STUDIES ON AGAR-DIGESTING BACTERIA[1]

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INTRODUCTION

During the course of studies on the bacteria responsible for changes brought about in an experimental trickling filter receiving a creamery waste, a number of microörganisms were encountered which were distinctive in that they digested the agar medium upon which they were grown. A study of these cultures was undertaken in the hope that it might throw some light upon their rôle in the purification process, as well as upon their ability to digest agar.

HISTORICAL

Agar has long been known by the peoples of the East as a food product. It was first mentioned in the bacteriological literature by Robert Koch (1882), and its introduction into bacteriological technique was no doubt due to the suggestions of Frau Hesse (Medical Research Council, 1930), the wife of one of Koch's early co-workers. It was introduced to overcome the difficulty encountered with gelatin due to its liquefaction at relatively low temperatures.

For many years it was thought that bacteria could not utilize this complex carbohydrate, but in 1902 Gran isolated an organism from sea water which liquefied seaweed agar and to which he gave the name *Bacillus gelaticus*.

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Lundestad in 1928 reported two varieties of this organism both of which were isolated from sea water. He also reported six species of the genus *Flavobacterium*, and one species of the genus *Bacterium* which had the power to dissolve seaweed agar and to liquefy fish-agar slants.

In 1905 Panek isolated from fermenting red beets a facultative anaerobe which liquefied agar.

Beijerinck in 1911 described *Microspira tyrosinatica*. Brief mention was made of the fact that the organism was "similar to the gelose vibrio in that it secreted the enzyme gelase which changes agar to sugar."

Biernacki in 1911 reported an organism isolated from grapes, which liquefied the agar slants upon which it was cultured.

Gray and Chalmers in 1924 reported the isolation of a motile curved rod which liquefied the agar medium upon which it was grown, and which grew well on $(NH_4)_2SO_4$ agar.

Van der Lek in 1929 reported that in the stock collection at Delft there had been for some time a culture which seemed to be the same as the organism described by Gray and Chalmers, though his seemed more vigorous in its action.

Aoi in 1924 reported his organism *Vibrio andoi*, which was isolated from manure and which liquefied ammonium sulphate agar slants.

Angst in 1929 reported 13 species of bacteria which disintegrated agar and which were placed in a new genus, *Agarbacterium*. The description of these species does not seem to warrant the creation of a new genus, since most of them could easily be placed in existing genera.

EXPERIMENTAL

The characteristics of the above mentioned bacteria were carefully compared with those of three newly isolated organisms, and since there were distinct differences, it was concluded that these organisms were new species.

Source and isolation of organisms

The organisms here described were isolated from an experimental trickling filter receiving creamery wastes. The filter was

constructed in six layers. Each layer was one foot in depth and made in the form of a box two feet square, fitted with a bottom consisting of 2- by 2-inch wooden strips, placed one inch apart. The boxes were filled with screened cinders and placed one above the other, leaving a 4-inch space between the layers. The creamery waste was distributed over the surface of the first layer by means of a tipping pan, and the material dripped from layer to layer in passing through the filter. The medium used in the isolation had the following composition.

$(NH_4)_2SO_4$	1 gram
K_2HPO_4	1 gram
NaCl	2 grams
$MgSO_4$	0.5 gram
Fe_2SO_4	trace
$MnSO_4$	trace
H_2O	1000 cc.

15 grams of agar
pH 7.2

Agar plates were poured, using as inoculum 1-cc. portions of a suspension of material washed from the cinders of the filter. The plates were incubated at 25°C. for five days. It was found upon examination under the low power of the microscope that nematodes were growing in the agar. They were congregated in groups in certain darkened and sunken areas.

The material in these sunken areas seemed to be liquid, as could be seen by the disturbance caused by the nematodes. Material from these areas was plated, using the above-described agar medium. After three days incubation small pits in the agar surface could be seen. At the end of five days some of the colonies had liquefied the agar medium down to the glass of the petri dish. This process of replating was repeated until pure cultures were obtained. In a similar manner two other organisms were isolated from the slime of the filter. In all 11 cultures were obtained, which fell into three definite groups. Table 1 indicates the differential characteristics of the three types, recognized as new species, to which the following names were given (Goresline, 1932): *Achromobacter pastinator*, *Pseudomonas lacunogenes*, and *Pseudomonas segne*.

The following characteristics were common to the three organisms.

Indol: Indol was not produced in trytophane broth.

Hydrogen sulphide: Not produced in lead acetate agar.

Oxygen requirement: Facultative; growth was much better aerobically.

Temperature: No growth at 42°C., moderate growth at 37°C., good growth at 25°C., moderate growth at 20°C., no growth at 10°C. Optimum about 28°C.

