ISOLATION AND CHARACTERIZATION OF HUMUS-UTILIZING BACTERIA FROM KANSAS SOILS

by

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INTRODUCTION

The importance of humus to the soil has long been recognized. Since the discovery of microorganisms and the realization of their varied powers of decomposition, soil bacteria have been implicated as both the source of humus formation and the cause of its destruction. Waksman, in his rather extensive book on the origin, composition and importance of humus (1938), discussed the possible role of soil bacteria, but he did not attempt to present any data on them, any previous work on the characterization of the suggested organisms, or any hypotheses on their classification.

Some work was done on the subject using humus extracted from the soil with concentrated alkali. Most authors obtained poor results from their efforts to isolate humus-utilizing bacteria.

Bremner and Lees (1949) devised a pyrophosphate extraction method which was less destructive to soil organic matter than either the concentrated alkali method or the concentrated acid method. Since their method was devised, several attempts to chemically characterize humus or its parts have been made by Savage and Stevenson (1961) and others.

A search of the literature since the pyrophosphate method of humus extraction was developed did not reveal any bacteriological studies on humus decomposition. The purpose of this thesis is to describe the isolation and characterization of some humus utilizing bacteria and to attempt to classify those cultures isolated.

REVIEW OF LITERATURE

History of Humus

The term "humus" came from the Romans. It usually designated the soil as a whole (Waksman 1938). The term was revived during the infancy of organic chemistry (early 1800's), but was applied inconsistantly to the total soil organic matter or to portions of the organic matter extracted from the total. Ollech (via Waksman) defined humus in 1890 as "all those substances which are formed in the decomposition and fermentation of organic matter of plant and animal origin, or through the action of certain chemical agents upon this organic matter in the form of amorphous, non-volatile, non-odorous, more or less dark colored organic compounds."

In 1938, Waksman stated that "Current concepts of humus contend that humus is practically insoluble in water, although a part of it may go into colloidal solution in pure water. Humus dissolves to a large extent in dilute alkali solutions, especially on boiling, giving a dark colored extract; a large part of this extract precipitates when the alkali solution is precipitated by mineral acids. Certain constituents of humus may also dissolve in acid solutions and be precipitated at the isoelectric point, which is at a pH of about 4.8."

Humus now denotes organic matter of plant, animal, or microbial origin that is not readily subject to decomposition. It is a complex mixture of complex organic compounds. Because it is difficult to decompose, humus forms a semistable reserve of organic carbon and nitrogen in the soil. The terms "humus" and "humic acid" are essentially equivalent.

Methods of Extraction

In 1786, Achard (via Bremner and Lees, 1949) reported using caustic alkali in extracting humus from soils. This method has been in general use from that time to the present, though some researchers have preferred to consider as humus the organic material that is insoluble in a concentrated acid extraction from soils. The alkali usually used are KOH, NaOH, or NH_4OH. The products of these extractions are substantially altered by the alkali.

Hamy and Leroy (1952) studied the efficiency of salts of organic acids as humus solvents. They tested oxalate, malonate, maleate, succinate, adipate, phthlate, fumarate, acetate, trichloroacetate, salicylate, and sulfosalicylate for comparative efficiency and also tested the effect of pH on the efficiency of humus extraction. The ammonium salts were used primarily. Organic acids have also been used as humus extractants. Fifty percent lactic acid was used in the preliminary step of humic acid extraction by Burges and Latter (1960).

Humic acids seem to be tightly complexed with metallic cations such as Ca^{++}, Fe^{+++}, Al^{+++}, and to clay minerals by di- and tri-valent ions (Chaudhri and Stevenson, 1957). In 1961, Sen found that the rate of humic acid decomposition was slowed when clay minerals were present in the growth medium.

Bremner and Lees (1949) tested several organic and inorganic salts, and were able to show that sodium or potassium salts of acids that form either insoluble precipitates or soluble coordination complexes with calcium yielded a significant increase in the extraction of organic matter. They found that sodium pyrophosphate gave the highest percent of humic acid extraction as measured by organic nitrogen. The duration of the extraction process, the temperature of the extraction, the volume of the extractant, shaking the extraction mixture,

or repeated extraction did not significantly affect the amount of humus extracted. Increased concentrations of sodium pyrophosphate gave increased yields of humus up to a concentration of 0.1 molar. Increased pH gave increased amounts of humus, but pH values above 8 were not recommended by the authors because of the danger of altering the humus. Chaudhri and Stevenson (1957) considered a pH of 9 to be optimum.

Composition of Humus

Alexander (1961) suggests that lignins or their derivatives form an important portion of humus. He points out their exceptional resistance to enzymatic degredation. Alexander also suggests that phytin, inositol hexaphosphate, may occur in large quantities though bound to iron and aluminum complexes. This was verified by Osgrove (1963), who was able to extract myoinositol hexaphosphate, and also dl-inositol and inositol pentaphosphate from humus.

Lynch and Lynch (1958) suggested that humus may be a protein-lignin complex. Their synthetic humus was attacked by microorganisms with difficulty. In 1961, Savage and Stevenson showed that amino acids were present in soil humic acids which had been extacted by the pyrophosphate method and subjected to H_2O_2 oxidation. They suggested that the amino acids were protected during oxidation by combination with other low molecular weight compounds. Their ultra-violet analysis of humus showed no azo- or diazo-nitrogen intermediates or rings with nitrogen as a member during the H_2O_2 oxidation. Infra-red analysis of humic acid by Wagner and Stevenson (1965) found phenolic OH in humus in addition to COOH groups which were close enough together to form cyclic anhydrides.

Savage (1961) showed that part of the humic acid nitrogen was present as acidic NH. He also found that carboxyl groups composed 10.2% to 17.8% of the humic material. Most of the carboxyls were attached to aromatic rings. Hydroxyl groups comprised less than 3% of the humic material.

A more comprehensive study, undertaken by Burges et al., in 1963, suggested that humic acids are heteropolycondensates of phenolic substances with the possible presence of amino acids. Substances such as phenolic compounds from plant flavenoids, lignins, or synthetic products of microorganisms were also suggested as follows:

flavenoids:	possible lignin units:	also proposed:
phloroglucinol	vanillic acid	pyrogallol
resorcinol	β-hydroxybenzoic acid	3:5-dihydroxybenzoic acid
methylphloroglucinol	syringic acid	
2:4-dihydroxytoluene	protocatechuic acid	
	guaiacylpropionic acid	
	syringylpropionic acid	

The resistance of humic compounds to microbiol degradation was shown by Birch and Friend (1961), who demonstrated that a high proportion of the organic carbon and nitrogen remained in a humus preparation after attack by microorganisms.

Humus-Utilizing Bacteria

Ladd (1964) isolated an Achromobacter sp. which would oxidize N-(O-carboxyphenyl)glycine. This compound was selected as a model humate because it contains an aromatic ring bonded to the nitrogen atom of an α-amino acid as proposed for humic acid polymers. He indicated the path of decomposition.

Ochilova (1961a) used soil as inocula into liquid humate media and found that members of the genus Pseudomonas were the predominant bacteria. In later work (1961b) he tested pure cultures and found that Pseudomonas fluorescens was the most active in decomposing humus. He used decolorization of the humus as his quantitative criterion of decomposition. He also listed Mycobacterium sp., Bact. herbicola, B. idosus, and B. sp. as humus decomposing organisms.

Pseudomonas as well as Achromobacter, Serratia, and Mycobacterium was isolated by Volkova (1961) from a liquid humic medium after soil inoculation. The humus was extracted by Tyurin's method (NaOH).

The ability of Pseudomonas fluorescens to decompose humus was studied by Nikitin (1960), who found that amino acids are stably connected to the main molecular groups of humus and that the degree of humate decolorization is related to the intensity of pyrocatechin oxidation. Mishustin and Nikitin (1961) were able to relate humus decolorization to the action of bacterial peroxidase and substantiated their hypothesis by testing the effect of a pure enzyme preparation on humus. They were able to show irreversable decolorization of the humic medium and partial loss of the humus sample. Nikitin later showed that added carbon in the form of glucose increased the rate of humus decomposition, but indicated that added nitrates inhibited the process (1962).

Kuznetsov and Dzyulan (1960) isolated fifteen species of Mycobacterium from water. Their studies indicated that the decolorization of humic substances is more rapid in the daylight.

Schönwalder (1958) tested several species of Nocardia, Corynebacterium, and Micrococcus for their ability to use humic acids as their sole source of carbon and nitrogen. He found that all organisms tested were able to use

humic acids as the sole nitrogen source, but that only two Nocardia species were able to use them as the sole carbon source. (His method of humic acid extraction was not available.) Schönwalder found that better results were obtained from synthetic "humic acids" prepared by reaction of quinones with ammonia than from natural humic acids.

