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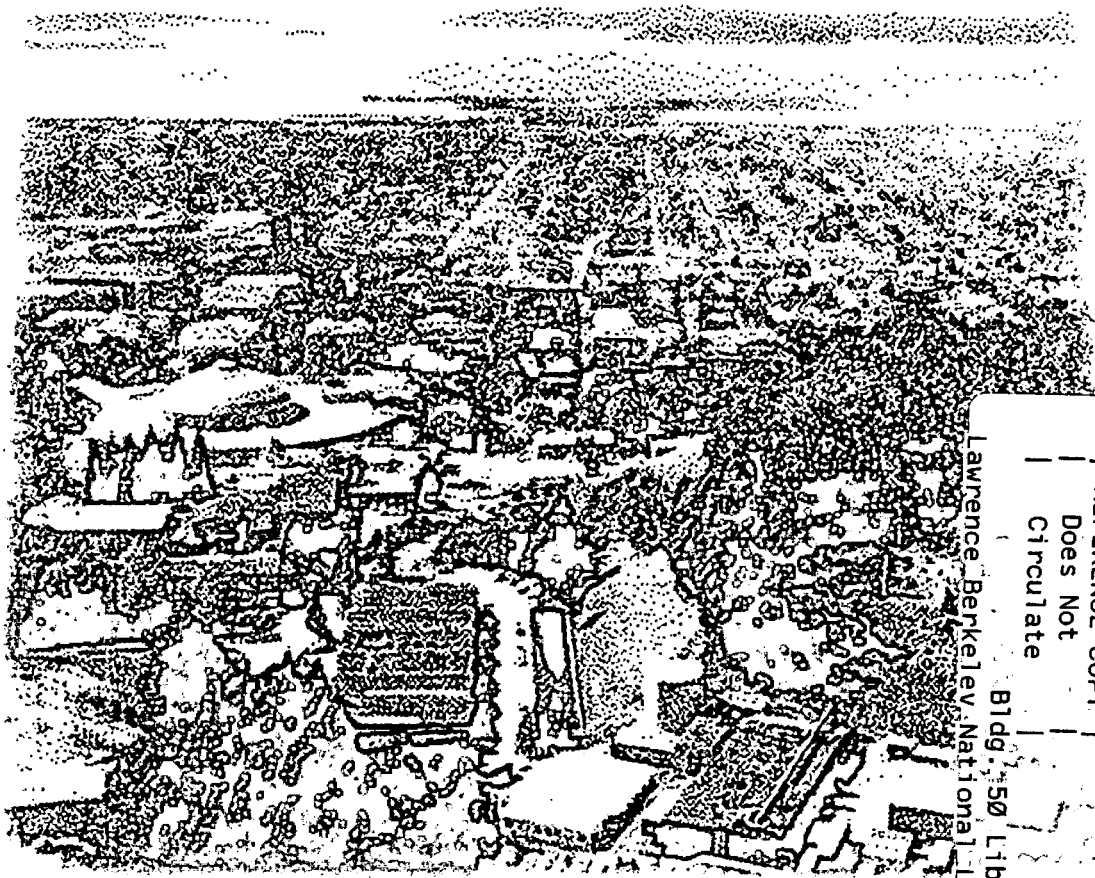

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ABSTRACT

This report is a description of the laboratory study undertaken to determine the biodegradability of Colloidal Silica (CS) and PolySiloXane (PSX), a new generation of barrier liquids employed by the Viscous Liquid Barrier (VLB) technology in the containment of subsurface contaminants. Susceptibility of either material to microbial degradation would suggest that the effectiveness of a barrier in the subsurface may deteriorate over time. Degradation may result from several different biological events. Organisms may consume the material as a carbon and/or energy source, organisms may chemically change the material as a detoxification mechanism, or organisms may erode the material by their physical penetration of the material during growth. To determine if degradation occurs, physical interactions between soil microbes and the barrier materials were analyzed, and the metabolic activity of individual organisms in the presence of CS and PSX was measured.

A set of 4 experiments were conducted. In Experiment 1 the soil microbial populations were characterized. The viable and CS- and PSX-tolerant microorganisms were selected in Experiment 2, and the barrier liquid effect on the microbial growth rates was determined in Experiment 3. The aerobic and anaerobic behavior of microbial colonies was visualized in Experiment 4 by using tetrazolium red, a dye which is clear in its oxidized state and becomes colored when reduced by biological activity. The results of the study indicated that PSX appeared to inhibit microbial growth despite its methyl and vinyl groups that could potentially serve as carbon sources for microbes. Biological activity in the CS appears to be higher than for PSX, and is attributed to the aqueous nature of CS which provides moisture for microbial growth. Such activity showed some effects (such as CS surface *dimpling*) that, under certain conditions, could be interpreted as indications of biosusceptibility. It is, however, unlikely that such *dimpling* will have any significant detrimental effect on the barrier permeability because of the limited nutrient and oxygen availability in the vicinity of, and within, the subsurface barrier. It is also quite possible that microbial growth on the barrier surface may have a beneficial effect by creating a low-permeability bacterial layer on the outer boundary surfaces of the barrier.

1. INTRODUCTION

Lawrence Berkeley National Laboratory (LBNL) staff have developed a permeation-based subsurface containment technology using a new generation of viscosity-sensitive liquids which, when set in porous media, cause the media to exhibit near-zero permeabilities and contain the contamination in the subsurface by entrapping and isolating both the waste source and the plume by a chemically inert physical barrier [*Moridis et al.*, 1993, 1994, 1995; *Persoff et al.*, 1994, 1995].

In order to create the barrier, a low-viscosity liquid is injected through a geometric array of injection points in the subsurface, which defines the barrier configuration in skeletal form. The injected liquid from the separate injection points merges to surround and isolate the contaminant source and/or plume. Once in place, it gels or cures to form a nearly impermeable barrier.

The LBNL Viscous Liquid Barrier (VLB) technology can be applied at a wide range of sites where hazardous wastes (radionuclides, heavy metals, organics, mixed) have contaminated the subsurface environment. These include isolation of ponds, buried tanks and other sources of contamination using a variety of barrier configurations (e.g. *box, inverted roof,* cylindrical and cone-shaped barriers. etc.), and either the creation or repair of landfill caps and liners. The technology can be applied in three ways: (1) permanent immobilization of contaminants, (2) creation of an impermeable container to surround and isolate the contaminated areas, or (3) sealing of permeable aquifer zones, thus helping to confine traditional cleanup techniques (pump and treat) in the difficult-to-treat zones.

1.1. Barrier Liquids in the VLB Technology

Two general types of barrier liquids have been tested within the framework of the VLB technology [*Moridis et al.*, 1994, 1995; *Persoff et al.*, 1994, 1995]. The first type belongs to the **P**oly**S**ilo**X**ane (PSX) family, and involves vinyl-terminated silanes with dimethyl side groups. The increase in viscosity in PSX is caused by cross-linkage, which is controlled by the quantities of catalyst, crosslinker, and (occasionally) retardant added to the PSX prior to injection. A special, low-viscosity PSX (called PSX-10) was developed for LBNL by Dow Corning, the industrial partner in this proposal. This material was shown to have exceptional barrier properties under field conditions [*Moridis et al.*, 1995].

The second barrier liquid is **C**olloidal **S**ilica (CS), i.e. an aqueous suspension of silica microspheres in a stabilizing electrolyte. It has excellent durability characteristics, poses no health hazard, is practically unaffected by filtration, and is chemically and biologically benign. The increase in viscosity of the CS following injection is due to a controlled gelation process induced by the presence of a neutralizing agent or a concentrated salt solution, either of which are added immediately prior to injection at ambient temperatures. The CS has a tendency to interact with the geologic matrix, and therefore, special formulations or techniques are required to minimize or eliminate the impact of such interactions.

1.2. Objective

The goal of this research was to determine the biodegradability of CS and PSX. Susceptibility of either material to microbial degradation would suggest that the effectiveness of a barrier in the subsurface may deteriorate over time.

Degradation may result from several different biological events: organisms may consume the material as a carbon and/or energy source, organisms may chemically change the material as a detoxification mechanism, or organisms may erode the material by their physical penetration of the material during growth. To determine if degradation occurs, physical interactions between soil microbes and the barrier materials were analyzed, and the metabolic activity of individual organisms in the presence of CS and PSX was measured.

2. MATERIALS AND METHODS

2.1. Soil Samples

The Hanford Site in Washington State is a candidate site for permanent waste containment using the injectable barriers. A sand and gravel quarry in Los Banos, California, with a geology similar to that of Hanford, had been established as an uncontaminated site for field tests of the barrier technologies. Uncontaminated soil samples from these two sites were used in this study.

The Hanford soil had been provided to LBNL as a part of the VLB project. This soil had been stored outside in a steel drum for approximately two years before use. The Los Banos soil sample had been obtained approximately two months before this study, and had been stored in a plastic container.

The pH of each soil was measured by shaking 5.0 g soil in 5 ml ddiH_2O (distilled, deionized H_2O) for 1 min and waiting 20 min before taking the reading. The electrode of the pH meter was positioned with the tip just below the surface of the soil.

2.2. Soil Extracts

In order to provide microorganisms with representative nutrients from the Hanford and Los Banos sites, soil extracts were prepared with the soils from each site (SE-H and SE-LB). Each soil extract was prepared as follows: 500 g soil were transferred into a double-layer cheesecloth. This "tea bag" was submerged in 2 l of ddiH_2O and allowed to steep for 25 min just below 100 °C. The resulting muddy solution was filter-sterilized

using a Corning disposable vacuum filter system with a 0.22-μm Cellulose Acetate filter. About 1800 ml of a clear soil extract was collected for each site. The pH of the filter-sterilized soil extracts was measured.

2.3. Nutrient Media

A variety of nutrient media were used. These ranged from minimal media to rich growth media, and are discussed in detail in **APPENDIX D**. The former provide cells with supplementary nitrogen and phosphate but no carbon, and are thus inadequate to support growth without an external carbon source. The rich media provide a complete spectrum of nutrients and are sufficient for cell growth. Some of the media used in this study have been designed to favor growth of a particular type of organism, such as actinomycetes or gram-negative bacteria. All media were sterilized by autoclaving for 15 minutes at 121 °C.

Two minimal media were prepared: **P**hosphate and **I**norganic **N**itrogen (PIN) and **P**hosphate and **O**rganic **N**itrogen (PON), according to recipes provided by *Hunter-Cevera* [1995]. Both were prepared 10 times concentrated to compensate for subsequent dilution in sample preparations.

The four rich media used in this study were modified versions of media described by *Hunter-Cevera et al.*, [1986]. Rich **S**oil **E**xtract (RSE) is a non-selective growth medium while **Y**east **M**alt **E**xtract (YME) favors the growth of fungi. Gram-positive bacteria tend to dominate a non-selective media plate, such as RSE. The other two rich media, **V**iolet-**R**ed **B**ile **A**gar (VRBA) and **A**rginine **G**lycerol **S**alts **M**edium (AGSM) are selective for gram-negative bacteria and actinomycetes, respectively. It was the manufacturer's recommendation (Difco) that VRBA not be autoclaved.

2.4. Experiment 1: Characterization of Soil Microbial Populations

To gain insight into the biodiversity of the soil at each site, the soils were plated on petri plates containing the above rich media and 1.5% Difco Bacto-Agar. Note that VRBA already contains the agar. The use of a variety of media allows more

community members to be cultivated, as some organisms may grow on some media but not on others.

2.4.1. Inoculation Methods

Two methods of inoculating the media plates were employed. A *stamping* method [*Hunter-Cevera et al.*, 1986] was used to transfer soil particles to the YME and AGSM plates. This method favors a greater diversity of fungi and actinomycetes and involves using a sterile sponge to stamp a small amount of ground soil over several petri plates.

To isolate bacteria in the second method, RSE and VRBA plates were inoculated by performing serial dilutions of a soil sample from each site, and by spreading 0.1 ml of each dilution on individual plates. The dilutions were prepared at room temperature by mixing 1.0 g Hanford soil in 10 ml of a 10% SE-H diluent and 1.0 g Los Banos soil in 10 ml of a 10% SE-LB diluent. These samples were then serial-diluted to 10^{-7} times the original concentration. The samples of the 10^{-2} through 10^{-7} dilutions were each spread-plated onto RSE and VRBA plates. This method of serial dilutions gives an indication of the levels of microbial populations in the soils, which are determined by counting the Colony-Forming Units (CFU's) that appear on a plate of a known dilution. All plates were incubated at 20 °C for 8 days.

2.5. Experiment 2: Selection of PSX-and CS-Tolerant Organisms

2.5.1. Barrier Liquid Specifications

The barrier liquids used in this investigation were as follows:

Polysiloxane (PSX):	Dow Corning (R) 2-7154-PSX10
Crosslinkage catalyst:	Dow Corning (R) 2-0707 INT (Pt 4)
Colloidal Silica (CS):	PQ Corporation, NYACOL® DP5110
Gelling agent:	Hill Brothers, HB98 Liquid $CaCl_2$ (3.5X dilute, ~0.4 M)

2.5.2. Preparation of Culture Plates With Barrier Materials

In order to encourage growth of soil microbes that can at least tolerate the barrier materials, plates of PSX and CS (supplemented with different nutrient media) were inoculated with soil and incubated until colonies formed. The PSX plates were prepared by combining 29.4 ml of PSX with 0.6 ml (2%) of the PSX crosslinking catalyst in a 50 ml Fisherbrand sterile polypropylene tube. This mixture was vortexed for 10 s and left to stand for 10 min. Twenty ml of nutrient media were added to the tube and then the mixture was vortexed for 20 s every 3 min until crosslinkage had sufficiently progressed (about 20-30 min). The aqueous phase of the nutrient media is not miscible with PSX, thus resulting in an emulsion of PSX droplets suspended in the aqueous phase. Before the solidification of PSX, the mixture was aliquoted into 9 wells of Corning Cell Well™ 6-well polystyrene plates.

The CS plates were prepared in a similar fashion, and by accounting for the faster gelation of CS. 25 ml CS solution was combined with 5 ml of the 0.4 M $CaCl_2$ gelling solution in a 50 ml Fisherbrand tube. The tube was vortexed for 10 s and left to settle for 5 min before adding 20 ml of media. The mixture was vortexed once more for 20 s before aliquoting the 50 ml into 9 wells of the Corning plates. The CS is an aqueous suspension and forms a homogeneous mixture with the aqueous nutrient media.

The final ratio of barrier material to nutrient media was 3:2 by volume. The different media used in these preparations were the minimal PIN and PON, and the rich RSE and YME. CS samples with minimal media were discarded because they failed to gel. This was attributed caused by the presence of phosphates in the nutrient media, which cause the precipitation of the divalent calcium ions needed to destabilize the colloid and effect the CS gelation. Because the YME accelerated the gelation of CS, the YME and CS mixture had to be prepared fast. RSE did not appear to have any measurable effect on the CS gelling rate.

2.5.3. Inocula:

Once the solidification of PSX and CS had advanced sufficiently, they were stamped with soil samples from the

Hanford and the Los Banos sites using a small sterile sponge. Of the 9 samples corresponding to all possible combinations of nutrient media and a barrier materials, 3 were inoculated with Hanford sand, 3 were inoculated with Los Banos soil and 3 were left blank. The sample plates were incubated at 20°C for 5 days.