Gelatin stab: No growth.

TABLE 1

Showing differential characteristics of agar-digesting bacteria

CHARACTERISTICS	GROUP I, ACHROMOBACTER PASTINATOR	GROUP II, PSEUDOMONAS LACUNOGENES	GROUP III, PSEUDOMONAS SEGNE
Chromogenesis on nutrient agar	Colorless to whitish	Light orange yellow	Orange yellow
Broth	Only a slight clouding	Pellicle, clouding sediment, viscous	Uniform clouding, no surface growth
Litmus milk	Slightly acid	Litmus reduced, alkaline	Litmus not reduced, becoming alkaline slowly
NO_2 from NO_3	+	−	−
Starch hydrolized	+	+	−
Fehling's solution reduced*	+	−	−
Nutrient agar	Rapid liquefaction	Slow liquefaction	Slow liquefaction
Utilization of $(NH_4)_2SO_4$ as nitrogen source	−	+	−
Growth on peptone water	−	+	+

* By liquefied agar.

Methods

Flagella. Flagella stains were made by Muir's modified Pitfield method as described by Stitt (1914).

Media. The media used in ascertaining the general character-

istics of these organisms were prepared according to the methods found in the Manual of Methods of the Society of American Bacteriologists (1930).

Utilization of carbohydrates. In order to study the carbohydrate requirements of these organisms the following medium was used:

Bacto peptone	10 grams
K_2HPO_4	5 grams
H_2O	1000 cc.
Carbohydrates	0.2–0.6 per cent
pH	7.2

Inoculations from water suspensions of growths from plain agar slants were made in triplicate, one tube containing Andrade's indicator and two tubes without an indicator. The tube containing indicator was used to record acid production by the organisms on the various carbohydrates.

One of the tubes not containing an indicator was used for periodic pH determinations, using a quinhydrone electrode of special design (Goresline, 1933), while the other was analyzed at the end of the experiment to determine the extent of carbohydrate utilization according to the procedure suggested by Stiles, Peterson, and Fred (1926). The non-reducing sugars were first hydrolyzed by the methods suggested by Browne (1912) and by the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists (1925) and then analyzed for reducing sugars by the above method.

Since *Pseudomonas lacunogenes* grew in the basal medium and produced an alkaline reaction, which masked any slight acidity which might have been produced from the carbohydrates, $(NH_4)_2SO_4$ was substituted for the peptone as a nitrogen source in order to study the acid production and the utilization of carbohydrates by this organism.

Change in viscosity. Agar solutions were inoculated with the test organisms, and after the period of incubation was over, the loss of weight due to evaporation was made up with distilled water. Since at room temperatures the control material was a solid jell, and therefore had a high viscosity, while the digested

material at the same temperature was a liquid of practically the same viscosity as water, readings had to be taken at temperatures at which both samples were liquids. Fifty degrees Centigrade was chosen as the starting point and the solutions were allowed to cool spontaneously, which resulted in an almost uniform rate of cooling for both solutions. The control sample was heated in an Arnold steamer just long enough to melt the agar, and after cooling to about 50°C., viscosity readings were made. The liquefied samples were given the same treatment, except that the material was strained through cheesecloth and then centrifuged just long enough to remove the bulk of the bacterial growth. The instrument used in these determinations was a Stormer Viscosimeter (Thomas, 1927). In recording results, relative viscosity was plotted against temperature. Relative viscosity was found by dividing the time in tenth-seconds taken by the revolving cylinder to perform 100 revolutions in the test solution, by the time taken for the same number of revolutions in distilled water at the same temperature.

Products of decomposition of agar. Fairbrother and Mastin (1923) said of agar, "It is a hemicellulose which upon hydrolysis with dilute acids, gives chiefly d-galactose; other hexoses and smaller amounts of pentoses are formed at the same time." Samec and Isajevic (1922) gave the formula for the free agar acid as $(C_6H_{10}O_5)_{54}SO_4H$ and showed that SO_4 was dializable only after the agar had been heated until it lost its power to jell. The molecular weight of agar was assumed to be about 9000. Hoffman and Gortner (1925) prepared the free agar acid by dialysis and found that as such it could not jell but had to be in the form of a calcium or other heavy metal salt. König and Gettels (1905) reported that upon acid hydrolysis agar gave galactose, glucose, levulose, pentoses, and methylpentoses.

It will be seen from the above that a number of compounds result from the chemical decomposition of agar. Without doubt the decomposition of agar by bacterial means follows somewhat the same path. The tests used for the detection of the various organic compounds in this study were those outlined by Hawk and Bergeim (1927), Browne (1912), and Mulliken (1905).