Other Humus-Utilizing Microorganisms

In 1960, Burges and Latter obtained cultures of Spicaria and Polystictus and also of Hypholoma and Trametes by percolation. These were able to grow on humic acid as extraced via their lactic acid method. They noted decolorization of humus also by Ganoderma, Collybra, Daedalia and Ustulina (Latter and Burges 1960). Hurst, Burges, and Latter later (1962) investigated the growth of 29 strains of fungi including basidiomycetes, fungi imperfecti, and an ascomycete. They found a correlation among the decolorization of humus, the reduction of the carboxyl group of m-hydroxybenzoic acid, and the oxygen content of the medium. They infer that the decolorization may be an aerobic oxidative process involving the reduction of aromatic carboxyl groups.

Lal and Mazumdar (1953) tested the effect of adding humus to soil. They found that Aspergillus fumigatus, A. niger, Penicillium glaucum, and Cephalosporim sp. were not able to use humus, though the growth of Helminthosporium nodulosum was stimulated. The authors suggested that increased growth of the fungi in the presence of bacteria might be due to preliminary decomposition of the humus by the bacteria.

Ochilova (1961 a,b) found that Penicillium sp. and actinomycetes (Actionmyces glaucus, A. griseus, and A. viridis) gave maximum decolorization of humus media in 30 or 60 days respectively, though the activity was less than that of bacteria.

In 1963, it was reported by Tepper that varieties of Bactoderma and Proactinomyces as well as Micromonospora were able to oxidize humates. He found that the addition of nitrogen as nitrate favored humate decomposition, though ammonia nitrogen inhibited the decomposition.

Volkova (1961) reported that though bacteria predominated in a liquid humic acid medium for the first two to three months after soil inoculation, actinomycetes appeared after four to five months and were the predominant organisms after ten to twelve months. Cultures isolated were Actinomyces violaceus chromogenes, A. cellulosae, A. longisporus ruber, and A. globisprous griseus.

MATERIALS AND METHODS

Humus was extracted from an alluvial deposit high in organic matter by a modification of the method of Bremner and Lees (1949). One kilogram of sieved and sorted soil was added to one liter of 0.1 molar sodium pyrophosphate. The mixture was stirred constantly for thirty minutes. The suspension was filtered through eight layers of cheese cloth to remove large particulate matter and then through Whatman #1 filter paper. The filtrate was collected and the colloidal humus was precipitated with concentrated HCl to a final pH of 2.0. The supernatant fluid was discarded after centrifugation of the suspension at 5000 x g in a Serval table centrifuge. The pellet was dissolved in pyrophosphate, and reprecipitated with concentrated HCl, and centrifuged in this manner for a total of three washings. After the third washing, the precipitated humus was adjusted to a pH of just below 6.5 to obtain the most nearly neutral pH while still retaining the floculant nature of the humus. The humus was sterilized by autoclaving at 15 lbs/in.^{2} pressure at 121 C for

25 minutes. Fifty-ml. culture flasks containing nine-ml. of mineral salts and one-ml. of humus were used for selection of humus-utilizing organisms by serial transfer. The formula for the mineral salts, to be designated as MS, is as follows:

K_2HPO_4	3.40 gram	10% CaCl	0.10 ml.
KH_2PO_4	1.35 gram	microelements	0.05 ml.
$(NH_4)_2SO_4$	2.00 gram	20% $FeCl_3$	0.0125 ml.
$MgSO_4$	0.10 gram	Distilled water	1.00 liter
NaCl	0.10 gram		

The microelement solution contained 0.5% of a non-nitrogenous salt, preferably the chloride of boron, copper, manganese, molybdenum, and zinc.

Samples of seven different soils from Riley Country, Kansas were gathered and kept under refrigeration until they were required for use as inocula. The characterization of these soils is as follows:

Sample 2	Geary silt loam
Sample 3	clay loam
Sample 4	clay loam
Sample 5	alluvial deposit
Sample 8	farmyard soil
Sample 9	Sarpee sandy loam
Sample 10	alluvial soil

After seven days of incubation at 27 C, one-ml. of a 1/100 dilution of each flask was transferred to a new flask. Five transfers were made. Plate

counts into nutrient agar were made from the humus - MS flasks at each transfer. Counts ranged from 10^8 to 10^7 cells per ml.

After the fifth transfer, 36 representative colonies were picked from the pour plates and streaked onto NA (nutrient agar) plates. Cultures were designated by two numbers. The first indicated the soil sample. The second designated the individual isolate from each soil sample. After 48 hours the colonial morphology was recorded and colonies were picked and transferred to NA slants. Gram stains were made from the slants after 24 hours. Staining reactions and cellular morphology were recorded.

A portion of the humus was dialized against MS. Each culture was tested against the dialysate and the dialysis fluid. Twenty-one cultures that showed good growth on either portion of the humus were selected for study. All cultures which were able to grow on MS only were eliminated by their slow or limited growth as compared to growth shown by humus-utilizing cultures. Additional cultural and biochemical characteristics were tested.

The Ashby's Mannitol agar was composed of the following:

Washed ion agar	10.0 gram	NaCl	0.10 gram
Mannitol	20.0 gram	10% $CaCl_2$, Micro-element Solution	0.10 ml. 0.05 ml.
K_2HPO_4	1.75 gram	20% $FeCl_3$	0.0125 ml.
KH_2PO_4	0.70 gram	Distilled Water	1.00 liter
$MgSO_4$	1.60 gram		

The contents of the microelements solution have been listed previously.

The trypticase broth, trypticase nitrate broth, carbohydrate fermentation broths, litmus milk, SIM (sulfide, indole, motility test agar) medium, motility

agar, starch agar, and tributyrin agar, were prepared according to Pelczar (1958). The amino acid mixture was a sterile commercial solution, TC Minimal Medium Eagle #5674, used for tissue culture, and obtained from Difro catalog #140, July, 1964.

Electron micrographs were made of each culture. The grids were prepared from 24 hour cultures. The preparations were not shadowed.

After the cultures were characterized, Bergey's Manual of Determinative Bacteriology, Seventh Edition (Breed, et al., 1957), was used as an aid to classification.

PHYSICAL CHARACTERIZATION OF THE UNKNOWN CULTURES

Cultural Characteristics of the Unknown Cultures

Streak plates were made to determine colonial morphology of the cultures isolated from the humus enrichments. The colonial morphology was observed and recorded after 48 hours. The data are presented in Table 1.

Pigment production is recorded if it occured on any medium. All pigments were water soluble except the pink pigment of culture 2-3.

Morphological Characteristics of the Unknown Cultures

In order to determine the cellular morphology of the unknown cultures, three Gram stains were made on each culture after 24 hours of incubation on nutrient agar slants. Acid fast stains were made after 5 days of incubation on nutrient agar slants. Motility test agar was stabbed and recorded. Electron photomicrographs were made of each culture after 24 hours of incubation on nutrient agar slants. In addition to gross morphology, the presence