2.5.4. Isolation and Identification of PSX and CS-Tolerant Organisms

Bacterial and fungal colonies that grew in the presence of PSX and CS were picked with sterile toothpicks and streaked onto petri plates of rich media (RSE or YME) and 1.5% agar. Purification of the organisms was achieved by subsequent picking of single colonies and streaking onto clean rich-media plates.

The bacteria were identified using the Biolog Identification System (BIS) [*Biolog*, 1995], in which an ELISA plate reader monitors the growth of a purified organism supplemented with different carbon sources. BIS can identify the species of bacteria based on the carbon sources which the organisms can (or cannot) metabolize. The fungi were classified based on morphology [*Hunter-Cevera and Torok*, 1995]. The identified organisms and their plates of origin are described in a table below.

2.5.4. Imaging of Samples

Images of fungi and bacteria growing in the presence of PSX and CS were captured using both scanning electron microscopy (SEM) and light microscopy. In preparing SEM samples, sections of barrier material were removed from the wells of the 6-well culture plates. Due to its relatively strong adhesion, the PSX had to be cut from the wells with a razor blade. CS samples were easy to obtain as it had already fragmented from drying (a process which occurs when the air relative humidity is lower than 85%).

The samples were first attached to a SEM mount with double-sided graphite tape and then coated with a thin layer of gold using a SEM sputter coater for 3 min at a current of 20 mA and a pressure of 0.06 torr. In subsequent experiments, shorter coating times (about 1.5 min) were used because the 3 min time was found to result in a slightly thicker-than-optimum gold layer. To assure good conductivity, a drop of graphite colloid was brushed between each sample and the SEM mount.

2.6. Experiment 3: Effect of PSX and CS on the Growth Rates of Organisms

In experiment 3 BIS was used to measure the biological activity of the bacterial isolates in the presence and in the absence of the barrier materials. BIS can monitor simultaneously the rate of change in optical density in each well of a 96-well plate. This change in optical density is caused by coloration of tetrazolium violet dye, present in the Biolog MT MictoPlates™. The dye indicates biological activity by acting as the terminal electron acceptor in the cellular metabolism and turning from clear to violet when reduced by electrons. This experiment gives a quantitative measure of the inhibition or promotion of cell growth in the presence of PSX and CS.

2.6.1. Inocula

The Biolog plates have 12 wells per row and 8 per column. Each row was inoculated with a single organism. Before inoculating the wells, the isolates were grown overnight on plates of solid media at 32 °C. Gram-negative bacteria were grown in a TSA+blood medium. Gram-positive bacteria other than *Bacillus spp.* were grown in a BUGM+blood medium, while *Bacillus spp.* were grown in a BUGM+glucose medium. These media and the protocols for identification and metabolic studies of organisms using the BIS are provided by *Biolog* [1995]. Cells from each plate were suspended in Biolog Tubed Suspension Saline. Cells were added until the suspension reached a specified level of turbidity on the Biolog Turbidimeter. 50 µl of this suspension were added to each well of a row.

2.6.2. Media and Barrier Material Combinations

To create a variety of growth conditions, individual strains were given a variety of nutrients. To place a demand on the cells for additional nutrients, cells were given only Soil Extract (SE) or nothing. To see how organisms responded to the barrier materials when ample nutrients were available for growth, RSE and YME were provided.

Of the 12 wells corresponding to each organism, 3 wells contained YME, 3 wells contained RSE, and 3 contained SE. Forty µl of these media were pipetted into the appropriate well. The

remaining 3 wells per row were left free of nutrient media. Thus, per organism, there were 4 different nutrient scenarios, with 3 wells per scenario. Of these 3 wells, one contained CS, the second PSX, and the third was left free of barrier material.

The PSX was first mixed with the crosslinking catalyst, and vortexed for 10 s before adding 50 µl of the mixture to each well. 50 µl of the CS suspension were added to the appropriate wells, followed by 10 µl of the gelling agent (brine). The final product allowed monitoring of the growth of each organism in the presence (and absence) of the barrier materials under 4 different nutrient scenarios.

One hour was allowed for crosslinkage/gelling before the first absorbance reading. Each multi-well plate was prepared in triplicate.

To ensure that the measured biological activity was that of the inoculum and not from contaminating organisms, the PSX and the CS, as well as the corresponding gelling agents and crosslinking catalysts, had been sterilized with propylene oxide gas. The barrier liquids were sealed in a chamber with propylene oxide gas for 3 days, and then left to vent for 3 days. Common heat sterilization was not used as it could alter the chemical properties of the PSX and CS. Propylene oxide gas is unlikely to have such an effect. Note that the barrier materials were only sterilized with propylene oxide for this experiment, in which the growth rates of individual organisms were measured. All other experiments were carried out with unsterilized barrier materials.

Only the growth rates of bacteria could be measured in this experiment because the growth of fungi and actinomycetes is inhibited by the tetrazolium dye. Furthermore, only purified, individual organisms could be tested, as inoculating the wells of a Biolog plate with an entire soil community could introduce too many nutrients from the soil into the culture. Under such a scenario it cannot be determined if growth in the minimal or no-media wells is due to organisms using the barrier materials as sustenance, or simply using nutrients from the soil.

2.7. Experiment 4: Tetrazolium Red Aerobic/Anaerobic Visualization

Like the tetrazolium violet dye used in the Biolog experiments, tetrazolium red (2,3,5-triphenyltetrazolium chloride dye) is clear in its oxidized state and becomes colored when reduced by biological activity. Thus, red coloration indicates growth. Controls include samples poisoned with 1% copper sulfate to show zero growth, and samples supplemented with YME for positive growth. YME was selected over RSE for the rich control because YME appeared to generate more growth of fungi **and** bacteria than RSE in the previous cultures. It seemed to be the *richer* of the two. Hanford organisms and SE-H were used in the controls.

2.7.1. Inocula

The community of microorganisms from the Hanford and the Los Banos sites were separated from the respective soil samples by shaking at room temperature (a) 1.0 g of Hanford soil in 10 ml of a 10% SE-H diluent and (b) 1.0 g Los Banos soil in 10 ml of a 10% SE-LB diluent, and collecting the supernatants (*H-orgs* and *LB-orgs*). These collections of cells were then mixed with the barrier materials and 0.3% 2,3,5-triphenyltetrazolium chloride dye.

2.7.2. Preparation of Culture Plates

Before the addition of the barrier materials, all ingredients of the various cultures were mixed and then vortexed for 5 s in 50 ml Fisherbrand tubes. The PSX solution was combined with the crosslinking catalyst (2% by volume), was vortexed for 10 s, left for 10 min, and then added to the other ingredients. This mixture was vortexed for 20 s every 3 min until sufficient crosslinkage had occurred (about 20-30 min). Before solidification, each sample was aliquoted into 3 wells of a Corning Cell Well™ 6-well polystyrene plate.

In the CS tests, 25 ml of ungelled CS suspension were mixed with 5 ml of the 0.4 M $CaCl_2$ gelling solution in a 50 ml Fisherbrand tube. The tube was vortexed for 10 s and left to settle for 5 min before adding it to the other ingredients. This mixture

was vortexed for 20 s before aliquoting each sample into 3 wells of a 6-well plate.

The ingredients for each sample are listed below, in the order that they were added in the sample preparation. The volume of each sample is 4 ml, to which 6 ml of PSX or CS were added, maintaining a 2:3 ratio of culture to barrier material.

(A) *killed control:*	2 ml H-orgs, 1.48 ml SE-H, 120 µl 10% tetrazolium sol., 400 µl 10% copper sulfate sol., 6 ml PSX
(B) *killed control:*	2 ml LB-orgs, 0.96 ml SE-LB, 240 µl 10% tetrazolium sol., 400 µl 10% copper sulfate sol., 6 ml CS
(C) *no inoculum:*	1.94 ml SE-H, 1.94 ml SE-LB, 120 µl 10% tetrazolium sol., 6 ml PSX
(D) *no inoculum:*	1.88 ml SE-H, 1.88 ml SE-LB, 240 µl 10% tetrazolium sol., 6 ml CS
(E) *H + PSX:*	2 ml H-orgs, 1.88 ml SE-H, 120 µl 10% tetrazolium sol., 6 ml PSX
(F) *LB + PSX:*	2 ml LB-orgs, 1.88 ml SE-LB, 120 µl 10% tetrazolium sol., 6 ml PSX
(G) *H + CS:*	2 ml H-orgs, 1.76 ml SE-H, 240 µl 10% tetrazolium sol., 6 ml CS
(H) *LB + CS:*	2 ml LB-orgs, 1.76 ml SE-LB, 240 µl 10% tetrazolium sol., 6 ml CS
(I) *rich control:*	1 ml H-orgs, 2.88 ml YME, 120 µl 10% tetrazolium sol., 6 ml PSX
(J) *rich control:*	1 ml H-orgs, 2.76 ml YME, 240 µl 10% tetrazolium sol., 6 ml CS

These samples will be referred to in Section 3 (Results and Discussion) by the above letters, A through J. Note that the muddy suspension of organisms that is used as inoculum contains many nutrients from the soil. Also note that, because CS is about 70% water (while PSX contains no water), more tetrazolium and copper sulfate were used in the CS samples in

order to keep the concentration of these compounds consistent in the aqueous phase of all samples.

The plates were incubated aerobically in the dark at 20°C and inspected periodically for 48 days. Photographs of the entire plates at day 1 and day 48 are provided below. Micrographs taken by using a light microscope are shown in **Figures 4.1** through **4.5** (**APPENDIX C**).

2.7.3. Anaerobic Experiment

The experiment was repeated, using the same media and sample preparation protocols, but differing in that the plates were incubated in an anaerobic jar (Difco). The conditions of the experiment favored the growth of those cells that exist under oxygen-deficient conditions in the deep subsurface. It is possible that anaerobic metabolisms will react differently to the barrier materials than aerobic metabolisms. One minor difference in the anaerobic protocol was that, in order to fit the anaerobic jar, the samples were poured into petri dishes to gel rather than into 6-well plates. The petri dishes were removed from the anaerobic chamber and viewed after 20 days of incubation at 20°C. These samples will be referred to by using the name convention of the analogous aerobic sample, but will be specified as anaerobic.

3. RESULTS AND DISCUSSION

3.1. pH of Soils and Soil Extracts

The pH of the Hanford and the Los Banos soils was 8.40 and 8.60 respectively. The Hanford soil extract (SE-H) had a pH of 7.23, and the Los Banos soil extract (SE-LB) had a pH of 7.34.

3.2. Experiment 1

The studies on the microbial communities of the Hanford and the Los Banos soils revealed a large population of bacteria, a smaller population of fungi, and a few actinomycetes. Viability was determined by counting the number of CFU's that result from a given quantity of a known dilution of the soil. Los Banos soil yielded about 10^7 CFU's per g. Hanford sand showed a concentration of about 4×10^5 CFU's per g. The lower viability of Hanford sample may be due to the more extreme storage conditions of the Hanford soil, or it may be due to the fact that the Hanford soil has a sandy consistency. The figures are in reasonable accordance with the claim that all soils and sediments contain about 10^9 cells per g but that viable counts, as performed above, may represent only 0.1% to 10% of the total community [*Tunlid and White*, 1991].

3.2.1. Test for Microbial Populations Preexisting in the Barrier Materials

In addition to the soil, PSX and CS were also tested for the presence of viable microorganisms. A volume of 0.1 ml of uncrosslinked PSX and ungelled CS, as well as the corresponding

crosslinking catalyst and gelling agent, were plated out on RSE, YME, AGSM, and VRBA plates (+1.5% agar). The ungelled PSX, its crosslinking catalyst, and the $CaCl_2$ solution yielded no growth on the media plates. The ungelled CS (without the $CaCl_2$ gelling electrolyte) produced growth on all four media plates, showing a variety of pre-existing fungi and bacteria in the material.

3.3. Experiment 2

3.3.1. Overview of Growth on Barrier Materials

Plates of barrier material supplemented with various media were inspected at 5 days with a dissecting microscope at a 20-40X magnification, and some growth was observed. All the figures related to Experiment 2 are located in **APPENDIX A**.

Three fungal colonies grew in PSX which had been supplemented with rich YME and inoculated with Hanford soil. One of them, *Fusarium sp.*, is shown in **Figure 2.1**. There was no other visible growth on any PSX plates, though streakings of the surface from the PSX + YME plates onto RSE + agar plates yielded some bacteria from both the Hanford and Los Banos sites. The supplemented PSX plates were inspected periodically up to 48 days, but no new growth appeared. Note that even after 48 days, there was no visible growth on PSX+RSE, PSX+minimal media, and PSX without nutrient media. This appears to indicate that PSX creates an unfavorable environment for microbial growth, at least under the conditions of the experiment.

After 5 days of incubation, the CS plates showed a variety of growth. All the plates supplemented with either YME or RSE, whether inoculated with soil or not, had collections of orange, yellow, and white bacterial colonies, as well as several types of fungi. **Figure 2.2**, photographed 48 days after inoculation, shows growth on CS supplemented with YME and inoculated with Hanford soil. Within 20 days white fungal colonies had even appeared on non-inoculated CS plates that contained no supplementary nutrients. In the latter case, no source of nutrients to support growth had been added to the system. No soil extract and no soil particles from an inoculum had been added, and CS was the only liquid present. This observation suggests that there are substances in the CS (i.e. sources of organic carbon in the water used in the manufacturing of CS), which preexisting organisms

can use as nutrients. A summary of growth occurrence in the various plates of this experiment is provided in **Table 2.1.**

Table 2.1. Growth (+) or No Growth (-) After 48 days

Barrier	With PIN	With PON	With RSE	With YME	Without Medium
PSX	(-)	(-)	(-)	(+)*	(-)
CS	N/A	N/A	(+)	(+)	(+)

(*) Growth on PSX with YME only for sample inoculated with Hanford soil. All other results apply to samples inoculated with Hanford soil, Los Banos soil and no soil.

3.3.2. Scanning Electron Microscope (SEM) Images

A SEM was used to investigate and photograph the barrier materials 47 days after inoculation. This was intended to reveal any visible degradation of the surface of the materials that may have been caused by growth of microorganisms.

PSX inoculated with Los Banos soil and without any supplementary nutrients showed soil particles on the PSX surface and no microbial growth (**Figure 2.3**). The SEM image of PSX inoculated with Hanford soil and with YME showed spherical (ball-like) globules of PSX that form in the presence of an aqueous-phase medium (**Figure 2.4**). These balls were in the vicinity of the growth shown in **Figure 2.1**, though no hyphae are visible in this SEM micrograph.

The texture of CS free of microbial growth is shown in **Figure 2.5**. This photograph was taken from a *clean* part of a CS sample inoculated with Hanford soil and with RSE; growth did occur elsewhere on this sample. In CS inoculated with Los Banos soil and with YME, a well-developed and sporulating fungal colony was observed (**Figure 2.6**). On the same sample, a fungal hyphae was shown possibly emerging from within the barrier material (**Figure 2.7**). **Figure 2.8** shows one of many well-

developed fungal colonies that appeared on a CS sample free of supplementary nutrients and without inoculum.