Tests were employed to detect the production of aldehydes, alcohols, and simpler carbohydrates from the agar. Among those employed for the latter determinations were Benedict's test for monosaccharides, the resorcinol reaction for keto sugars, and the mucic acid test for galactose and lactose. The phloroglucine reaction was employed to detect such compounds as arabinose, xylose, rhamnose, levulose, and sorbinose; as was, also, the aniline acetate test for the production of furfural from these compounds. The phenyl hydrazine reaction and the B naphthol hydrazone tests (Hilgen and Rothenfussen, 1902) were both used.

Utilization of organic acids. The same basal medium employed for the utilization of carbohydrates was used in this study. The various organic acids in 0.2 per cent concentration were substituted for the carbohydrates, and the reaction of the medium adjusted to pH 6.6. Growth and change in acidity were used to detect the utilization of the organic acid.

Since *Pseudomonas lacunogenes* grew in the basal medium and produced an alkaline reaction, $(NH_4)_2SO_4$ was substituted for the peptone as a source of nitrogen in the study of this organism.

The following organic acids were used:

Acetic	Lactic	Palmetic
Aconitic	Lauric	n-propionic
n-butyric	Malonic	Succinic
Caproic	d- and l-malic	Tartaric
Citric	Oleic	Valeric
Formic	Oxalic	

Utilization of nitrogen compounds: The following basal solution was prepared to study the utilization of compounds as a nitrogen source.

K_2HPO_4	5 grams
NaCl	2 grams
H_2O	1000 cc.
Nitrogen compounds	0.2 per cent
pH	7.2

To one-half of this solution was added 0.2 per cent glucose, to act as the carbon source, while nothing was added to the other

half. This arrangement tested the ability of the organism to utilize the various compounds as both nitrogen and carbon sources, or as sources of nitrogen alone, when augmented by some fermentable carbohydrate as the carbon source. Utilization of the compounds was determined by growth.

The following compounds were employed: Ammonium sulphate, sodium nitrite, sodium nitrate, ammonium chloride, ammonium phosphate, urea, cystein, asparagin, aspartic acid, lactamide, leucine, uric acid, tyrosine, alanine, glutaminic acid, ammonium succinate, and peptone.

RESULTS

Group I. Achromobacter pastinator nov. sp.

This microörganism was a non-spore-forming, Gram-negative, short rod, occurring singly or in pairs, and very constant in size, about 0.4μ by 1.5μ. Motility was by means of peritrichous flagella, two to five in number.

Cultural characters. All materials were incubated at 28°C.

Nutrient agar colonies: After incubation for forty-eight hours small dents appeared in the agar surface, and the colonies were located with the eye with difficulty. After three days the colonies were about the size of a pinhead, almost colorless, and situated in the bottom of a funnel-shaped depression. The colonies sank rapidly through the agar, and generally at the end of five days had reached the glass of the petri dish. At this stage the growth formed in a ring and widened the bottom of the crater, leaving the glass bare. The outside of the crater was often more than an inch in diameter. When the colonies were close together, the liquefaction was much reduced.

Agar slant: On nutrient agar slants there was good growth, the agar being liquefied along the streak often to the depth of one fourth of an inch. The growth was flat and did not seem to be very thick. The medium was not darkened nor changed except for the liquefaction, and a pocket was formed at the bottom of the slant which was filled with a rather viscous liquid, yellowish in color.

Potato: No growth on potato: probably no suitable nitrogen available.

Nutrient broth: The medium showed slight clouding after five days. There was no growth on the surface and no sediment at the bottom. The best growth seemed to be well toward the surface.

Nutrient gelatin stab: Very scanty growth on the surface, no liquefaction.

Physiological characters:

General characteristics:

Litmus milk: After twenty days the milk was slightly acid. No curd was formed and only a trace of reduction took place at the bottom of the tube.

pH limits of growth: The upper limit of growth was between pH 8.8 and 9.0, while the lower limit was between pH 5.9 and 6.1.

Utilization and decomposition of organic carbon compounds:

Utilization of carbohydrates: Acid production was never evidenced by a deep red color, but a pink color was obtained with Andrade's indicator, indicating only a moderate production of acid.

No growth took place in the plain peptone-phosphate broth. This indicated that the organism could not utilize the peptone as a source of both nitrogen and carbon, but when certain carbohydrates were added good growth occurred, showing that when a suitable carbon source was supplied, the peptone could be utilized as a nitrogen source. Growth was obtained on arabinose, glucose, galactose, lactose, levulose, maltose, mannose, melezitose, pectin, raffinose, rhamnose, salicin, sucrose, starch, and dextrin, while no growth was obtained in dulcitol, erythritol, mannitol, sorbitol, glycerol, xylose, and inulin. It is interesting to note that none of the five higher alcohols used supplied an available carbon source.