PLATE I

GROWTH IN SOLUBLE HUMUS

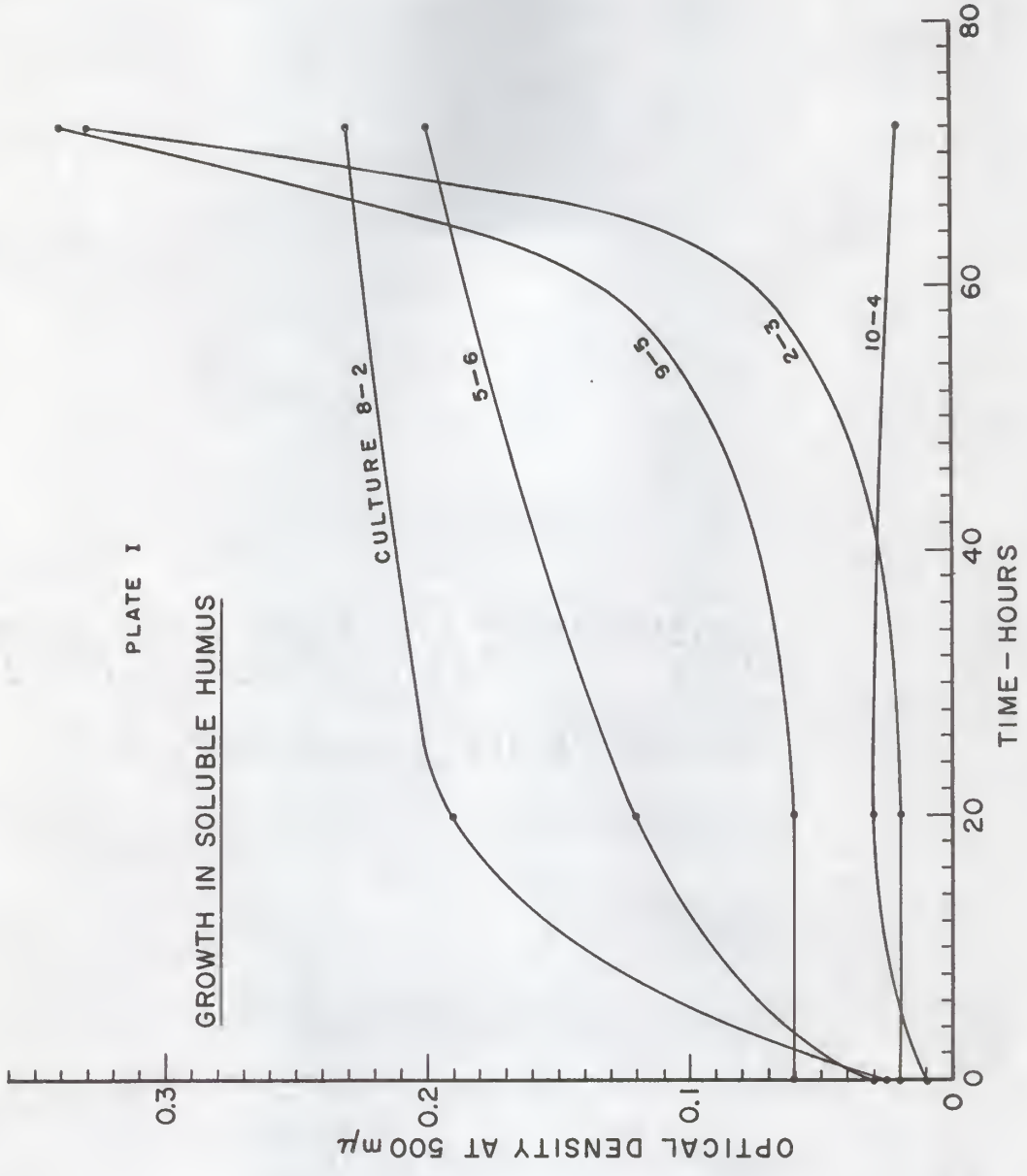


PLATE I
GROWTH IN SOLUBLE HUMUS
CULTURE 8-2
5-6
5-6
2-3
10-4
OPTICAL DENSITY AT 500 mμ
0.3
0.2
0.1
0
0
20
40
60
80
TIME-HOURS

of flagella, granules, or capsule formation was noted. The data are recorded in Table 2. The description of cells is a summary of the data gained by each of the methods mentioned.

BIOCHEMICAL CHARACTERIZATION OF THE UNKNOWN CULTURES

Selection of Cultures Giving Vigorous Growth

For this experiment, 50-ml of humus extracted with sodium pyrophosphate by the method described and adjusted to a pH of 6.1 was dialyzed against 100 ml. of freshly made MS of a pH of 7.0 for 24 hours. Following dialysis the insoluble humus from within the dialysis tubing was autoclaved. The soluble humus was used in growth experiments as follows.

Two ml. of MS + two ml. of the soluble humus were put into calibrated tubes, capped with aluminum foil and autoclaved. A pre-incubation reading of the optical density (OD) was taken at 500 mμ using the Bausch and Lomb Spectronic 20 spectrophotometer, immediately after inoculation of the tubes from nutrient agar slants of the 36 pure cultures isolated from the serial enrichment flasks. The tubes were incubated at 27 C on a rotary shaking device. Readings were taken at 20 hours and at 72 hours. Cultures were selected for study if they reached an OD of 0.12 or more. This was equivalent to at least 100 million cells per milliliter. Values recorded ranged from 0.00 to 0.34. Cultures selected were 2-1, 2-3, 2-4, 3-2, 3-3. 3-4, 3-5, 4-3, 5-1, 5-6, 8-1, 8-2, 8-4, 9-2, 9-3, 9-5, and 10-5.

The sterile insoluble humus from within the dialysis tubing was used in growth studies as follows:

Fifty-ml. culture flasks were sterilized. Sterile MS (9.9 ml.) and

TABLE 1. Cultural Characteristics of the Unknown Cultures

Culture	Colony Diameter	Colony Type	Additional Description	Pigment
2-1	1mm	entire	white, shiny	--
2-3	punctiform	entire	pink, shiny	pink
2-4	2-3mm	lobate	grey, shiny	green
2-5	1mm	entire	white, shiny	--
3-2	2mm	entire	white, shiny, mucoid	--
3-3	1mm	entire	translucent to white	yellow
3-4	1mm	entire	cream, shiny	--
3-5	punctiform to $\frac{1}{2}$mm	entire	translucent to white,shiny	--
4-2	1-2mm	entire	cream, shiny	--
4-3	2-3mm	lobate	cream	yellow
5-1	2-4mm	lobate	translucent to cream	yellow
5-3	2-3mm	entire	white, some shine	--
5-6	2-4mm	lobate	cream to white, some shine	yellow
8-1	punctiform to 1mm	entire	translucent to cream,shiny	--
8-2	1mm	entire	translucent	yellow
8-4	1-2mm	entire	white, shiny	--
9-2	2mm	entire	cream-white, shiny	--
9-3	1mm	entire	translucent to white,shiny	yellow
9-5	1-2mm	entire	white, shiny	--
10-3	punctiform to 1mm	entire	cream to white, shiny	--
10-5	1-1.5mm	entire	white, shiny	--

TABLE 2. Morphological Characteristics of the Unknown Organisms

Culture	Gram Stain	Acid Fast Stain	Mobility	Flagella	Description of Cells
2-1	neg	-	-	-	rods, L=3 to 4W, slightly bipolar, easily autolyzed
2-3	neg	-	+	-	rods, very bipolar
2-4	neg	-	+	-	small rods, L=3 to 4W
2-5	neg	-	+	polar	small rods, L=2 to 3W
3-2	neg	-	+	-	small rods, L=4W, slightly bipolar
3-3	neg	-	-	-	small rods, L=2 to 3W
3-4	neg	-	+	-	small, thin rods, L=4W, slight pleomorphism
3-5	neg	-	+	-	small rods, L=2 to 3W
4-2	neg	-	-	polar	tiny thin rods, L=3 to 4W
4-3	neg	-	-	-	rods, L=4W, slight pleomorphism
5-1	neg	-	+	polar	coccoid rods, L=2W, pleomorphic
5-3	neg	-	+	-	small rods, L=2 to 3W, slightly bipolar, much capsule
5-6	neg	-	+	polar	rods, L=2 to 4W
8-1	neg	-	+	-	small rods, L=1 to 4W, easily autolyzed
8-2	neg	-	+	-	coccoid rods, L=2 to 3W
8-4	neg	-	+	-	small rods, L=2W, slightly pleomorphic
9-2	neg	-	+	-	small rods, L=1.5 to 3W
9-3	neg	-	+	polar	rods, L=1.5 to 3W
9-5	pos	-	-	-	rods, curved, pleomorphic
10-3	neg	-	+	-	rods, L=3 to 4W
10-5	neg	-	+	-	small thin rods, curved, or clubbed

L = Length
W = Width

sterile, insoluble humus (0.1 ml.) were pipetted aseptically into each flask and allowed to mix. A total inoculum of approximately 10^6 cells from a suspension in sterile distilled water was used. Plate counts were made at 24 hours and at 72 hours. Cultures showing more than 10^{10} cells/ml. were selected for study. The selected cultures were 2-4, 2-5, 3-4, 4-2, 5-1, 5-3, 8-4, and 10-3.

Twenty-one cultures were selected in this manner for further study.

Utilization of Sugars

The inocula for this experiment were prepared by suspending one loopful of culture from a nutrient agar slant in a sterile, 100 ml. distilled water blank. The bottles were shaken to separate and suspend the bacterial cells immediately after the addition of the cells and again just prior to the inoculation of the media. Media were inoculated with one drop of the cell suspension. The media inoculated were glucose, maltose, and lactose broths in tubes equipped with Durham fermentation tubes and incorporating 0.018g phenol red/l. as a pH indicator, and glucose, maltose, lactose, xylose, and cellobiose as 1.0 percent in MS.

The tubes of the first series were read and recorded using A for acidic reaction, G for gas production, B for basic reaction and NC for no change. In the second series, growth was recorded as relative + or -. Results were read after 48 hours of incubation at 27 C.

Results are presented in Table 3.

