uninoculated (but incubated) controls.

I next determined whether these aerobic organisms (C and 2E) could utilize polyacrylamide J279 as sole nitrogen source. This polymer has a molecular weight of 3 x 10^6 daltons and is 1-4% hydrolyzed. It

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[5] Length of Individual carbon chains may vary--hence the range is given.

[6] Designated as J333, this polymer is produced by, and was a gift to us from Dowell Division, Dow Chemical USA, Tulsa, Oklahoma.

TABLE XXI

EFFECT OF POLYMER J 333 (0.05% & 1.0%) ON GROWTH OF STRAINS C & 2E IN BASAL MEDIUM WITH LACTATE & 0.1% PEPTONE

Concentration of Polymer	Strain	Absorbance at 540 nm* 8 hrs.	18 hrs.
No Polymer	C	1.35	0.4 X 5
0.05%	C	1.40	0.4 X 5
0.1%	C	1.30	0.42 X 5
No Polymer	2E	1.60	0.85 X 5
0.05%	2E	1.50	0.80 X 5
0.1%	2E	1.50	0.79 X 5

* Five fold dilution was made to obtain absorbance reading. Zero time absorbance was 0.02 units in the inoculated controls.

was used at a higher concentration than the polymer of the previous experiment, i.e. 0.2% (W/V). The medium used was defined basal medium of pH 7.0, with lactate as carbon-energy source, and without NH_4Cl. The inocula used were ten drops of washed cell suspension (24 hrs. old and an O.D. of 0.5) per one hundred ml of medium. The flasks were incubated with shaking at 34°C. The results are presented in Figure 9. These results show that these strains are able to grow to a limited extent with J279 as sole nitrogen source. However, much heavier growth occurs if both NH_4Cl and J279 are in the medium (XXII). It is believed that this small amount of growth with polymer only results from the ammonia released by spontaneous hydrolysis of the polymer (6). In the past, growth of similar strains with polymer as sole nitrogen source has not resulted in any loss of viscosity of the polymer solutions. It was desirable to confirm this observation using the pigmented aerobic strains; therefore we determined screen factors of culture supernatants after growth had taken place. The data are given in Table XXII. This table also gives the measurements of growth of these strains. Neither strain C nor 2E produced a decrease in screen factor of J279 in presence or absence of NH_4Cl. J279 appears to enhance growth of both strains at 90 hrs.

Effect of Polyacrylamide J332 on Growth of Strains C and 2E in Postgate's Medium B

Polyacrylamide J332 (highest average molecular weight; 9-10 x 10^6 daltons) was tested for possible ability to enhance the growth of these strains C & 2E. The medium used was Postgate's medium B. One reason for using this medium was simply to detect whether the medium might

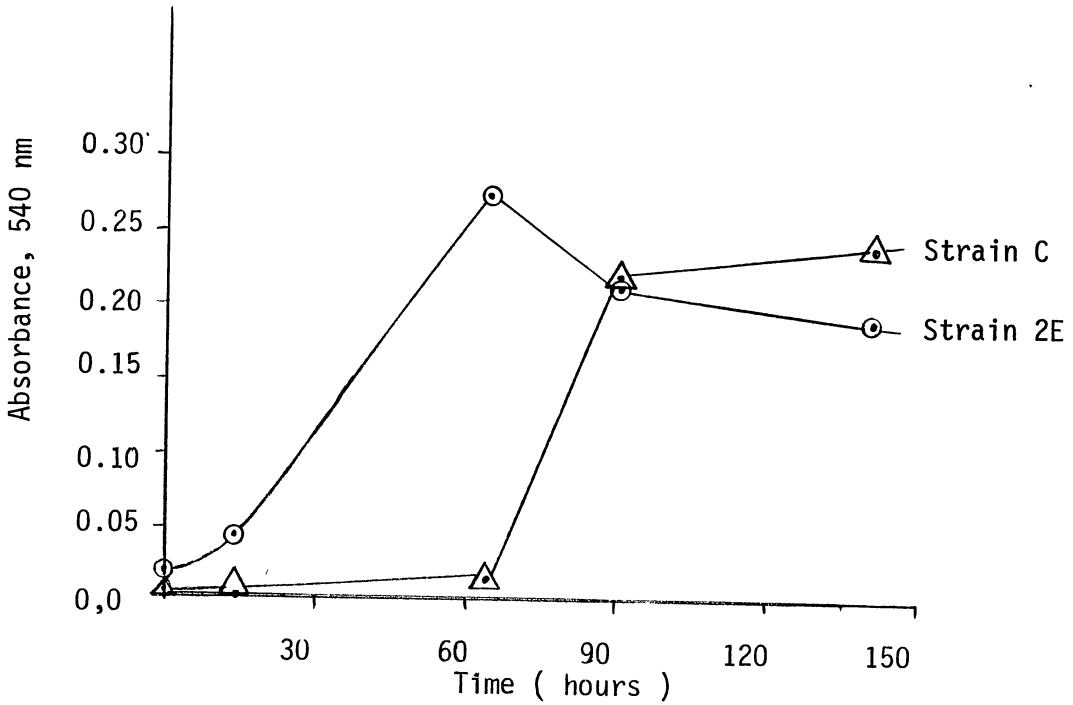

Figure 9. Growth of strains C and 2E with polyacrylamide J279 as a sole nitrogen source in chemically defined medium. Concentration of J279 is 0.2% (w/v).

TABLE XXII

EFFECT OF POLYMER J279 ON GROWTH OF STRAINS C AND 2E IN BASAL MEDIUM

Strain	Medium*	Absorbance** at 540 nm		Screen factor 144 hrs.
		14hrs	90 hrs.	
None	Basal + J279 (w/o NH_4Cl)	0.01	0.01	4.74
None	Basal + J279	0.01	0.01	4.11
C	Basal + NH_4Cl	0.00	0.80	-
C	Basal + J279 (w/o NH_4Cl)	0.00	0.22	5.12
C	Basal + J279 + NH_4Cl	0.02	1.55	4.17
2E	Basal + NH_4Cl	0.06	0.78	-
2E	Basal + J279 (w/o NH_4Cl)	0.00	0.21	4.99
2E	Basal + J279 + NH_4Cl	0.09	1.13	4.21

* Lactate was added as carbon and energy source

** Zero time absorbance was 0.02 units in inoculated samples

influence the ability of the polymer to stimulate growth. It has been observed that J332 enhances the growth of Desulfovibrio in medium B, especially if sulfate concentration was a limiting factor. Because of the mutualistic relationship between the "pink" strains and Desulfovibrio, it was thought worthwhile to use medium B.

The final concentration of polyacrylamide in the medium was 0.1% (W/V). Results are given in Table XXIII. At 20 hr, there appeared to be somewhat less growth (both strains) in the presence of the polymer than in its absence in Postgate's medium B.

Interactions of Aerobic Pigmented Strains With Xanthan Gum, A Biopolymer

Xanthan gums are extracellular, heteropolysaccharides produced by various strains of the genus Xanthomonas. This particular Xanthan gum used in our experimentation is produced by the organism Xanthomonas campestris NRRL B-1459. This is the strain used the most as a source of commercial Xanthan gum for both the food industry and the oil industry. Our source of Xanthan gum was Sigma Chemical Company. It was in the form of a dry, amorphous powder. It can be easily dissolved in aqueous solution with proper care. At a given weight concentration, solutions of Xanthan gum are more viscous than polyacrylamide solutions of the same concentration.