3.3.4. Isolates:

The original pool of purified isolates consisted of 28 bacteria and 12 fungi. After visual inspection many of these were discarded as duplicates and the final pool was narrowed to the 7 bacteria and 9 fungi listed in **Tables 2.2** and **2.3**. Identified organisms are listed below with the barrier material, medium, and inoculum used in the plate from which each organism was isolated.

Among the organisms that appeared on non-inoculated CS were *Rhodococcus erythropolis, Aureobacterium saperdae,* a *Penicillium, sp.* an *Aspergillus sp.*, and an unidentified fungus. *Rhodococcus erythropolis* and the unidentified fungus were the isolates able to grow without supplemented nutrients. The unidentified fungus had the interesting characteristic that it grew as slowly on a medium typically used to grow fungi (Potato Dextrose Agar), as it did on the unsupplemented CS.

3.4. Experiment 3

All the tables and figures related to Experiment 3 appear in **APPENDIX B. Tables 3.1 through 3.4** list the values of the absorbance at 590 nm at time 0 h and time 48 h for each organism in the presence or absence of barrier materials. Each value represents the average of 3 trials. Samples in **Table 3.1** were given no supplementary nutrients, those in **Table 3.2** were given soil extract, those in **Table 3.3** were supplemented with RSE, and **Table 3.4** shows values for organisms given YME. The difference in absorbance between time 0 h and time 48 h, caused by the coloration of tetrazolium violet dye, represents the extent of biological activity in each sample. These differences are depicted, by organism, in **Figures 3.1** through **3.7**.

The change in absorbance at 590 nm for the non-inoculated control wells is shown in **Figure 3.1**. The barrier materials had been sterilized with propylene oxide gas. Note that, even in the absence of biological activity, the absorbance of the CS increases slightly at 590 nm. This is probably caused by an increase in opacity of the gel as it sets. In the absence of biological activity, no change in absorbance occurs in the wells with no barrier material, nor in the wells with PSX.

Table 2.2. Biological Identification of Bacteria

Organism Name	Barrier material	Nutrient Medium	Inoculum
Flavobacterium indologenes	PSX	YME	Hanford
Variovorax paradoxus	PSX	YME	Los Banos
Pseudomonas mendocina	CS	YME	Los Banos
Ochrobactrum anthropi	PSX	YME	Hanford
Rhodococcus erythropolis	CS	none	none
Aureobacterium saperdae	CS	YME	none
Aureobacterium saperdae	PSX	YME	Hanford

Table 2.3. Morphological Classification of Fungi

Organism Name	Barrier Material	Nutrient Medium	Inoculum
Fusarium sp.	PSX	YME	Hanford
Aspergillus niger	PSX	YME	Hanford
Penicillium sp.	PSX	YME	Hanford
Streptomyces sp. (filamentous)	CS	RSE	Los Banos
Streptomyces sp. (asporogenous)	CS	YME	Hanford
Penicillium sp.	CS	YME	none
Aspergillus sp.	CS	RSE	Los Banos
Streptomyces sp.	CS	RSE	Los Banos
Unidentified fungus	CS	none	none

The effects of the barrier materials on the growth rates of the bacterial isolates are depicted in **Figures 3.2** through **3.7**. The change in absorbance (at 590 nm) of the CS wells was omitted for the first 4 organisms because no staining was observed in the CS wells inoculated with those organisms.

None of the 6 organisms tested showed significant biological activity when given no media (bars labeled "none") or only soil extract (bars labeled "SE"). This was the case whether or not barrier material was provided.

Flavobacterium indologenes and *Variovorax paradoxus* showed marked staining of the dye for the cultures supplied with the rich media (RSE and YME). This indication of biological activity was observed both in the presence and in the absence of PSX, although the test wells with PSX appeared to have less activity than those without barrier material (**Figures 3.2 and 3.3**). Both of these genera are aerobic, Gram-negative rods [*Holt et al.*, 1994). One possible explanation for the retarded growth is that PSX obstructs these aerobic organisms' access to oxygen.

Pseudomonas mendocina and *Ochrobactrum anthropi* also showed significant change in absorbance at 590 nm in the presence of rich media. These organisms seem to grow as rapidly in the presence of PSX as they do without it (**Figures 3.4 and 3.5**). These genera are also Gram-negative rods and typically aerobic. In some cases, *Pseudomonas spp.* can use nitrate as an alternative electron acceptor, allowing anaerobic growth [*Holt et al.*, 1994]. This ability could explain why *P. mendocina* can grow better in the presence of PSX than the strict aerobes, if PSX does indeed obstruct oxygen uptake by the cell.

Rhodococcus erthropolis had the interesting result that, when supplemented with RSE, appeared to grow more rapidly in the well with CS than in the well without barrier material (**Figure 3.6**). Perhaps the barrier material provided the organism with a favorable support matrix, or perhaps something in the CS material was used by the organism as additional nutrients. The genus *Rhodococcus* is composed of Gram-positive aerobes that may exist as rods or may form an extensively branched vegetative mycelium, and are particularly abundant in the soil [*Holt et al.*, 1994].

Aureobacterium saperdae showed no measurable growth under any scenario. In fact, it appears to perform much like the non-inoculated control (**Figure 3.7**). Members of this genus are

aerobic Gram-positive rods that require nutritionally rich media [*Holt et al.*, 1994].

3.5. Experiment 4

3.5.1. Aerobic Incubation

All the figures related to Experiment 4 appear in **APPENDIX C.** Photographs of the tetrazolium plates before and after 48 days of aerobic incubation (at 20 °C) are shown in **Figures 4.1** through **4.5.** Plates of samples C through H - in black and white - showed no visible coloration of the tetrazolium dye under the naked eye. Their coloration after 48 days is similar to that of the killed control samples (A and B), shown in color in **Figure 4.1.b**. The rich control samples (I and J) did show a red coloring, even after 5 days (**Figure 4.5**).

Plates were viewed after 5 days under a 20-40X magnification with a dissecting microscope. At this time, red coloration - indicating biological activity - was evident only in the rich-medium (YME) control plates (I, J). In the PSX + YME wells (I), growth was contained within pockets of the medium. **Figure 4.6** shows some hyphae and one red bacterial colony on CS + SE-LB inoculated with Los Banos organisms (H). **Figure 4.7**, from the same culture (H), shows a developing fungal colony that was clearly growing *within* the CS. **Figures 4.5.b, 4.8,** and **4.9** show localized growth (dark areas) which appears unable to penetrate and spread within the PSX material.

Many red colonies within the barrier material were observed in the wells of CS + YME (J). **Figure 4.10** shows these red colonies (appearing black) in CS. Note a *dimpling* on the CS where the colonies are just below the surface. **Figure 4.11** shows a fungus - not yet visible by day 5 - on the rich CS control (J).

All aerobic tetrazolium images were taken after 26 days of incubation in the dark at 20°C. The plates of the barrier materials and soil extract alone had not provided enough nutrients to result in visible growth within 5 days. By the time the plates were photographed (day 26) some fungi and a few bacterial colonies had appeared on the plates of CS and soil extract (D, G, H). There was no growth on the plates of PSX and soil extract (C, E, F) at that time. **Figure 4.12** (actually from an anaerobic sample,

i.e. anaerobic C), is a typical image of the PSX samples with no growth (compare with **Figure 4.9**).

3.5.2. Anaerobic Incubation

Under reducing anaerobic conditions, the tetrazolium dye may undergo some coloration even in the absence of biological activity. For this reason, resulting patches of red were carefully analyzed at 1000X magnification with a light microscope to determine whether or not growth had occurred.

No red coloration was observed on the killed control plates (anaerobic A, B). The CS plates (anaerobic D, G, H, and rich control J) all contained an even distribution of red forms like those shown in **Figure 4.13** (appearing black). At a higher (1000X) magnification these red objects appeared to be fungal and/or bacterial colonies. The PSX plates with soil extract (anaerobic C, E, F) all appeared similar to the killed control and showed no evidence of red coloration (**Figure 4.12**). The PSX rich control (anaerobic I) in **Figure 4.14** contained concentrated patches of red balls of the barrier material such as that seen in **Figure 2.4** (red appears black). At 1000X magnification (not shown), these patches resembled more closely a crystalline, non-biological structure than they do microbial growth. It is not known why such a non-biological red staining is observed in a *rich* control and not in the other anaerobic PSX plates.

4. CONCLUSIONS

The research project spanned only a few months, with the longest incubation of organisms and barrier materials lasting 48 days. This is a brief period compared to the decades of endurance required of the materials in the field. For this reason, evidence of resistance to degradation by either PSX or CS in these experiments is not a definite sign of resistance to degradation in the field over the long term. Thus, caution needs to be exercised in extending the applicability of the findings of these laboratory experiments to the biodegradation performance of barrier materials in the field.

In order to make possible the observation of microbial interactions with a barrier material in the brief period of this laboratory study, accelerated growth conditions were created for the organisms in these experiments. The organisms were provided with *rich* media or *minimal* media (with supplementary nitrogen and phosphate), as well as ample water and, in most cases, oxygen. These are favorable growth factors not present in the vadose zones (unsaturated subsurface environments) of semiarid sites such as Hanford [*Kieft et al.*, 1993]. Microorganisms can have very different biochemical capabilities in a short period of rapid growth - which characterized the conditions in these experiments - than in a long period of slow growth, as is likely to be the case in the field. The ability of an organism to break down a compound depends also on which other nutrients are available at the time. In some cases, a substance cannot be broken down without supplementary nutrients. In other cases, the presence of other nutrients will cause an organism to ignore a less desirable substance.

A summary of the findings of this study follows.

4.1. Polysiloxane

It was originally theorized that if one of the barrier materials was more supportive of microbial growth than the other, this would be the PSX. This was expected because PSX has methyl and vinyl groups that could potentially serve as carbon sources for microbes. The experimental observations, however, showed the opposite. Under the conditions of these experiments, PSX appeared to inhibit growth compared to CS and the no-barrier controls. Cell growth in the presence of PSX only occurred when the richest of the media, YME, was provided as a supplement. Even in this case, however, the growth was localized and could not penetrate (and spread into) the material; it was instead confined to pockets of aqueous media. This can be explained by the fact that PSX is neither an aqueous system nor water-miscible, and thus the YME nutrients are not solubilized but remain confined to the aqueous pockets.

Further evidence of the inability of PSX to biodegrade comes from Dow Corning Corporation, the PSX manufacturer. Dow Corning studies [*Dow Corning*, 1991] show that polydimethylsiloxane fluids - as is the ungelled PSX - do not bioaccumulate, meaning that the material cannot enter a cell membrane. This is due to the their large molecular size, which precludes membrane permeability. This finding appears to lessen the possible mechanisms of biodegradation of the material to those involving extracellular enzyme activity. Dow Corning has also carried out laboratory experiments analyzing biological oxygen demand (B.O.D.) and has found no evidence of biodegradation of polydimethylsiloxane fluids in soil [*Frye*, 1988; *Dow Corning*, 1991].

4.2. Colloidal Silica

Biological activity in the CS appears to be higher than for PSX, and is attributed to the aqueous nature of CS which provides moisture for microbial growth. This hypothesis is supported by the fact that growth in the non-aqueous PSX was only observed in aqueous pockets of externally-supplied nutrients. Biological activity in CS showed some effects that, under certain conditions, could be interpreted as indications of biosusceptibility. One such effect was the *dimpling* of the surface of the CS just above bacterial colonies suspended in the material (**Figure 4.10**). This was observed on the *rich* control plate of the

aerobic incubation with tetrazolium red (sample J, experiment 4). Such indentations could be the result of the concentrated growth of cells in a microbial colony.

The effect of such dimpling (produced under conditions of abundance of nutrients and oxygen, i.e. a highly improbable scenario in the subsurface) on the permeability of the CS-impregnated soil is not known. It is, however, unlikely that such *dimpling* will have any significant detrimental effect on the barrier permeability because of the limited nutrient and oxygen availability in the vicinity of and within the subsurface barrier. If there is any adverse effect of dimpling, this is expected to be limited to the outer boundaries of the barrier where the transfer rates of nutrients and oxygen are the highest due to higher permeability. The extremely low permeability within the barrier create practically anaerobic conditions, under which no dimpling was observed. The usual design thickness of the barrier (about 1 m) is such that the possible localized effects of dimpling (if they are indeed detrimental) are not expected to be important. It is also quite possible that such dimpling may have a beneficial effect by creating a low-permeability bacterial layer on the outer boundary surfaces of the barrier.

The growth observed in CS samples in which no external sources of nutrients had been provided indicates that certain microorganisms can derive the necessary nutrients from energy sources in the CS material. This is intriguing since pure CS contains no carbon. Such carbon sources are likely to preexist in the water used in the manufacturing process, or may be due to low-level CS contamination by organic substances in the course of CS production. Photosynthesis is not a likely source of energy because in this study the samples were incubated in the dark.

The observed penetration of the material by fungal hyphae could have positive or negative implications: these hyphae may enhance a barrier system by reducing its permeability with their physical presence in the form of a microbial layer. Physical erosion of the material due to his biological activity, though possible, is not likely due to the limited potential extent of such fungal colonies on the outer surfaces of the barrier and only very close to the surface. No fungal growth is expected in the deeper subsurface or under the near-anaerobic conditions within the barrier.

While some visual results may be interpreted as the product of degradation, there is currently no verification of

change in the chemical composition or permeability of the CS, but such an investigation is currently being planned. If organisms can grow favorably in the presence of CS without degrading it, such as by using the material solely as a support matrix, this could be advantageous. In addition to the potentially low permeability of a microbial film on its outer fringes, a barrier may facilitate the processes of bioremediation of the wastes they contain by providing a surface favorable for microbial colony growth.

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APPENDIX A

FIGURES OF EXPERIMENT 2

A1. Figure Short Descriptions and/or Clarifications

Figure 2.1. The red hyphae of a *Fusarium sp.*, growing on a ball of PSX submerged in YME and inoculated with Hanford soil. Visible within 5 days of inoculation.

Figure 2.2. A variety of fungal colonies (black, white, and grey spots) and bacteria (yellow background) on CS supplemented with YME and inoculated with Hanford soil. Photographed 48 days after inoculation.

Figure 2.3. PSX with no supplementary nutrients, inoculated with Los Banos soil. This image shows soil particles on surface of PSX and no growth of microorganisms.

Figure 2.4. PSX with YME, inoculated with Hanford soil. This Image shows balls of PSX that form in the presence of an aqueous-phase medium. These balls were in the vicinity of the growth shown in Figure 2.1, though no hyphae are visible in this SEM micrograph.

Figure 2.5. CS with RSE, inoculated with Hanford soil. This image shows texture of CS free of microbial growth. Growth did occur elsewhere on this sample.

Figure 2.6. CS with YME, inoculated with Los Banos soil. Image shows a well-developed and sporulating fungal colony growing on CS. Photographed after 47 days of incubation at room temperature.

Figure 2.7. Same sample as Figure 2.6. A fungal hyphae on CS, possibly emerging from within the barrier material.

Figure 2.8. CS without supplementary nutrients and without inoculum. A well-developed fungal colony able to grow on the CS material alone.

