

ISOLATION AND PARTIAL CHARACTERIZATION  
OF A BIO-EMULSIFIER PRODUCED  
BY A CLOSTRIDIAL ISOLATE

By

DENNIS LYNN BRYANT

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Oklahoma State University

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Thesis Approved:

*Glean W. Todd*

Thesis Adviser

*Mary M. Grula*

*George V. Odell*

*Norman A. Dunham*

Dean of the Graduate College

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## CHAPTER I

### INTRODUCTION

The increased awareness of the world's dwindling oil reserves and the subsequent rise in oil prices over the past decade can be seen in the heightened interest in enhanced oil recovery technologies.

Enhanced oil recovery refers to any technology which is used to recover oil remaining in a formation following primary and secondary techniques. In primary recovery naturally occurring forces, such as those associated with gas and liquid expansion or influx of water from aquifers, are used to produce the oil. Secondary recovery methods normally involve water flooding of reservoirs in order to supplement the original reservoir pressures and force more oil from the reservoir. One of the technologies under study is Microbially Enhanced Oil Recovery (MEOR), which involves the use of microorganisms and their metabolic products as enhanced oil recovery agents.

Microbially Enhanced Oil Recovery involves the injection of microorganisms into an oil reservoir. A medium for growth of the organisms is injected into the reservoir either along with the organisms or following the injection of the organisms. The metabolic products from the organisms then become the chemical and physical agents causing the release of additional oil from the inoculated well.

The bacteria to be used in MEOR must be able to grow under anaerobic conditions, and depending on the individual reservoir, grow

under conditions of high saline concentrations, high pressure and high temperatures (McInerney, 1982). A possible drawback to MEOR is the difficulty of finding bacteria which are able to grow will under the constraints of oil reservoir environments and still produce high yields of end products in situ which would contribute to oil recovery. The desired metabolic characteristics for bacteria to be used in MEOR include:

- a) The production of large amounts of gas to repressurize the reservoir ( $\text{CO}_2$  is especially useful because of its ability to lower the viscosity of oil when dissolved in the oil).
- b) The production of large amounts of low molecular weight organic acids. Sufficient amounts of such acids should act to dissolve limestone (producing additional  $\text{CO}_2$ ), as well as to dislodge oil that was attached to such rock.
- c) The production of low molecular weight solvents, primarily alcohols and acetone. The presence of these compounds in sufficient quantities should result in easier removal of crude oil adhering to the rock or trapped in rock pores.
- d) The production of large amounts of low molecular weight non-ionic emulsifiers that would form oil-in-water emulsions and thus result in "solubilization" of the crude oil from rock surfaces and pores (Grula et al., 1982).

As stated above, among the microbial metabolic products which may prove useful in MEOR are surfactants and hydrocarbon-emulsifying compounds; surfactants have been used in enhanced oil recovery since the late 1920's and are used in one of two ways. One is to inject the well with a large volume of solution having a low concentration of a surfactant so that the interfacial tension between the oil and aqueous solution is lowered and the large volume of the solution passing through the reservoir eventually removes most of the oil. The other method is to inject the well with a small volume of a solution having a

high concentration of a surfactant, which results in a stable hydrocarbon-in-water emulsion which is pushed out of the reservoir often by a high-viscosity polymer flood (Gogarty, 1976). ZoBell (1947) was the first to speculate that bacterially produced surfactants may be useful in oil recovery. In conjunction with this work ZoBell was also the first to describe a system in which bacteria could lower the surface tension of the growth medium (ZoBell, 1947). Since this time many bacteria have been described which lower the surface tension of the growth medium. The vast majority of these systems involve aerobic bacteria with the ability to utilize hydrocarbons for growth.

As stated earlier, MEOR will require the use of anaerobic bacteria (McInerney, 1983). Only two bacteria have been reported to date which will reduce the surface tension of the growth medium while growing under anaerobic conditions, Desulfovibrio desulfuricans (ZoBell, 1947; LaRiviere, 1954) and Clostridium pasteurianum (Cooper et al., 1980). Perhaps this apparent scarcity of anaerobic emulsifier- and surfactant-producing bacteria is due to a lack of investigation. Research into the feasibility of MEOR provides a new and increasing impetus for investigation into the production of surface active and hydrocarbon-emulsifying compounds by anaerobic bacteria.

#### Terms Used in the Study of Surfactants and Emulsifiers

The study of surfactants and emulsifiers, like that of most fields, has its own terminology. The following are brief definitions of the major terms. A more detailed explanation of the terms used in the study of surfactants and emulsifiers is given in the Appendix.

The surface tension of a liquid is the force exerted on the molecules at the surface of the liquid due to the unbalanced attractive forces present at the surface. Unlike the molecules in the interior of the liquid, which are attracted equally from all directions; the molecules at the surface are not attracted equally, this imbalance has the effect of "stretching" the molecules at the surface, much like a thin film. The "tension" caused by this stretching is the surface tension.

There is a similar imbalance of forces present at the interface of two immiscible liquids. The force resulting from this imbalance is referred to as the interfacial tension.

A surface active agent, or surfactant, is a substance which alters the surface or interfacial properties of an aqueous solution. The molecules which alter the surface properties of liquids to the greatest extent are known as amphipathic molecules. These molecules contain both a hydrophobic portion, such as a non-polar hydrocarbon group, and a hydrophilic portion, which is normally a polar functional group, such as a carboxylic acid group or a quaternary ammonium salt.

An emulsion is a system of two immiscible liquids, one being dispersed as fine droplets in the other.

An emulsifier is an agent which acts to stabilize an emulsion system. Many emulsifiers are the above mentioned amphipathic molecules, however several other types of compounds have been shown to stabilize emulsions. These are discussed in greater detail the Appendix.

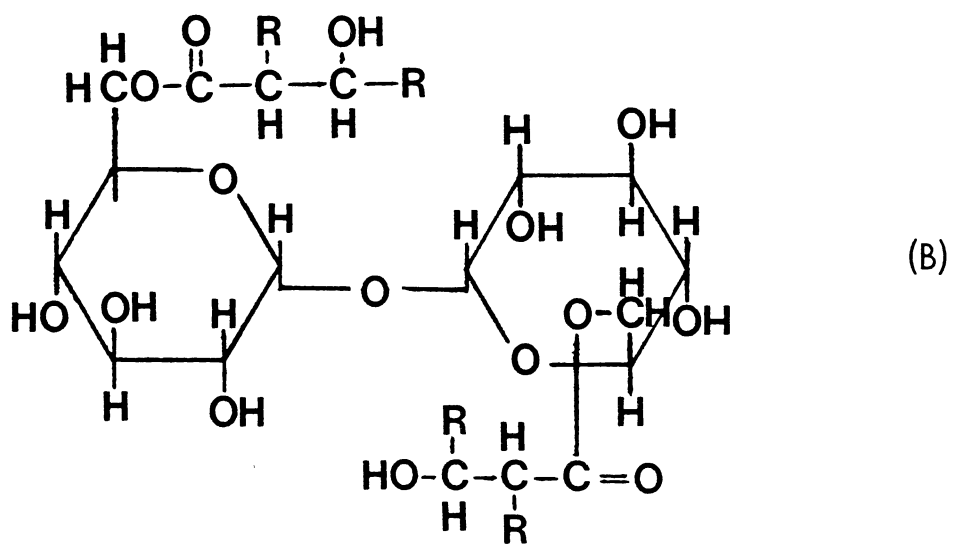
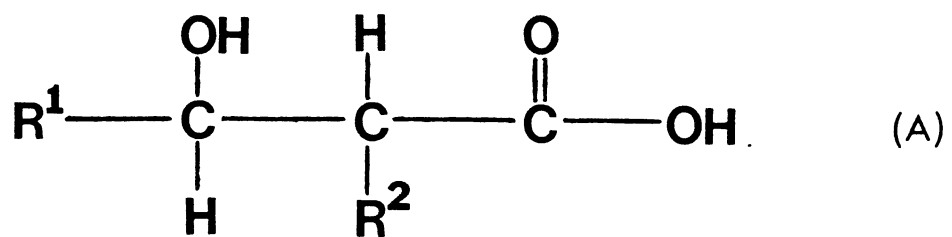
## Bacterially Produced Surfactants and Emulsifiers

As stated earlier, the first reduction of the surface tension of an aqueous solution due to a bacterium was reported by ZoBell in 1947. Since then many microorganisms have been shown to produce compounds with surface activity or with the ability to emulsify hydrocarbons. Most of these compounds contain a long chain fatty acid portion associated with a more hydrophilic biological compound such as a carbohydrate or peptide. Many organisms have been shown to produce the emulsifier compound only when growing on hydrocarbon substrates and often the lipid portion of the molecule is determined by the lipids present in the growth medium (Zajic and Panchal, 1976).

The most commonly isolated microbial surfactants and emulsifiers are the glycolipids. Glycolipids are complex lipids combined with a carbohydrate. Perhaps the most studied of the glycolipid bacterial surfactants are the cord factors (Cooper and Zajic, 1980). Cord factors belong to a group of glycolipids known as trehalose lipids. These molecules contain the disaccharide trehalose which is ester-linked to two corynomycolic acid groups (Figure 1). While the site of the ester bond is unknown in most trehalose lipids, the cord factors have been shown to be 6,6' dimycolates of the trehalose (Figure 1). Cord factors were originally discovered in mycobacteria and since have been found in many other organisms including the genera Corynebacterium and Nocardia. Other trehalose lipids have been isolated from Arthrobacter, Mycobacterium, Corynebacterium, and Nocardia (Cooper and Zajic, 1980). The cord factors and other trehalose lipids have been cited as good emulsifiers and have been

Figure 1. Structures of Corynomycolic acid and a Cord Factor.

- a) Structure of Corynomycolic acid.  
 $R^1$  and  $R^2$  are alkyl groups  
totalling fewer than 40 carbons.
- b) Structure of a cord factor.



reported as instrumental in the growth of the aforementioned microorganisms on hydrocarbon substrates (Cooper and Zajic, 1980).

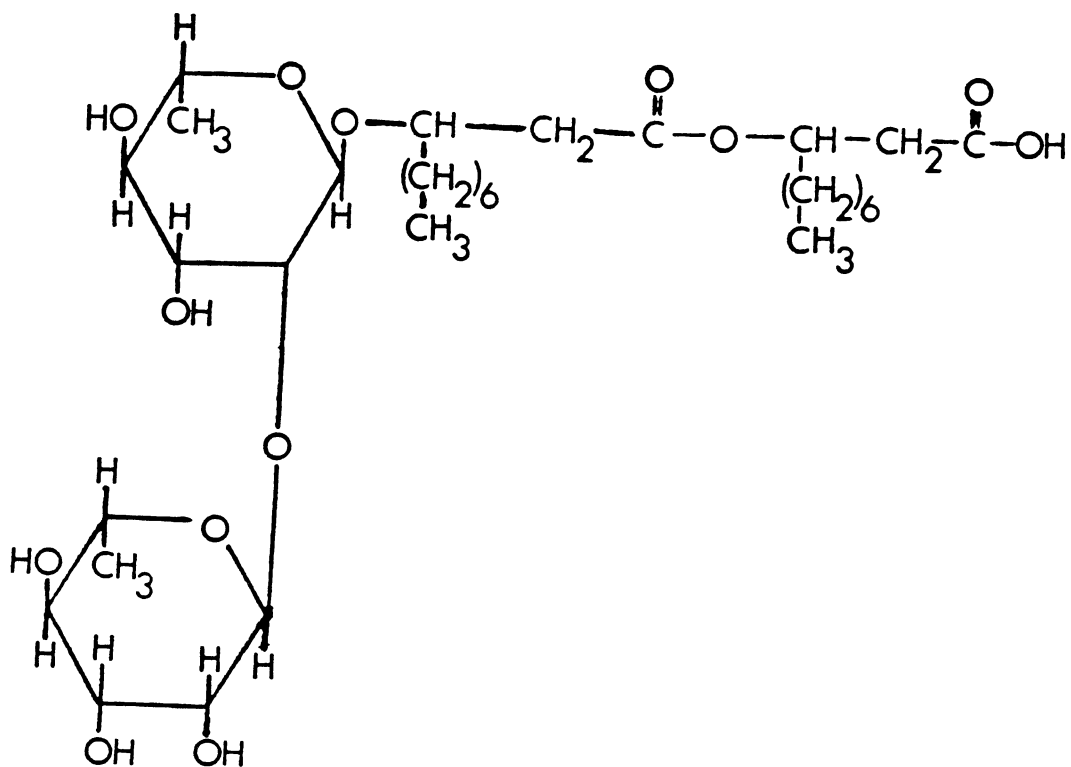
Other glycolipids have been isolated from microorganisms growing on hydrocarbon substrates. The rhamnolipids have been isolated from Pseudomonas aeruginosa and Arthrobacter paraffineus and are illustrated in Figure 2. The rhamnolipids contain the sugar rhamnose and  $\beta$ -hydroxydecanoic acid. Rhamnolipids have been shown to excellent emulsions stabilizers and are thought to emulsify hydrocarbons used as substrates by Ps. aeruginosa (Itoh et al., 1971).

The hydrocarbon-utilizing yeast Torulopsis has been shown to produce a glycolipid containing the carbohydrate sophorose. The first of the sophorose lipids isolated contained 17-L-hydroxyoctadecanoic acid. Further studies on Torulopsis have shown however, that the lipid moiety of these compounds is greatly influenced by the fatty acids present in the medium (Tulloch et al., 1962).

Another class of carbohydrate containing bio-emulsifiers are the polysaccharide-lipid complexes. One that has received a great deal of recent attention has been isolated from Acinetobacter RAG-1 and termed "Emulsan" by Rosenberg and co-workers (1979). This compound is composed primarily of D-galactosamine and an amino uronic acid, with the remainder being composed of D-glucose and fatty acids. As with most other bio-emulsifiers, emulsan has been shown to be produced in conjunction with growth on hydrocarbons by Acinetobacter (Rosenberg et al., 1979).

Many bio-surfactants and bio-emulsifiers are composed of one or more amino acids complexed with a fatty acid component. The most effective bio-surfactant reported to date is a peptide-lipid complex

Figure 2. Structure of a Rhamnolipid Isolated  
from Arthrobacter paraffineus.



isolated from Bacillus subtilis known as surfactin (Arima et al., 1968). Surfactin is composed of a seven amino acid peptide bound to a  $\alpha$ -hydroxy fatty acid (Figure 3). In concentrations as low as 0.005%, surfactin lowers the surface tension of 0.1 molar  $\text{NaHCO}_3$  from 71.6 dynes/cm to 27.9 dynes/cm (Arima et al., 1968).