Acid production on the various carbohydrates was determined electrometrically at intervals during the period of incubation in order to see if any fluctuations in the pH would take place, indicating a possibility of the utilization of any acid by-products by this organism. In carbohydrates in which growth occurred, a

small amount of acid was formed. In general the pH change was more than 0.4 pH but never more than 0.7 pH. Arabinose, rhamnose, and melezitose supported good growth, but very little acid was formed as shown by pH readings. Since the weak acid production gave no indication to what extent the carbohydrates were utilized, a series of tubes was inoculated, and after twenty days incubation, an analysis was made for reducing sugar. The results of these determinations are shown in table 2, which indicates that there was a marked reduction in the sugar content of a solution when fermented by this organism.

TABLE 2

Showing utilization of various carbohydrates

CARBOHYDRATE	INITIAL PER CENT OF CONCENTRATION AS GLUCOSE	PER CENT UTILIZATION BY		
		ACHROMOBACTER PASTINATOR	PSEUDOMONAS LACUNOGENES	PSEUDOMONAS SEGNE
Arabinose	0.238	44.1	69.0	65.3
Glucose	0.645	27.6	95.5	73.0
Galactose	0.252	51.5	55.2	95.0
Lactose	0.419	31.6	36.4	17.65
Levulose	0.676	33.9	42.2	27.7
Maltose	0.290	80.0	100.0	56.2
Mannose	0.276	90.0	37.4	60.1
Xylose	0.268	No growth	94.5	13.8
Sucrose	0.677	15.8	58.9	74.3
Starch	0.158	38.7	77.2	5.0
Melezitose	0.111	20.7	27.9	53.1
Raffinose	0.184	49.4	83.2	65.2

All tubes incubated for twenty days at 28°C.

Decomposition of agar:

Change in viscosity: An agar medium of the following composition was used to study the changes in viscosity brought about by this organism:

$NaNO_3$	5 grams
K_2HPO_4	2 grams
NaCl	2 grams
$MgSO_4$	0.5 gram
Agar	10 grams
H_2O	1000 cc.
pH	7.2

This medium was sterilized and just before solidification inoculated with a culture of *Ach. pastinator* and weighed. By the end of the third day small colonies could be seen all through the agar, although the growth at the surface was heaviest. Nineteen days after inoculation all of the agar had become liquid, although the major portion had liquefied by the twelfth day. This solution showed no tendency to solidify even when stored for twenty-four hours at 5°C. The results of the viscosity determinations are shown in figure 1. It is improbable that exact viscosity

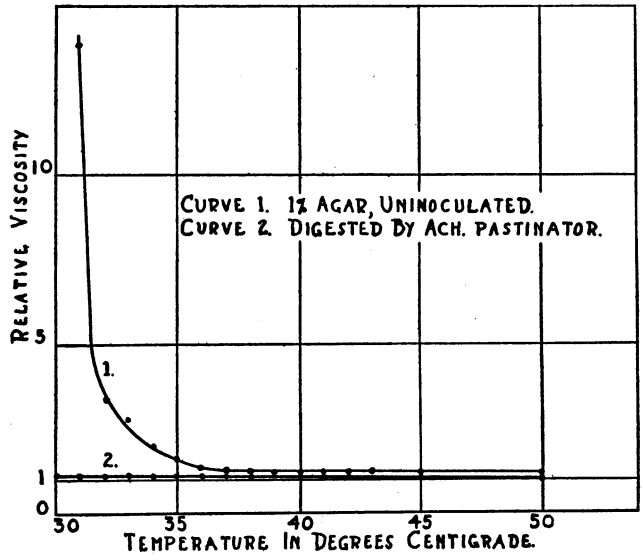

FIG. 1. SHOWING CHANGE IN RELATIVE VISCOSITY IN A 1 PER CENT AGAR SOLUTION

values were obtained for the undigested agar at some of the temperatures close to the solidifying point, since each reading broke up the jell structure that had formed, thus preventing the material from taking a firm set, and because of this disturbing action the control agar solution remained liquid to a much lower temperature than its ordinary solidifying point. The difference in readings obtained between the digested and the undigested agar shows clearly the effect of this organism upon the jell structure of agar.

Products of agar decomposition: *Ach. pastinator* digested agar, and a reducing substance was found in the resulting liquid. It was found difficult to identify any one product formed by this decomposition, since each occurred in conjunction with other carbohydrates as well as in the presence of other complex organic compounds. Because the original concentration of the agar was only 1.5 per cent, the resulting products of decomposition were present in small amounts. The total amount of reducing compounds was only 0.24 per cent.