Figure 2.1
PSX + YME + Hanford soil inoculum, Day 5.
(Field width approximately 0.1 cm)

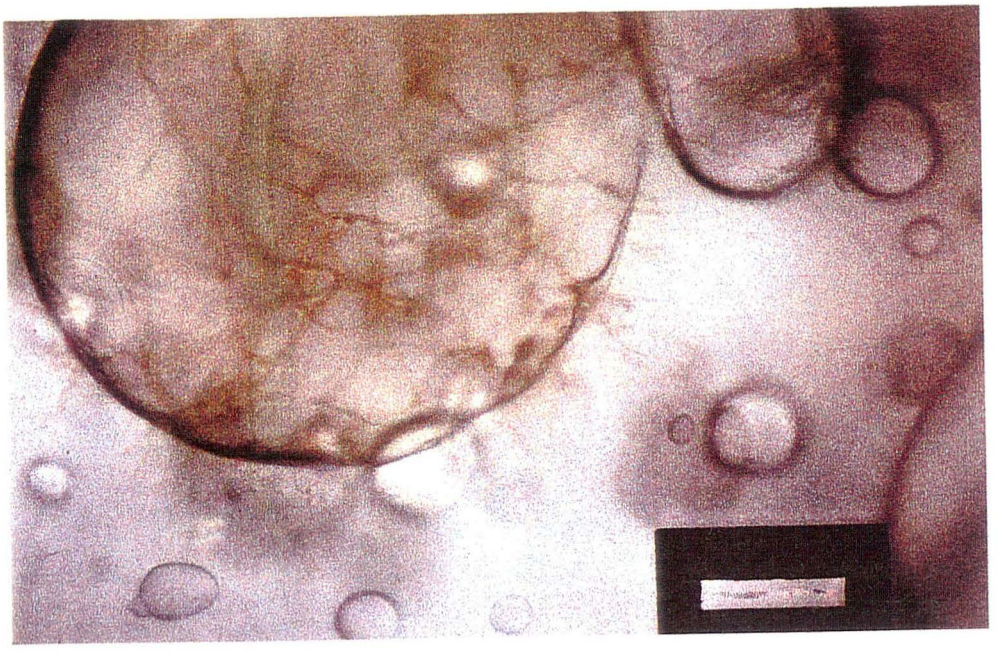


Figure 2.2
CS + YME + Hanford soil inoculum, Day 48.
(Field width approximately 3 cm)

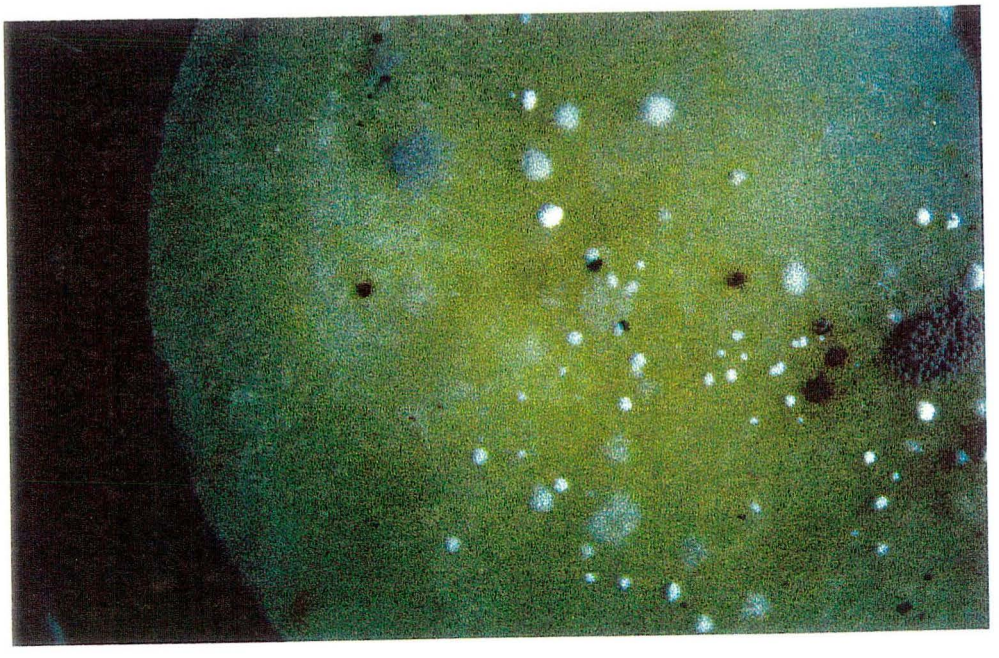


Figure 2.3: SEM micrograph. PSX + no supplementary nutrients + Los Banos soil inoculum.

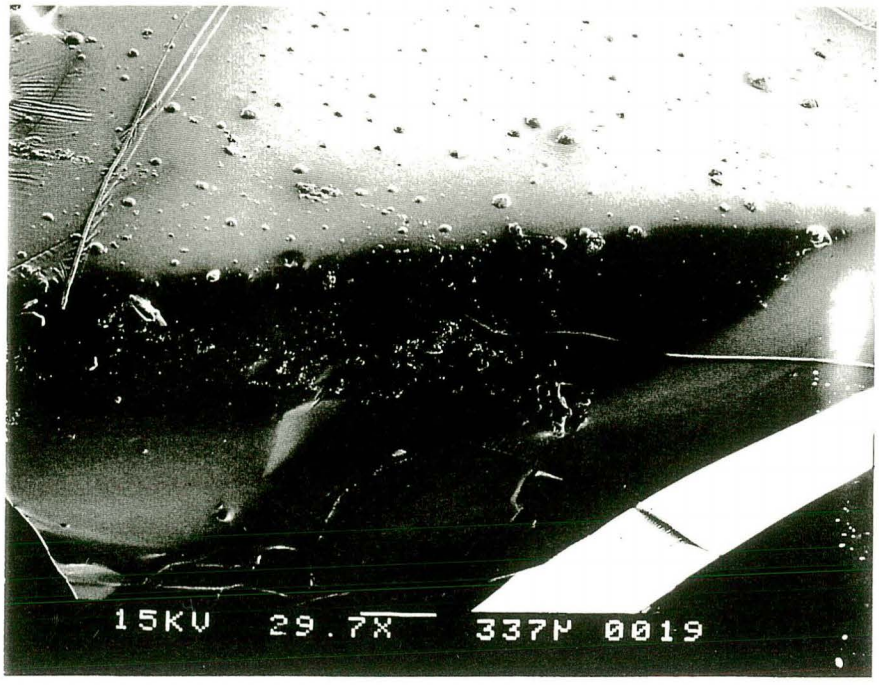


Figure 2.4: SEM micrograph. PSX + YME + Hanford soil inoculum.

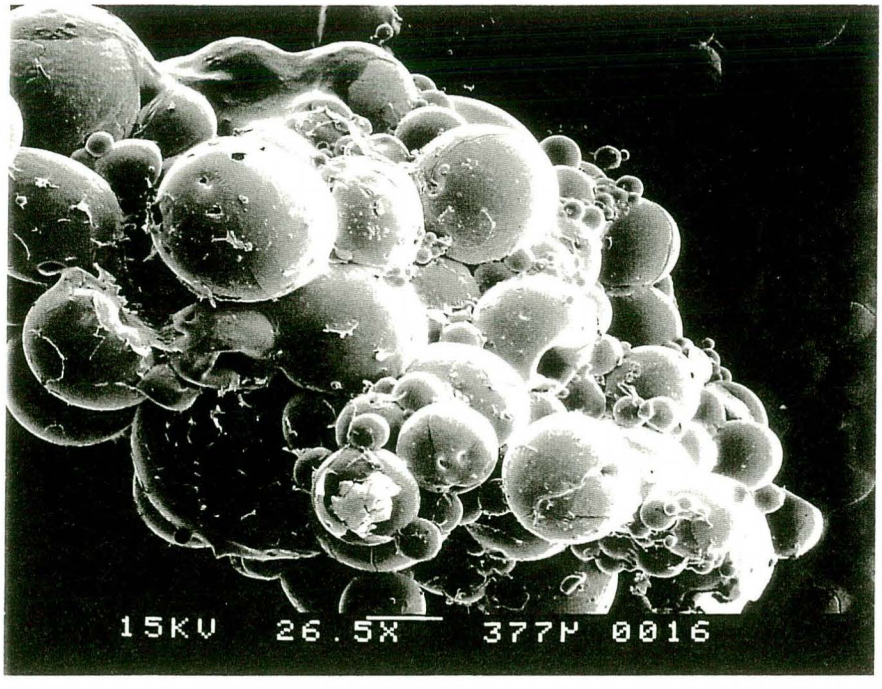


Figure 2.5: SEM micrograph. CS + RSE + Hanford soil inoculum. (An area without growth.)

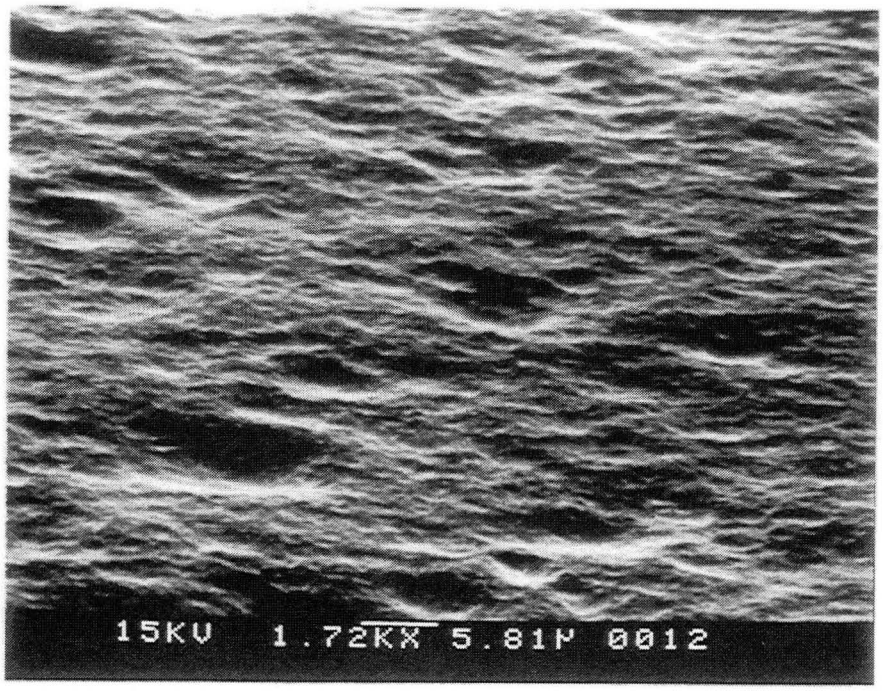


Figure 2.6: SEM micrograph. CS + YME + Los Banos soil inoculum.

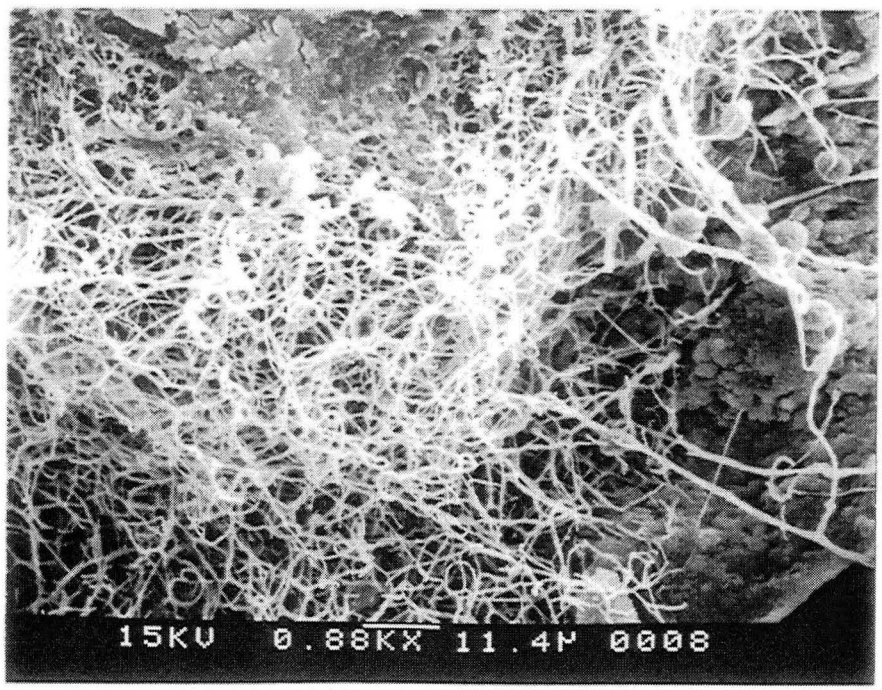


Figure 2.7: SEM micrograph. CS + YME + Los Banos soil inoculum.

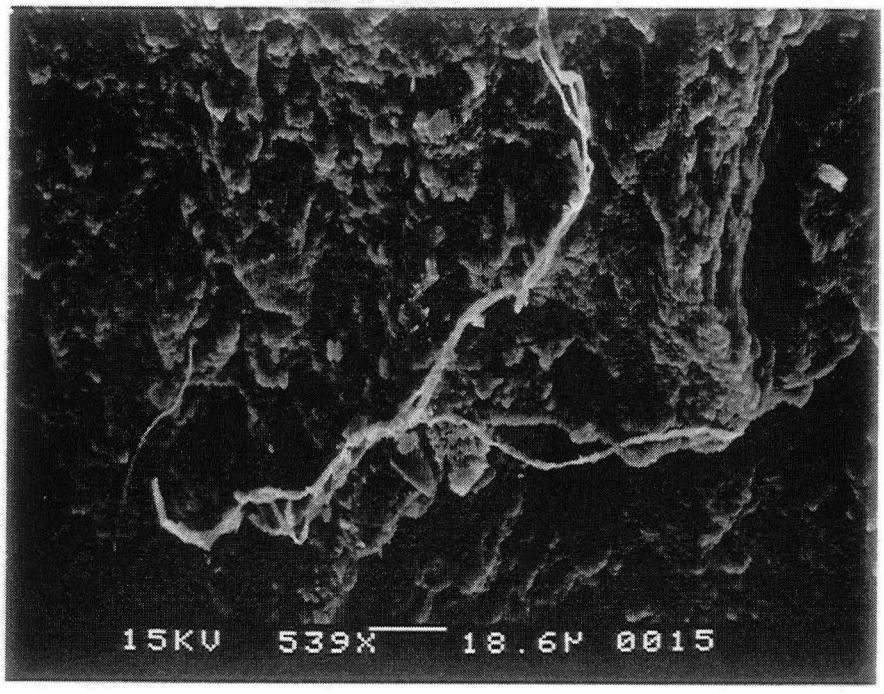
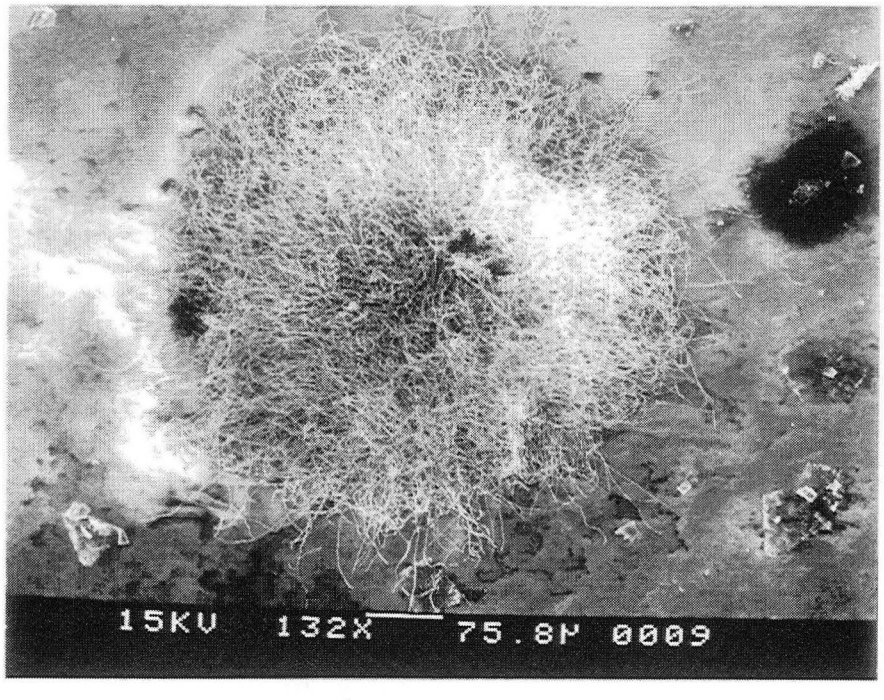


Figure 2.8: SEM micrograph. CS + no supplementary nutrients + no inoculum.