Other surface active peptide-lipid complexes have been isolated from other species of Bacillus (Asselineau, 1966 p. 190), Nocardia (Asselineau, 1966 p. 190), Streptomyces (Cooper and Zajic, 1980) and Corynebacterium (Cooper, 1979).

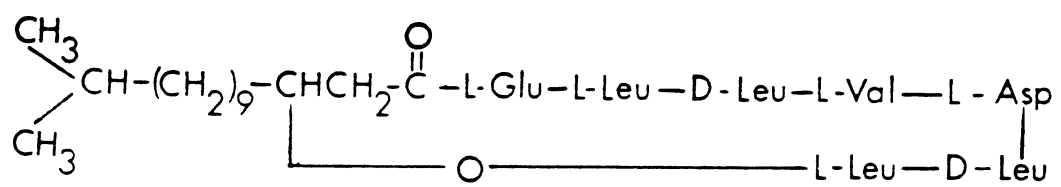
Surface active molecules composed of the amino acid ornithine and a lipid complex have been isolated from several bacterial genera including Thiobacillus (Knoche and Shivelely, 1972), Agrobacterium (Tahara et al., 1976), Pseudomonas (Wilkinson, 1972) and Gluconobacter (Tahara et al., 1976).

A protein isolated from Ps. aeruginosa was shown to emulsify hexadecane in water (Hisatsuka et al., 1972, 1975, 1977). This protein is composed of 147 amino acid residues and had a molecular weight of 14,300. The protein stimulated growth of Ps. aeruginosa on hexadecane, and the removal of the protein greatly impaired the growth of the organism on the hexadecane.

All microbial cells contain some type of lipid compound and many produce extracellular lipids. Most of these lipids have been shown to possess some surface activity and like most other bio-surfactants these lipids are usually associated with microbial growth on hydrocarbon substrates.

The neutral surface active lipids isolated from bacteria have recieved little attention as surface active agents. Among those that

Figure 3. Structure of Surfactin.



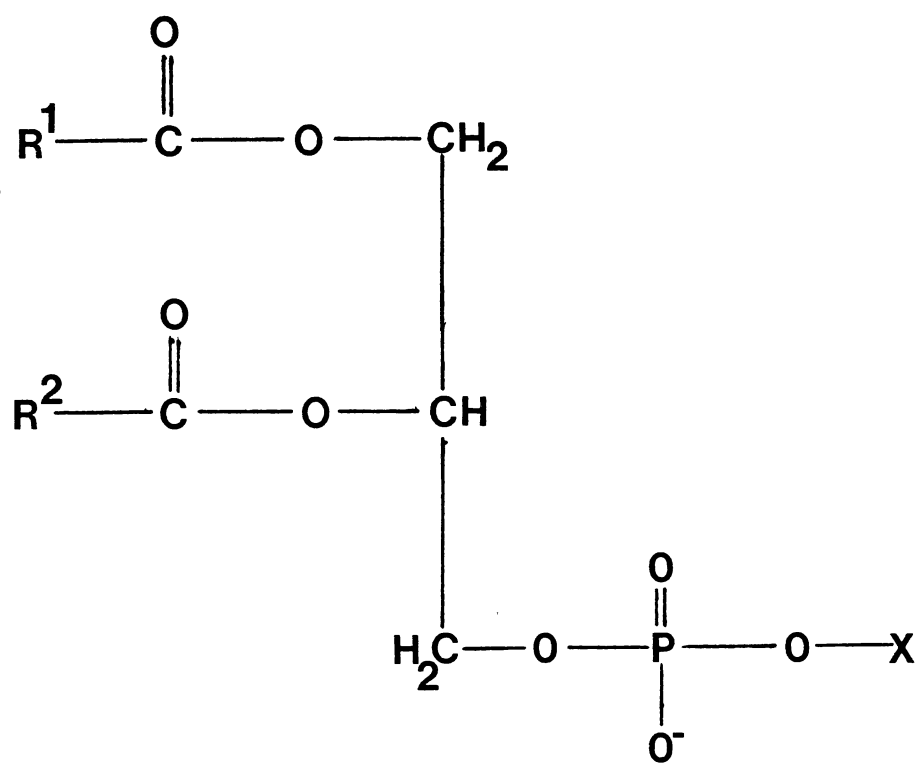
have been reported are a surface active ether produced from Mycobacterium rhodocrous (Holdorn and Turner, 1969) and fatty alcohols produced by Mycobacterium laticolum (Mil'ko et al., 1976) and Arthrobacter paraffineus (Suzuki and Ogawa, 1972). The surfactant isolated by Cooper et al. from Clostridium pasteurianum is thought to be an ether (1976). A species of Acinetobacter has been shown to produce a variety of lipids when grown on hexadecane including mono- and diglycerides along with wax esters (Makula et al., 1975). Thiobacillus thiooxidans has also been shown to produce several neutral lipids, including glycerides and hydroxy fatty acids, which act as wetting agents for elemental sulfur (Beebe and Umbreit, 1979).

The more polar surface active lipids produced by bacteria are the long chain carboxylic acids and the phospholipids. Many organisms are known to produce extracellular fatty acids while growing on hydrocarbon substrates. The genera Pseudomonas, Myococcus, Penicillium, Aspergillus, and Acinetobacter were reported by Odier (1976) to produce extracellular fatty acids while growing in fuel tanks. Micrococcus cerificans has been reported to produce extracellular fatty acids while growing on hexadecane which were absent when the organism was grown on non-hydrocarbon substrates (Makula and Finnerty, 1972). A number of yeasts have also been shown to produce extracellular fatty acids when grown on hydrocarbons. Candida cloacae was shown to produce the largest amounts of extracellular fatty acids among the yeasts tested. Phospholipids are present in all microorganisms but reports of extracellular phospholipid production by bacteria are rare (Cooper and Zajic, 1980). Phospholipids are composed of two fatty acids esterified to a glycerol unit with a phosphate group esterified to the third

carbon of the glycerol (Figure 4.) In all but phosphatidic acid a positively charged group, such as choline or ethanolamine, is linked to the phosphate. Among the microorganisms known to produce extracellular phospholipids are Corynebacterium lepus (Cooper et al., 1979), Corynebacterium alkanolyticum (Nakao et al., 1973), Micrococcus cerifcans (Makula and Finnerty, 1970) and Candida tropicalis (Mishina et al., 1977). All of these organisms produce phospholipids while growing on hydrocarbons, and the fatty acid component of the phospholipids is known to be dependent on the hydrocarbon being degraded by the organism (Cooper and Zajic, 1980). Thiobacillus thiooxidans has also been reported to produce extracellular phospholipids which act as wetting agents for elemental sulfur (Schaeffer and Umbreit, 1963; Jones and Benson, 1965; Beebe and Umbreit, 1971).

It is obvious that nearly all of the research into microbially produced surfactants and emulsifiers has concerned itself with aerobic, hydrocarbon-degrading organisms. The goal of MEOR is not to degrade hydrocarbons, but to obtain higher yields of oil intact. The aim of this study therefore, is to examine the production of hydrocarbon-emulsifying agents by anaerobic bacteria. The work presented here reports on the production and characterization of a bio-emulsifier by an isolates of the genus Clostridium, under study for use in MEOR.

Figure 4. Structure of a Phospholipid.  
Where "x" can be choline, ethanolamine,  
inositol, serine, etc.



## CHAPTER II

### MATERIALS AND METHODS

#### Surface and Interfacial Tension Determinations

Surface and interfacial tensions were determined using a Cenco-DuNouy Interfacial Tensiometer, using a 6-centimeter platinum-iridium alloy tensiometer ring (Fisher Scientific).

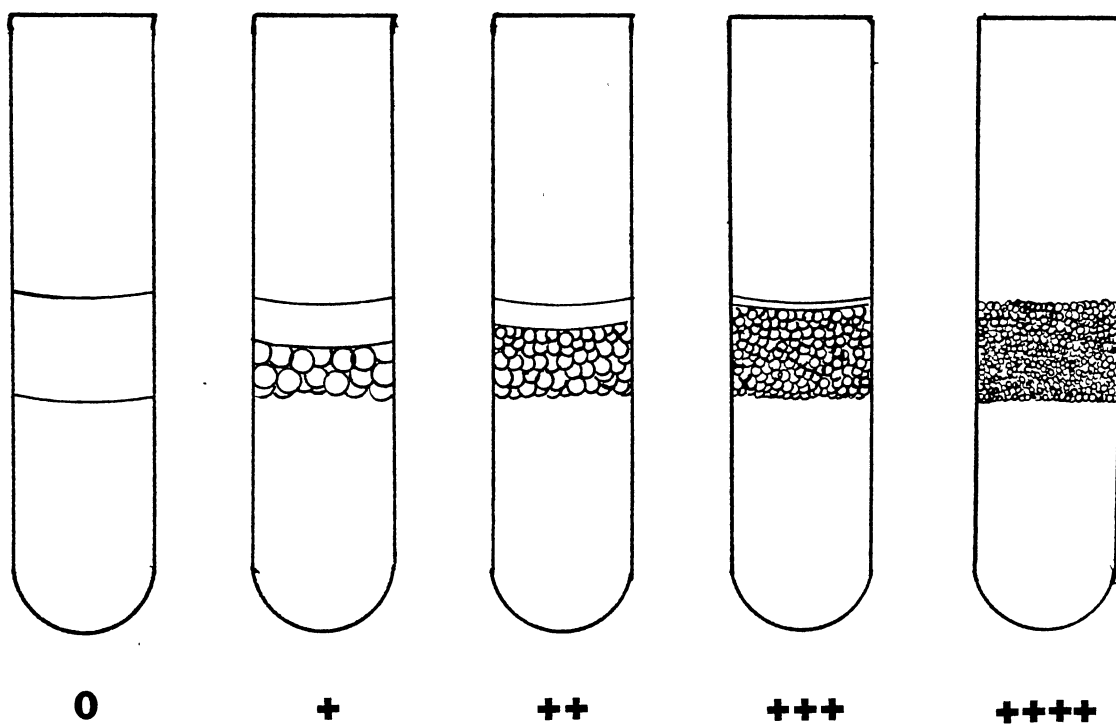
#### Estimation of Emulsifying Ability

The emulsifying ability of the various samples was determined by layering 1.5 ml of either kerosene (Fisher) or paraffin oil (Fisher) onto 3.5 ml of the sample to be tested in a 16 x 100 mm test tube. The two phases were then homogenized using a Lab Line Supermixer vortex mixer at high speed for one minute. The mixture was allowed to stand at room temperature for approximately 24 hours, at which time the emulsion was subjectively assessed and assigned values ranging from 0 (no emulsification) through ++++ (excellent emulsification) as seen in Figure 5.

#### The Bacterium

The organism used in the study was isolated at Oklahoma State University by Hugh Russell. The organism was isolated from molasses, which was being evaluated as a source of carbohydrates for the growth

Figure 5. Guide to Estimation of Emulsifying  
Ability.



media to be used in MEOR. The organism was isolated from conditions which selected for anaerobic bacteria and was found to be unable to grow aerobically. The organism was designated HR-3. HR-3 was observed to produce a stable foam at the surface of liquid media, a trait common to surfactant producing bacteria. When the spent medium was tested in the above manner for emulsifying ability it produced a ++++ emulsion. This was the first organism isolated at Oklahoma State University in conjunction with the MEOR project which had this ability and was thus chosen for the study of the production of an emulsifier by an anaerobic bacterium in detail. Due to the organism's ability to produce the emulsifier the organism was later designated HR-3E. The identity of the organism was investigated using the ANA-STAT system of Scott Laboratories.

## Fermentation

### Medium

The fermentation characteristics of HR-3E were determined in two media. One was Tryptic Soy Broth (Difco) with 1% (w/v) sucrose (Fisher). A better defined medium was composed of 1% (w/v) sucrose (Fisher), 0.5% (w/v) enzyme-hydrolyzed casein (Sigma), and the basal salts used by Grula (1960) for the growth of Erwinia caratovora (Table 1). Seven milliliters of the medium to be used was placed into Hungate tubes (Bellco), the tubes were then purged with nitrogen gas and autoclaved.

The media were inoculated with two drops of a heavy suspension of washed HR-3E cells and incubated at 37° C.

TABLE I  
MINERAL COMPONENTS OF MEDIA

Component	Stock Concentration (per 100 ml)
Phosphate Buffer:	
$K_2HPO_4$	870 mg
$KH_2PO_4$	680 mg
Trace Minerals:	
$HBO_3$	2.5 $\mu$ g
$CaCO_3$	50.0 $\mu$ g
$CuSO_4 \cdot 5 H_2O$	5.0 $\mu$ g
$FeSO_4 (NH_4) SO_4 \cdot 6 H_2O$	250 $\mu$ g
KI	5.0 $\mu$ g
$MnSO_4 \cdot H_2O$	10.0 $\mu$ g
$MoO_3$	5.0 $\mu$ g
$ZnSO_4 \cdot 7 H_2O$	25.0 $\mu$ g
Other Components:	
$MgSO_4 \cdot 7 H_2O$	100 mg
$NH_4Cl$	1.0 g

Components were added to the media as follows:

Phosphate buffer --- 20 ml/100 ml medium

Trace mineral solution --- 20 ml/100 ml medium

$MgSO_4 \cdot 7 H_2O$  --- 3 ml/100 ml medium

$NH_4Cl$  --- 10 ml/100 ml medium

### Growth

The growth of the organism was monitored with a Bausch and Lomb Spec 100 spectrophotometer. At the specified intervals three tubes were selected at random and the absorbance at 540 nanometers was measured. The three readings were averaged and the absorbance reading of the tube nearest the average was recorded as the amount of growth that had taken place during that time interval. This tube was also used to determine the amount of acids, solvents and emulsifier that been produced during that time interval.

### Fermentation Products

The amount of gas produced by HR-3E was determined using a 3.0 liter batch culture of HR-3E in half-strength Tryptic Soy Broth with 1% sucrose. The culture vessel, a 4-liter Erlenmeyer flask, was vented to a gas trap filled with a saturated sodium chloride solution. The saline forced from the gas trap by the gas produced by the organism was collected and the volume measured (a more detailed description of the large batch culture apparatus is given below, under Emulsifier Compound: Growth of Organism). When the 3-liter batch no longer evolved gas, the culture was supplied with 40 grams of sucrose and 11.5 grams of dehydrated Tryptic Soy Broth dissolved in one liter of distilled water. Again, the saline forced from the gas trap was collected and the volume measured.