Effect of Autoclaving on Viscosity of Xanthan Gum in Absence of Added Sodium Lactate, in Defined Basal Medium

During my experimentation, I found that sodium lactate somehow

TABLE XXIII

EFFECT OF POLYACRYLAMIDE J332 (DOW) ON GROWTH OF STRAINS C AND 2E IN POSTGATE'S MEDIUM B *

Strain	Medium	Absorbance, 540 nm** 20 hrs.
None	Medium B + J332	0.00
C	Medium B	0.74
C	Medium B + J332	0.48
2E	Medium B	1.20
2E	Medium B + J332	1.10

* Concentration of polyacrylamide is 0.1% (w/v).
** Zero time absorbance was 0.01 units in the inoculated controls.

protects the viscosity of Xanthan gum while autoclaving. Figures are given in Table XXIV. When Xanthan gum was autoclaved in defined basal medium without lactate, the viscosity (Brookfield--in centipoises) was decreased to less than one-third of its value in the unautoclaved medium. If the medium was autoclaved with lactate, the viscosity was decreased by about 25 percent. In view of this reason, I adopted the method of autoclaving Xanthan gum separately (as stated in materials and methods) and adding it to the sterile medium.

Effect of Xanthan Gum on Growth of Strains C and 2E in Defined Basal Medium

The original goal of this work was to determine if the aerobic ("pink") strains of bacteria could degrade Xanthan gum directly, or if they could influence its degradation in any indirect way. Since active degradation of a substance by a microorganism usually involves utilization of some of the intermediate products of degradation (we are excluding from consideration, at this time, co-oxidation), I set out to determine if Xanthan gum could serve as a sole carbon source in a chemically defined medium. Additionally, if Xanthan gum could not serve as a sole carbon source, it seemed worthwhile to determine if the polymer could, possibly non-specifically, stimulate the growth of the pink organisms under conditions otherwise allowing some growth of the microorganisms.

If the latter proved to be the case, there would be some significance in this finding for the oil industry. Stimulation of growth of these aerobic organisms, which appear to be fairly widely distributed in reservoir environments, could be deleterious for oil recovery because of

TABLE XXIV

EFFECT OF LACTATE ON VISCOSITY OF XANTHAN GUM IN BASAL MEDIUM DURING AUTOCLAVING

Medium	Viscosity (centipoise)
Basal medium with lactate (Unautoclaved)	31.5
Basal medium with lactate (Autoclaved)	23.0
Basal medium without lactate (Autoclaved)	8.5

increased corrosion, increase in growth of sulfate-reducing bacteria, or both.

Finally, it may be that growth of the pink organisms in the presence of Xanthan gum would produce a loss of viscosity of the gum solution. Even a relatively minor alteration in the polymer structure may result in a loss of the rheological properties that make it useful in oil recovery.

These experiments were done using 100 ml culture volumes in 250 ml Erlenmeyer flasks. The inocula used were 10 drops of cell suspension (24 hr, O.D 0.5). The incubation was at 34°C, with shaking on a rotary shaker. Xanthan gum was sterilized separately and added to sterile basal medium aseptically. From these experiments, I found that Xanthan gum stimulates the growth of these strains through decreasing the length of lag phase. Figure 10 shows the early growth stimulation of strain 2E by 0.1% Xanthan gum. Similar results were obtained using strain C (Figure 11).

As stated before, Xanthan gum shows a decrease in viscosity when autoclaved in the basal medium devoid of lactate. I next tested if this Xanthan gum (with loss of viscosity) would stimulate the growth of strains C and 2E. Sterile lactate was added as carbon-energy source to the autoclaved medium which contained 0.1% Xanthan gum. Figure 12 shows the effect on growth of strain 2E by autoclaved gum. As shown in the figure, Xanthan gum autoclaved separately from the medium stimulated early growth, resulted in a significantly higher level of growth, and less loss of absorbance as the culture ages. Xanthan gum autoclaved in the medium (lactate was added later) did stimulate early growth, but did not significantly increase the level of growth attained. Figure 13

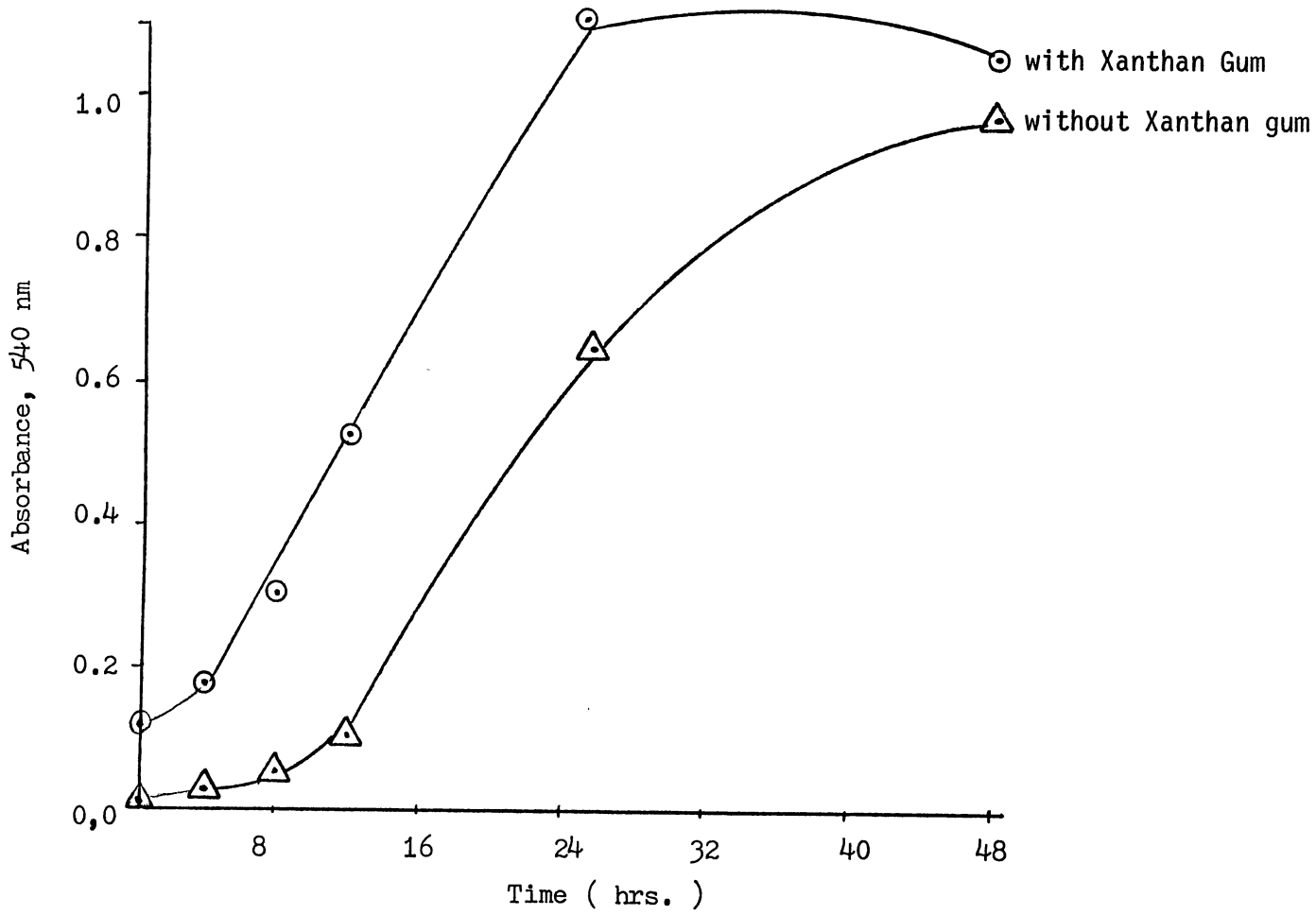

Figure 10. Growth stimulation of strain 2E in defined basal medium by 0.1% Xanthan gum.