The material used was obtained by digesting 1.5 per cent agar with *Ach. pastinator*. Various methods were employed in clarifying the resulting solution, but filtration through a Berkefeld filter was found to be the most satisfactory. Repeated attempts to concentrate the material resulted in a dark brown liquid, which made the reading of delicate tests very difficult. Even slight heating, such as was necessary to distill in a vacuum, was sufficient to produce a dark liquid with a caramel odor. The reducing substance was not volatile.

Aldehydes, acids, and alcohols were not found upon analysis of the liquefied agar. After employing the various carbohydrate tests discussed under methods, it was concluded that the liquefaction of agar by *Ach. pastinator* gave rise to a number of simpler carbohydrates.

It was shown that members of the monosaccharide group were present. The pentoses were represented in this group, as were no doubt methylpentoses, although this was not proved conclusively. The hexoses were represented, since the test for ketose group was positive. The only two ketoses listed by Browne (1912) are levulose and sorbose, which are both hexoses.

Members of the disaccharide group were present, since a positive test was obtained with Benedict's reagent after testing with Barfoed's reagent.

Galactose could not be demonstrated by the mucic acid test, although there was a possibility that the concentration of the sugar was not high enough, since considerable is necessary to obtain a good test. Only an amorphous mass could be obtained with phenylhydrozine, and therefore no carbohydrates could be identified by their osazone crystals.

Utilization of organic acids: The various organic acids were used in 0.05 per cent concentration. Since this organism could not utilize the peptone as a source of carbon as well as nitrogen, this experiment was designed to test the ability of the organism to utilize the organic acid as its sole source of carbon.

Repeated inoculations into the various media failed to induce growth. It was thought possible that 0.05 per cent concentration of these acids was toxic to this organism. To test this possibility, a few drops of sterile glucose solution were introduced into each of the tubes and growth was obtained with this organism, showing that toxicity was not the explanation, but rather that the acids were not available as carbon sources when peptone was supplied as the nitrogen source.

Utilization of nitrogen compounds:

Reduction of nitrates: Nitrates were reduced.

Utilization of nitrogen sources: No growth was obtained with any of the nitrogen compounds alone, but when glucose was added to them growth was obtained with cystein, asparagin, aspartic acid, glutaminic acid, ammonium succinate, and peptone. This showed that these compounds were available as nitrogen sources when the carbon was supplied. It was peculiar that no growth could be obtained with $(NH_4)_2SO_4$ or $NaNO_3$ when glucose was used as the carbon source, while good growth was obtained when agar was substituted for the glucose. No growth could be obtained on agar alone.

Group II. Pseudomonas lacunogenes nov. sp.

This microörganism was a non-spore-forming, Gram-negative, short rod with pointed ends, occurring singly or in pairs, and varying in size from 0.2μ to 0.3μ in width, by 1μ to 1.2μ in length. It was motile by means of a single polar flagellum, ranging from 2μ to 15μ in length.

Cultural characters. All cultures were incubated at 28°C.

Nutrient agar colonies: After forty-eight hours incubation small yellow colonies appeared. Each colony was surrounded by a circle that seemed to refract the light differently from the rest of the agar. The surface of the agar was pitted or dimpled.

After five days the surface colonies were about 5 to 7 mm. in diameter and surrounded by a depression, the outside diameter of which was about three times that of the colony. The colonies were orange yellow in color and slightly raised. They did not sink through the agar to the glass but settled into a shallow depression and seemed to remain stationary. There was no liquid in the depression, as it was evidently absorbed by the surrounding agar.

Agar slant: The growth was heavy and of a light orange yellow color, the consistency about that of warm butter, and the edge was entire, slightly raised. A shallow depression, which extended about 3 mm. on each side of the streak, was produced. No liquid was found in the depression, but the agar was softened beneath the growth. The medium was not darkened.

Potato: Moderate growth on potato, orange yellow and smooth. Potato not darkened.

Nutrient broth: The medium was clouded in 48 hours. A light orange yellow pellicle was formed and considerable viscous sediment was developed.

Nutrient gelatin stab: The growth was brownish yellow and could be traced about half way down the stab, but was much heavier at the surface. No liquefaction was produced.

Physiological characters:

General characteristics:

Litmus milk: After two days incubation the top one-third of the tube became faintly alkaline. Upon further incubation the alkaline reaction spread to the entire tube, and a butter-colored pellicle formed on the surface. After about ten days incubation the bottom third of the tube became reduced and fresh litmus recorded a marked alkaline reaction. No digestion was noticed and no curd was formed.

pH limits of growth: The upper limit of growth was about pH 10.0, while the lower limit of growth was between pH 5.4 and 5.8.