The organic acids and solvents in the spent media were detected by gas-liquid chromatography using a Perkin-Elmer Sigma II Gas Chromatograph equipped with a hydrogen flame detector (FID). For the

free fatty acids a 6 ft. x 2mm glass column packed with 60/80 Carbopack C/0.3% Carbowax 20M/0.1%  $H_3PO_4$  (Supelco) was used. Nitrogen gas at a flow rate of 50 ml/min was the mobile phase of the system. The solvents in the spent media were detected using a 6 ft. x 2 mm glass column packed with 80/100 Carbopack C/0.1% sp-1000 (Supelco). The areas of the peaks generated by the compounds when chromatographed were determined using a Perkin-Elmer M-2 calculating integrator, and concentrations of the acids and solvents in parts per million were determined using a standard curve.

The pH's of the spent media were determined using an Orion Ionalyzer 501 pH meter.

#### Emulsifier Compound

##### Growth of Organism

Medium. The medium used for the production of the emulsifier compound by HR-3E was the sucrose/enzyme-hydrolyzed casein/basal salts medium described earlier.

Large Batch Culture. In order to obtain an appreciable amount of the emulsifier compound, the organism was grown in large volume batch cultures. The inoculum was prepared by growing HR-3E cells in Tryptic Soy Broth plus 1% sucrose. The cells were grown in a Gas-Pak anaerobic chamber (BBL) at 37° C. When the cells reached early stationary phase a ten milliliter aliquot of the cell-containing whole broth was used to inoculate the large batch. Sixteen liters of the sucrose/casein/basal salts medium was prepared in a 5-gallon Pyrex carboy.

To establish anaerobic conditions the culture vessel was sealed

with a rubber stopper through which two lengths of glass tubing were passed. One tube was connected to a sintered glass sparger below the surface of the medium, through which five times the vessel volume (approx. 100 liters) of nitrogen gas was bubbled through the medium to purge the vessel of oxygen. Once the system was purged the gas inlet tube was clamped shut. The other glass tube was connected with Tygon tubing to another 5-gallon carboy which contained a saturated saline solution. This allowed the escape of gas produced by HR-3E while preventing the entry of atmospheric oxygen into the system. The gas trap not only served to maintain anaerobic conditions but allowed the amount of gas produced during the growth of HR-3E to be determined by collecting the saline solution forced out of the gas trap and measuring the volume. The large batch apparatus is diagrammed in Figure 6.

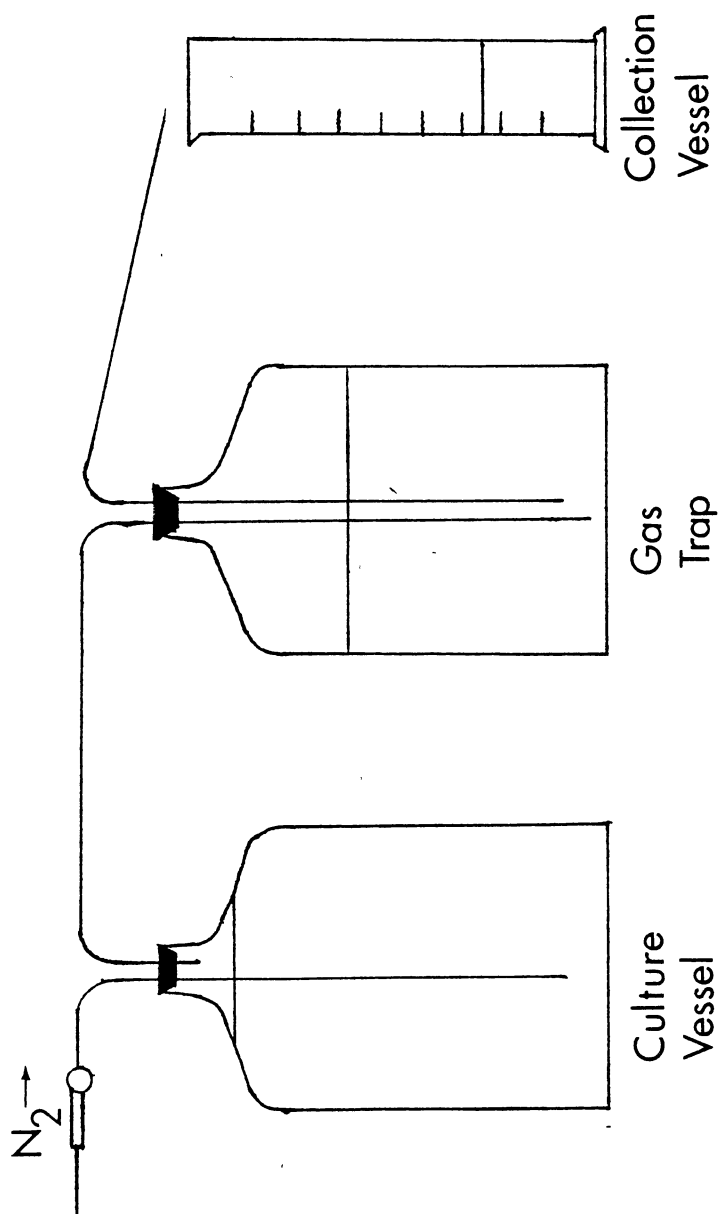
The cells were allowed to grow until gas no longer evolved from the culture vessel. At this time the culture was supplied with four liters of the medium with 200 grams of sucrose and 100 grams of enzyme-hydrolyzed casein, insuring that the concentrations in the 20 liter batch would be at least 1.0% (w/v) for sucrose and 0.5% (w/v) for casein. The cells were again allowed to grow until gas was no longer evolved from the culture vessel.

#### Concentration and Isolation of the Emulsifier Compound

When growth was complete the cells were removed from the medium with a Sharples Super Centrifuge.

Once the medium was cleared of cells, the emulsifier compound was concentrated by foaming. A glass cylinder with an inner diameter of 3.5 cm and length of 150 cm was constructed with a coarse frit at the

Figure 6. Diagram of the Large Batch Culture Apparatus.



bottom. Two hundred fifty milliliters of the spent medium was placed in the cylinder and aerated through the frit at a rate of 500 ml of air/minute. The resulting foam rose vertically through the cylinder and the foam reaching the top of the cylinder was collected and allowed to collapse. The culture fluid remaining in the column was discarded. The procedure was repeated with the collapsed foam until the volume of the emulsifier-containing fluid was approximately 250 ml.

When the foaming procedure was completed, the collapsed foam was placed in dialysis tubing with a molecular weight cut-off of 1000 daltons (Spectrapor). This was dialyzed four times against 3 liters of phosphate buffer of the same concentration as was present in the medium, changing the buffer every 24 hours, and once for 24 hours against 3 liters of deionized water. In order to concentrate the compound remaining in the dialysis tubing, water was removed from the tubing by dialysis against a saturated solution of polyethylene glycol (M.W.=8,000, Sigma).

The emulsifier-containing fluid retained by the dialysis tubing was examined using thin-layer chromatography. This was carried out using Whatman K5 silica gel plates. The uninoculated sterile medium was used as a control. The following compounds were used as standards; heptadecane (Sigma), olive oil, sodium lauryl sulfate (Sigma), Triton X-100 (Sigma) and Tween 80 (Fisher). The following nitrogenous lipids were also used as standards; phosphatidyl inositol, phosphatidyl choline, phosphatidyl serine and phosphatidyl ethanolamine (all from Sigma).

Two solvent systems were used for the development of the thin-layer chromatograms. One consisted of hexane-carbon

tetrachloride-glacial acetic acid (15:10:1) (Cooper et al., 1980). The other system was prepared by allowing 10 parts of chloroform to equilibrate with one part of ammonium hydroxide for two hours, and then mixing 97 parts of the ammoniacal chloroform and 3 parts methanol for the solvent system (Mangold, 1961). The developed plates were visualized by exposing them to iodine vapors (subliming iodine crystals).

Amino acids present in the emulsifier-containing fluid were detected by subjecting the sample to acid hydrolysis and analyzing the hydrolyzed sample using thin-layer chromatography. Equal volumes of the sample and concentrated (6N) hydrochloric acid (100  $\mu$ l) were combined, sealed under vacuum and incubated at 110<sup>o</sup> C for 20 hours. The acids was removed by breaking the vacuum seal and placing the tube in an evacuated desiccator until all liquid was evaporated. The sample was rehydrated with 150  $\mu$ l deionized water. Forty  $\mu$ l of the sample were deposited onto 20 x 20 cm cellulose powder thin-layer plates. The solvent system Jones and Heathcote (1966) was used to develop the chromatograms. The amino acids were visualized by spraying the developed chromatograms with 0.4% ninhydrin (Sigma) in acetone.

In order to detect and characterize the fatty acid portion of the emulsifier-containing fluid, a methyl ester of the fatty acid component was prepared. Two drops of the emulsifier-containing fluid were mixed with 4 ml of sodium-dried benzene, 0.4 ml of 2,2-dimethoxy propane, and 0.5 ml of methanolic HCl. This mixture was allowed to stand overnight at room temperature and was then dried by passing a stream of air over the mixture and the residue was resuspended in 0.5 ml of benzene (Mason and Waller, 1964). A sample of a commercial vegetable oil and sample of

ascorbyl palmitate were also esterified in this manner in order to discern the retention time of the methyl esters of known fatty acids. The sterile uninoculated medium was used as a control.

The fatty acid esters were then detected on a 1.8 m x 2.8 mm glass column packed with 20% diethylene glycol succinate (DEGS). Nitrogen gas at a flow rate of 45 ml/min. was the mobile phase of the system. The column was used with a Perkin-Elmer 990 Gas Chromatograph, equipped with a hydrogen flame detector (FID).

#### Nutritional Requirements of HR-3E

The nutritional requirements of HR-3E were investigated by preparing several defined media and testing each for the ability to support growth by HR-3E. Each medium contained 1% (w/v) sucrose and the mineral components described earlier (Table I). In order to determine the organic nutrient requirements of HR-3E the media various combinations of amino acids and vitamins. The amino acids used were placed into groups according to the ultimate precursor of their biosynthetic pathway or some other outstanding chemical characteristic, and the amino acids used to prepare the various media were added in these groups. The amino acids (all obtained from Sigma) and the concentrations at which they appeared in the various media are shown in Table II. The amount of amino acids present is intended to simulate a 5% casein solution, with the amino acids present in the proportions reported in the analysis of casein reported by Harrow (p. 49, 1946). The vitamins used (all obtained from Sigma) and the concentrations at which they appeared in the various media are shown in Table III. The concentrations of vitamins were those suggested by Campbell and Frank

TABLE II  
AMINO ACIDS USED IN DEFINED MEDIA

Group	Precursor or Characteristic	Amino Acid	Final Concentration (g/100 ml)
I	5-phosphoribosyl- 1-pyrophosphate	Histidine	0.38
II	Aromatic group	Tyrosine	0.33
		Tryptophan	0.11
		Phenylalanine	0.29
III	3-phosphoglycerate	Serine	0.29
		Cysteine	0.02
		Glycine	0.02
IV	pyruvate	Alanine	0.09
		Valine	0.40
		Leucine	0.49
V	$\alpha$ -ketoglutarate	Glutamic acid	1.09
		Proline	0.40
		Arginine	0.26
VI	Other precursors	Aspartic acid	0.21
		Isoleucine	0.49
		Lysine	0.13
		Methionine	0.18
		Threonine	0.20

TABLE III  
VITAMINS USED IN DEFINED MEDIA

Vitamin	Concentration (per 100 ml)
Biotin	0.5 µg
Riboflavin	0.08 mg
Thiamine	0.4 mg
Pyridoxal	0.5 mg
Niacin	1.0 mg
Pantothenic acid	1.0 mg
<u>para</u> -Aminobenzoic acid	10.0 µg
Folic acid	10.0 µg
Vitamin B <sub>12</sub>	10.0 µg

(1956) for the preparation of a synthetic medium for putrefactive anaerobic bacteria.

The inoculum for these experiments was prepared by growing HR-3E in the sucrose/enzyme-hydrolyzed casein/mineral salts medium described earlier. When the culture had reached early stationery phase the cells were removed from the medium by centrifugation, and washed three times in a sterile solution of 0.85% sodium chloride. The washed cells were resuspended in a sterile 0.85% sodium chloride solution so that an optical density (measured with a Bausch and Lomb Spec 100 at 540 nm) of 0.5 was obtained. An inoculum size of 2 milliliters per 100 milliliters of medium was used in all experiments.

The media were prepared as described in Table IV. In one group of media all of the amino acids in Table II were present and the vitamin content was varied. In the other group of media all of the vitamins in Table III supplied were present and the amino acid content was varied.

All nutritional experiments were carried out in Gas-Pak anaerobic chambers at 37°C.

TABLE VIII  
NUTRIENT COMPOSTIONS OF DEFINED MEDIA

Medium	Amino acid group						Vitamin								
	1	2	3	4	5	6	BI	RI	TH	PY	NI	PA	PB	FA	B <sub>12</sub>
1	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
4	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
6	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
8	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
9	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+
13	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+
14	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+
15	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+
16	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

BI - Biotin                      RI - Riboflavin                      TH - Thiamine  
 PY - Pyridoxal                  NI - Niacin                              PA - Pantothenic acid  
 FA - Folic acid                  B<sub>12</sub> - Vitamin B<sub>12</sub>                      PB - para-Aminobenzoic acid

## CHAPTER III

### RESULTS

#### Identification of HR-3E

HR-3E is a Gram-positive, anaerobic, spore-forming rod. The cells of HR-3E range from 3.2 to 4.5 micrometers in length and are 1.1 micrometers in width (Figure 7). The spores formed by HR-3E range from 1.8 to 2.3 micrometers in length and 1.1 to 1.6 micrometers in width.

HR-3E is somewhat aerotolerant, in that it can be stored and transferred in air, but the organism requires anaerobic conditions for growth.

When subjected to the tests in the ANA-STAT system the following carbohydrates were fermented; glucose, sucrose, melibiose, cellibiose, arabinose, maltose, ribose, esculin and glycogen. Mannitol, inositol, amygdalin and glycerol were not fermented. Ammonia was not produced from arginine. A curd was formed in milk but the curd was not further digested. Starch was hydrolyzed. Gelatin was not liquified. Neither a lipase nor a lecithinase was present. The organism grew well in the presence of bile.

From these results the organism most closely resembles Clostridium butyricum, but bears some resemblance to Clostridium beijerinckii.

Figure 7. Photomicrograph of HR-3E.



### Fermentation Characteristics of HR-3E.

The fermentation characteristics of HR-3E are shown in Figures 8a through 8e).