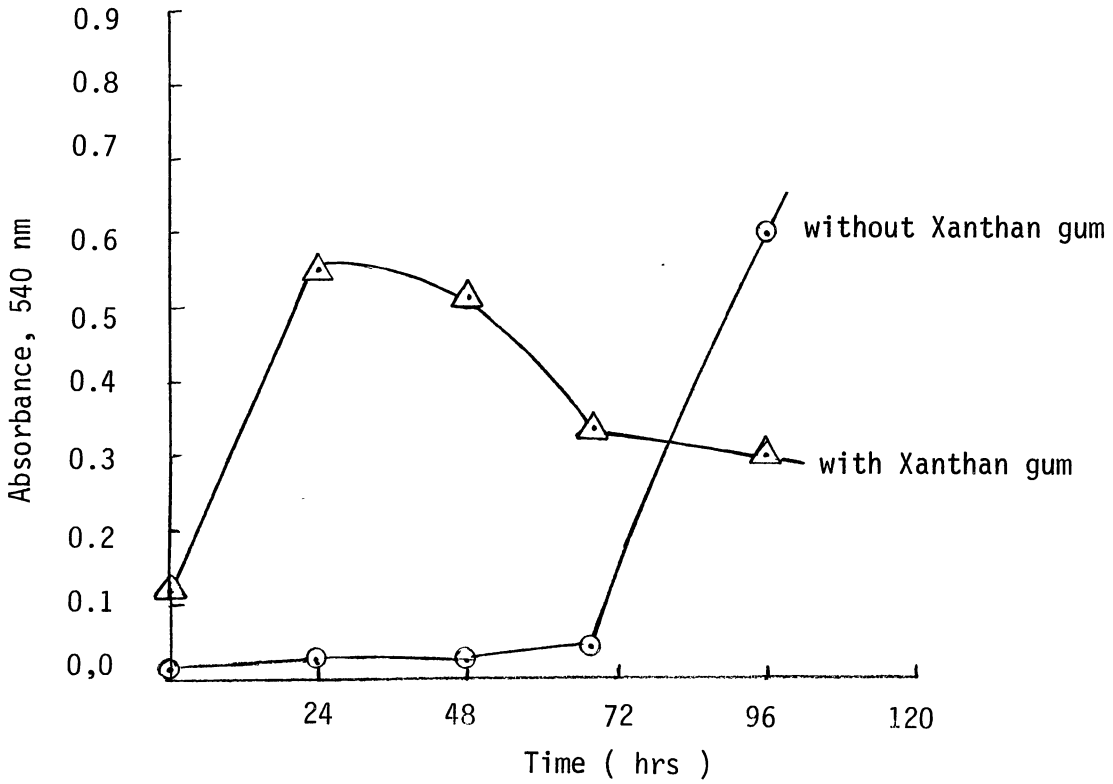

Figure 11. Growth stimulation of strain C in defined basal medium by 0.1% Xanthan gum.

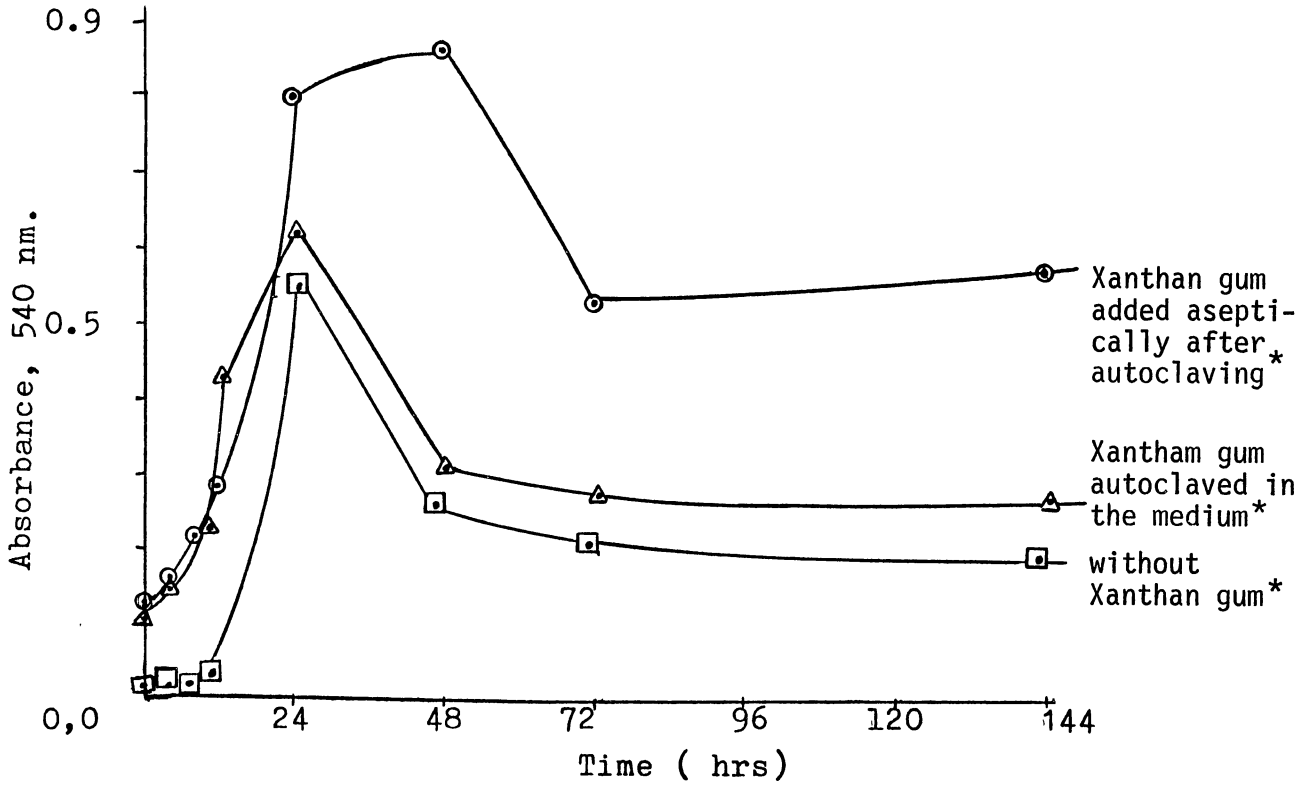

Figure 12. Effect of Xanthan gum (when autoclaved in medium without lactate) on growth of strain 2E in defined basal medium.
*Sterile lactate was added to the medium aftter autoclaving.