Utilization and decomposition of organic carbon compounds:

Utilization of carbohydrates: After two days incubation a slight pink tinge was noticed in glucose, maltose, levulose, and

sucrose, but this color was sustained only for a day or two and then disappeared entirely. It was found that this organism produced an alkaline reaction from the peptone, which masked any slight amount of acid which might have been formed from the carbohydrate.

A series of pH determinations was undertaken to find if any variation could be detected in the rate of pH change in the various carbohydrates. The pH readings showed that an alkaline reaction was produced in all of the cultures. A reaction more alkaline than the control was produced in arabinose, galactose, glycerol, lactose, levulose, maltose, melezitose, raffinose, starch and xylose; while glucose, mannose, and sucrose remained about the same pH as the control. This was also true with dulcitol, erythritol, pectin, rhamnose, salicin, sorbitol, dextrin, and inulin. The pH change was insufficient to warrant any definite conclusion.

Since neither Andrade's indicator nor pH determinations had given any clue to the carbohydrates that had been utilized, reducing sugar determinations were made on inoculated and uninoculated materials after twenty days incubation. Table 2 shows the extent of utilization of the various carbohydrates by the organism.

In order to test the availability of the carbohydrates as a sole source of carbon, a medium was used in which $(NH_4)_2SO_4$ was the sole source of nitrogen. The carbohydrates were added to this solution and inoculated with *Ps. lacunogenes*.

Good growth was obtained with the following carbohydrates: glucose, galactose, lactose, levulose, maltose, mannose, melezitose, raffinose, rhamnose, sucrose, agar, pectin, and salicin. This showed that this organism was capable of utilizing these carbohydrates as sources of carbon, with $(NH_4)_2SO_4$ acting as the nitrogen source. Growth was obtained in arabinose and xylose only when a heavy inoculum was used, and then the growth was not luxuriant. No growth was obtained with the following carbohydrates: dulcitol, erythritol, glycerol, sorbitol, mannitol, and inulin.

Decomposition of agar:

Change in viscosity: A medium consisting of $(NH_4)_2SO_4$,

K_2HPO_4, NaCl, water, and agar was used. That a marked change might be recorded, a 0.5 per cent agar solution was employed. After twenty days incubation the agar material had become quite liquid, and viscosity measurements were made. Figure 2 shows the difference in viscosity between the uninocu-

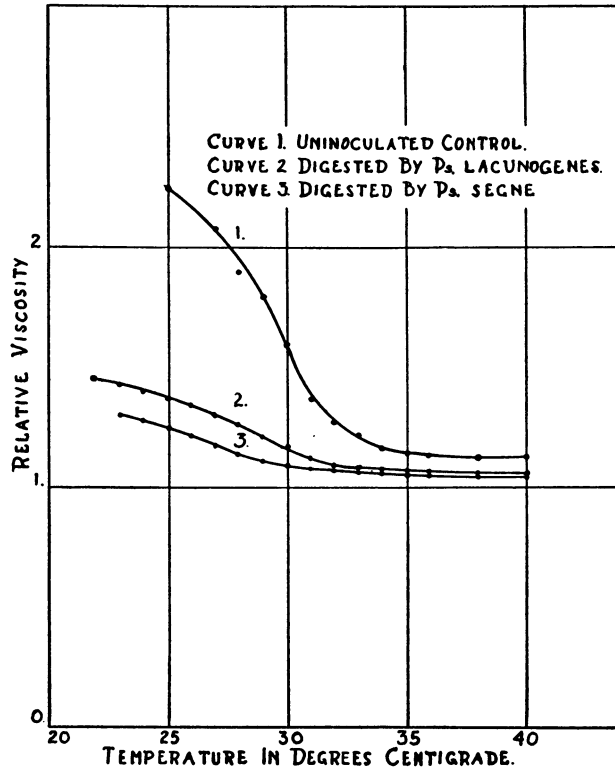

FIG. 2. SHOWING CHANGE IN VISCOSITY OF 0.5 PER CENT AGAR SOLUTION

lated control and the inoculated material. It will be noted that about 70 per cent change in the viscosity of the agar at 25°C. was effected by the action of *Ps. lacunogenes*. The solution never became watery, but the viscosity changed until the jell structure was lost.

Products of decomposition of agar: Determinations were made

for aldehydes, alcohols, and reducing carbohydrates and all gave negative results. The agar solution used was 0.5 per cent and any by-products that might have been formed were probably in very small amounts.

Utilization of organic acids: Since good growth took place in the peptone solution without any added organic acid, the appearance of growth in the tubes could not be taken as an index to the utilization of the organic acids. The change in pH was then determined, using brom-thymol blue as an indicator, to find if the acidity of the acids utilized changed faster than the pH of the control solutions.