Figure 8a shows the gas production by HR-3E. The final volume of gas produced in the four liter batch was 13.8 liters. The primary gas produced is carbon dioxide, with lesser amounts of hydrogen gas present (Grula et al., 1983).

The major acids produced by HR-3E are acetic acid and butyric acid. Propionic acid is also produced early in the fermentation (Table V). The concentration of all of the acids combined 48 hours after inoculation is approximately 910 parts per million (Fig. 8b).

The pH of the medium begins to decrease three hours after inoculation and steadily decreases until a pH of 4.5 is reached about nine hours after inoculation (Fig. 8c). At this point, although acid production and possibly growth continue to increase the pH of the medium remains constant.

The major solvent produced is ethanol (ethyl alcohol) and later in the fermentation 2-methyl-1-propanol (isobutyl alcohol) begins to appear (Table VI). The concentration of the solvents present 48 hours after inoculation is approximately 380 parts per million (Fig. 8d).

Figure 8e shows the degree to which the spent medium was able to emulsify kerosene at the various times during the fermentation.

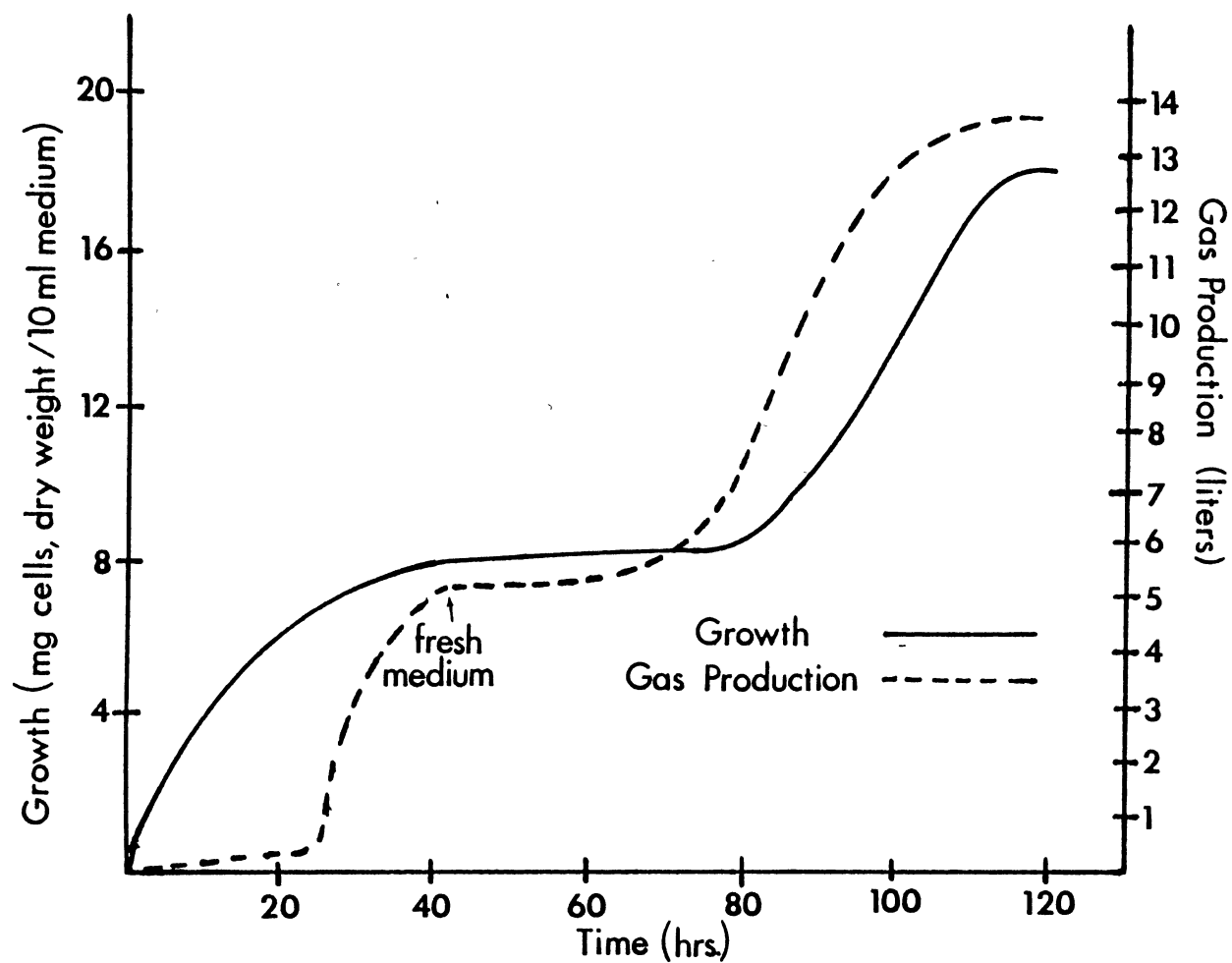
### The Emulsifier Compound

Table VII shows the concentrating effect that foaming the spent medium had on the surface active agent present. When the collapsed foam

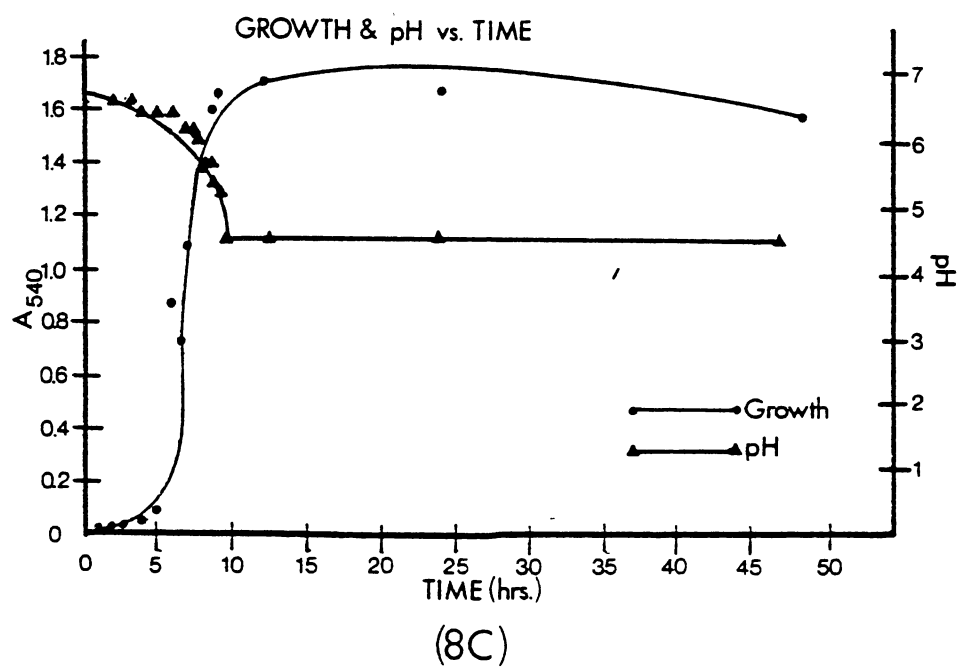
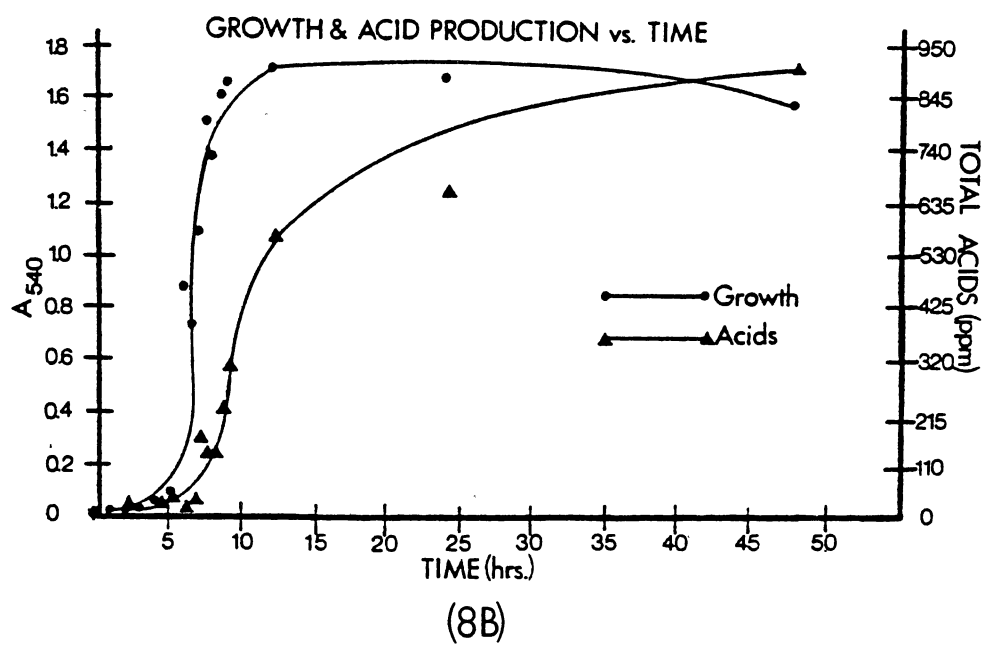
Figure 8. Fermentation Products by HR-3E.

- A) Gas production
- B) Acid production
- C) pH
- D) Solvent production
- E) Emulsifier production

# Gas Production by HR-3E



(8A)



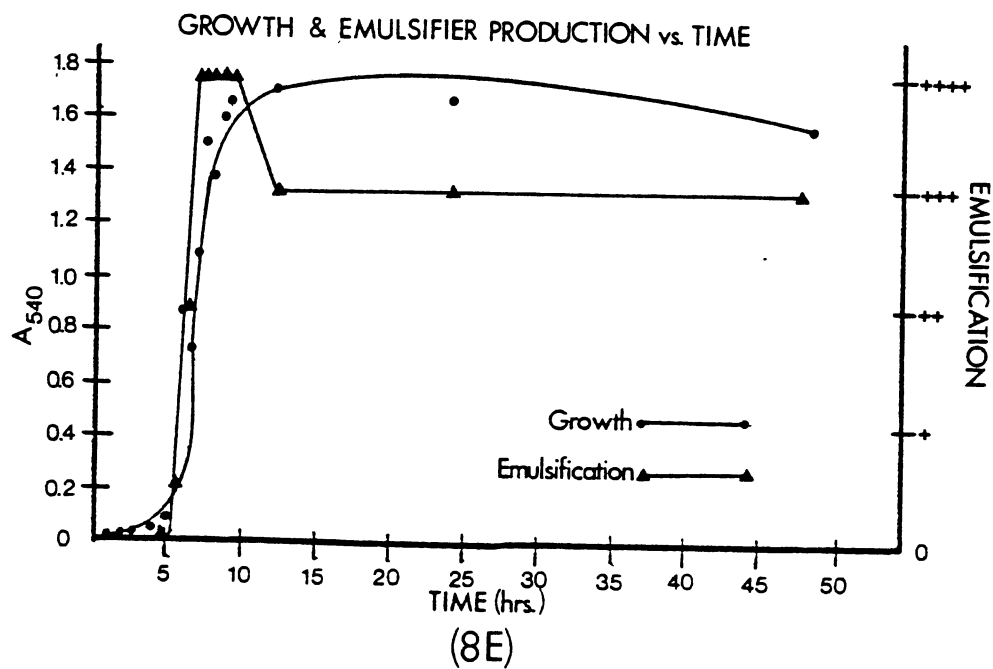
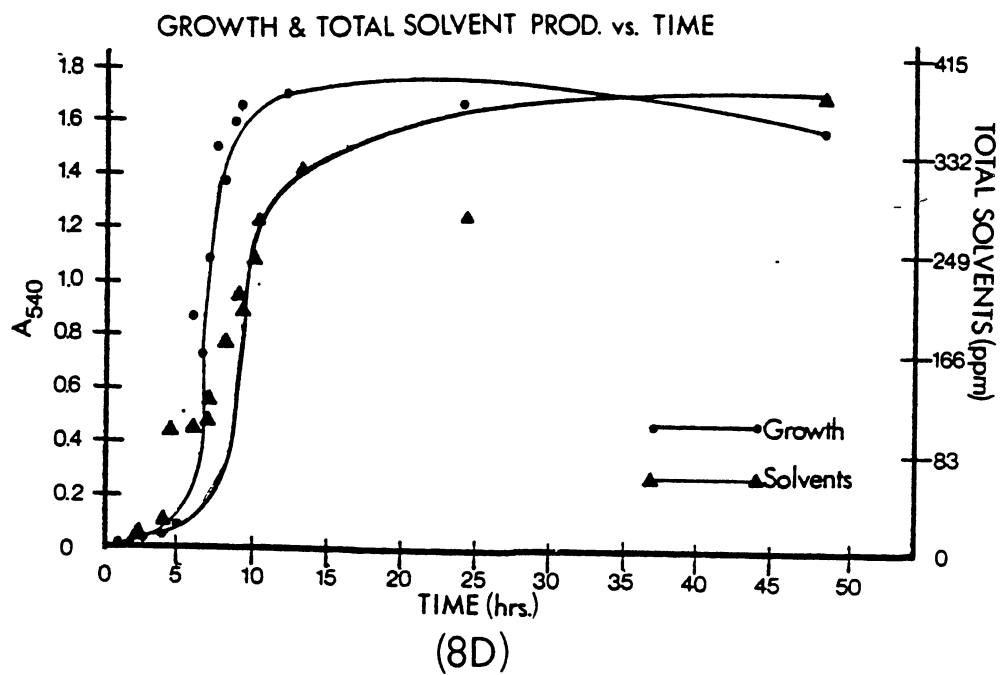


TABLE V  
ACIDS PRODUCED BY HR-3E

TIME (hrs)	ACID	% OF TOTAL *
1	Acetic Propionic	trace trace
2	Acetic	100
3	Acetic Propionic	46.3 53.7
4	Propionic	100
5	Propionic	100
6	Acetic Propionic	52.5 47.5
7	Acetic Propionic Butyric	45.8 41.5 12.7
7.5	Acetic Propionic Butyric	39.1 4.0 56.9
8	Acetic Propionic Butyric	49.4 11.7 38.9
8.5	Acetic Propionic Butyric	30.4 11.5 58.1
9	Acetic Propionic Butyric	27.5 trace 72.5
12	Acetic Propionic Butyric	21.8 trace 78.2

TABLE V (cont.)