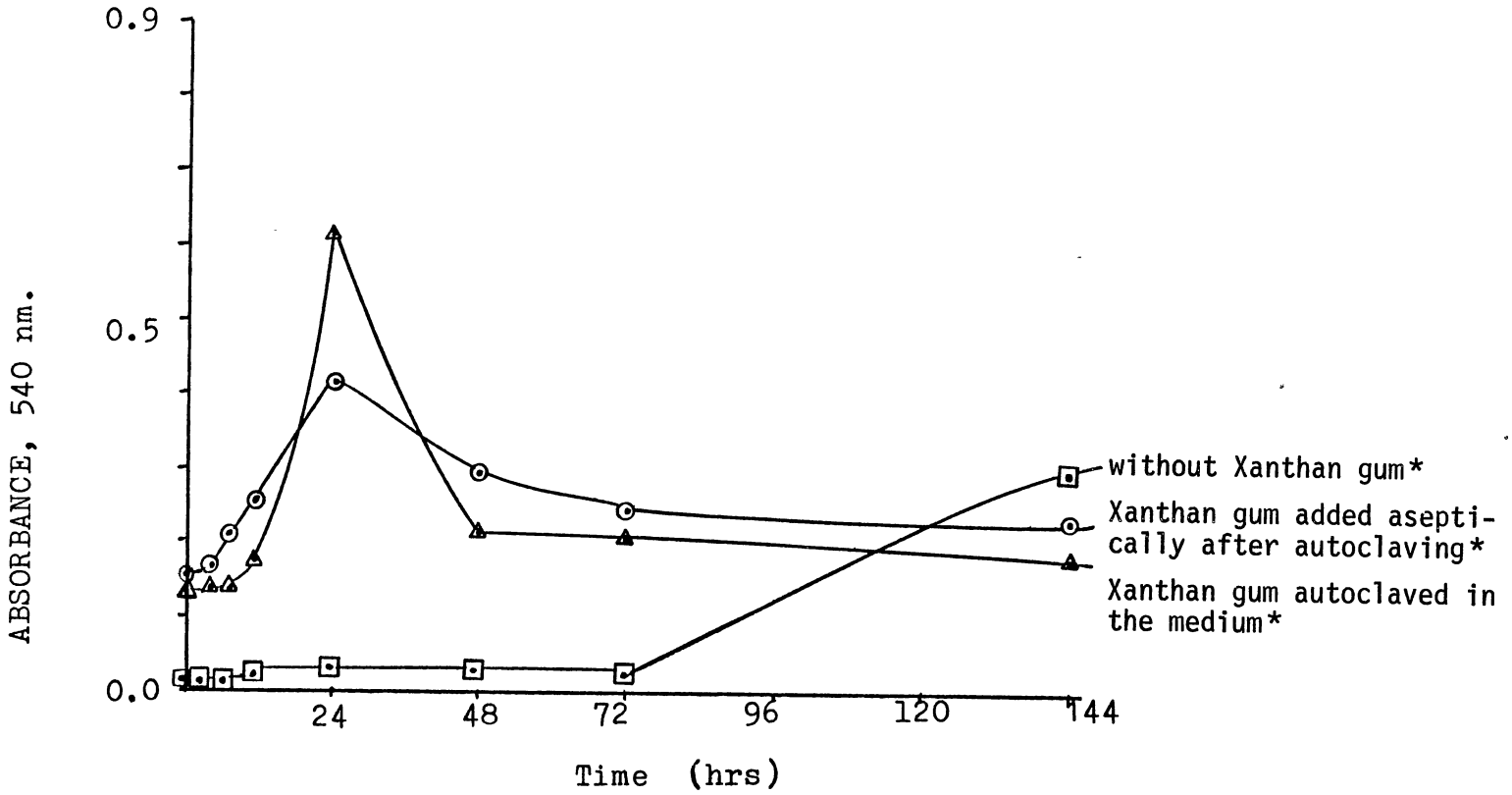

Figure 13. Effect of Xanthan gum (when autoclaved in medium without lactate) on growth of strain C in defined basal medium.
* Sterile lactate was added to the medium after autoclaving.

gives the results obtained using strain C. Strain C grows very slowly in basal medium plus lactate without any Xanthan gum. Xanthan gum when autoclaved in the basal medium without lactate (but lactate was added later), does not stimulate the early growth as well as when added aseptically after autoclaving. Effects on strain C were less pronounced, and a large loss in absorbance was noted with Xanthan gum autoclaved in the medium. Growth of strain C in the chemically defined medium with lactate is slow; maximum absorbance was not attained until 144 hr of incubation.

After each experiment, the media were tested for loss of viscosity possibly resulting from the degradation capabilities of these organisms. Such reductions were not observed.

Effect of Dialyzed Xanthan Gum and Flocon on Growth of Strains C and 2E

If these organisms are able to grow faster in presence of Xanthan gum without causing any damage to the molecular structure of the polymer (such as loss of viscosity), the question arises as to whether impurities possibly present in the Xanthan Powder or Flocon (a liquid form of Xanthan gum consisting basically of crude fermentation medium with formaldehyde added) may be stimulating growth. In order to clarify this question, Xanthan Powder and Flocon, treated by dialysis to remove low molecular weight impurities, were tested for their ability to stimulate growth of these strains. The pore size of the dialysis bag was 12,000 (molecular weight cut off). The samples were dialyzed in 0.01 M phosphate buffer, for 24 hours. This procedure should eliminate any inorganic impurities, if present in the preparation. The experiments were conducted in exactly the same way as previously, except for the use of

dialyzed Xanthan gum. The results using Xanthan Powder and Flocon are given in Tables XXV and XXVI respectively. The concentration of Xanthan gum used were 0.1% (W/V). Again, there is a significant early growth stimulation of these strains by both dialyzed Xanthan Powder and dialyzed Flocon. The viscosity of Xanthan gum was not affected by these organisms. This indicates that a dialyzable impurity is not responsible for early growth stimulation. In addition, it shows that toxic formaldehyde (added at a level of 3000 ppm) in Flocon is completely removed by dialysis.

Stimulation of Growth by Xanthan Gum in the Presence of Sodium Chloride

Oil reservoirs often contain brine. So, it is important to study what effect Xanthan gum may have on the growth of these strains in presence of sodium chloride. The medium used for this experiment was basal medium with lactate as carbon-energy source. A concentration of 2.5% (W/V) sodium chloride was added to the medium except in some control flasks. Concentration of Xanthan gum used was 0.1% (W/V). Both strains were tested in this experiment for their ability to grow in the medium and to alter the viscosity of the Xanthan gum solution. The results are presented in Tables XXVII and XXVIII. These tables give the measurements of viscosity of the culture supernatants in addition to absorbance (growth).

The stimulation of growth of strain C, and to a lesser extent, of strain 2E, by 2.5% NaCl alone was not unexpected. When freshly isolated, several of the"pink"strains were moderately stimulated by 2.5-3.0% added NaCl. Both strains C and 2E could well be of marine origin since

TABLE XXV

EFFECT OF DIALYZED XANTHAN GUM (SIGMA) ON GROWTH OF STRAINS C AND 2E IN DEFINED BASAL MEDIUM

Strain	Medium *	Absorbance, 540 nm**		
		12 hrs.	21 hrs.	90 hrs.
None	Basal + Xanthan gum	0.11	0.11	0.11
C	Basal (w/o Xanthan gum)	0.03	0.05	0.40
C	Basal + Xanthan gum	0.41	1.40	1.62
2E	Basal (w/o Xanthan gum)	0.28	0.42	1.20
2E	Basal + Xanthan gum	0.44	1.51	1.55

* Lactate was added as sole carbon-energy.
** Zero time absorbance was 0.01 units in the inoculated controls.

TABLE XXVI

EFFECT OF DIALYZED FLOCON (0.1%) ON GROWTH OF STRAINS C AND 2E IN DEFINED BASALMEDIUM

Strain	Medium	Absorbance, 540 nm*	
		12 hrs.	72 hrs.
None	Basal + Flocon	0.23	0.25
C	Basal (w/o Flocon)	0.01	0.71
C	Basal + Flocon	0.52	0.81
2E	Basal (w/o Flocon)	0.06	0.20
2E	Basal + Flocon	0.82	1.25

* Zero time absorbance was 0.01 units in the inoculated controls.

TABLE XXVII

EFFECT OF 2.5% SODIUM CHLORIDE ON GROWTH OF STRAIN C IN DEFINED BASAL MEDIUM (WITH LACTATE) PLUS 0.1% XANTHAN GUM

Strain	Medium	Absorbance at 540 nm*		Viscosity (centipoise)
		18 hrs	42 hrs	42 hrs
none	Basal + Xanthan gum	0.12	0.12	28.0
none	Basal + Xanthan gum + NaCl	0.12	0.12	30.0
C	Basal (W/O Xanthan gum)	0.02	0.02	-
C	Basal + NaCl (W/O Xanthan gum)	0.05	0.30	-
C	Basal + Xanthan gum	0.21	0.96	30.0
C	Basal + Xanthan gum + NaCl	0.98	1.25	30.0

* Zero time absorbance was 0.01 units in the inoculated controls.