When a 0.2 per cent concentration of the organic acid was used, n-butyric, caproic, lauric, oleic, oxalic, palmetic, n-propionic, and valeric were toxic and no growth was obtained. When the concentration was dropped to 0.05 per cent, they were no longer toxic and growth was obtained. The following acids showed a pH change: acetic, n-butyric, formic, lactic, malonic, d- and l-malic, succinic, and tartaric. In order to prove conclusively that this pH change indicated utilization of the acid, a medium consisting of $(NH_4)_2SO_4$, K_2HPO_4, NaCl, and distilled water was prepared to test the ability of this organism to utilize the organic acids when an inorganic nitrogen source was supplied. To this medium were added the various organic acids.

The observations on the peptone solution were borne out when $(NH_4)_2SO_4$ solution was used as a nitrogen source. Those acids which had shown a marked pH change in the peptone medium, also showed growth in the ammonium sulphate medium. Aconitic, caproic, citric, lauric, oleic, oxalic, palmetic, n-propionic, and valeric acids were not available as carbon sources.

Utilization of nitrogen compounds:

Reduction of nitrates: Nitrates were not reduced.

Utilization of nitrogen sources: It was found that certain of the test compounds were capable of furnishing both nitrogen and carbon *to Ps. lacunogenes,* while others served only in the capacity of a nitrogen source. Of the nitrogen compounds tried, cystein, asparagin, aspartic acid, tyrosine, alanine, glutaminic acid, ammonium succinate, and peptone were available as both

nitrogen and carbon sources, while a carbon source had to be supplied to make urea, lactamide, leucine, and uric acid available. Lactamide and uric acid supported only a moderate growth. Ammonium sulphate, ammonium chloride, and ammonium phosphate were available as a nitrogen source when glucose was supplied, while sodium nitrate and sodium nitrite failed to support growth.

Group III. Pseudomonas segne nov. sp.

This microörganism was a non-spore-forming Gram-negative, short rod with pointed ends, occurring singly or in pairs, and varying between 0.2μ to 0.3μ in width by 1μ to 1.2μ in length. It was motile by means of a single polar flagellum.

Cultural characters. Nutrient agar colonies: After forty-eight hours incubation, very small light yellow colonies appeared. Around each colony was a small circle that seemed to refract the light differently from the rest of the agar. The surface of the agar was pitted. After five days the colonies on the surface were about 5 mm. in diameter, and the outside diameter of the depression in the agar about twice that of the diameter of the colony. The colony did not sink through the agar to the glass, and there was no liquid in the depression, probably due to the absorption by the drying agar.

Agar slant: The growth was heavy on the nutrient agar slants, orange-yellow in color and having about the consistency of warm butter. The growth was only slightly raised and the edge was entire. A slight depression was made in the surface of the slant, extending about 5.5 mm. beyond the edge of the growth. No liquid was present, as it evidently was absorbed by the drying agar, but the agar was softened below the growth. The medium was not darkened.

Potato: Scant orange-yellow growth on potato, and then only with large inoculum. No darkening of the potato.

Nutrient broth: The medium was clouded in forty-eight hours. There was neither pellicle nor surface growth and only a moderate amount of sediment. Old cultures showed a yellow ring at the surface, and in some cases a loose membrane.

Nutrient gelatin stab: The yellow growth was best at the surface, but growth could be traced almost half way down the stab. No liquefaction took place.

Physiological characteristics:

General characteristics.

Litmus milk: The milk was slightly alkaline at the end of ten days incubation, and there was no evidence of surface growth.

pH limits of growth: The upper limit of growth was between pH 8.8 and 9.0, while the lower limit was between pH 5.8 and 6.0.

Utilization and decomposition of organic carbon compounds:

Utilization of carbohydrates: This organism grew well in the control material consisting only of the peptone and phosphate, and produced a marked alkaline reaction, which tended to mask any slight acidity which might have been produced from the various carbohydrates. After two days incubation a slight pink tinge was noted in glucose, levulose and sucrose, but this color remained for only a day or two and then disappeared entirely. A series of pH determinations was undertaken to find if any variations could be detected in the rate of pH change in the various carbohydrates. The pH changes did not give sufficient information to draw conclusions as to the utilization of the carbohydrates, and since Andrade's indicator did not give an indication of what carbohydrates were utilized by this organism, a series of chemical determinations was made on uninoculated material and upon inoculated material incubated for twenty days. Table 2 shows the extent of utilization of the various carbohydrates.