TIME (hrs)	ACID	% OF TOTAL *
24	Acetic	20.1
	Butyric	79.9
48	Acetic	18.2
	Butyric	81.8

\* -- based on parts per million amounts from standard curves

TABLE VI  
SOLVENTS PRODUCED BY HR-3E

TIME (hrs)	SOLVENT	% OF TOTAL *
1	MeOH-CH <sub>2</sub> O	trace
	EtOH	trace
	t-BuOH	trace
	2-BuOH	trace
2	MeOH-CH <sub>2</sub> O	57.7
	EtOH	42.3
3	EtOH	57.2
	t-BuOH	10.6
	2-BuOH	7.5
	n-BuOH	24.7
4	EtOH	48.6
	t-BuOH	16.4
	n-BuOH	35.0
5	MeOH-CH <sub>2</sub> O	24.3
	EtOH	45.1
	Acetone	9.4
	t-BuOH	3.0
	2-BuOH	7.0
	n-BuOH	11.2
6	EtOH	80.4
	t-BuOH	6.4
	n-BuOH	13.2
6.5	MeOH-CH <sub>2</sub> O	7.8
	EtOH	92.2
	t-BuOH	trace
	n-BuOH	trace

TABLE VI (cont.)

TIME (hrs)	SOLVENT	% OF TOTAL *
7	MeOH-CH <sub>2</sub> O	14.4
	EtOH	72.0
	t-BuOH	5.1
	n-BuOH	8.5
7.5	MeOH-CH <sub>2</sub> O	7.6
	EtOH	91.4
	t-BuOH	1.0
8	MeOH-CH <sub>2</sub> O	8.5
	EtOH	74.4
	t-BuOH	4.2
	n-BuOH	4.3
8.5	MeOH-CH <sub>2</sub> O	12.8
	EtOH	74.2
	t-BuOH	4.2
	i-BuOH	8.8
	n-BuOH	trace
9	MeOH-CH <sub>2</sub> O	11.9
	EtOH	68.3
	PrOH	1.9
	t-BuOH	4.4
	i-BuOH	9.51
	n-BuOH	4.5
12	MeOH-CH <sub>2</sub> O	12.4
	EtOH	72.6
	t-BuOH	trace
	i-BuOH	15.0
	n-BuOH	trace

TABLE VI (cont.)

TIME (hrs)	SOLVENT	% OF TOTAL *
24	EtOH	85.5
	t-BuOH	0.8
	i-BuOH	11.0
	n-BuOH	2.7
48	MeOH-CH <sub>2</sub> O	31.9
	EtOH	60.9
	t-BuOH	trace
	i-BuOH	7.2
	n-BuOH	trace

MeOH-CH<sub>2</sub>O -- Methanol-Formaldehyde    EtOH -- Ethanol  
 PrOH -- Propanol    2-BuOH -- 2-Butanol  
 t-BuOH -- 2-Methyl-2-propanol    n-BuOH -- 1-Butanol  
 i-BuOH -- 2-Methyl-1-propanol

\* -- based on parts per million amounts from standard curves

TABLE VIII  
EFFECT OF FOAMING ON SPENT MEDIUM

Sample	Surface tension (dynes/cm)	Interfacial tension* (dynes/cm)
Uninoculated Medium	68.5	not tested
Spent Medium	60.4	29.0
Collapsed Foam	44.2	18.3

\* Against kerosene.

was subjected to the emulsification test, a very stable ++++ emulsion was formed.

Figure 9 shows the effect of dialysis on the organic solvents present in the spent medium. From this it can be seen that many of the low molecular weight compounds, such as alcohols, are essentially removed from the spent medium by the dialysis procedure. Also, thin-layer chromatography revealed no appreciable amounts of free amino acids present in the dialyzed emulsifier-containing fluid (Fig. 12). While the spent medium retained by the dialysis membrane did maintain the ability to form an emulsion with kerosene, the ability was somewhat diminished.

Figure 10 represents the thin-layer chromatograms of the emulsifier-containing fluid when chromatographed in the ammoniacal chloroform/methanol system. The spots drawn were present after exposing the chromatograms to iodine vapors (subliming iodine crystals). This procedure detects lipoidal and nitrogenous compounds. The emulsifier-containing fluid contained one component using this system. This component had an  $R_f$  value of 0.54. The uninoculated medium was resolved into three components using this system. The  $R_f$  values of the components in the control were 0.16, 0.31, and 0.49. The emulsifier-containing fluid contained no components that were mobile in the hexane/carbon tetrachloride/acetic acid system.

Following the exposure to iodine crystals and outlining the resulting spots, the chromatograms were sprayed with 0.4% ninhydrin in acetone to detect any free amino groups. The only ninhydrin-positive spot present was at the origin of the uninoculated medium, indicating that the emulsifier-containing fluid contained no free amino groups.

Figure 9. Effect of Dialysis on the Level of Low-Molecular Solvents in the Spent Medium.  
(Gas chromatographic data; same system used for detection of solvents in "Fermentation Products")

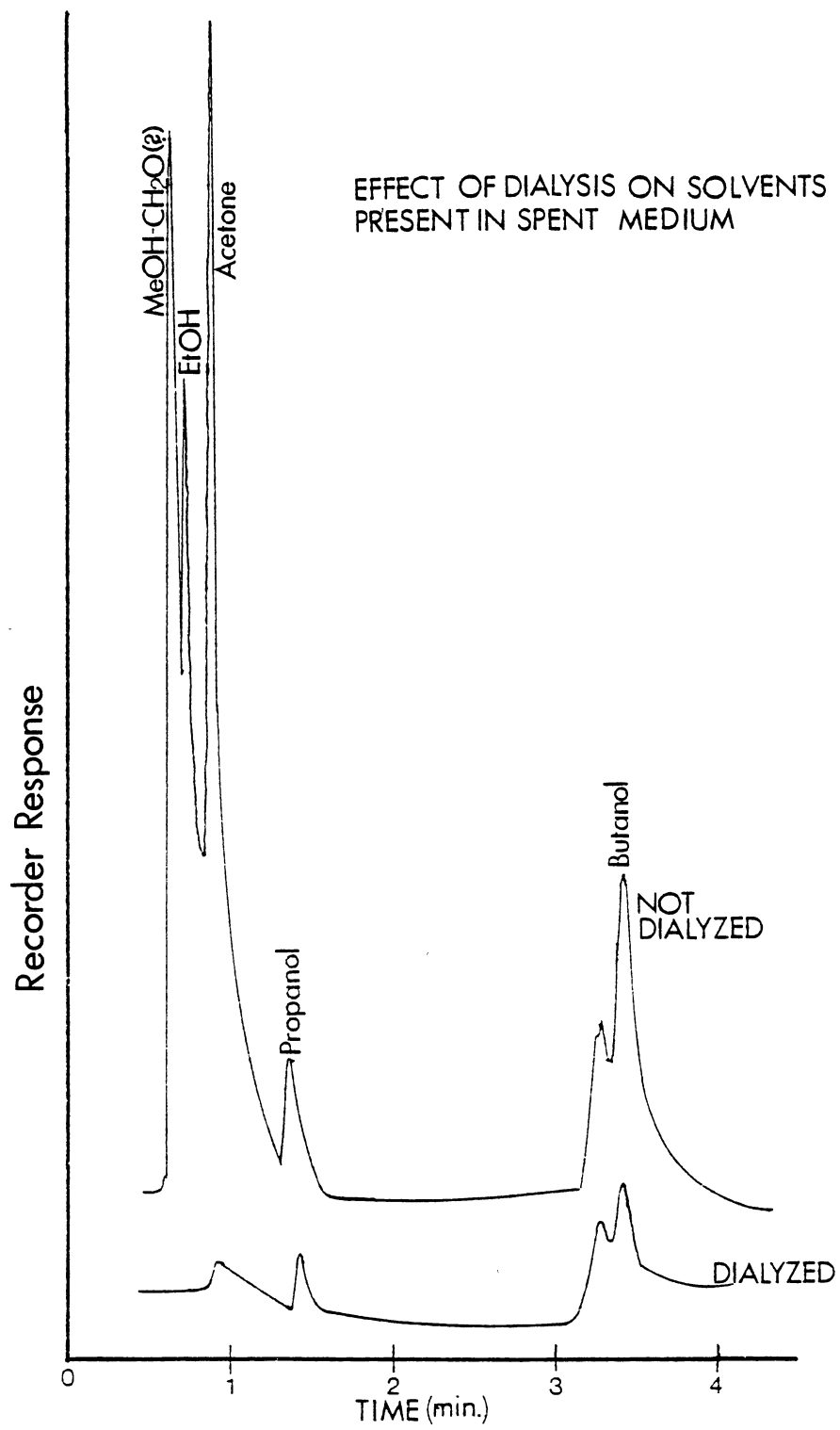


Figure 10. Diagram of Thin-layer Chromatograms  
(silica gel) of Emulsifier-containing  
Fluid.

- P - Emulsifier-containing fluid
- C - Uninoculated medium
- (N)- Ninhydrin positive
- LP- Lyophilized Emulsifier-containing  
fluid

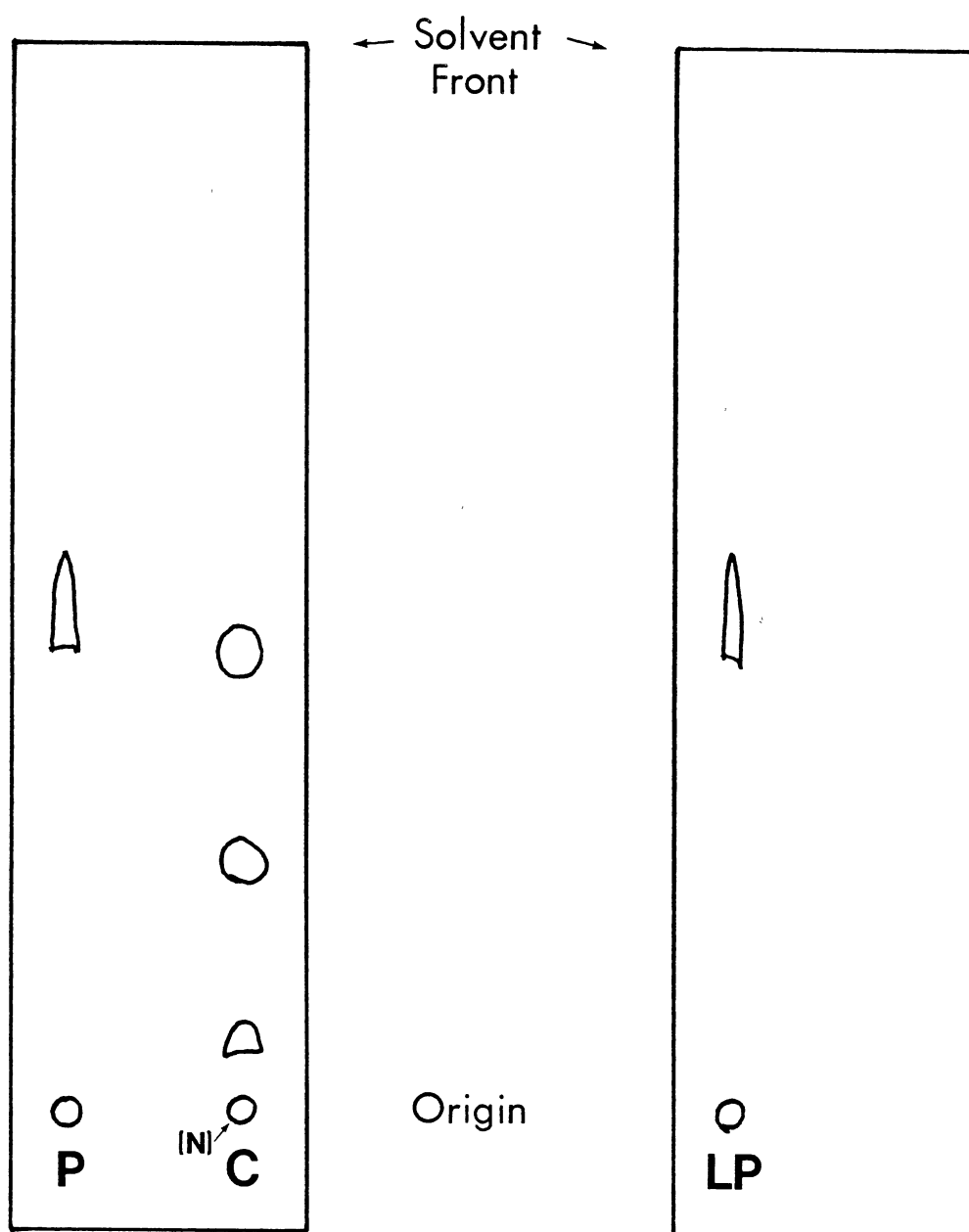


Figure 11 shows the resulting chromatograms when the standard lipids were chromatographed in the above system. Sodium lauryl sulfate did not move in this system. Tween 80 and Triton X-100 showed only slight movement, most of the compound remaining at the origin with a "spike" shaped spot appearing. Heptadecane moved with the solvent front (an  $R_f$  of 0.92). The olive oil was resolved into three components, one a "spike" at the origin, one a large spot with an  $R_f$  value of 0.69 and a smaller spot with an  $R_f$  value of 0.81 when exposed to iodine vapors.

When the emulsifier-containing fluid was subjected to acid hydrolysis and thin-layer chromatography performed on the hydrolyzed sample the chromatograms shown in Figure 12 were obtained. The acid hydrolyzed sample clearly shows the presence of at least ten amino acids including; leucine, isoleucine, threonine, alanine, proline, glutamic acid, glycine, lysine, either valine or methionine (possibly both) and possibly aspartic acid. The non-hydrolyzed emulsifier-containing fluid, as with the above systems, contained no components with a free amino group. The chromatogram of the non-hydrolyzed emulsifier-containing fluid clearly shows the absence of any free amino acids, and any other low molecular weight amino-compounds.

Figure 13a shows the gas chromatograms of the methyl ester of palmitic acid from the ascorbyl palmitate. The methyl palmitate has a retention time of 6.4 minutes.

Figure 13b shows the gas chromatogram of the methyl esters of the fatty acids present in the commercial vegetable oil. The vegetable oil was resolved into five components using this system, with retention

Figure 11. Diagram of Thin-layer Chromatograms  
(silica gel) of Standard Lipids.