TABLE XXVIII

EFFECT OF 2.5% SODIUM CHLORIDE ON GROWTH OF STRAIN 2E IN DEFINED BASAL MEDIUM (WITH LACTATE) PLUS 0.1% XANTHAN GUM

Strain	Medium	Absorbance at 540 nm*		Viscosity (cps)
		18 hrs	44 hrs	44 hrs
None	Basal + xanthan gum	0.10	0.10	23.0
None	Basal + xanthan gum + NaCl	0.12	0.12	23.0
2E	Basal (W/O xanthan gum)	0.23	0.78	-
2E	Basal + NaCl (W/O xanthan gum)	0.20	1.20	-
2E	Basal + xanthan gum (W/O NaCl)	0.85	1.10	23.5
2E	Basal + xanthan gum + NaCl	0.55	1.00	23.0

* Zero time absorbance was 0.01 units in the inoculated controls.

they were isolated from water samples obtained from the Wilmington field, which is located near Long Beach, California, near the Pacific Ocean. Xanthan gum and NaCl together result in what appears to be an additive effect with strain C (Table XXVII). With strain 2E, it is possible that I missed the time of maximum absorbance because of infrequent observations. In no case was any loss of viscosity of Xanthan gum observed.

Testing for Ability of Xanthan Gum to Serve as a Sole Source of Carbon and Energy

Experiments were done to study whether Xanthan gum could serve as a sole source of carbon and energy for these organisms. I used chemically defined basal medium devoid of lactate. The concentration of Xanthan gum used was 0.1% (W/V). Under every condition I used, there was no growth with Xanthan gum as sole source of carbon and energy. The most likely manner in which this polymer could supply carbon-energy for a growing bacterium would be for the organism to remove the terminal pyruvylacetal group on the side chains. Such a reaction would severly damage or destroy the rheological properties of the polymer solution.

Growth of Strain 2E, with 0.2% Xanthan Gum and 2 ppm DBNPA (A Biocide) in Defined Basal Medium

Although I did not have time in this study for extensive study of the effects of Xanthan gum in the medium on the toxicities of the biocides tested earlier, I thought that at least one experiment in this area would be interesting. Therefore, I tested whether 0.2% Xanthan gum would stimulate the growth of strain 2E in defined basal medium in

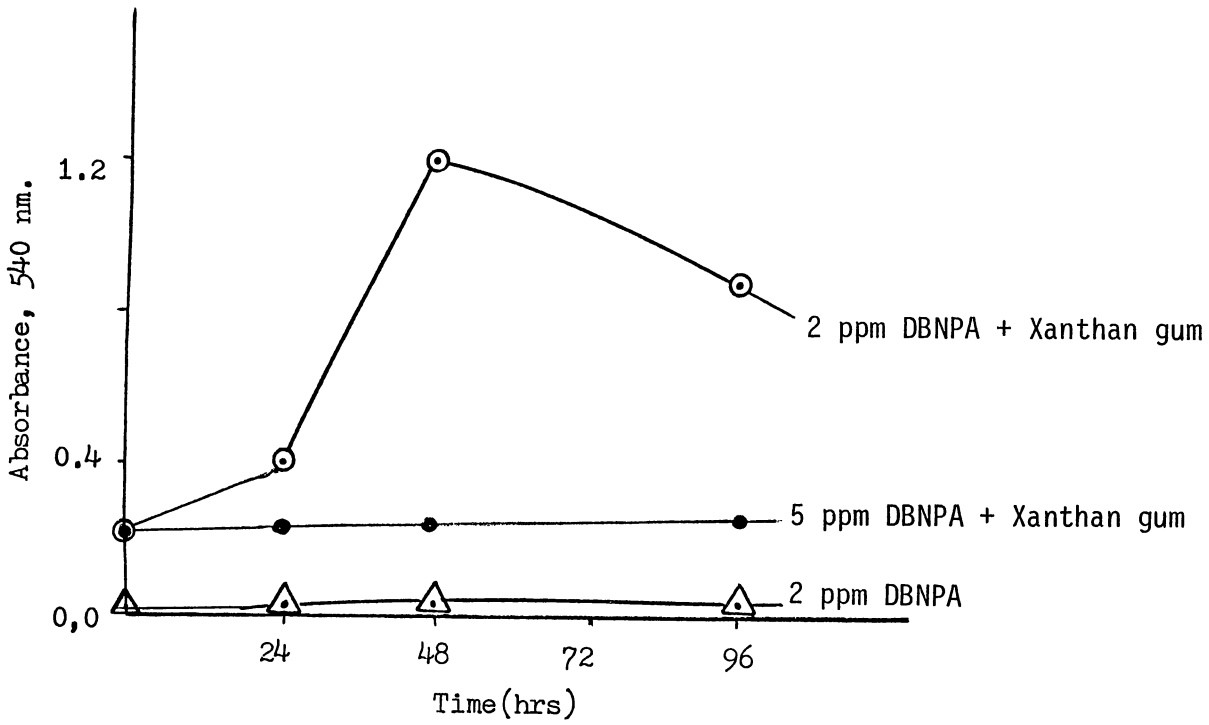

Figure 14. Effect of Xanthan Gum on Toxicity of DBNPA for Strain 2E in Defined Basal Medium.

presence of 2 ppm and 5 ppm DBNPA. From my previous experiments, I had found that DBNPA at a concentration of 2 ppm would inhibit the growth of strain 2E completely in defined basal medium (without Xanthan gum). Results from this experiment are plotted in Figure 14. Xanthan gum at 0.2% does decrease the toxicity of DBNPA in the defined basal medium. However, the decrease is only by a factor of two, as compared to the roughly 100-fold decrease resulting from the use of a complex medium such as TSB. The viscosity of the culture supernatants was not altered. Because of the comparatively small decrease in toxicity of the biocide, and the need to pursue other lines of investigation, further experiments in this area were not done.

Effect of Strains C and 2E on Viscosity Of Xanthan Gum in Postgate's Medium C

I have already shown that the composition of the growth medium influences the effects of Xanthan gum on growth of strans C and 2E. Since these strains grow well in Postgate's Medium C, a rich medium used for growing sulfate-reducing bacteria, it was thought worthwhile to test the effects of Xanthan gum on growth of the strains in this medium.

Both strains produce very noticeable amounts of a pink pigment while growing in Postgate's Medium C. Strain C appears to be a darker pink in this medium than 2E; both grow very well--at a faster rate than in any other medium tested. Postgate's Medium C was prepared as in Table III. To this medium, sterile Xanthan gum solution was added to get a final concentration of 0.1% (W/V). The culture volumes were 100 ml (in 250 ml Erlenmeyer flasks). The inoculum was prepared by washing the cells off a TSA slant (washed two times). The O.D of inoculum was 0.5. Ten drops

of the suspension was used to inoculate each 100 ml medium. The flasks were incubated with shaking at 34°C. Absorbance readings were taken at 12, 48, 72 hr, and at five times thereafter. Viscosity measurements were also made at these intervals. Figure 15 shows the growth curves obtained for strain 2E in Postgate's Medium C. In this medium, no early stimulation of growth by Xanthan gum, as manifest by a shortening of the lag phase, was observed; this is in marked contrast to its effect in the basal defined medium. However, a significantly smaller loss of absorbance from its maximum value, attained at 72 hr, was observed in the presence of Xanthan gum. This can not be attributed to the absorbance of the Xanthan gum itself; the latter has a value of only about 0.10 absorbance units at a concentration of 0.1%. The difference in absorbance shown in Figure 13 is at least 0.3 units. Similar results were obtained in the case of strain C also (Figure 16).