Repeated attempts were made to find a medium in which this organism would grow only when given a carbon source, such as the carbohydrates, or one in which an alkaline reaction would not be produced, thus masking the acid produced from the carbohydrates. These experiments did not meet with success. Seventeen nitrogen sources were tried, but not one of them would serve as a nitrogen source for this organism. Glucose was added to each of the nitrogen compounds to serve as a carbon source, but no growth could be obtained. Because of this peculiarity,

the exact number of carbohydrates that this organism will attack cannot be given, but as shown by the chemical tests in table 2, arabinose, glucose, galactose, lactose, levulose, maltose, mannose, xylose, sucrose, melezetose, and raffinose were utilized.

Decomposition of agar:

Change in viscosity: When grown in a 0.5 per cent agar solution *Ps. segne* produced a change in the viscosity of the solution. After twenty days incubation the agar material was quite liquid. Figure 2 shows the difference in viscosity between the uninoculated control and the inoculated materials. It will be noted that about an 80 per cent change in the viscosity at 25°C. was effected by this organism.

Products of decomposition of agar: Determinations were made upon digested agar material to determine if possible the products of decomposition. Fehling's solution was not reduced by the digested material, and although all of the previously described tests for carbohydrates were made, no positive results were obtained.

Utilization of organic acids: As indicated under the utilization of carbohydrates, repeated attempts were made to obtain a nitrogen source with which it would be possible to show the utilization of the various compounds as indicated by acid production without interfering-substances being formed. These series of experiments were not successful, and therefore the utilization of the organic acids was studied by a comparative method, in which the pH change produced in the medium, and the magnitude of growth were taken as the indication of the utilization of the organic acids. In a concentration of 0.2 per cent n-butyric, caproic, citric, lauric, oleic, palmetic, n-propionic, and valeric acids were toxic; but when the concentration was reduced to 0.05 per cent, they were not toxic. The following organic acids were utilized by *Ps. segne:* acetic, n-butyric, formic, lactic, malonic, d- and l-malic, succinic, and tartaric, while aconitic, caproic, citric, lauric, oleic, oxalic, palmetic, n-propionic, and valeric acids were not.

Utilization of nitrogen compounds:

Reduction of nitrates: Nitrates were not reduced.

Utilization of nitrogen sources: The same nitrogen compounds

listed before were used and repeated inoculations were made, but growth could not be induced in any of the compounds other than peptone. Because of this peculiarity, only peptone could be used as a basic medium for the various tests.

GENERAL DISCUSSION AND SUMMARY

Many microörganisms are associated in the purification process of a trickling filter receiving a creamery waste, as has been shown by Levine and Soppeland (1926) and by Frye and Becker (1929). The exact nature of the association of the various forms is not known.

No doubt the ability of *Achromobacter pastinator* to utilize lactose and galactose accounts for its presence in the trickling filter. Since *Ps. lacunogenes* and *Ps. segne* utilized lactose, galactose, and lactic acid, their presence in the filter no doubt was due to their ability to utilize these compounds. Since these organisms attacked the complex colloid agar, their function may have been that of breaking down the colloidal jell-like material, which made up the organic growth of the filter.

Without doubt such organisms as have been described here may be found in many places in nature in connection with digestion of naturally occurring gums and jells. Since the work on this paper was finished, Nicol (1931) has described agar-softening organisms from garden soils in England.

Many of the agar-digesting microörganisms described in the literature were reported as having no action on carbohydrates. It is possible that these organisms were similar to *Ps. lacunogenes* and *Ps. segne* in producing an alkaline reaction in the peptone medium which masked any slight acidity that might have been produced from the carbohydrates. Perhaps if an inorganic source of nitrogen were used or a chemical determination of the sugar before and after fermentation were made, the organisms thus described might be shown to utilize many of the carbohydrates.

CONCLUSIONS

1. Three groups of microörganisms capable of digesting agar were isolated from an experimental trickling filter. A survey

of the literature indicated that they were distinctly different from previously described species.

2. These three groups were recognized as new species as follows:

Group I. This microörganism was very energetic in its attack on agar and quickly liquefied an agar medium. Because of the marked excavation of the agar along a streaked culture and because no pigment was produced, the name *Achromobacter pastinator,* meaning the one who digs a trench, was given this bacterium.

Group II. The name *Pseudomonas lacunogenes* was given to this microörganism because of the dimpled appearance of the colonies on an agar plate and because of the motility by means of a single polar flagellum.

Group III. Because of its backward or non-energetic action toward the various compounds and because of its motility by means of a single polar flagellum, the name *Pseudomonas segne,* meaning non-energetic or backward in doing anything, was given to this microörganism.

3. Viscosity determinations showed that marked change was effected in the jell structure of agar by growth of these organisms upon an agar medium.

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