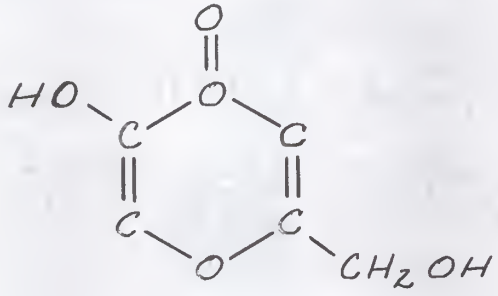
TABLE 3. Utilization of Sugars

Culture	Glucose Broth	Maltose Broth	Lactose Broth	Glucose + MS	Xylose + MS	Lactose + MS	Maltose + MS	Cellobiose + MS
2-1	B	B	B	-	+	+	+	+
2-3	NC	NC	NC	-	-	-	-	+
2-4	B	B	B	++++	-	-	+	+
2-5	B	B	B	-	-	-	+	-
3-2	B	B	B	-	-	-	-	-
3-3	B	B	B	+++	-	-	+	-
3-4	NC	B	B	+	-	-	+	-
3-5	B	B	B	++++	+++	++++	++++	++++
4-2	B	B	B	-	-	-	-	-
4-3	B	B	B	++++	-	-	+	-
5-1	B	B	B	++++	-	+	+	-
5-3	B	B	B	-	-	-	-	-
5-6	B	B	B	+++	+	++	++	+
8-1	B	B	B	+++	++	+++	+++	+++
8-2	B	B	B	+	-	+	++	+
8-4	B	B	B	+++	+++	++++	++++	++++
9-2	B	B	B	-	-	-	+	-
9-3	B	B	B	++++	-	-	+	-
9-5	NC	NC	NC	+	+++	++	++	+++
10-3	B	B	B	-	-	-	-	-
10-5	B	B	B	-	-	-	-	-

B = basic reaction; NC = no change; + = growth; - = no growth

Utilization of Organic Acids as a Source of Carbon

The media for this experiment were inoculated as described for the carbohydrate experiment. Growth media were prepared by adding the organic substrate to MS to obtain a total concentration of one percent weight/volume. The pH was adjusted to 7.0 with NaOH. Results were read after 48 hours of incubation at 27 C and recorded as relative + or -. The formula for kojic acid is given below. Results are presented in Table 4.



Utilization of Additional Carbon Sources

Dodecane, mineral oil, tributyrin, starch, phenol, and cellulose were tested for their ability to support the growth of the humus isolates. The dodecane and mineral oil media were prepared by overlaying a thin layer (one to two drops) of the hydrocarbon onto MS in the tubes before autoclaving. The phenol medium contained one percent phenol in MS, volume/volume. The cellulose medium was prepared by placing $\frac{1}{4}$" x $2\frac{1}{2}$" filter paper strips in test tubes. MS were added to a depth of $1\frac{1}{2}$". The tubes were then autoclaved. The starch and tributyrin were prepared in agar. All these media

were inoculated by the drop method as described. Care was taken not to invert the agar plates until the surfaces were dry.

Dodecane and mineral oil tubes were read at two and five days. Phenol tubes were read at four days. Cellulose tubes were read weekly up to $4\frac{1}{2}$ weeks. The plates of starch and fat agar were observed daily for six days. Growth in the tubes or cleared zones of hydrolysis on the plates are marked + in Table 5, regardless of the amount of time required to obtain results.

Effect of Carbon Compounds on Growth Rates

In this experiment, the effect of the form of the carbon source on the growth rate and on the total growth of the cultures was tested. One percent glucose in MS, one percent sodium succinate in MS, one-half percent ethanol in MS and MS only were placed in sterile, foil-covered spectrophotometer tubes. Glucose and ethanol were sterilized by filtration. Succinate and MS were sterilized by autoclaving. The media were inoculated by the drop method. Optical densities were read at 500 mμ and recorded for each tube immediately after inoculation and at 14 hours, $25\frac{1}{2}$ hours, 48 hours, and 72 hours after inoculation. The tubes were incubated at 27 C on a rotary shaking device to aid aeration.

Typical results are shown on the growth curves for cultures 4-3, 5-6, 8-2, and 9-5 shown in Plates II, III, IV, and V.

TABLE 4. Utilization of Organic Acids as Carbon Sources

Culture	Formate	Acetate	Lactate	Succinate	Citrate	Kojate
2-1	-	-	++	+	++	-
2-3	-	-	-	+	-	+
2-4	-	++++	++++	+++	+	-
2-5	-	-	+++	+++	++++	-
3-2	-	-	-	-	-	-
3-3	+	++	++++	++	+++	-
3-4	-	+	+	-	-	+
3-5	-	++	++++	++	++++	-
4-2	-	-	-	-	-	-
4-3	-	++	++++	+++	+++	-
5-1	-	+	++++	+++	+	-
5-3	-	-	+++	++	-	-
5-6	-	++++	++++	+++	++++	-
8-1	-	-	+	+	++	++
8-2	+	+	+	+	-	-
8-4	-	-	+	++++	++	-
9-2	-	-	-	-	-	-
9-3	-	++++	++++	+++	++++	-
9-5	-	+	++	+	-	+
10-3	-	-	++	-	-	-
10-5	-	-	-	-	-	-

+ = growth
- = no growth

TABLE 5. Utilization of Other Carbon Sources

Culture	Dodecane	Mineral Oil	Tributyrin	Starch	Phenol	Cellulose
2-1	+	-	+	-	-	-
2-3	+	-	+	-	-	-
2-4	+	-	+	-	-	-
2-5	+	-	-	-	-	-
3-2	-	-	-	-	-	-
3-3	+	-	-	-	-	-
3-4	+	+	-	-	-	-
3-5	-	-	+	-	-	-
4-2	-	-	+	-	-	-
4-3	+	-	-	-	-	-
5-1	+	-	-	-	-	-
5-3	+	-	+	-	-	-
5-6	+	+	-	-	-	-
8-1	+	-	-	-	-	-
8-2	+	-	-	-	-	-
8-4	+	+	-	-	-	-
9-2	+	-	-	-	-	-
9-3	+	-	-	-	-	-
9-5	+	-	+	-	-	-
10-3	+	-	+	-	-	-
10-5	+	-	-	-	-	-

+ = growth

- = no growth

PLATE II

EFFECT OF CARBON SOURCES
ON GROWTH OF CULTURE 4-3

PLATE II

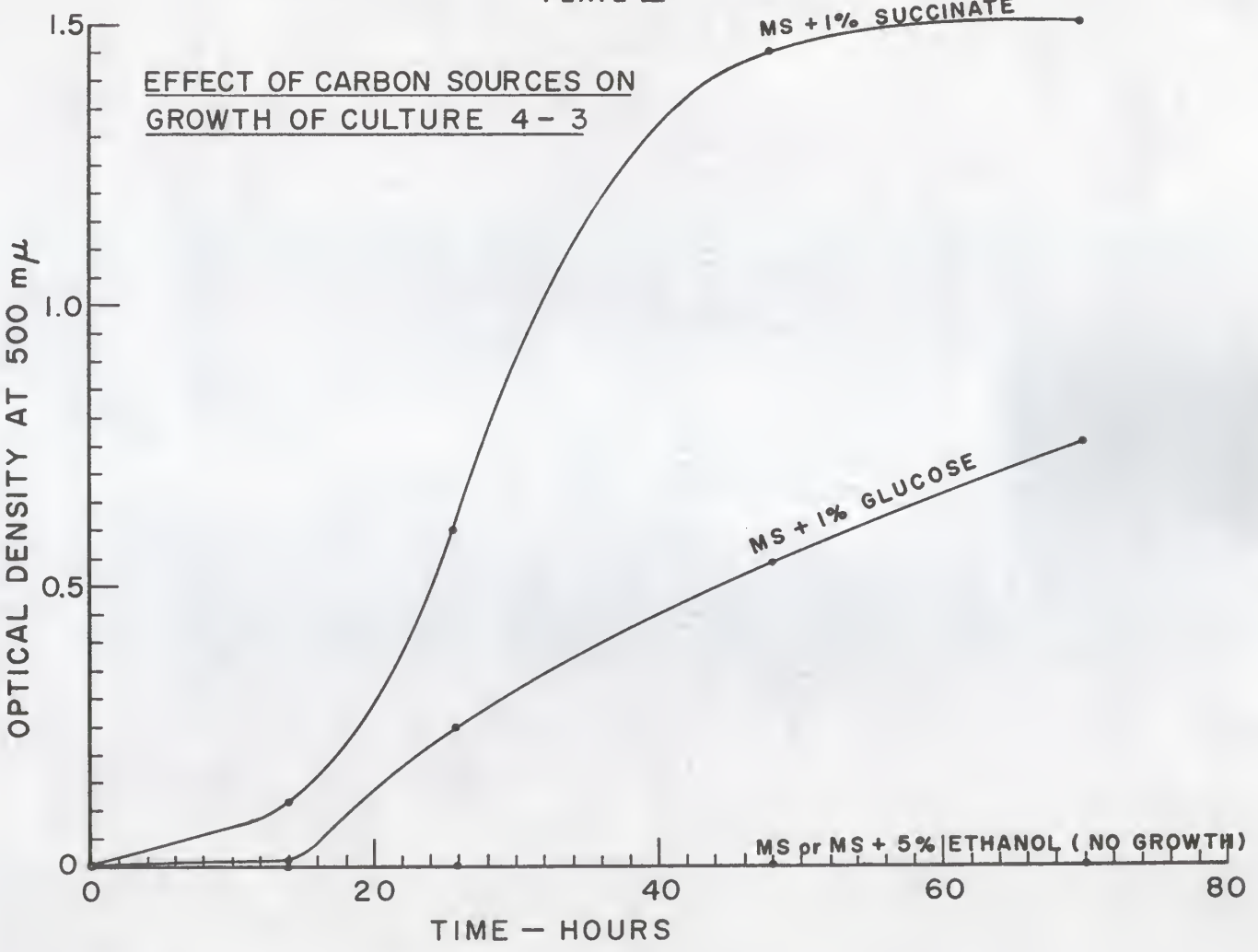

EFFECT OF CARBON SOURCES ON
GROWTH OF CULTURE 4 - 3
MS + 1% SUCCINATE
MS + 1% GLUCOSE
MS or MS + 5% ETHANOL (NO GROWTH)
OPTICAL DENSITY AT 500 mμ
1.5
1.0
0.5
0
0
20
40
60
80
TIME — HOURS