APPENDIX B

TABLES AND FIGURES OF EXPERIMENT 3

B1. Table and Figure Short Descriptions and/or Clarifications

Tables 3.1 through 3.3. These list the values of the absorbance at 590 nm at time 0 h and time 48 h for samples of individual organisms in the presence or absence of barrier materials. The difference in absorbance between time 0 h and time 48 h, caused by the coloration of tetrazolium violet dye, represents the extent of biological activity in each sample. Each value represents the average of 3 trials.

Table 3.1: Organisms were given no supplementary nutrients.

Table 3.2: Organisms were given soil extracts.

Table 3.3: Organisms were supplemented with RSE.

Table 3.4: Organisms were given YME.

Figures 3.1 through 3.7. Differences in absorbance at 590 nm between time 0 h and time 48 h are depicted, by organism, representing the extent of biological activity in each sample. In **Figure 3.1** the samples had not been inoculated and were used as a no-growth control.

Table 3.1. Change in absorbance at 590nm - No supplemental nutrients

Organism	Barrier	t=0 hr	t=48 hrs	Change
Blank Control	MT	0.05	0.05	0.00
	CS	1.90	1.93	0.03
	PSX	0.06	0.06	0.00
Flavobacterium indologenes	MT	0.17	0.12	-0.05
	PSX	0.19	0.17	-0.02
Variovorax paradoxus	MT	0.13	0.10	-0.03
	PSX	0.17	0.15	-0.02
Pseudomonas mendocina	MT	0.11	0.09	-0.02
	PSX	0.11	0.12	0.01
Ochrobactrum anthropi	MT	0.17	0.15	-0.02
	PSX	0.18	0.19	0.01
Rhodococcus erythropolis	MT	0.27	0.21	-0.06
	CS	2.44	2.49	0.05
	PSX	0.27	0.27	0.00
Aureobacterium saperdae	MT	0.19	0.18	-0.01
	CS	2.43	2.48	0.05
	PSX	0.18	0.16	-0.02

Note: The CS sterile control wells showed no coloration of the tetrazolium dye, yet absorbance at 590 nm increased 0.03 in 48 hours. Also, the change in absorbance of the CS wells for the top four organisms was omitted because these organisms caused no coloration of the tetrazolium dye in the presence of CS.

Table 3.2. Change in absorbance at 590nm - Supplemented with SE (1/2 SE-H, 1/2 SE-LB)

Organism	Barrier	t=0 hr	t=48 hrs	Change
Blank Control	MT	0.05	0.05	0.00
	CS	2.40	2.49	0.09
	PSX	0.05	0.05	0.00
Flavobacterium indologenes	MT	0.12	0.13	0.01
	PSX	0.13	0.13	0.00
Variovorax paradoxus	MT	0.13	0.13	0.00
	PSX	0.17	0.16	-0.01
Pseudomonas mendocina	MT	0.12	0.12	0.00
	PSX	0.12	0.12	0.00
Ochrobactrum anthropi	MT	0.20	0.18	-0.02
	PSX	0.19	0.18	-0.01
Rhodococcus erythropolis	MT	0.29	0.30	0.01
	CS	2.62	2.70	0.08
	PSX	0.29	0.29	0.00
Aureobacterium saperdae	MT	0.18	0.17	-0.01
	CS	2.68	2.72	0.04
	PSX	0.18	0.17	-0.01

Table 3.3. Change in absorbance at 590nm - Supplemented with RSE

Organism	Barrier	t=0 hr	t=48 hrs	Change
Blank Control	MT	0.04	0.05	0.01
	CS	2.27	2.32	0.05
	PSX	0.10	0.10	0.00
Flavobacterium indologenes	MT	0.24	0.74	0.30
	PSX	0.27	0.53	0.26
Variovorax paradoxus	MT	0.60	1.14	0.54
	PSX	0.57	0.98	0.41
Pseudomonas mendocina	MT	0.81	2.32	1.51
	PSX	0.66	2.11	1.45
Ochrobactrum anthropi	MT	0.51	1.15	0.64
	PSX	0.50	1.06	0.56
Rhodococcus erythropolis	MT	0.29	0.33	0.04
	CS	2.51	2.71	0.20
	PSX	0.32	0.35	0.03
Aureobacterium saperdae	MT	0.18	0.17	-0.01
	CS	2.47	2.52	0.05
	PSX	0.19	0.18	-0.01

Table 3.4. Change in absorbance at 590nm - Supplemented with YME

Organism	Barrier	t=0 hr	t=48 hrs	Change
Blank Control	MT CS PSX	0.05 2.34 0.07	0.05 2.44 0.07	0.00 0.10 0.00
Flavobacterium indologenes	MT PSX	0.29 0.26	0.72 0.52	0.43 0.26
Variovorax paradoxus	MT PSX	0.52 0.54	1.21 1.03	0.69 0.49
Pseudomonas mendocina	MT PSX	0.73 0.63	2.55 2.51	1.82 1.88
Ochrobactrum anthropi	MT PSX	0.60 0.52	1.34 1.27	0.74 0.75
Rhodococcus erythropolis	MT CS PSX	0.30 2.65 0.32	0.34 2.79 0.38	0.04 0.14 0.06
Aureobacterium saperdae	MT CS PSX	0.19 2.78 0.18	0.18 2.62 0.17	-0.01 0.04 -0.01