Figure 17 shows the viscosity measurements obtained from this experiment. As indicated, there was a slight decrease in the viscosity of Xanthan gum below that of the control, but only after 96 hours of incubation. There was an increase in viscosity of the uninoculated (but incubated) controls. This increase is thought to be a result of unwinding of the Xanthan gum helix (5). Growth of the microorganisms apparently prevents this increase.

Table XXIX shows the effect of Xanthan gum on growth of the strains C and 2E in various media. Results of viscosity were also given in this table. Effects of Xanthan gum on growth in various media are complex and varied. I can conclude that the polymer is unable to serve as a sole carbon-energy source. Under most conditions it shortens the lag phase, thus appearing to stimulate early growth. The mechanism of this

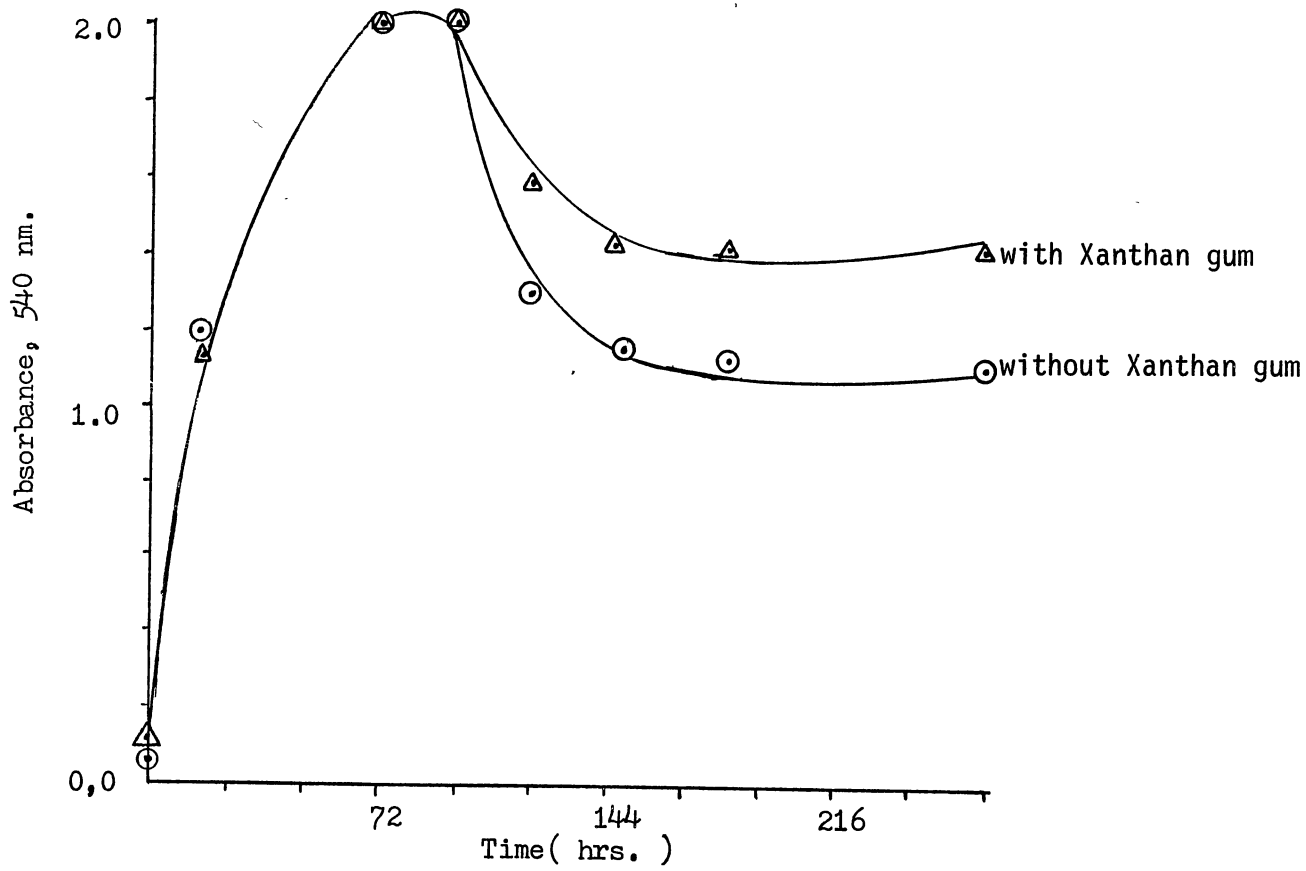

Figure 15. Growth of strain 2E in Postgate's Medium C with Xanthan gum

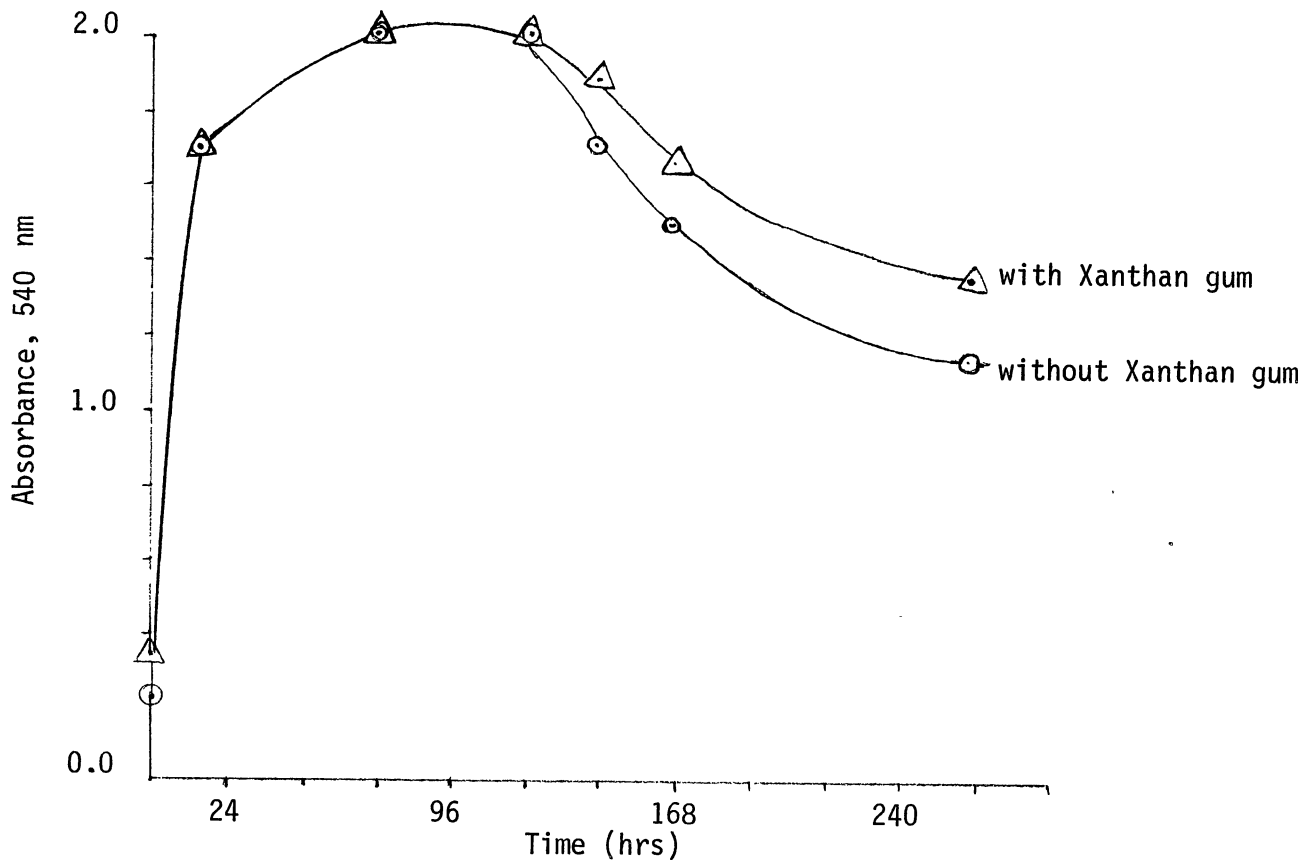

Figure 16. Growth of strain C in Postgate's Medium C with Xanthan gum.