PLATE III

EFFECT OF CARBON SOURCES
ON GROWTH OF CULTURE 5-6

PLATE III

EFFECT OF CARBON SOURCES ON GROWTH OF CULTURE 5—6

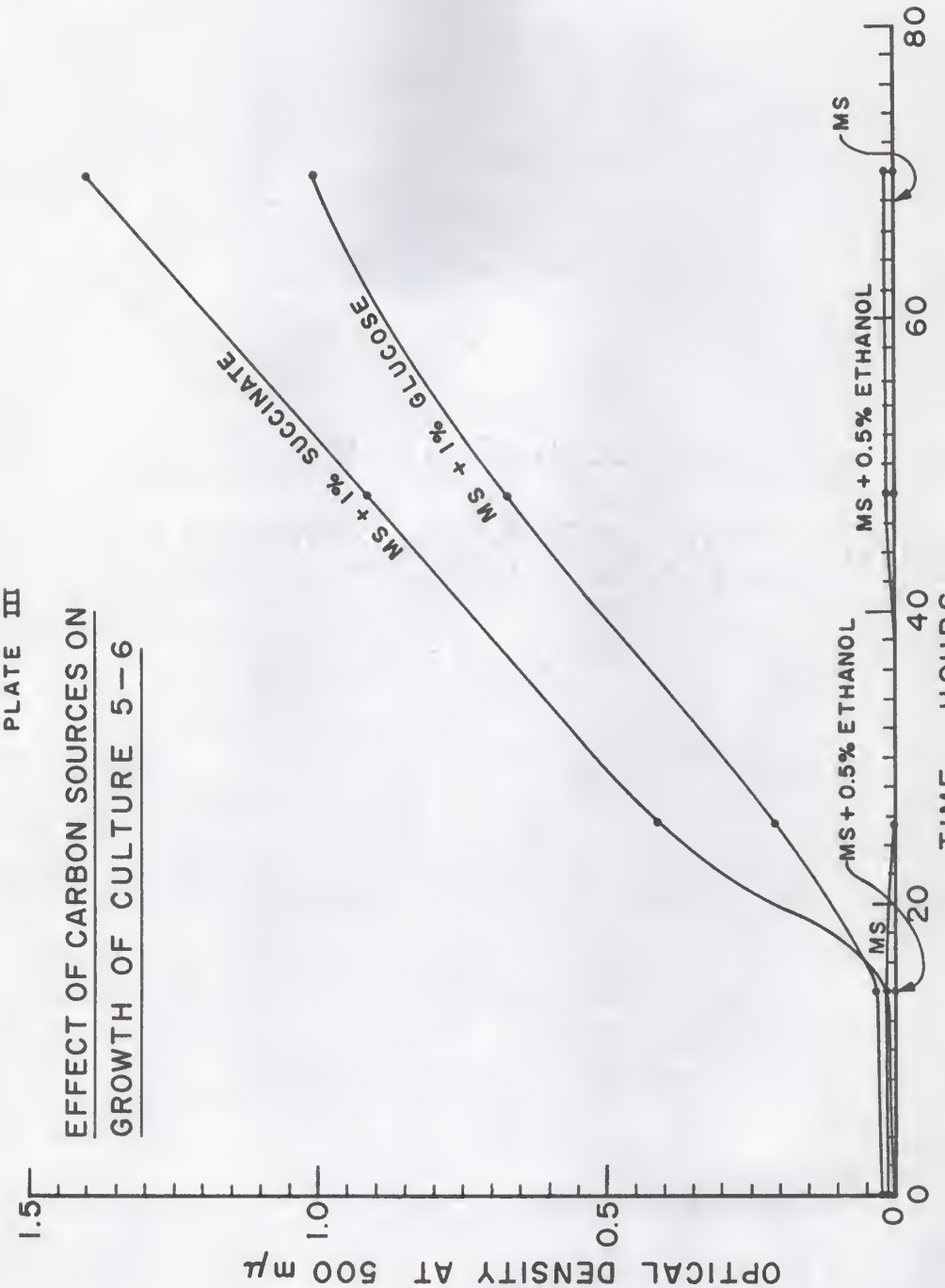


PLATE IV

EFFECT OF CARBON SOURCES
ON GROWTH OF CULTURE 8-2

PLATE IV

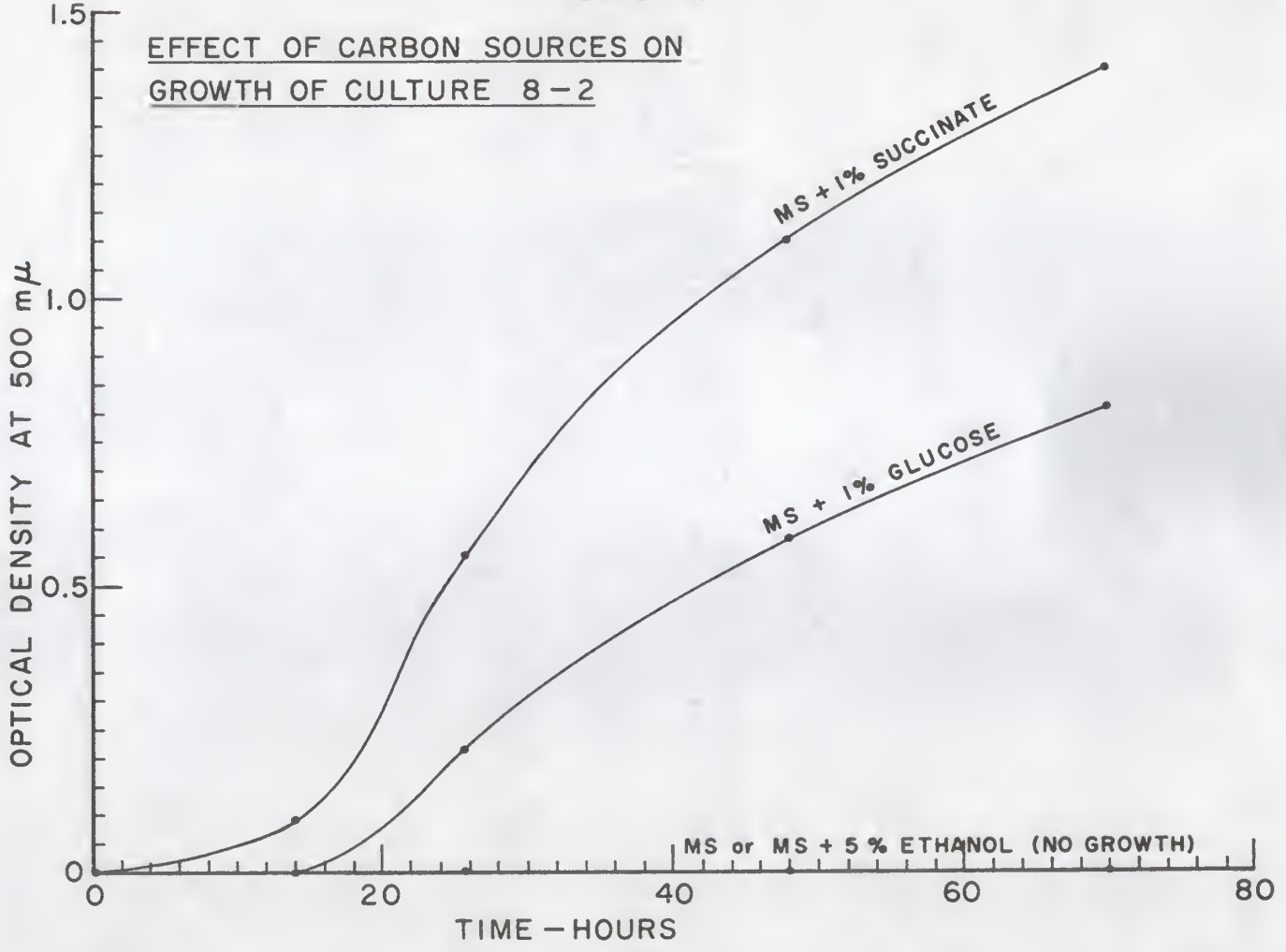
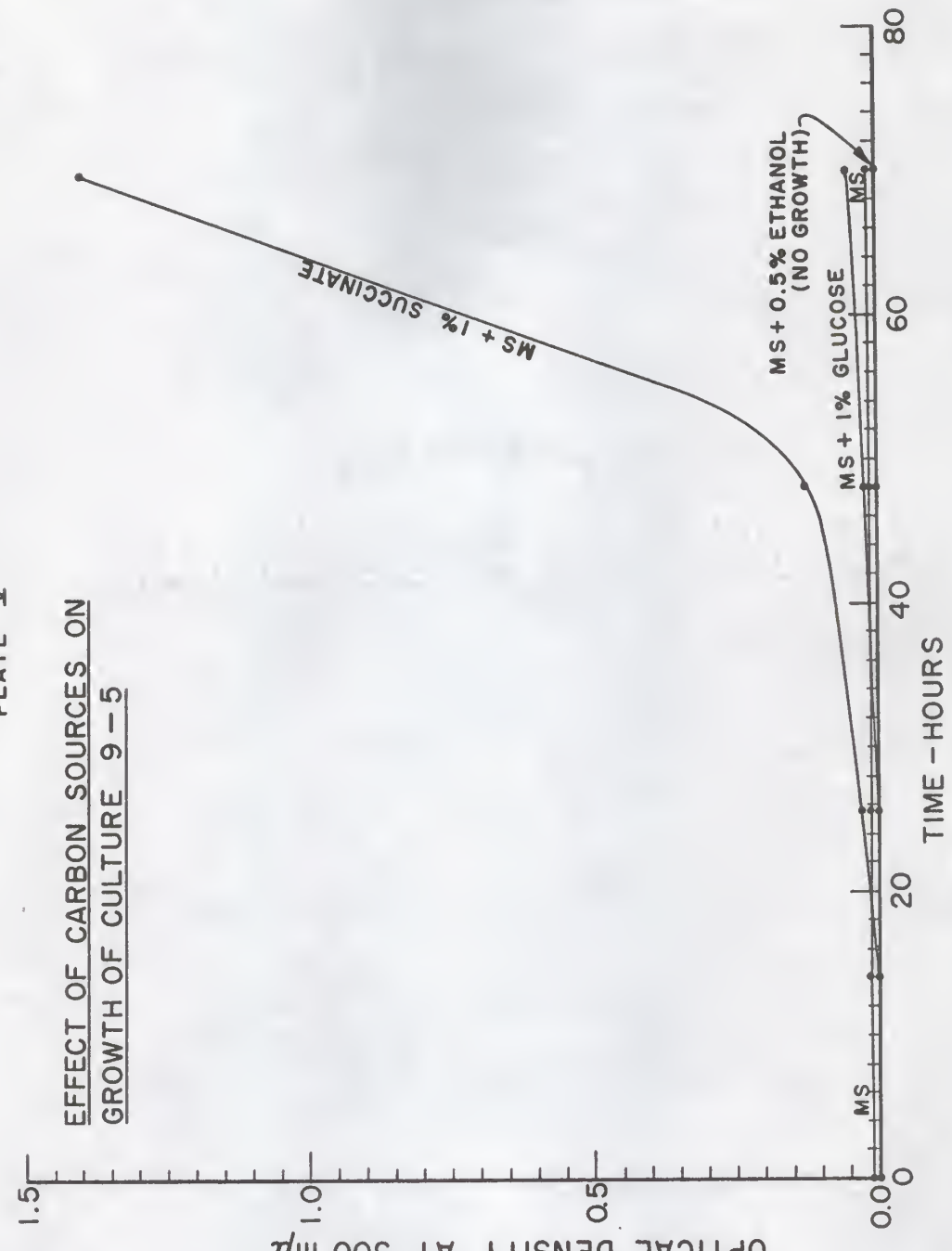

EFFECT OF CARBON SOURCES ON
GROWTH OF CULTURE 8-2
OPTICAL DENSITY AT 500 mμ
1.5
1.0
0.5
0
0
20
40
60
80
TIME - HOURS
MS + 1% SUCCINATE
MS + 1% GLUCOSE
MS or MS + 5 % ETHANOL (NO GROWTH)

PLATE V

EFFECTS OF CARBON SOURCES
ON GROWTH OF CULTURE 9-5

PLATE V

EFFECT OF CARBON SOURCES ON GROWTH OF CULTURE 9-5



Growth With Respect to Inorganic Nitrogen

Plates of Ashby Mannitol agar were streaked from nutrient agar slant cultures of the 21 unknown humus-utilizing bacteria. The plates were incubated for 10 days at 27 C. Cultures from each plate were restreaked to eliminate the possibility of nitrogen being present from the nutrient agar. After four days of incubation, growth was recorded as ++, +, ±, and -. Only cultures showing ++ growth were accepted as nitrogen fixing bacteria.

Trypticase-nitrate broth was used to test the ability of these cultures to reduce nitrate. After four days of incubation, the tubes were tested for the presence of nitrate by the addition of sulfanilic acid and dimethyl-α-naphthalamine. The production of a red color was interpreted as a positive test. Zinc powder was added to the negative tubes. The formation of the red color was interpreted as evidence that the nitrate had not been reduced, while the absence of color was interpreted as evidence that the nitrate had been completely reduced to ammonia.

Nitrogen-fixing ability and degree of nitrate reduction are shown in Table 6.

Utilization of Other Nitrogen Sources

In this experiment, KNO_3, $(NH_4)_2SO_4$, gelatin, Eagles' medium, peptone, soy protein, skim milk, casein hydrolysate, native sheep serum, denatured sheep serum, native egg white, and denatured egg white were each added to 1.5% agar to a final concentration of one percent. Glucose was provided as a carbon source for the KNO_3 and $(NH_4)_2SO_4$ media.