- A) Sodium lauryl sulfate
- B) Tween-80
- C) Triton X-100
- D) Heptadecane
- E) Olive oil

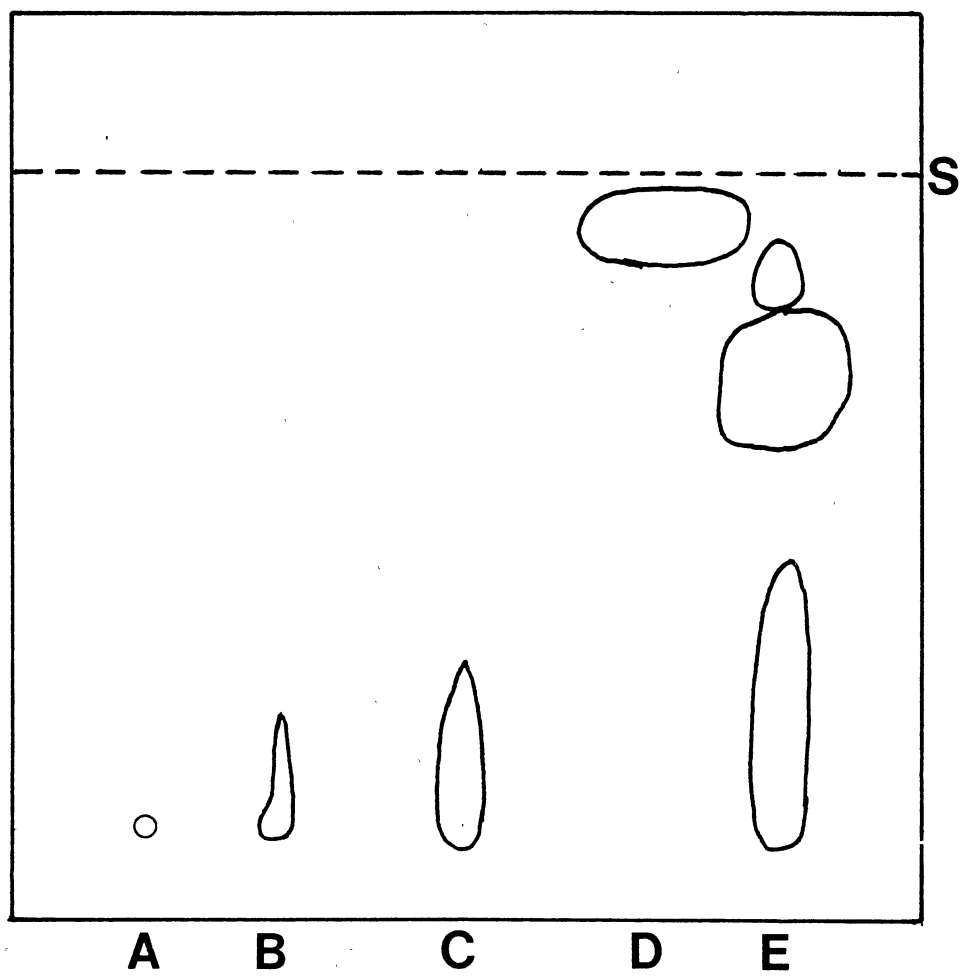
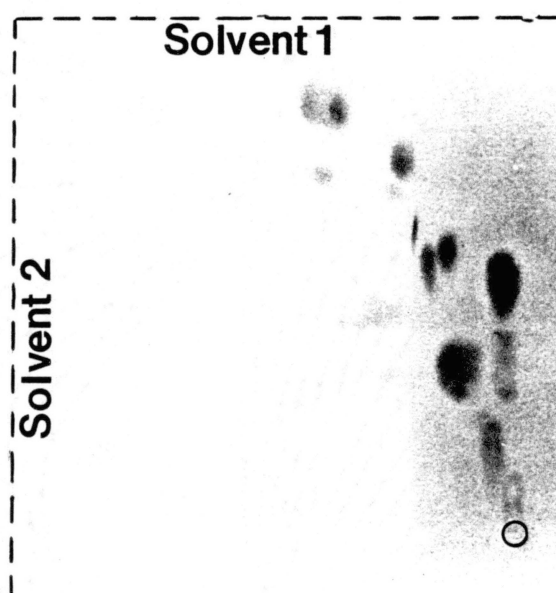
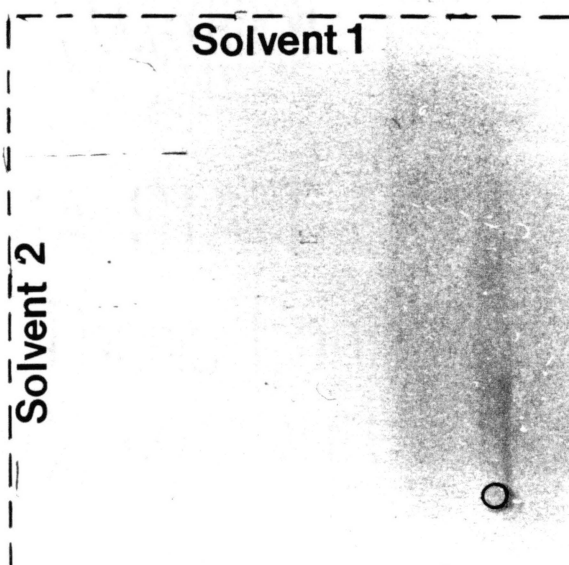


Figure 12. Thin-layer Chromatograms (cellulose powder) of Acid Hydrolyzed Emulsifier-containing Fluid.

- a) Acid hydrolyzed
- b) Not acid hydrolyzed



(A)



(B)

times of 6.2, 11.5, 12.8, 17.0 and 22.8 minutes. The compounds that normally have these retention times in this system and similar systems are methyl palmitate, methyl stearate, methyl oleate, methyl linoleate and methyl linolenate respectively (Hawke et al., 1959).

Figure 13c shows the gas chromatogram of the uninoculated medium.

Figure 13d shows the gas chromatogram of the methyl ester of the fatty portion of the emulsifier-containing fluid. This chromatogram shows a broad peak at a retention time of 17.8 minutes, indicating a compound similar to linolenic acid. A smaller peak does appear at a retention time of 5.5 minutes, however a similar peak is also present in the uninoculated medium.

#### Nutritional Requirements

None of the defined media described was able to support the growth of HR-3E.

Figure 13. Gas Chromatograms of the Methyl Esters  
of Various Lipid Samples.

- A) Ascorbyl Palmitate  
Attenuation = 256X
- B) Vegetable oil  
Attenuation = 256X
- C) Uninoculated medium  
Attenuation = 4X
- D) Emulsifier-containing  
fluid  
Attenuation = 8X

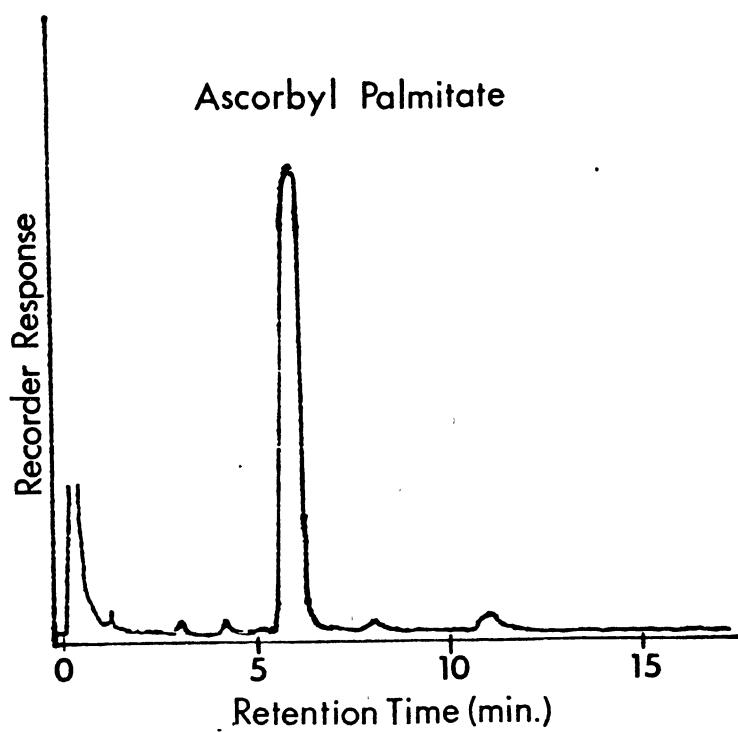


Figure 13A

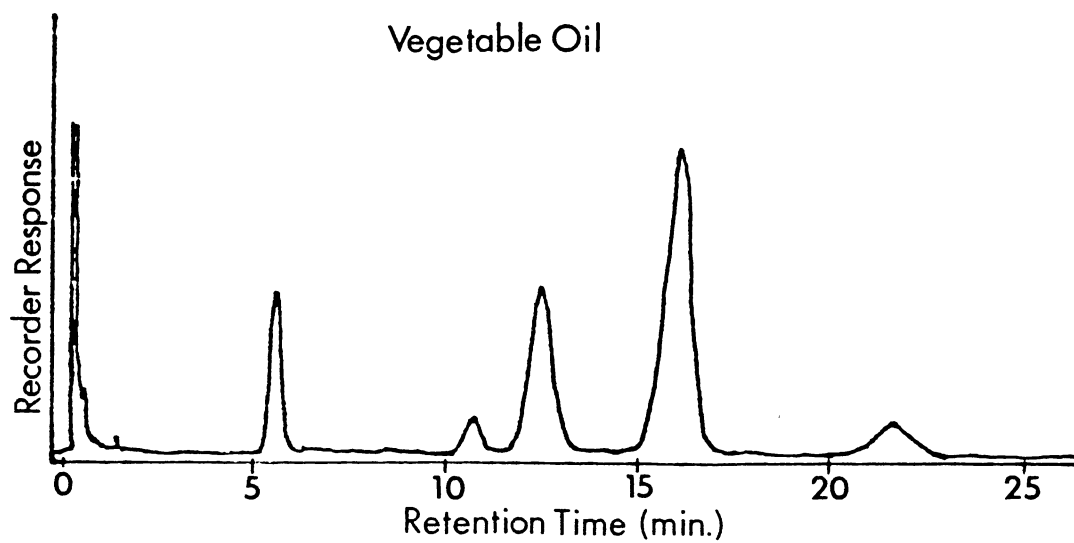


Figure 13B

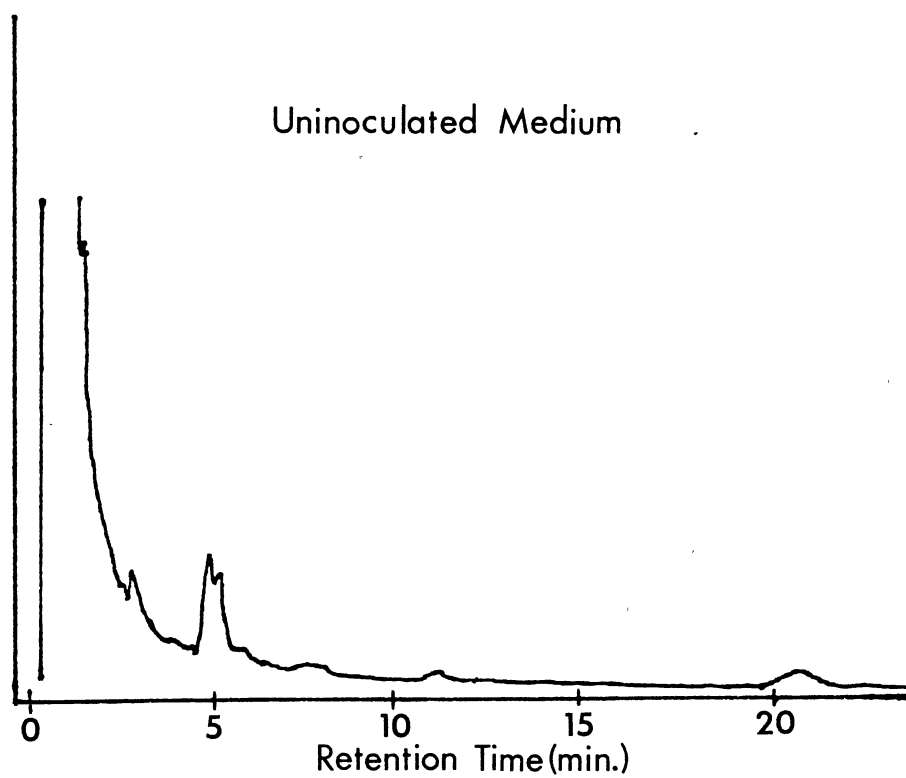


Figure 13C

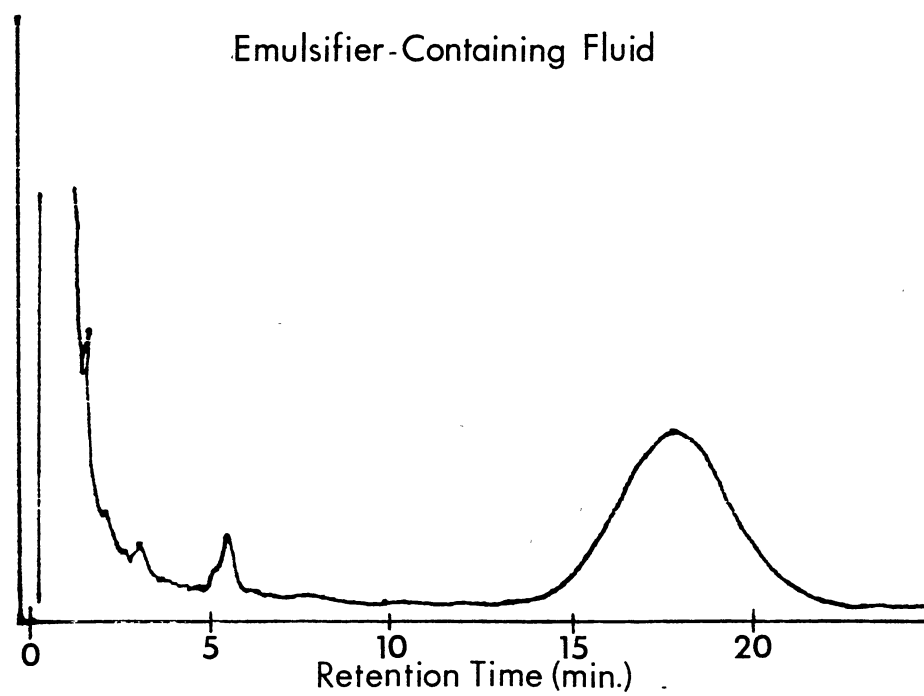


Figure 13D

## CHAPTER IV

### DISCUSSION

A bacterium has been described which produces a substance with the ability to lower the surface tension of the growth medium, and to stabilize a hydrocarbon-in-water emulsion. Two unusual features regarding the production of this substance are that the bacterium is an anaerobe and that the bacterium does not produce the substance in response to the presence of hydrocarbons in the growth medium.