Figure 3.1

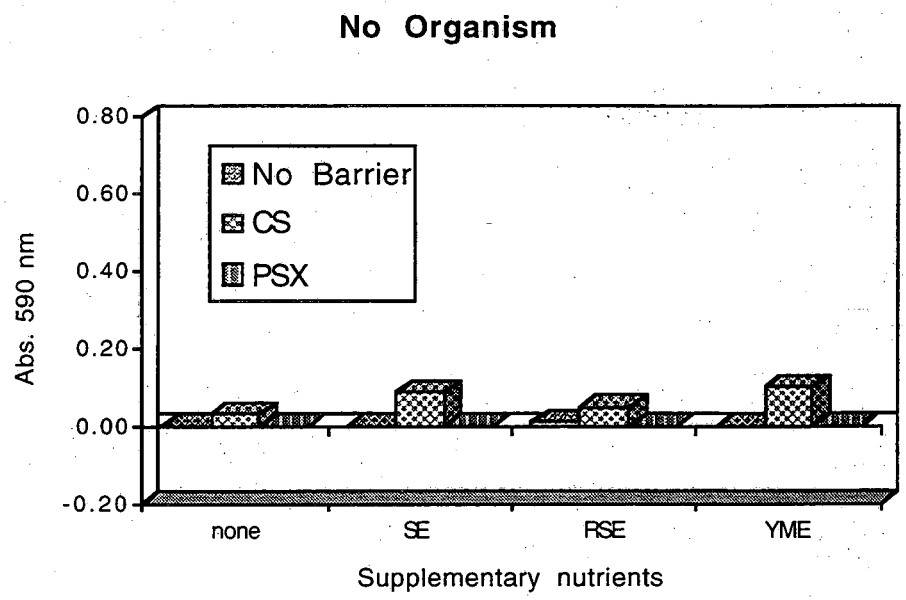


Figure 3.2

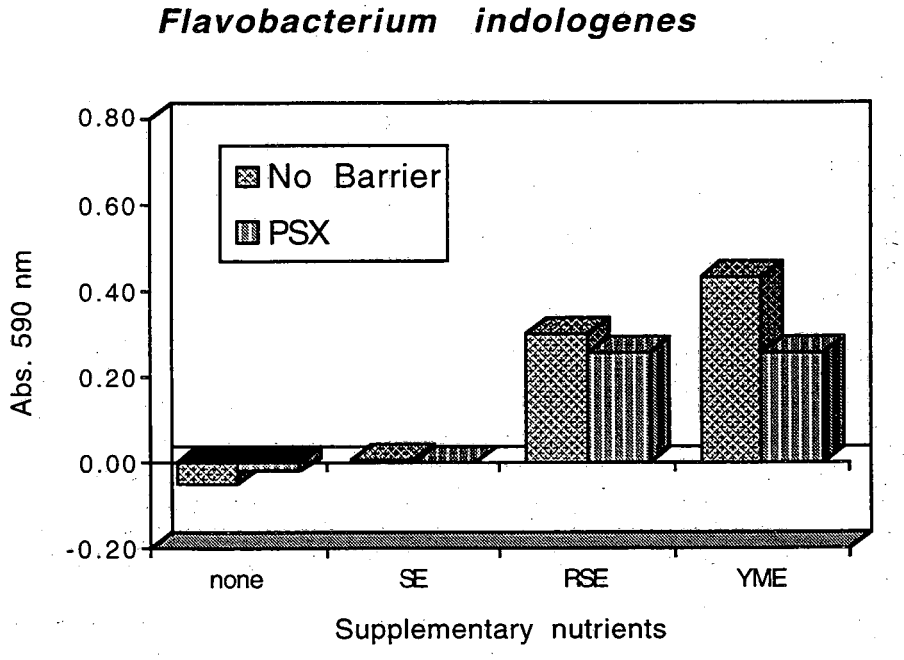


Figure 3.3

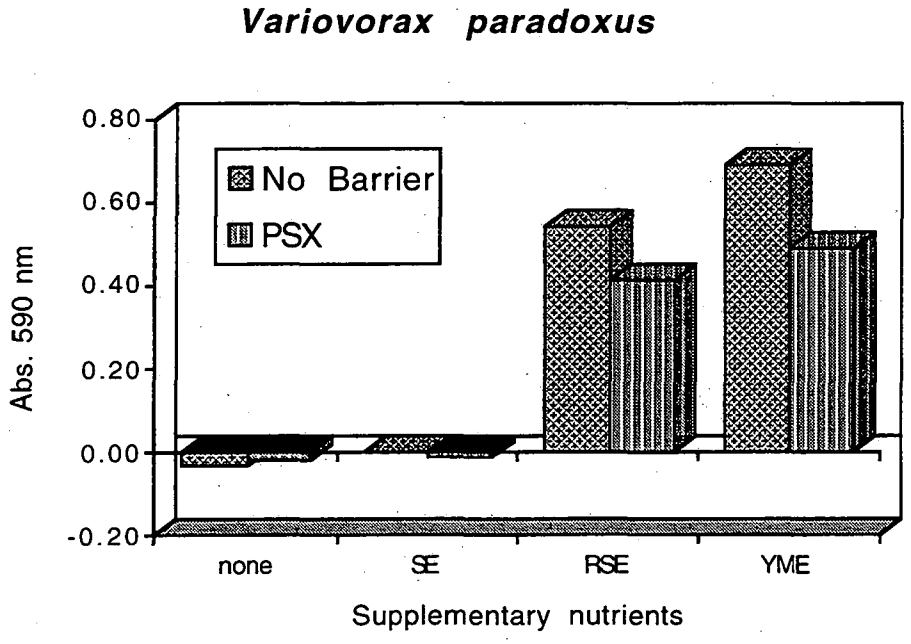


Figure 3.4

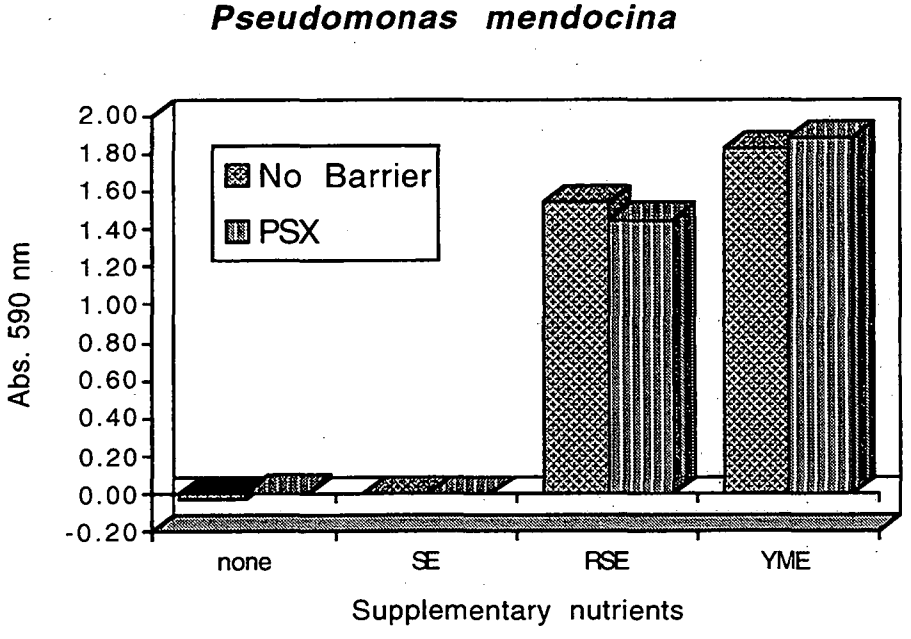


Figure 3.5

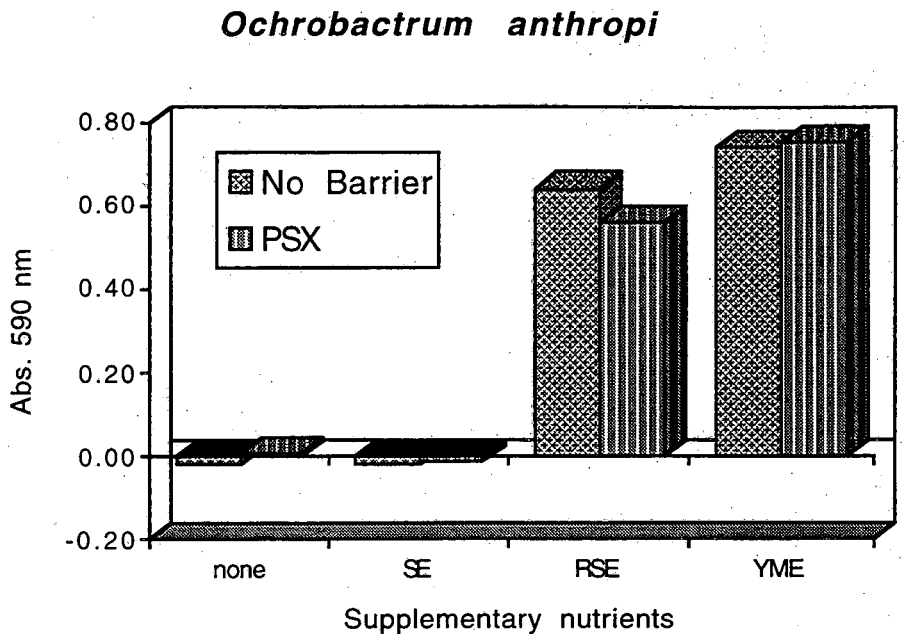


Figure 3.6

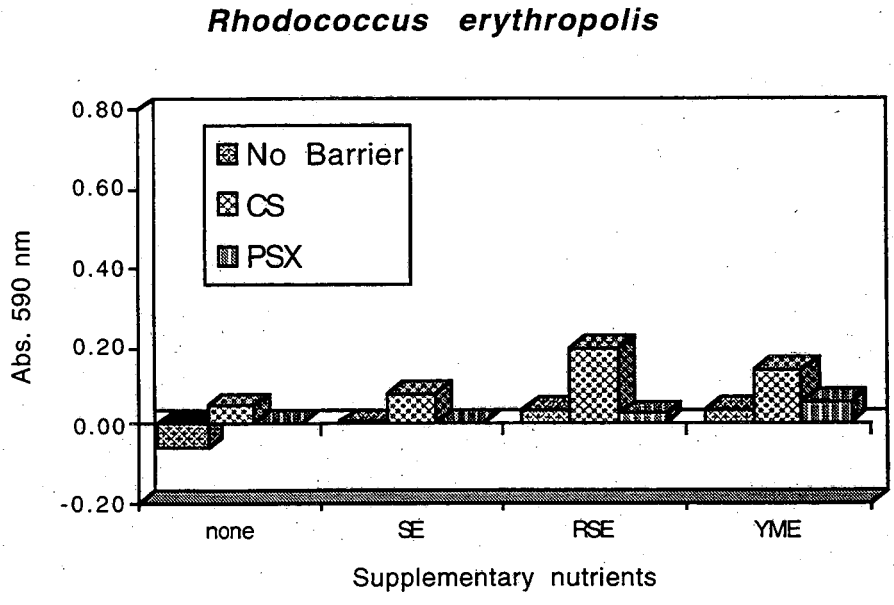
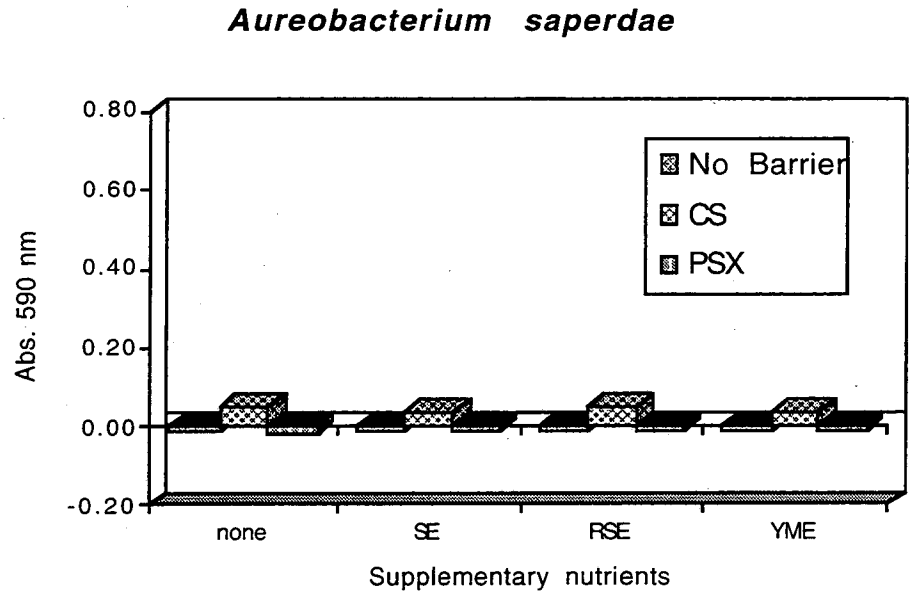


Figure 3.7


Aureobacterium saperdae
0.80
0.60
0.40
0.20
0.00
-0.20
Abs. 590 nm
No Barrier
CS
PSX
none
SE
RSE
YME
Supplementary nutrients

APPENDIX C

FIGURES OF EXPERIMENT 4

C1. Figure Short Descriptions and/or Clarifications

Figures 4.1 through 4.5. Photographs of tetrazolium plates before and after 48 days of aerobic incubation. Plates of samples C through H - in black and white - showed no visible coloration of the tetrazolium dye under the naked eye. Their coloration after 48 days is like that of the killed control samples (A and B), shown in color in **Figure 4.1.b**. The rich control samples (I and J) did show a red coloring, even after five days (**Figure 4.5**).

Figure 4.6. Hyphae and one red bacterial colony on CS + SE-LB inoculated with Los Banos organisms (H).

Figure 4.7. From the same culture (H), shows a developing fungal colony that was clearly growing *within* the CS.

Figures 4.8 and 4.9. In the PSX+YME wells (I), growth (dark areas) was contained within pockets of the medium.

Figure 4.10. The wells of CS + YME (J) had many colonies (black dots) suspended within the barrier material. Note a "dimpling" on the CS where the colonies are just below the surface.

Figure 4.11. A fungus on the rich CS control (J).

Figure 4.12. From an anaerobic sample (anaerobic C). A typical image of the PSX samples with no growth (compare with figure 4.9).

Figure 4.13. The CS plates (anaerobic D, G, H, and rich anaerobic control J) all contained an even distribution of red forms (appearing black). At a closer look - 1000X magnification- these red objects appeared to be fungal and/or bacterial colonies.

Figure 4.14. The PSX rich control (anaerobic I) contained red forms permeating balls of the barrier ma-

terial (red appears black). At 1000X magnification (not shown), these patches resembled more closely a crystalline, non-biological structure than microbial growth.

Figure 4.1.a: Killed control, Day 1.
Top row: PSX (A), Bottom row: CS (B)
(Approximately 3/4 scale)

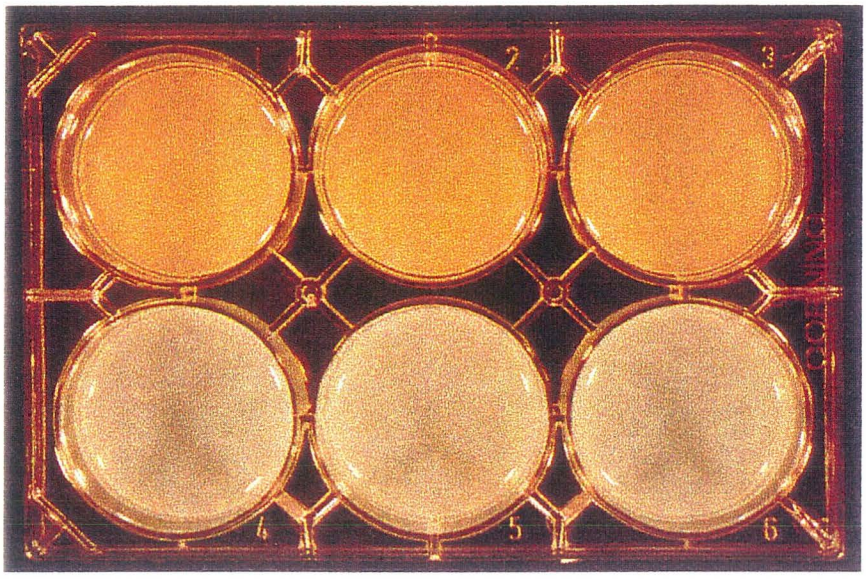


Figure 4.1.b: Killed control, Day 48.
Top row: PSX (A), Bottom row: CS (B)
(Approximately 3/4 scale.)

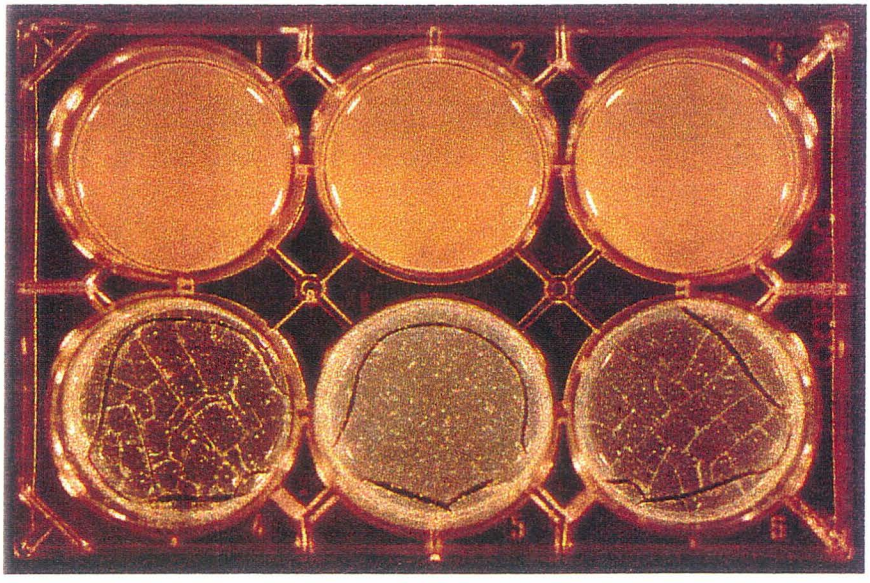


Figure 4.2.a: SE, No inoculum, Day 1.
Top row: PSX (C), Bottom row: CS (D)
(Approximately 3/4 scale)

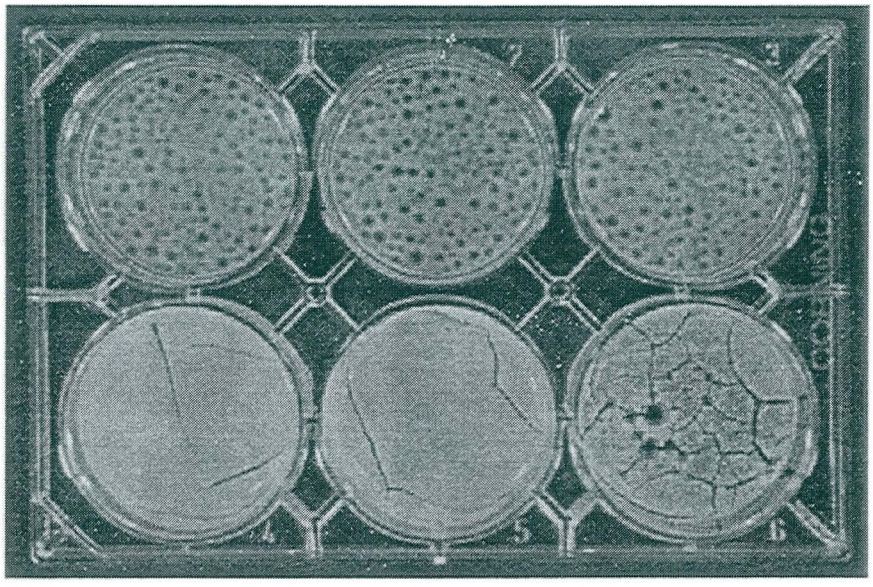


Figure 4.2.b: SE, No inoculum, Day 48.
Top row: PSX (C), Bottom row: CS (D)
(Approximately 3/4 scale.)

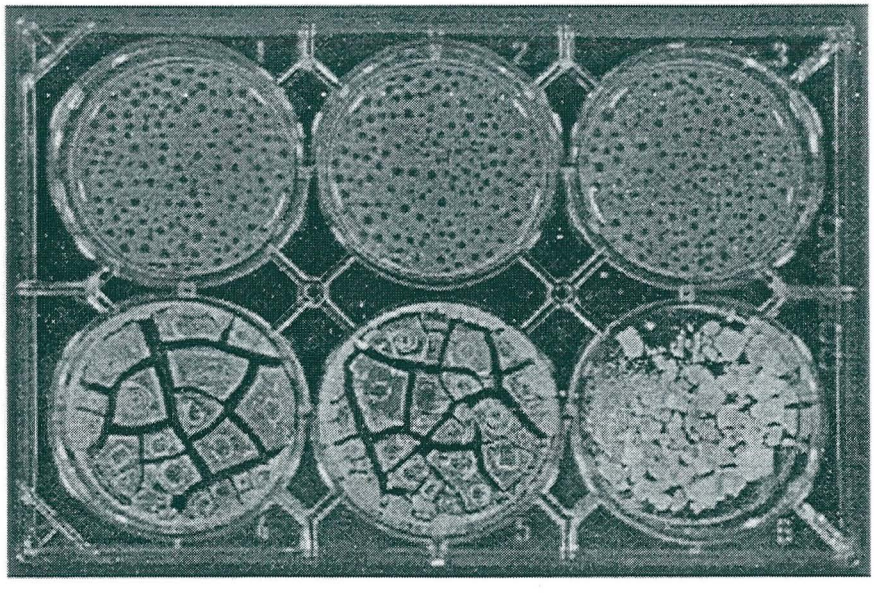


Figure 4.3.a: SE, PSX, Day 1.
Top row: H-orgs (E), Bottom row: LB-orgs (F)
(Approximately 3/4 scale)

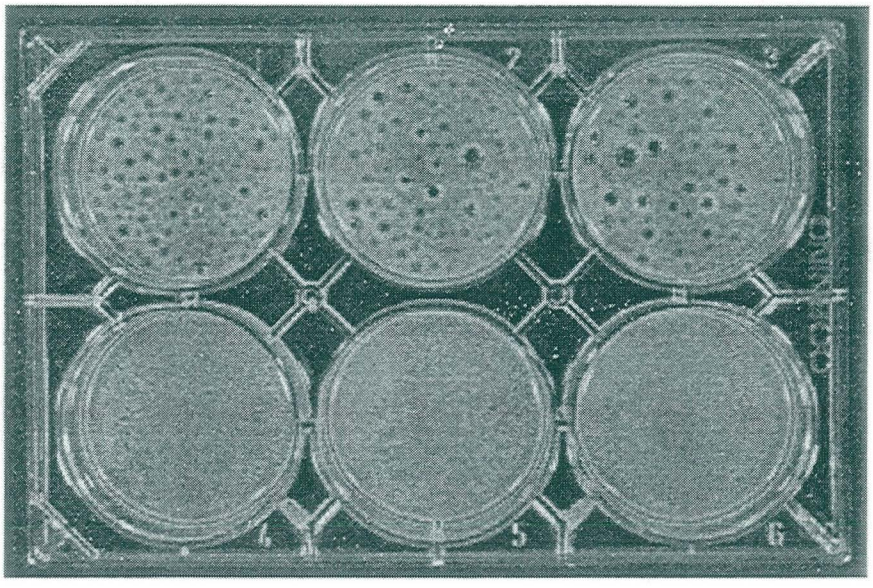


Figure 4.3.b: SE, PSX, Day 48.
Top row: H-orgs (E), Bottom row: LB-orgs (F)
(Approximately 3/4 scale.)