TABLE 6. Growth with Respect to Inorganic Nitrogen

Culture	Growth on Ashby's Mannitol	Amount of NO_3^- Reduction
2-1	++	$NO_2^=$
2-3	+	-
2-4	+	NH_3
2-5	±	$NO_2^=$
3-2	++	-
3-3	+	NH_3
3-4	-	-
3-5	-	-
4-2	-	NH_3
4-3	-	-
5-1	±	-
5-3	-	$NO_2^=$
5-6	-	NH_3
8-1	++	NH_3
8-2	-	NH_3
8-4	++	NH_3
9-2	-	NH_3
9-3	-	NH_3
9-5	-	NH_3
10-3	-	NH_3
10-5	-	NH_3

Streak inoculations were made directly onto the agar from nutrient agar slants of the cultures. The plates were incubated at 27 C for 48 hours. Growth was recorded as relative + or -. Results are presented in Table 7.

Additional Differential Tests

Additional differential tests were made on each culture. Indole production was tested by using trypticase broth and Kovac's reagent. Inoculations were made by the drop method previously described. Readings were made 48 hours after inoculation. Each culture was tested for the ability to produce H_2S by stab inoculation into SIM (sulfur-indole-motility) agar. Readings were made after 48 hours of incubation at 27 C. Stab inoculations into nutrient gelatin were used to test for the ability to liquify gelatin. Cultures were incubated at 27 C. Tubes were refrigerated for 30 minutes after 48 hours and after five days to check for solidification of the gelatin. One percent $Na_2S_2O_3$ in MS was used to test for oxidation of thiosulfate. The thiosulfate was prepared by filter sterilization to decrease the amount of decomposition, and was added with autoclave sterilized MS to sterile flasks. Inoculations were made by the drop method. Readings of pH were taken after five days of incubation at 27 C. Four controls were used. Data were recorded as pH values. Litmus milk was inoculated by the drop method and data were read at 24 hours and at five days. Data for these experiments are given in Table 8.