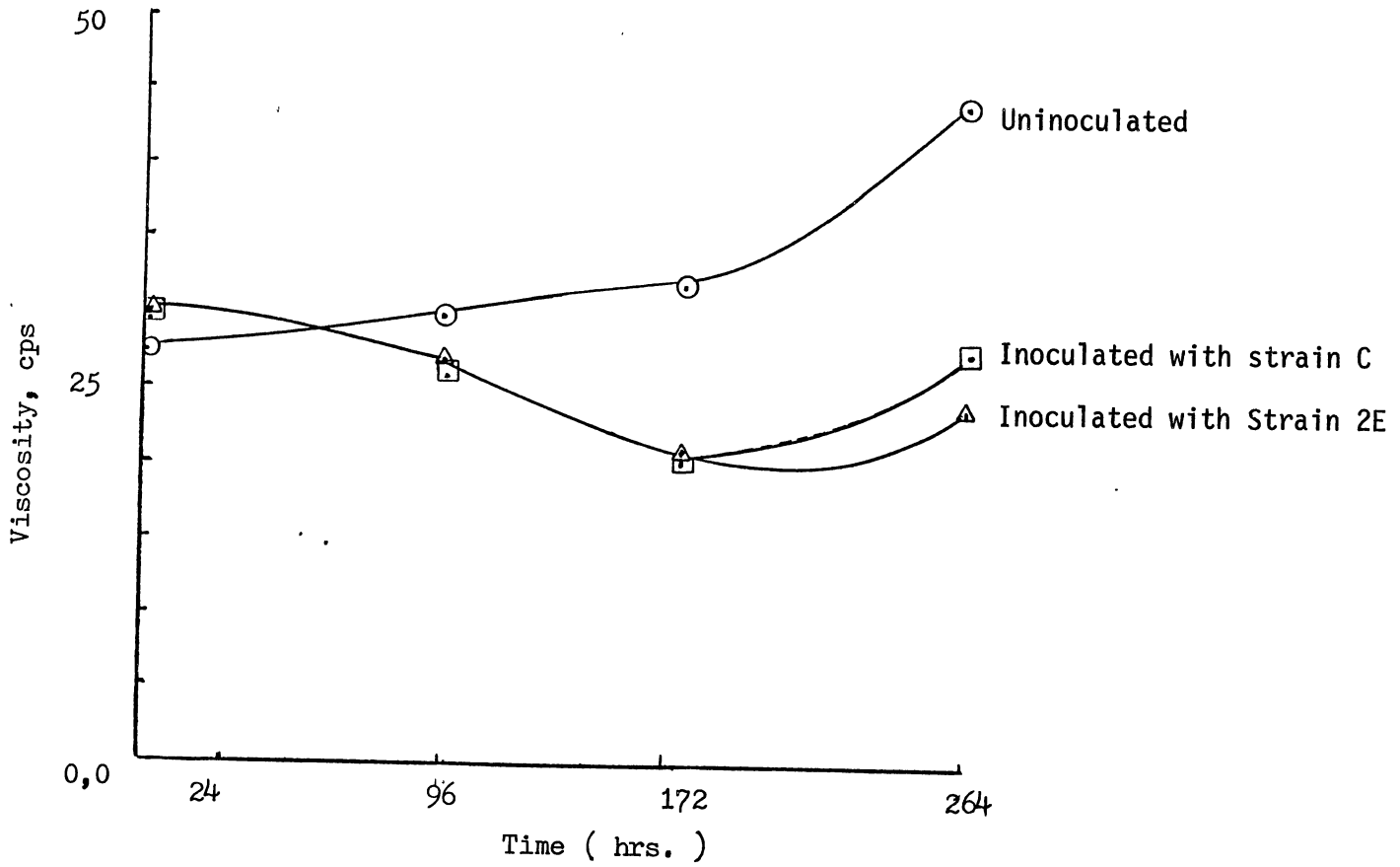

Figure 17. Effect of Strains C and 2E on Viscosity of Xanthan Gum in Postgate's Medium C.

TABLE XXIX

SUMMARY OF EFFECTS OF XANTHAN GUM ON GROWTH OF STRAINS C AND 2E IN VARIOUS MEDIA, AND EFFECT OF GROWTH ON VISCOSITY OF XANTHAN GUM SOLUTIONS

Medium*	Strain	Growth	Reduction in viscosity
Tryptic soy broth	2E, C	Maximum growth	None
Basal + 1% peptone	2E, C	Maximum growth	None
Basal medium	2E, C	Early growth stimulated	None
Basal + 2.5% NaCl	2E, C	Early growth stimulated	None
Basal + 1% peptone (without lactate)	C	Less than maximum growth	None
Basal (without lactate)	2E	No growth	None
Postgate's Medium C	2E, C	Maximum growth	Yes

* All media contained Xanthan gum at 0.1% (w/v)

effect and of the increase in maximum absorbance attained, and decrease in loss of absorbance in old cultures, remain unknown. The fact that the solutions of the polymer did not (except in the case of old cultures in Medium C) show any decrease in viscosity as compared to the uninoculated control indicates that no appreciable biodegradation of Xanthan gum by these strains is taking place.

Experiments Designed to Obtain Direct Evidence for Degradation of Xanthan Gum by Strains C & 2E

Since ability to remove individual monomers of Xanthan gum (particularly from the side chains) by a metabolic reaction would probably constitute the initial reactions in degradation, I sought to obtain direct evidence that this may have occurred.

My first test was for the ability of the monomers found in the side chains to support the growth of these organisms. Pyruvate (the terminal group on most of the side chains) has already been shown to support good growth in the basal chemically defined medium. Acetate (attached to first D-mannose residue of side chain as acetyl group) supports good, but somewhat delayed growth. So it seemed logical to test D-mannose and D-glucuronic acid (the central residues in the side chains) along with acetate and pyruvate for ability to support growth of these organisms. Table XXX shows the results obtained from this experiment. These organisms are able to use pyruvate as well as lactate as sole carbon and energy source. D-glucuronic acid, and D-mannose did not allow growth of these organisms. Acetate allowed very little growth of strain 2E but not strain C.

TABLE XXX

EFFECT OF DIFFERENT COMPOUNDS ON GROWTH OF STRAINS C & 2E IN DEFINED BASAL MEDIUM AS SOLE CARBON AND ENERGY SOURCE

Strain	Carbon and energy Concentration, 0.1M	Absorbance at 540 nm*	
		17 hrs	90 hrs
C	None	0.00	0.00
C	Glucose	0.00	0.00
C	Sodium lactate	0.02	0.90
C	Sodium acetate	0.00	0.00
C	Pyruvate	0.03	1.10
C	D-glucuronic acid	0.01	0.01
C	D-mannose	0.02	0.02
2E	None	0.02	0.02
2E	Glucose	0.00	0.02
2E	Sodium lactate	0.09	1.93
2E	Sodium acetate	0.02	0.22
2E	Pyruvate	0.20	1.98
2E	D-glucuronic acid	0.01	0.01
2E	D-mannose	0.02	0.02

* Zero time absorbance was 0.01 in all the inoculated controls.