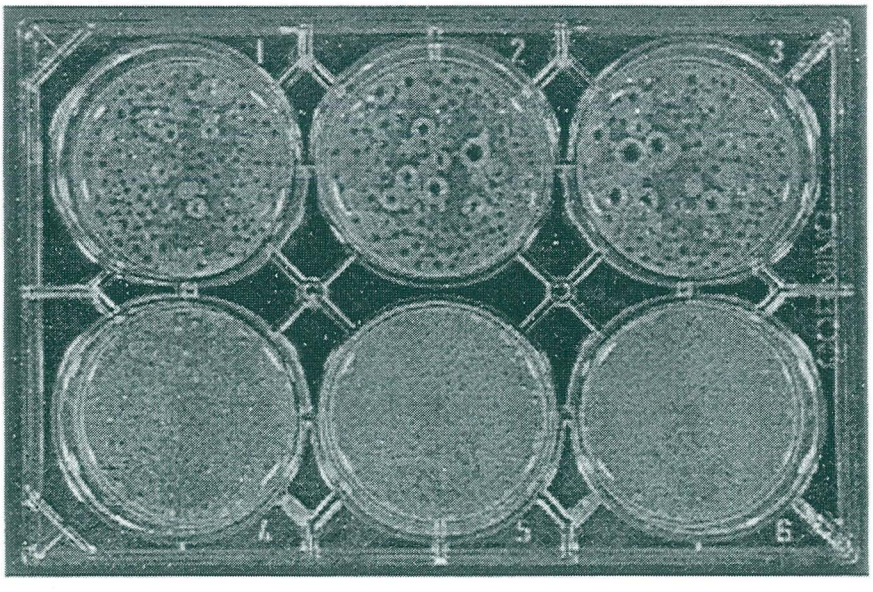


Figure 4.4.a: SE, CS, Day 1.
Top row: H-orgs (G), Bottom row: LB-orgs (H)
(Approximately 3/4 scale)

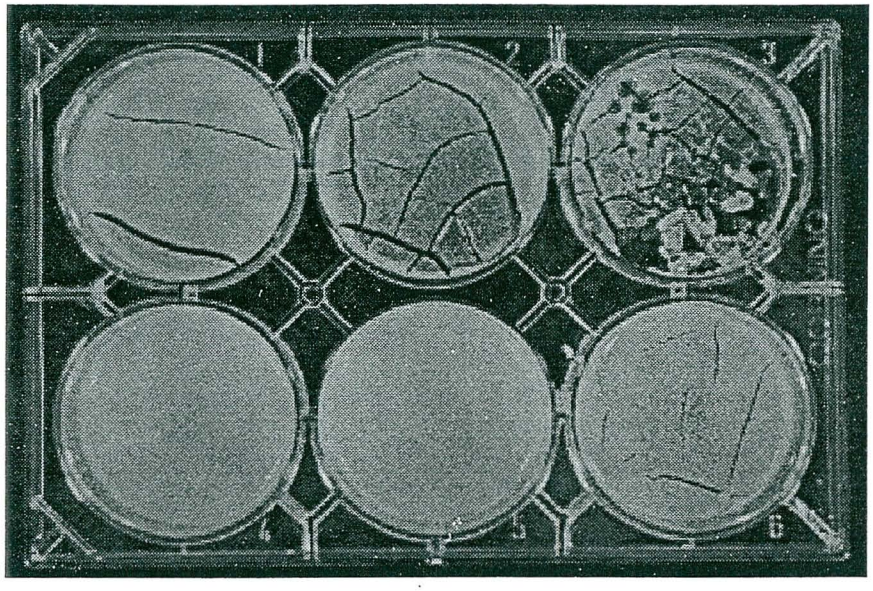


Figure 4.4.b: SE, CS, Day 48.
Top row: H-orgs (G), Bottom row: LB-orgs (H)
(Approximately 3/4 scale.)

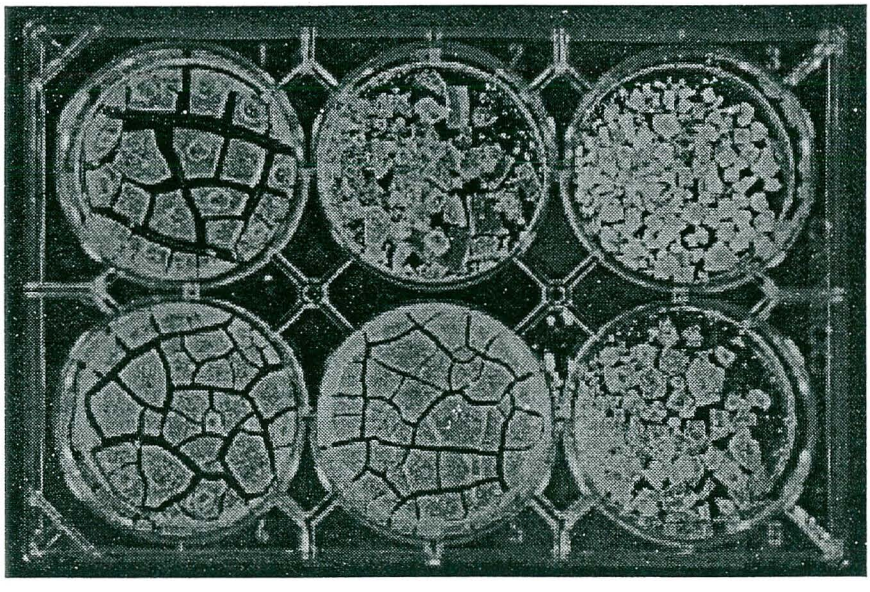


Figure 4.5.a: Rich control, Day 1.
Top row: PSX (I), Bottom row: CS (J)
(Approximately 3/4 scale)

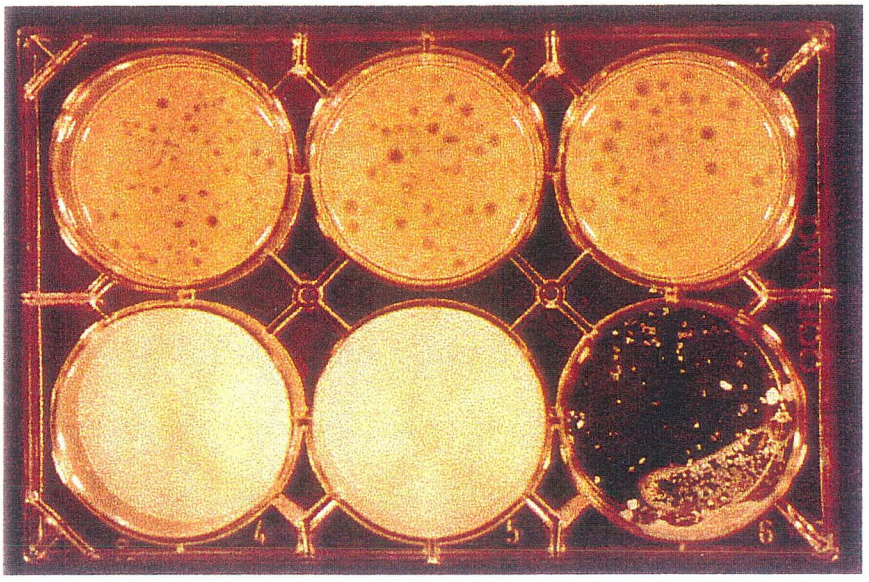


Figure 4.5.b: Rich control, Day 48.
Top row: PSX (I), Bottom row: CS (J)
(Approximately 3/4 scale.)

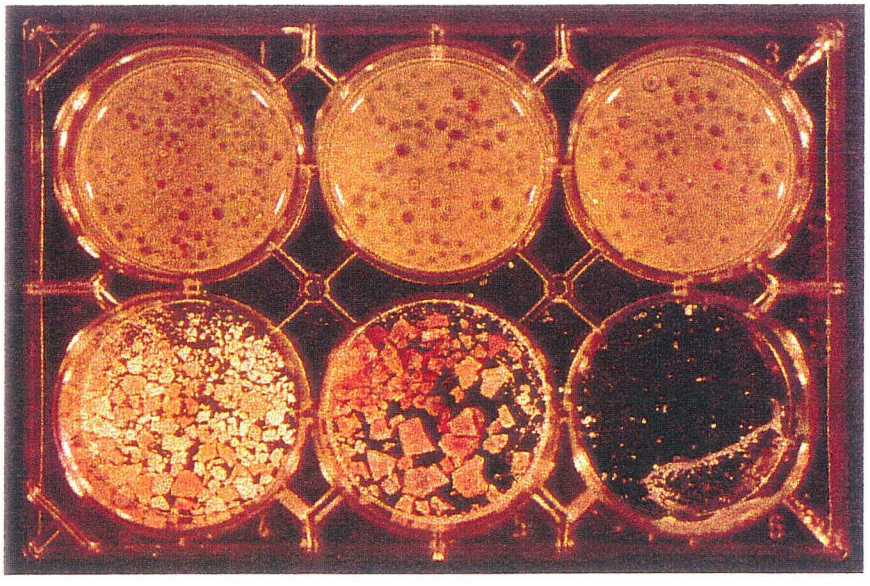


Figure 4.6
Aerobic CS + SE-LB + LB-orgs (H), Day 26.
(Field width approximately 0.3 cm)

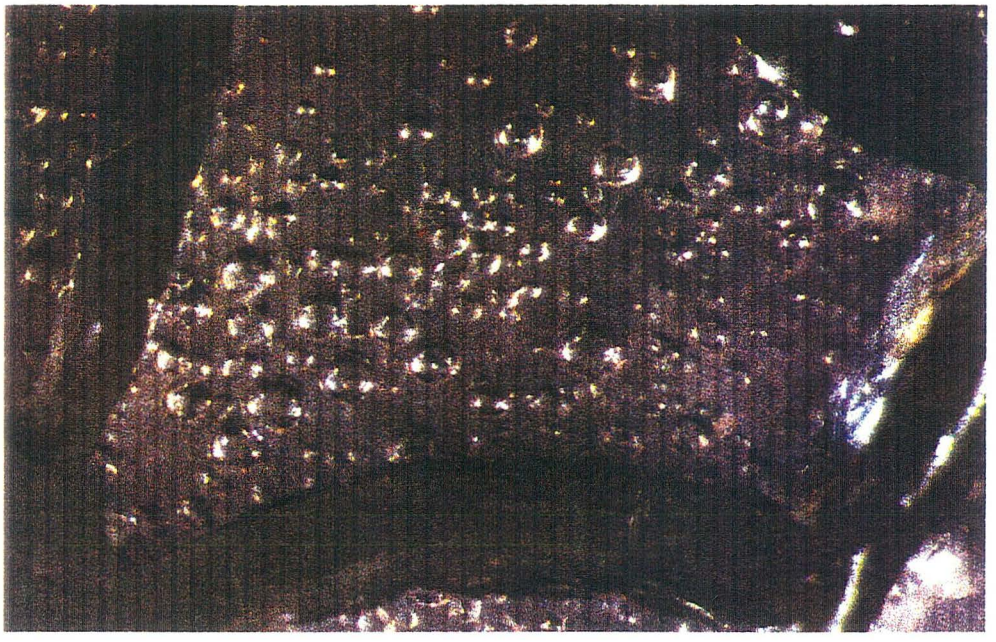


Figure 4.7
Aerobic CS + SE-LB + LB-orgs (H), Day 26.
(Field width approximately 0.3 cm)

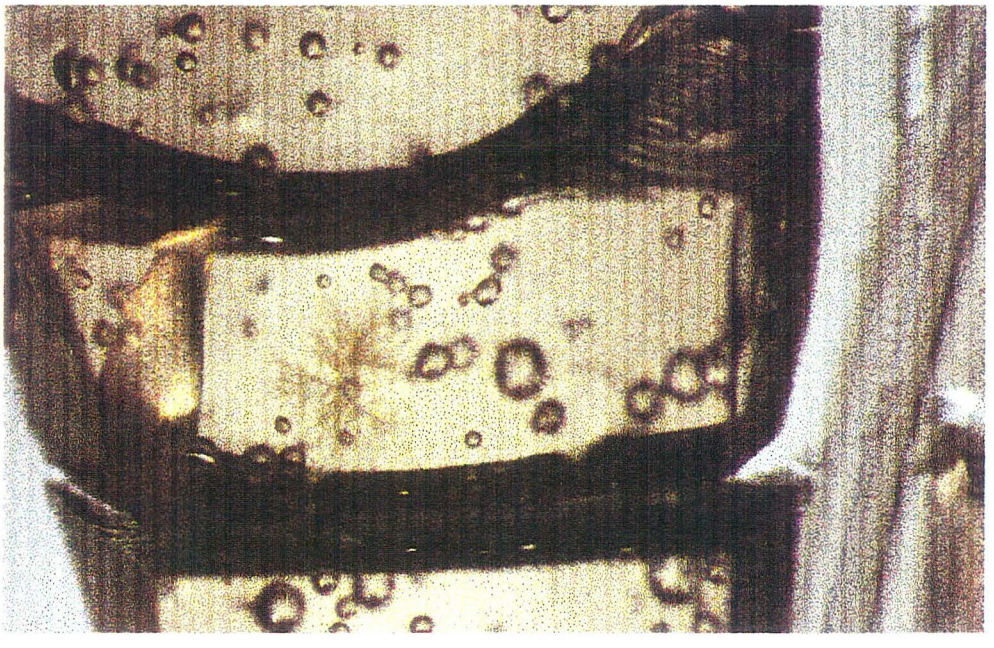


Figure 4.8
Aerobic PSX + YME + H-orgs (I), Day 26.
(Field width approximately 2 cm)