TABLE 7. Utilization of Other Nitrogen Sources

Culture	NH_4^+	$NO_3^=$	Geletin	Peptone	Soy Protein	Skim Milk	Casein Hydrolysate	Eagles' Medium	Native Sheep Serum	Denatured Sheep Serum	Native Egg White	Denatured Egg White
2-1	++	++++	-	++	+++	+	++	++++	++	++	++	+
2-3	+	++	-	++	++	+	++	+	-	-	-	+
2-4	+++	++++	+	++	+	+	++++	++	+	-	-	++
2-5	++	++	-	++++	++	+	++++	++++	++	++	+	+
3-2	++	++	+	++++	++++	++	+++	++	++	++	++	++
3-3	++++	++++	+	++	++	+	++++	++++	+	+	+	+
3-4	-	-	-	+	++	+	+	-	+	-	-	-
3-5	+++	++++	-	++++	++	++	+++	+++	+	-	+	-
4-2	+	+	+++	+++	+++	+++	++++	+++	+	+	+	+++
4-3	++++	+++	-	+++	++	+	++++	+++	+	+	+	+
5-1	++++	++++	-	++	++	-	++++	++++	+	-	-	-
5-3	-	-	-	++	-	+++	++++	++	-	-	-	-
5-6	+++	+++	+++	+++	+++	+++	+++	++	+	+++	++	++
8-1	+++	++++	-	++	+++	+++	+++	+++	++	-	+++	++
8-2	+++	+++	+	++++	+++	++++	++++	+++	+++	+++	+++	++
8-4	++++	++++	+++	++	+++	-	+++	+	+++	++++	+++	-
9-2	++	+++	+	++++	++++	++	++	+++	++	+++	++	++
9-3	++++	++++	+	++++	++++	++	+++	++++	++	+++	++	+
9-5	+++	++++	+	+++	+++	+++	++++	+++	+	+	+	+
10-3	++++	-	++	+++	++	++++	++++	++	+	+++	+	+++
10-5	++	+	-	++++	+	+	++	++	+	+	+	+

TABLE 8. Additional Differential Tests

Culture	Indole Production	Geletin Liquefaction	H_2S Production	Thiosulfate Utilization pH	Litmus Milk 24 hr.	Litmus Milk 5+ days
2-1	-	-	-	7.00	NC	ALK
2-3	-	-	-	7.85	NC	ALK
2-4	-	+	-	7.77	ALK PEP	PEP
2-5	-	-	-	6.80	ALK	ALK
3-2	-	-	-	6.85	NC	ALK
3-3	-	-	-	7.15	ALK	ALK
3-4	-	-	-	6.90	NC	NC
3-5	-	-	-	6.85	NC	ALK
4-2	-	+	-	6.85	ALK PEP	ALK PEP
4-3	-	-	-	7.10	ALK	ALK
5-1	-	-	-	7.00	ALK	ALK PEP
5-3	-	-	-	7.50	ALK	ALK
5-6	-	-	-	7.80	ALK	ALK
8-1	-	-	-	6.90	ALK	ALK
8-2	-	-	-	6.90	ALK	ALK
8-4	-	-	-	6.90	ALK	ALK PEP
9-2	-	-	-	6.90	NC	ALK
9-3	-	-	-	7.10	ALK	ALK
9-5	-	-	-	6.85	NC	NC
10-3	-	+	-	6.80	ALK PEP	ALK PEP
10-5	-	-	-	6.80	ALK	ALK

ALK = alkaline reaction
PEP = peptonization
NC = no change

PLATE VI

ELECTRON PHOTOMICROGRAPHS OF
SELECTED CULTURES

Figure A. Culture 5-1 showing typical granulation. 6500x.

Figure B. Culture 5-3 showing plasticity of the cell wall and capsule formation. 4400x.

Figure C. Culture 2-5 showing granulation and vacuolation. 6500x.

Plate VI.

Figure A

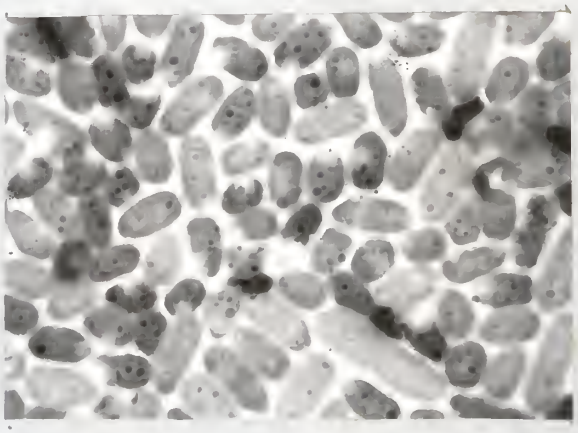


Figure B

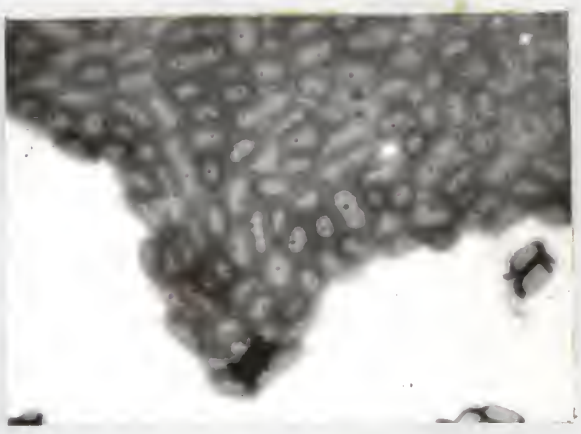
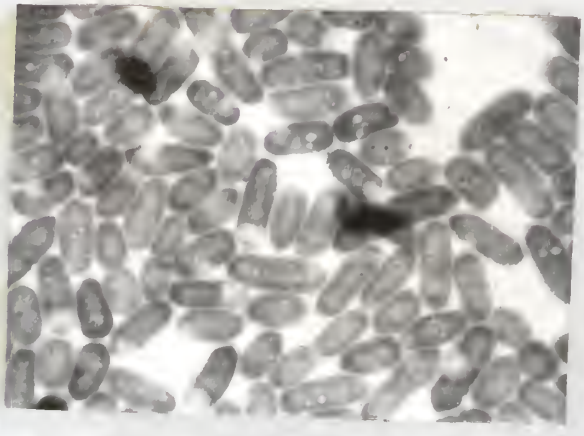


Figure C



DISCUSSION

The soil isolates showed unusual similarity, although they came from seven different soil types and were selected from pour plates on the basis of colonial differences. With the exception of culture 2-3 all the isolates which were later selected as humus users, ranged in color from translucent through white to gray. Only culture 2-4 produced a water soluble pigment, noticable at the time of the primary isolation. It produced a teal-colored pigment which became green, then brown. Cultures 3-3, 4-3, 5-1, 5-6, 8-2, and 9-3, produced slight yellowing of nutrient agar slants after aging. The production of the yellow pigment was most apparent in the succinate-MS medium. The pigment of culture 2-3 was pink and non-water-soluble. All colonies were either convex or raised and entire or lobate. Colonies ranged in diameter from punctiform to four mm. Many colonies were shiny and mucoid.

All cultures were comprised of rod shaped bacteria. With the exception of culture 9-5, all cultures gave negative reactions to the Gram stain. Pleomorphism was frequent, so acid-fast stains were made. None of the cultures were acid-fast. Varying degrees of bipolar staining were shown by 24% of the cultures. Organisms from culture 2-3 were consistently bipolar.

Motility was determined by stab inoculation into motility agar and confirmed by observation of electron photomicrographs. In several instances, the stab inoculation tests indicated that the organisms were motile, yet flagella were not observed on the micrographs. Emphasis was given to the data from the former tests because the micrographs were unshadowed.

Cultures 2-1 and 8-1 showed extreme autolysis in the micrographs. These cultures were somewhat difficult to analyze morphologically.

A notable characteristic of these cultures was the granulation shown by a large number of cultures. Each cell contained both electron-dense and electron-light granules. Cells from culture 2-3 did not show granulation. Flexibility of cell walls is indicated in Plate VI, Figure B.

None of the cultures produced acid or gas in the carbohydrate broth media. The basic reaction of the phenol red indicator suggests, instead, that the cultures were using the peptones in the broth as a source of carbon. When these cultures were placed in a MS medium with a carbohydrate as the only available carbon source, 24% were unable to grow. Another 29% gave moderate to good growth. Growth of all other cultures was slight or was shown on less than half of the carbohydrates tested.

More cultures were able to grow on organic acids than on carbohydrates. Lactate supported the most growth followed by succinate. Acetate and citrate each supported growth in approximately 50% of the cultures. Formate did not support even moderate growth of any culture. Greater use of organic acids might have been expected from the reports by Savage (1961) and Wagner and Stevenson (1965) that carboxyl groups were present and might comprise as much as 10.2% to 18.8% of the humic material.

The lack of growth in the presence of phenol and kojic acid indicates that the phenolic OH suggested by Wagner and Stevenson (1965) and analyzed as approximately 3% of the humic material by Savage (1961) was not the site of attack on the humus complex.