#### Identity of HR-3E

The organism described has been shown to closely resemble Clostridium butyricum and, to a lesser extent also resembles Clostridium beijerinckii. The major difference between HR-3E and Cl. beijerinckii is that HR-3E is able to ferment ribose where Cl. beijerinckii is not.

The difference between HR-3E and Cl. butyricum is not so clear cut. Bergey's Manual of Determinative Bacteriology reports that Cl. butyricum "does not require amino acids or vitamins, other than biotin, for growth." As stated earlier HR-3E will not grow in a defined medium in which many growth factors, including biotin, were present.

#### Fermentation Products

A profile of the fermentation products of HR-3E is given. HR-3E

produces large amounts of gas in comparison to most of the anaerobes tested at Oklahoma State University for use in MEOR (Grula et al., 1983). HR-3E also produces moderate amounts of acids and solvents. Physiologically, the production of these compounds probably are not related to the production of the emulsifier. Gases, acids and solvents are normally the end products or intermediates in the energy yielding catabolism of carbohydrates. Bio-emulsifiers are normally produced by the organism in response to hydrocarbons present in the growth medium. Although this is not the case with the bio-emulsifier produced by HR-3E, it is obvious that the compound which appears to be responsible for hydrocarbon emulsification is not simply a product of the breakdown of sucrose.

Once produced by the organism the gases, acids, solvents and the emulsifier do seem to interact with each other. It was stated that HR-3E was first selected for this study because of the stable foam produced at the surface of the growth medium. Many of the organisms tested produce gas in quantities comparable to HR-3E, but few foam the medium in this manner. From the graphic representation of the fermentation characteristics it seems that the production of emulsifier begins before that of copious gas production. What apparently occurs is that the presence of the emulsifier stabilizes the foam produced by the evolution of gas by the organism.

Also, the acids and solvents may aid in the stabilization of emulsions by the spent media. Solutions of organic acids and solvents have lower surface tensions than pure water and compounds of this type have been used as co-surfactants in enhanced oil recovery procedures involving surfactant flooding (Taber, 1969).

## The Emulsifier Compound

### Purification

The simple purification procedure described is apparently sufficient to rid the spent medium from all components except the emulsifier compound. It was assumed that the emulsifier compound would be a relatively large (molecular weight greater than 1000 daltons) molecule. The growth medium was constructed of small components which could be easily separated from the emulsifier compound using dialysis.

Both gas and thin-layer chromatography show that most, if not all of the low-molecular weight compounds present in the uninoculated medium, and those produced by the organism have been removed by the dialysis procedure. Gas chromatography shows the virtual absence of low-molecular weight solvents. Thin-layer chromatography shows that no free amino acids or any other compounds with free amino groups (dipeptides, etc.) are present in the dialyzed emulsifier-containing fluid.

Apparently the small compounds present in the spent medium have been removed, if not completely, at least to the point where they are undetectable by chromatographic techniques. The question remains, how many large compounds are present in the emulsifier-containing fluid? Again gas and thin-layer chromatographic methods indicate that one single compound is present. The thin-layer chromatography method used to detect polar lipids and lipids with nitrogenous groups resolved the dialyzed emulsifier-containing fluid into a single component. The uninoculated medium was resolved into three components in this system, with a ninhydrin positive spot remaining at the origin. Gas

chromatography indicates the presence of only one fatty acid component in the emulsifier containing fluid. The uninoculated medium contained no fatty acids that chromatographed in the system used.

### Identification

From the data provided by the chromatographic techniques used on the emulsifier-containing fluid, the compound responsible for the stabilization of the emulsions formed appears to be a peptide-lipid.

The peptide portion of the molecule contains ten, or perhaps eleven, amino acids. From the amino acids present it is apparent why this molecule possess the ability to emulsify hydrocarbons. Of the ten (or eleven) amino acids present, half contain hydrophobic side chains and the other half contain hydrophilic side chains. While neither the sequence of the amino acids present nor the three-dimensional structure of the peptide is known, it is easy to envision the hydrophobic side chains of the amino acids possessing them (leucine, isoleucine, alanine, proline, and valine) becoming associated with an oil droplet while the hydrophilic side chains of the remaining amino acids (threonine, glutamic acid, glycine, lysine, methionine, and aspartic acid) remain at the surface of the oil droplet, forming a hydrophilic "coating" which would prevent the oil droplets from coalescing. This is the mechanism by which most proteins and peptides are thought to stabilize emulsions (Zajic and Panchal, 1976).

The lipid portion of the molecule is not so easily identified. The methyl ester of the lipid portion of the emulsifier compound has a retention time very near that of linoleic acid in the gas chromatography system used. Linoleic acid, however, contains two

double bonds, and fatty acids with more than one double bond are not known to exist in bacteria (Lehninger p. 281, 1975; Rawn p.453, 1983). Therefore the lipid present is not likely to be linoleic acid.

There are any number of possibilities as to the identity of the lipid. Most lipid components of microbially produced bio-emulsifiers are derivatives of straight-chain fatty acids, such as branched-chain fatty acids, hydroxy fatty acids, amino fatty acids, etc. (Cooper and Zajic, 1976).

From the gas chromatography data, reports of lipid chromatography using similar systems (to the one used in this study), literature concerning the lipids known to exist in similar bacteria, and literature concerning the types of lipids known to be present in microbially produced emulsifiers, it appears that the lipid from the emulsifier produced by HR-3E is likely to be from one of four classes of lipids. These classes of lipids include straight-chained, odd-numbered fatty acids, branched-chained fatty acids, hydroxy fatty acids, and cyclopropane fatty acids.

If the logarithm (base 10) of the retention time is plotted against the carbon chain length of a straight-chain, saturated fatty acid, a linear relationship is obtained. From this plot an "equivalent chain length" can be obtained. The equivalent chain length of the HR-3E emulsifier lipid is 19.4, and is therefore unlikely that this lipid is either nonadecanoic acid (19 carbons) or eicosanoic acid (arachidic acid, 20 carbons). Further evidence that the emulsifier lipid is neither nonadecanoic acid nor eicosanoic acid is that neither of these acids have been shown to exist in the "butyric acid" group of the genus Clostridium (Johnston and Goldfine, 1983).

Branched-chained fatty acids are common components of microbially produced emulsifiers (Cooper and Zajic, 1976). Iso-fatty acids have slightly lower retention times than the straight-chain isomer and as the methyl group is located to the carbonyl end of the lipid the retention time decreases. The retention time of the emulsifier lipid relative to palmitic acid is 2.87. The retention time of 18-methyl nonadecanoic acid (iso-arachidic acid) relative to palmitic acid can be predicted to be 2.78 from the data of Hawke, et. al. (1959) obtained from a chromatography system similar to that used in this study. These values are certainly comparable and could indicate the presence of a similar compound. However, no reports of branched-chain fatty acids in Clostridium were found in the literature.

Another possible identity of the emulsifier lipid is a hydroxy fatty acid.  $\beta$ -hydroxy fatty acids are common in microbially produced emulsifiers (Cooper and Zajic, 1976) and have been reported to be synthesized by Clostridia (Thomas, 1964). O'Brien and Rouser (1964) have analyzed all positional isomers of hydroxy palmitic acid using a system similar to the one used in this study, and found  $\beta$ -hydroxy palmitic acid to have a retention time of 2.62 relative to that of palmitic acid. This value is also comparable to the value of the emulsifier lipid.

Another possibility is that of a cyclopropane fatty acid. While no microbially produced emulsifier has been reported as containing a cyclopropane acid (Cooper and Zajic, 1976), these compounds are known to be produced in many bacteria, including clostridia (Johnston and Goldfine, 1983). Lactobacillic acid (cis-11,12-methylene octadecanoic acid) has a retention time relative to palmitic acid of 2.82 in a

system similar to the one used in this study (Brian and Gardner, 1967). The equivalent chain length of lactobacillic acid is 19.5 and the equivalent chain length of the emulsifier lipid is 19.4. Another similarity of these compounds is the peak shape. Both compounds give very broad peaks.

The above discussion is intended only to present some possibilities as to the chemical nature of the lipid portion of the emulsifier molecule produced by HR-3E, the actual identity of the compound is unknown.

#### The Lipid Chromatogram

As can be seen in Figure 13d, the peak given by the emulsifier lipid is quite broad. Again several explanations are possible for this occurrence. One is that the peak represents a mixture of two compounds that elute from the column together. Several microbially produced emulsifiers contain a mixture of lipid compounds (Cooper and Zajic, 1980).

Another possibility is that a small amount of water is present. As stated earlier, a portion of the emulsifier compound is made up of a peptide which contains hydrophilic amino acids. One end of the peptide is probably bound to the lipid at the carbonyl group in peptide bond, which is cleaved during esterification. However a second site of attachment is possible as seen in surfactin (Fig. 3). If any of the peptide remains bound to the lipid during esterification, especially a hydrophilic group carrying water a broad peak may result.

Perhaps the most plausible explanation has to do with the retention time of the compound and the attenuation of the chromatograph. The

longer a compound is in the mobile phase of a column the broader the peak will be. This can be seen in the chromatogram of vegetable oil, the compounds with higher retention times give broader peaks in the chromatogram. The attenuation must also be taken into account, to obtain a definite peak the attenuation of the machine was set at an attenuation of 4X. This sensitive setting will cause any peak to appear broad, as can be seen by comparing the benzene solvent peaks at the beginning of the chromatograms. Using the same time scale the peak for benzene is much more narrow in the vegetable oil chromatogram (att.= 256X) than in the emulsifier-containing fluid chromatogram (att.= 8X).

While it would have been desirable to define the chemical nature of the emulsifier from HR-3E to greater extent, this study does show that a compound is produced by this anaerobic organism that will stabilize an emulsion of hydrocarbons in an aqueous phase. Since this organism does not appear to utilize hydrocarbons for carbon or energy, the physiological reason for the production of the compound is unknown.

The contribution of this and similar compounds to Microbially Enhanced Oil Recovery is also unknown. It is thought that very low interfacial tensions are required for the displacement of oil from reservoir formations (Taber, 1969), and few, if any, microbially produced surfactants are effective enough to attain these low interfacial tensions. If other microbial products are successful in dislodging the oil present in reservoirs, the bacterially produced emulsifiers may serve to "hold" the oil in the flood water phase and thus contribute to the ease of removal of the oil.

It is also apparent from the study of this organism and other

organisms involved the study of MEOR that the production of emulsifiers by anaerobic bacteria is not as rare as the literature would indicate, but is simply unstudied. Since anaerobic fermentations are less costly than aerobic fermentations, if the production of bacterially produced surfactants is to become commercially feasible, as is expected (Cooper and Zajic, 1980), the anaerobic production of bio-emulsifiers should become the subject of increased study in the near future.

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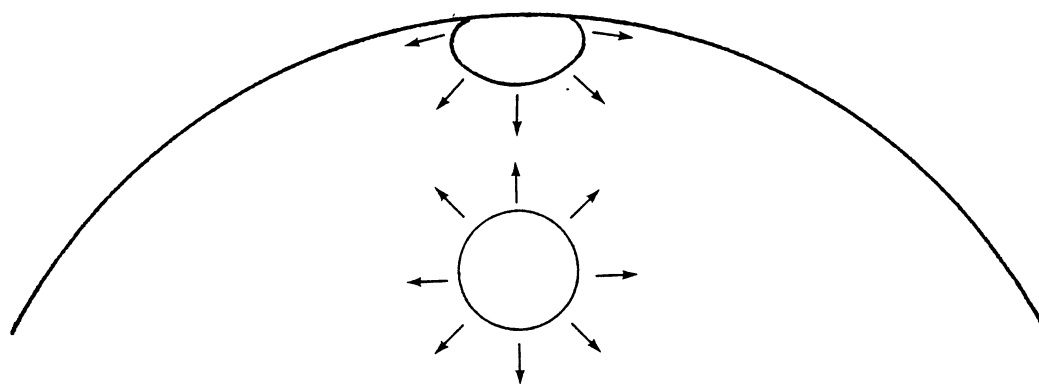
## APPENDIX

### TERMS USED IN THE STUDY OF SURFACTANTS AND EMULSIFIERS

The study of surface active agents and emulsifiers, like that of most fields, has its own terminology. The following is a brief explanation of the major terms.

The surface tension of a liquid is the force caused by the difference in the attractive forces between molecules at the surface of the liquid and the attractive forces between the molecules within the interior of the liquid. The interior molecules of the liquid are equally attracted from all directions by the surrounding molecules, but the molecules at the surface of the liquid are subjected to "imbalanced" attractive forces, as shown in Figure 14. This imbalance creates a situation similar to stretching a thin film. The force caused by "stretching" this "film" of surface molecules is the surface tension. Surface tension is normally given in units of dynes per centimeter, which results from the measurement of the work (in ergs or dynes·cm) to form the surface per unit of surface area (in  $\text{cm}^2$ ). Surface tension is the force responsible for the formation of droplets. The molecules of a liquid are more strongly attracted to other like liquid molecules than they are to the molecules of the surrounding medium. This difference in attractive forces causes the liquid molecules to "pull" themselves into a sphere, where the surface area

Figure 14. Diagram of Imbalanced Forces  
Responsible for Surface Tension  
in a Liquid Droplet.



per volume is as small as possible. Compounds with a high intermolecular attraction, such as water, have relatively high surface tensions, while those compounds with a low intermolecular attraction, such as chloroform and other organic solvents, normally have lower surface tensions (Table 5).

Surface tensions deal with the interface between the liquid and air. The analogous situation between two immiscible liquids is known as the interfacial tension. As with surface tension, a greater intermolecular attraction exists between like molecules than exists between the molecules of the different liquids. The asymmetric attractive forces experienced by the molecules at the interface of the two liquids causes the interfacial tension.