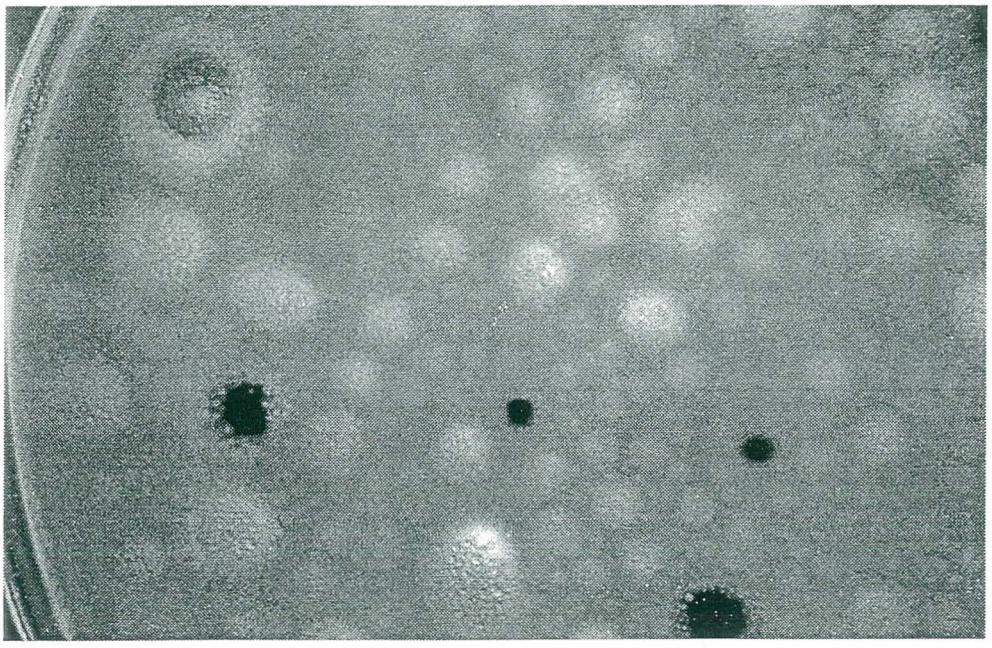


Figure 4.9
Aerobic PSX + YME + H-orgs (I), Day 26.
(Field width approximately 0.5 cm)

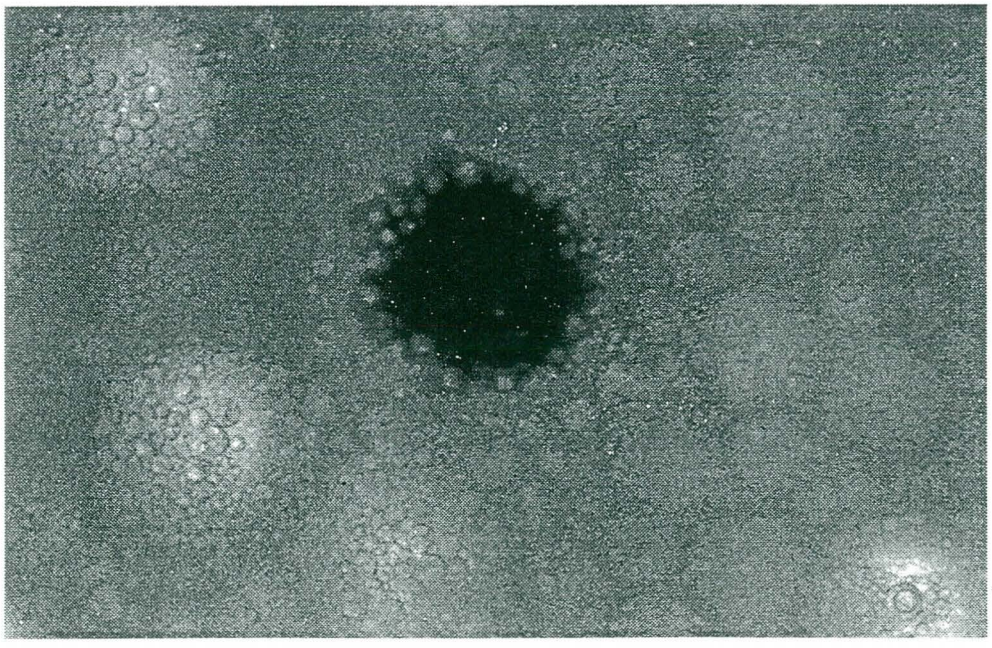


Figure 4.10
Aerobic CS + YME + H-orgs (J), Day 26.
(Field width approximately 0.1 cm)

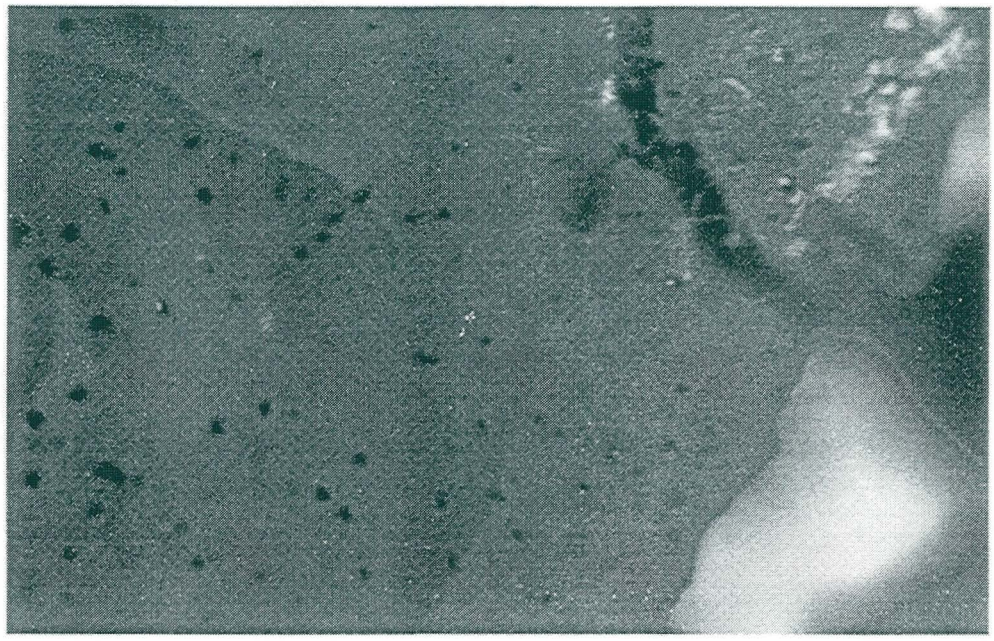


Figure 4.11
Aerobic CS + YME + H-orgs (J), Day 26.
(Field width approximately 0.5 cm)

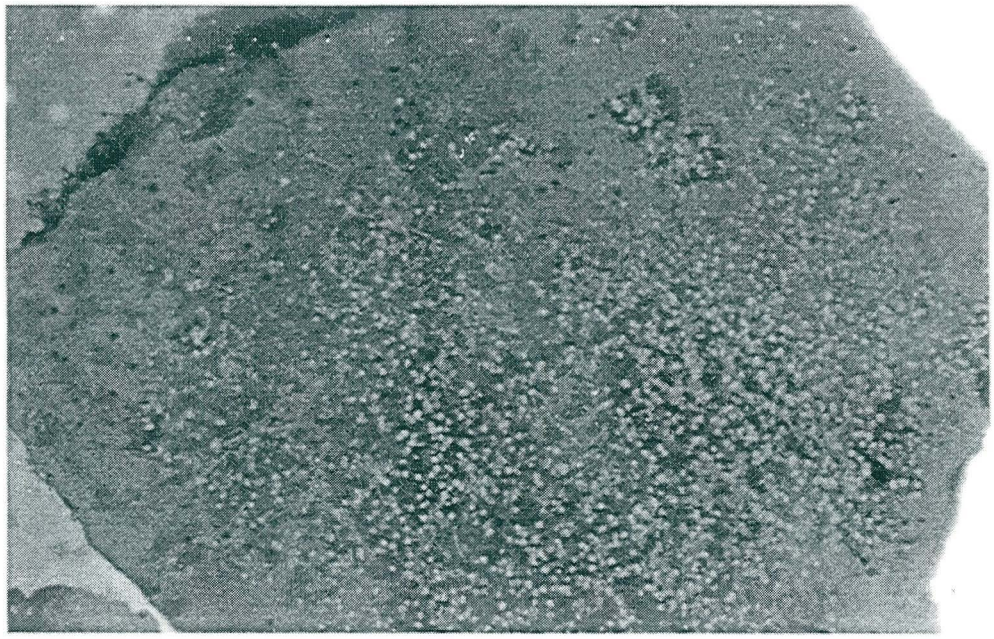


Figure 4.12
Anaerobic PSX + SE + no inoculum (C), Day 20.
(Field width approximately 0.5 cm)

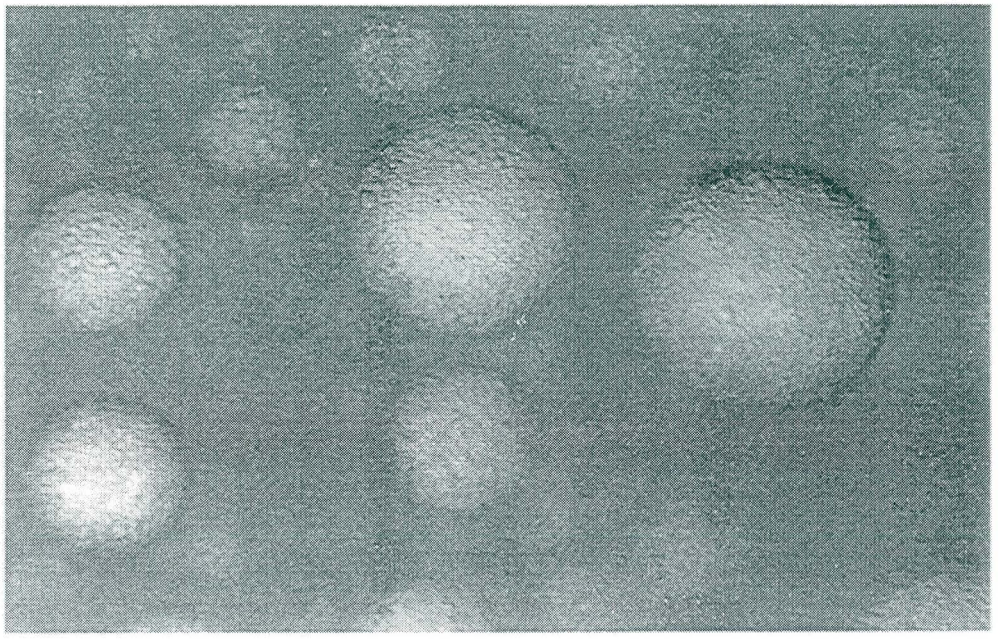
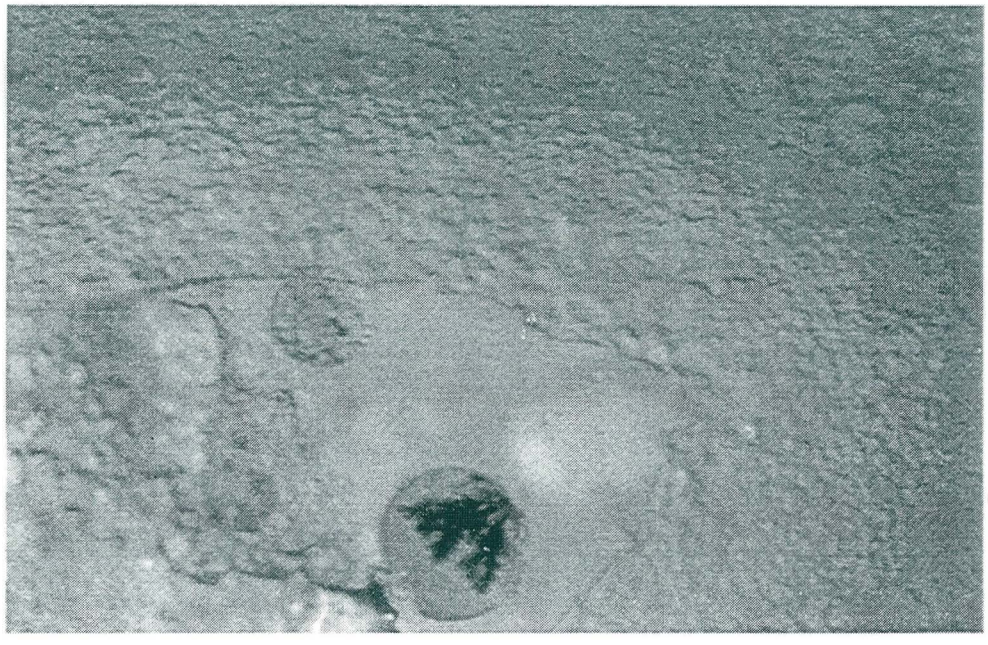


Figure 4.13
Anaerobic CS + SE-LB + LB-orgs (H), Day 20.
(Field width approximately 0.1 cm)

Figure 4.14
Anaerobic PSX + YME + H-orgs (I), Day 20.
(Field width approximately 0.3 cm)



APPENDIX D

NUTRIENT MEDIA

D1. Minimal Media

D.1.1. Phosphate and Inorganic Nitrogen (PIN)

K_2HPO_4 ..... 25.0 g
KH_2PO_4 ..... 15.0 g
$NaNO_3$ ..... 20.0 g
$ddiH_2O$* ..... 1,000 ml
NB: This preparation is 10 times concentrated.

D.1.2. Phosphate and Organic Nitrogen (PON)

K_2HPO_4 ..... 25.0 g
KH_2PO_4 ..... 15.0 g
Difco Yeast Extract ..... 1.0 g
$ddiH_2O$ ..... 1,000 ml
NB: This preparation is 10 times concentrated.

D2. Rich Media

D.2.1. Rich Soil Extract (RSE)

K_2HPO_4 ..... 0.5 g
KH_2PO_4 ..... 0.5 g
Difco Yeast Extract ..... 3.0 g
$MgSO_4 \cdot 7H_2O$ ..... 0.5 g
Glucose ..... 7.5 g
SE-H** ..... 200 ml
SE-LB*** ..... 200 ml
$ddiH_2O$ ..... 600 ml

D.2.2. Yeast Malt Extract (YME)

Difco Yeast Extract ..... 3.0 g
Difco Malt Extract ..... 3.0 g
Glucose ..... 10.0 g
Peptone ..... 5.0 g
$ddiH_2O$ ..... 1,000 ml

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* Distilled, deionized water.
** Hanford Soil Extract.
*** Los Banos Soil Extract.

YME favors the growth of fungi.

D.2.3. Violet-Red Bile Agar (VRBA)

Difco Violet-red Bile Agar .....28.0 g
Agar .....7.0 g
SE-H ..... 125 ml
SE-LB ..... 125 ml
ddiH_2O ..... 750 ml
VRBA is selective for Gram-negative bacteria.

D.2.4. Arginine Glycerol Salts Medium (AGSM)

Arginine monohydrochloride .....1.0 g
Glycerol (specific gravity not less than 1.249 at 25 °C) .....12.5 g
K_2HPO_4 .....1.0 g
NaCl .....1.0 g
$MgSO_4 \cdot 7H_2O$ .....0.5 g
$Fe_2(SO_4)_3 \cdot 1H_2O$ .....0.0082 g
$CuSO_4 \cdot 7H_2O$ .....0.001 g
$ZnSO_4 \cdot 7H_2O$ .....0.001 g
$MnSO_4$ H_2O .....0.001 g
ddiH_2O ..... 1,000 ml
AGSM is elective for Actinomycetes.

Adjust to pH 6.9-7.1. After autoclaving for 15 minutes at 121 °C, add:
Cycloheximide .....0.075 g
By dissolving in a small amount of warm water and filter sterilizing. Also add:
Nystatin (Sigma) .....0.027 g
Dissolve in a small amount of water by bringing pH to 11 with 1 M NaOH, filter-sterilizing, and dropping the pH down to 7.0 immediately with HCl (nystatin is unstable at high pH).

(All rich media are modified from media described in *Hunter-Cevera et al.*, [1986])

Appendix D

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