Growth in dodecane was recorded for 86% of the cultures. Only 14% were able to use mineral oil as a carbon source. Tributyrin agar showed zones of hydrolysis by 38% of the cultures. In no instance did a culture which grew on mineral oil show hydrolysis of tributyrin.

The inability of starch and cellulose to support growth is in opposition to the ability of maltose and cellobiose to support at least minimal growth of 71% and 43% of the cultures respectively.

All cultures gave more growth in the presence of succinate as a carbon source than in the presence of glucose or ethanol. For some cultures, the growth was delayed. This may indicated the presence of inducible enzymes rather than constitutive enzymes in those cultures. Some cultures grew well on succinate that showed no growth on glucose or ethanol. None of the cultures were able to utilize ethanol.

Four of the cultures were able to grow on Ashby's mannitol agar, indicating their ability to fix atmospheric nitrogen. Three of the cultures reduced nitrate to nitrite when grown on trypticase nitrate broth while 12 (57%) were able to reduce nitrate to ammonia. Six cultures did not attack nitrate. There seemed to be no significant relationship between utilization of inorganic nitrogen and utilization of humus.

All cultures except 3-5 grew better on the protein media than on the carbohydrate media. These data are supported by the reports of Lynch and Lynch (1958), of Savage and Stevenson (1961), and of Burges et al. (1963) who suggested the presence of amino acids or proteins in the humus complex.

Gelatin supported the least growth of any organic nitrogen source tested as shown in Table 7. This may be due to a lack of ring amino acids. Of the cultures which were able to utilize gelatin, only 2-4, 4-2, and 10-3 were able to bring about liquefaction. Indole was not produced by any of the isolates.

The native and denatured sheep sera and the native and denatured egg white showed less bacterial growth than other proteins. This may have been caused by antibacterial agents which are naturally present in these proteins,

or by a lack of proper proteolytic enzymes.

Sulfide was not produced from sulfur-containing amino acids by any culture. A lack of ability to oxidize sulfur in the form of sodium thiosulfate ($Na_2S_2O_3$) was shown by all cultures as indicated by the lack of the decrease in pH anticipated from the formula

$$5\ Na_2S_2O_3 + 4\ O_2 + H_2O \longrightarrow 5\ Na_2SO_4 + H_2SO_4 + 4\ S.$$

The action of the cultures on litmus milk could have been anticipated from the data already presented. Those cultures that did produce a change in the litmus milk gave an alkaline reaction indicating that milk proteins rather than milk carbohydrates were used.

It can be seen from the data presented that the lytic powers of the humus utilizing bacteria correspond well with, and are perhaps limited by, the characteristics of humus. Complex compounds are more preferred growth sources than simple compounds. Dicarboxylic acids and monocarboxylic acids are more readily attacked than monosaccharides or disaccharides. Nitrogenous material is more readily attacked that carbohydrate material. The role of the humus-decomposing bacteria in the breakdown of complex organic matter is to alter the reserve carbon and nitrogen in the soil to simpler forms which are available to plants. The data indicate that the capabilities of the humus-utilizing bacteria are rather limited and that humus itself seems to be a specialized energy source.

CLASSIFICATION OF THE ISOLATES

Each culture was traced through the comprehensive key contained in the Seventh Edition of Bergey's Manual of Determinative Bacteriology (Breed, et al., 1957). The most plausible results are listed in Table 9.

Cultures 2-1, 3-2, 8-1, and 8-4 were classified as Azotobacter sp. on the basis of their profuse growth on Ashby's Mannitol agar and were classified as Azotobacter indicus on the basis of both morphological and biochemical tests.

Pigmentation was the primary basis for the original classification of cultures 2-4, 3-3, 4-3, 5-1, 5-6, 8-2, and 9-3, though they also yielded the Pseudomonas sp. if treated as achromogenic cultures.

Flagellation and the litmus milk reaction were the main criteria for designating cultures 3-4, 4-2, and 5-3 as Alcaligenes sp.

Culture 9-5 keyed to Brevibacterium sp. or Corynebacterium sp. Separation of those genera at that point was based on the degree of pleomorphism. Corynebacterium was the most plausible genus into which this culture could have been placed.

Two cultures, 2-3 and 10-5, keyed to the genus Vibrio. Culture 10-5 most nearly fitted the description of the species Vibrio percolans. The author states that Vibrio percolans may be an alternate form of Alcaligenes viscolactis as differentiation is based on the degree of curvature of the cells. Culture 2-3 was most nearly classified in the genus Vibrio and matched well biochemically with Vibrio extorquens. However, under normal staining procedures, as well as under electron microscopy, the cells appeared to be straight and definitely bipolar.

TABLE 9. Classification of the Isolates

Culture	Identification
2-1	*Azotobacter indicus*
2-3	*Vibrio* (*extorquens*)
2-4	*Pseudomonas sp.*
2-5	*Pseudomonas sp.* or *Alcaligenes sp.*
3-2	*Azotobacter indicus*
3-3	*Pseudomonas sp.*
3-4	*Alcaligenes sp.*
3-5	*Pseudomonas sp.* or *Alcaligenes sp.*
4-2	*Alcaligenes sp.*
4-3	*Pseudomonas sp.*
5-1	*Pseudomonas sp.*
5-3	*Alcaligenes sp.*
5-6	*Pseudomonas sp.*
8-1	*Azotobacter indicus*
8-2	*Pseudomonas sp.*
8-4	*Azotobacter indicus*
9-2	*Pseudomonas sp.* or *Alcaligenes sp.*
9-3	*Pseudomonas sp.*
9-5	*Brevibacterium sp.* or *Corynebacterium sp.*
10-3	*Pseudomonas sp.* or *Alcaligenes sp.*
10-5	*Vibrio percolans* (*Alcaligenes viscolactis*)

Four cultures could not be designated as either Pseudomonas sp. or as Alcaligenes sp. due to insufficient evidence on the type of flagellation. Each of these cultures was achromogenic.

SUMMARY

Humus was extracted from an alluvial soil with 0.1 molar sodium pyrophosphate, washed and neutralized. Thirty-six cultures were isolated from seven soil samples. Twenty-one of these cultures which actively utilized the humus were selected for characterization.

Gram staining reactions, acid-fast reactions, colonial morphology, and cellular morphology were observed. Gram negative, non-acid-fast, nonpigmented cultures were predominant. Seven cultures produced water soluble pigments. Non-rigidity of the cell wall and granulation were observed in many of the electron photomicrographs.

Cultures were tested for utilization of sugars, organic acids, and other carbon sources, for reduction of nitrate, for nitrogen fixing ability, for utilization of nitrogen sources, and other differential tests.

Good growth was obtained from natural carbon-nitrogen sources. Little growth was obtained from carbohydrates.

The cultures were classified according to Bergey's Manual of Determinative Bacteriology, Seventh Edition, (Breed, et al., 1957). Pseudomonas and Alcaligenes were the dominant genera. Vibrio and Brevibacterium or Corynebacterium were also represented.

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ISOLATION AND CHARACTERIZATION OF HUMUS-UTILIZING BACTERIA FROM KANSAS SOILS

by

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ABSTRACT

Humus was extracted from the soil with 0.01 molar sodium pyrophosphate, washed and neutralized. This material was used for the isolation of bacteria that were able to utilize humus as their only source of carbon. Thirty-six cultures were selected from seven soil samples by five serial transfers.

The cultures isolated were tested for their ability to utilize both soluble and insoluble humus. These humic materials were obtained by 24 hour dialysis of extracted humus. Twenty-one cultures were selected for characterization. Fifteen cultures were eliminated due to their slow growth. In this manner, organisms which used the diluent, mineral salts, rather than the humus were discarded.

Each of the 21 cultures was characterized physically. Gram staining reactions, acid-fast reactions, colonial morphology, and cellular morphology were observed. Only one Gram positive culture was included in the cultures tested. None of the cultures were acid-fast. One culture produced pink colonies with a non-water soluble pigment. All other cultures produced non-pigmented colonies. Seven cultures produced yellow or green water soluble pigments.

Electron photomicrographs were made of each culture. A high percentage of the cultures showed cells with intense granulation of both electron-light and electron-dense material. One culture showed extreme capsule formation. In several micrographs extreme non-rigidity of cell walls led to pleomorphism where cells were in contact with each other.

Each culture was characterized biochemically. They were tested for utilization of sugars, utilization of organic acids, utilization of other carbon sources, nitrogen fixing ability, reduction of nitrate, utilization of nitrogen sources, and other differential tests.

As a group the cultures displayed a lack of ability to utilize carbohydrates. Some grew on organic acids. Best growth was obtained from natural carbon-nitrogen sources.

The cultures were classified according to the key contained in Bergey's Manual of Determinative Bacteriology, Seventh Edition.(Breed, et al. 1957). Pseudomonas and Alcaligenes were the dominant genera. Vibrio and Brevibacterium or Corynebacterium were also represented.