Cadmus (1), isolated a Bacillus species which could degrade Xanthan gum into its smaller components. In the analysis, they found the end products to be D-glucuronic acid, D-mannose, pyruvylated mannose, acetyl D-mannose, and β (1→4) linked glucose backbone. I used methods similar to those used by Cadmus (1) to determine whether strains C and 2E were capable of producing any of the above end products from the Xanthan molecule. Strains C and 2E were grown in Postgate's Medium C (since best viscosity reduction had occurred in this medium) containing 0.1% (W/V) Xanthan gum. Culture volumes were 125 ml in 250 ml Erlenmeyer flasks. Inocula and methods of incubation were the same as given earlier. The viscosity of the medium was measured before incubation. After one week of incubation, measurements with the Brookfield viscometer showed that a significant decrease in the viscosity of Xanthan gum below that of the control had occurred. Data are given in Table XXXI. There was 7-8 centipoises decrease in the viscosity of the media, after one week of incubation.

At this point, the culture was centrifuged at 6000 RPM for 10 minutes, removing the cells and associated extracellular polysaccharide, and the supernatant was saved for further experimentation. If the Xanthan molecule had lost its viscosity because of extracellular enzymatic activity of these strains, the supernatant should contain the non-assimilated end products of degradation (probably side chain monomers) and the non-degraded β -glucose 1, 4 linked backbone along with the extracellular enzymes. In order to separate the high molecular weight molecules from small fragments or molecules, the supernatant (50 ml) was dialyzed in 200 ml (0.01 M) phosphate buffer (pH 7.0) for 36 hr, in a 12,000 molecular cut-off dialysis bag. After dialysis, 100 ml of the dialysate

(i.e., the phosphate buffer) was collected and concentrated to 10 ml using a lyophilizer. This 10 ml samples was analyzed for presence of free sugars such as D-mannose, D-glucose, and D-glucuronic acid, using thin layer chromatography (TLC). I also analyzed for pyruvate, using the Haidle-Knight assay for carbonyl-containing compounds. Methods of these analyses are given in detail in the Materials and Methods section. The results for these assays turned out to be negative (i.e., no free sugars or pyruvate were detected). I then lyophilized the remainder of the above sample to powder, and diluted in 1 ml of deionized distilled water. This 1 ml of sample was again analyzed for presence of D-mannose, D-glucose, and D-glucuronic acid, using TLC. Again, the results were negative. If any degradation involving release of side chain monomers had occurred, all products had been assimilated.

In order to detect acetic acid--which could have been produced by deacetylation of the proximal D-mannose group, or be a result of pyruvate oxidation, I analyzed the concentrated dialysate by gas chromatography. Details are given in the Materials and Methods section. I found considerate amounts of acetic acid in the samples that were inoculated with strain C or strain 2E. The results are given in Table XXXI.

It was not clear from this experiment whether the acetic acid produced was because of the degradation of Xanthan gum. It is possible that the acetic acid may be an end product of lactate oxidation, which would release acetate to the medium. To check this possiblity, I analyzed for presence of acetic acid in a separate experiment, using as controls Postgate's Medium C, with and without Xanthan gum, inoculated with strain C or 2E. After incubating for 10 days, the supernatant was

TABLE XXXI

EFFECT OF STRAINS C AND 2E ON VISCOSITY OF XANTHAN GUM IN POSTGATE'S MEDIUM C

Strain	Medium*	Absorbance at 540 nm		Viscosity in cetipoise		Concentration of acetic acid (u mol/ml supernatant)
		0 hrs.	7 days	0 hrs.	7 days.	
None	Without Xanthan gum	0.00	0.00	-	-	not done
None	With Xanthan gum	0.10	0.10	22.0	24.5	trace
C	Without Xanthan gum	0.02	1.8	-	-	not done
C	With Xanthan gum	0.12	1.91	22.0	15.0	1.03
2E	Without Xanthan gum	0.03	1.88	-	-	not done
2E	With Xanthan gum	0.13	2.00	22.0	14.0	0.864

* Concentration of Xanthan gum present in the medium is 0.1% (w/v)

analyzed for presence of acetic acid (the supernatant was not dialyzed or concentrated). I found acetic acid in all the inoculated controls i.e., samples without Xanthan gum. However, I did not find any significant additional accumulation of acetic acid in the inoculated samples that contained Xanthan gum, when compared to those without Xanthan gum. The acetic acid detected in all the inoculated controls may be due to lactate oxidation. I did not observe any correlation between the reduction of viscosity and an increase in acetic acid accumulation. From the data obtained, I can not conclude that Xanthan gum is being degraded by the "pink" organisms, under the conditions provided.

Preliminary tests of the ability of these strains to produce one or more extracellular enzymes which could alter the viscosity of Xanthan gum solutions were done. Cells of both strains were grown in Postgate's Medium C with Xanthan gum. After one week of incubation (i.e., when the viscosity of Xanthan gum was reduced below that of the control), a 5 ml aliquot of the culture supernatant was added to 25 ml of fresh Xanthan gum (0.1%) solution and incubated for 24 hours. After the incubation, the Xanthan solutions were tested for reduction of viscosity. No reductions were observed. The data are given in Table XXXII.

Although the "pink" strains are highly aerobic, they will grow to a limited extent under anaerobic conditions with nitrate as an electron acceptor. It is known that Xanthan gum can be degraded anerobically (A. Laskin, Personal Communications to M. Grula), but the nature of the microorganism or consortium of microorganisms involved, is largely unknown. As a final check, I thought that an experiment looking into the possibility of anaerobic degradation of Xanthan gum by the pink organisms might be worth doing. Two media, Postgate's Medium C and a

TABLE XXXII

EFFECT OF CRUDE CULTURE SUPERNATANT CONTAINING STRAIN C OR 2E ON VISCOSITY OF XANTHAN GUM SOLUTION

Xanthan gum solution (0.1%)	Viscosity (cps) 0 hrs.	24 hrs.
Without culture supernatant	22.5	22.0
With culture supernatant (containing strain C)	23.0	23.0
With culture supernatant (containing strain 2E)	23.0	23.0
With autoclaved culture supernatant (containing strain C)	18.0	18.0
With autoclaved culture supernatant (containing strain 2E)	15.0	15.0

medium used to isolate a "xanthanase" producing strain by Cadmus et al., (1) were used, but neither strains grew anaerobically in either medium. The composition of this medium is given in the Materials and Methods section.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The aerobic strains of bacteria isolated by the petroleum microbiology group at Oklahoma State University, from nine or ten oil reservoirs in different geographical locations are characterized by the production of a pink or orange pigment and an extracellular polymer which causes the cells to cohere in clumps. The nature of this sticky polymer is unknown at present. All strains examined so far are aerobic facultative methylotrophs. They most closely resemble the genus Pseudomonas, although none have been completely identified yet.

Strains C and 2E, although they grow much faster in rich complex media than a minimal media, do not have an absolute requirement for any vitamin, amino acid, or other organic growth factor. Lactate and pyruvate are preferred carbon-energy sources; glucose is unable to serve as a sole carbon-energy source in a chemically defined medium. Certain vitamins or groups of amino acids will markedly stimulate growth in an ammonium lactate medium.

It does not appear that these strains constitute a threat to the polymer Xanthan gum in situ. Although Xanthan gum in concentrations in the range 0.1-0.2% (W/V) stimulates growth of these aerobic strains C and 2E under some conditions, no evidence either direct or indirect, for an ability of the strains to degrade Xanthan gum was obtained.

The enhancement of growth of these strains by a combination of sodium chloride and Xanthan gum, and the possiblity of an increase in the minimum inhibitory concentration of biocide in the presence of Xanthan gum, indicate that the biopolymer could indirectly contribute to problems resulting from the growth of these microbes in oil reservoirs.

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