A surface active agent, or surfactant, is a substance which alters the surface or interfacial properties of the solvent in which they are present, even when present in small amounts. Although many compounds when mixed with water will lower the surface tension of the water not all are commonly thought of as surfactants. While many water miscible organic solvents are able to lower the surface tension of water, "conventional" surfactants create a much greater reduction in the surface tension of water even though the concentration of the surfactant is quite low when compared to the concentration of the solvent (Table 6). Normally when the term surfactant is used, it refers to a class of molecules which contain both a hydrophilic, polar portion and a hydrophobic, non-polar portion. A molecule of this type is known as an amphipathic molecule. The hydrophobic portion of the molecule is driven to the surface of the liquid by the repellant forces of the water. These molecules essentially create a new surface for the

TABLE VIII  
SURFACE TENSIONS OF VARIOUS LIQUIDS AT 20° C

Liquid	Surface tension (dynes/cm)
Water	72.7
Benzene	28.9
Acetic acid	27.6
Chloroform	27.2
Carbon tetrachloride	26.8
Acetone	23.5
Ethanol	22.5
n-Hexane	18.4

TABLE IX  
SURFACE TENSIONS OF AQUEOUS SOLUTIONS  
OF VARIOUS COMPOUNDS

Solution	Surface tension (dynes/cm)
Solvents:	
1% 2-propanol	65.0
5% 2-propanol	50.5
1% Acetone	69.8
5% Acetone	59.8
Surfactants:	
0.05% Triton X-100	31.2
0.1% Triton X-100	32.3
0.05% Sodium dodecyl sulfate	33.1
0.1% Sodium dodecyl sulfate	35.2

liquid in which the molecules are not subjected to the same attractive forces as the surface molecules of the pure liquid. This altered molecular attraction results in a lowered surface tension (Figure 15).

The reduction of the interfacial tension between two immiscible liquids is analogous to the reduction in surface tension. In the case of interfacial tension the hydrophobic and hydrophilic portions of the molecule create a "bridge" between the two immiscible liquids (Figure 16). With this new interface the asymmetric attractive forces are lessened and the interfacial tension is decreased.

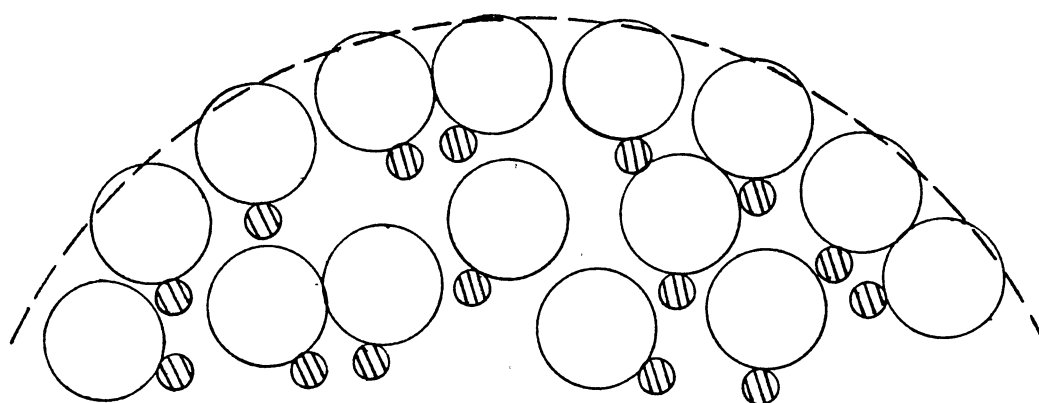
A micelle is a grouping of amphipathic molecules in water where the hydrophobic moieties are turned toward the interior of the cluster and the hydrophilic portions remain turned outward, toward the water (Figure 17).

The critical micelle concentration (CMC) of a surfactant is the concentration at which the surface of the surfactant solution becomes saturated with the surfactant and micelles begin to form. At the CMC the surface tension of the solution does not decrease further even if more surfactant is added to the system.

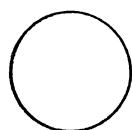
An emulsion is a system of two immiscible liquids, one dispersed as droplets throughout the other. The liquid which is broken into droplets is the dispersed, or discontinuous phase, the liquid surrounding the droplets is the external or continuous phase. There are two basic types of emulsions, oil-in-water where the water is the continuous phase, and water-in-oil, where water is the discontinuous phase.

An emulsifier is a substance which stabilizes an emulsion system. The type of emulsion formed (water-in-oil or oil-in-water) is dependent

Figure 15. Diagram of the Action of a Surfactant  
at the Surface of an Aqueous Solution.



----- Liquid Surface

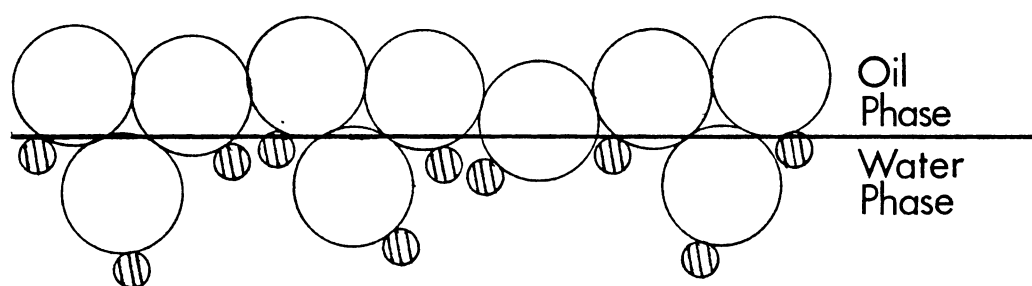


Hydrophobic Portion



Hydrophilic Portion

Figure 16. Diagram of the Action of a Surfactant  
at the Interface Between Immiscible  
Liquids.

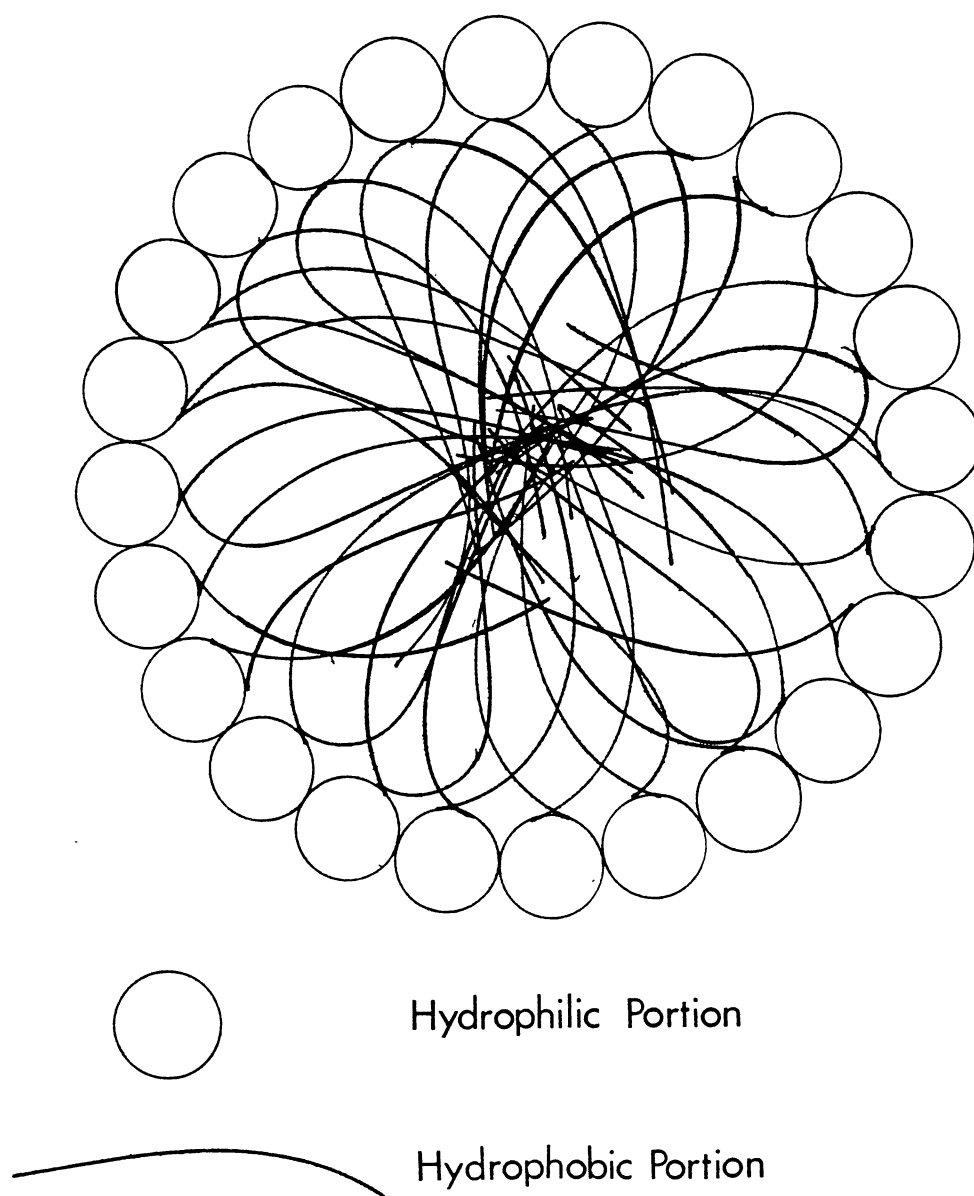


Oil/Water Interface

Hydrophobic Portion

Hydrophilic Portion

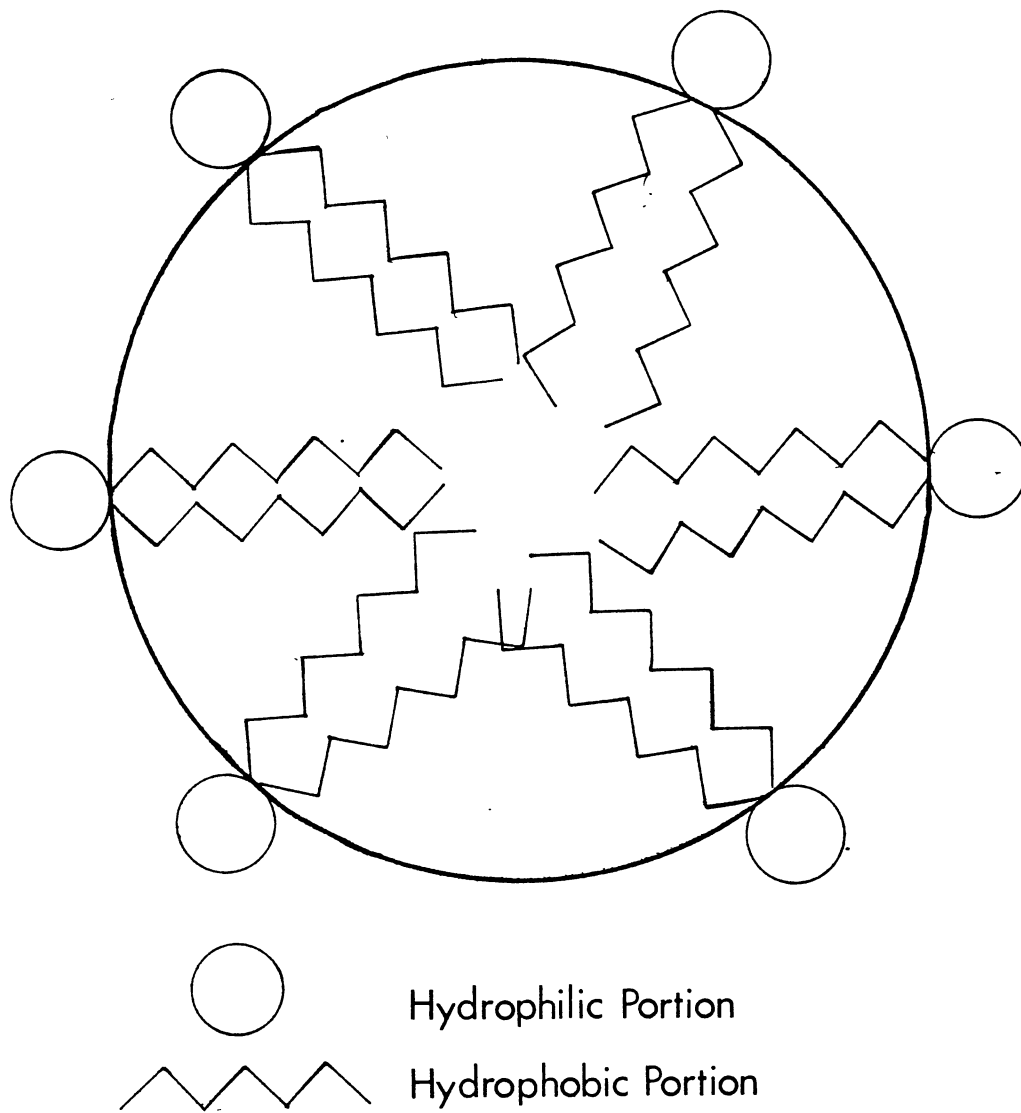
Figure 17. Diagram of the Cross-section of a Micelle.



on the emulsifier. The best emulsifiers are the amphipathic surfactant compounds. With these compounds the hydrophobic portion of the molecule becomes associated with the oil phase of the emulsion system, leaving the hydrophilic portion of the molecule on the outside of the oil droplet. The hydrophilic groups of the molecules act as a barrier, preventing the droplets from coalescing, thus the emulsion is maintained (Figure 18).

Other types of substances have been known to act as emulsifiers. Among these substances are macromolecules such as proteins (i.e. bovine serum albumen), various gums and finely divided solids (i.e. quartz) (Zajic and Panchal, 1976). The mechanisms by which these compounds stabilize an emulsion is not understood as well as the mechanism by which the amphipathic surfactants act, however it is generally believed that these substances in some way "coat" the droplets and prevent the droplets of the discontinuous phase from coalescing.

Figure 18. Diagram of the Mechanism of a  
Surfactant Stabilized Emulsion.



VITA

- - Dennis Lynn Bryant

Candidate for the Degree of  
Master of Science

Thesis: ISOLATION AND PARTIAL CHARACTERIZATION OF A BIO-EMULSIFIER  
PRODUCED BY A CLOSTRIDIAL ISOLATE

Major Field: Microbiology

Biographical:

Personal Data: Born in Denison, Texas, August 8, 1957, the son of  
Gerald E. and Mary L. Bryant.

Marital Status: Single.

Education: Graduated from U. S. Grant High School, Oklahoma City,  
Oklahoma, in May 1975; attended South Oklahoma City Junior  
College from August 1975 to May 1977; received Bachelor of  
Science Degree in Microbiology from Oklahoma State University  
in December 1979; completed requirements for Master of  
Science degree at Oklahoma State University in July, 1984.

Professional Experience: Teaching Assistant, Department of  
Microbiology, Oklahoma State University, January, 1980, to  
May, 1980; Research Assistant, Department of Microbiology,  
Oklahoma State University, June, 1980, to August, 1983;  
Teaching Assistant, Department of Botany and Microbiology,  
Oklahoma State University, January, 1983 to December 1983.  
Research Assistant, Department of Food Science, University of  
Illinois, January, 1984, to present.