



Studies on aerobic cellulose-decomposing bacteria  
by Jane Nishio

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree  
of Master of Science in Bacteriology at Montana State College  
Montana State University  
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**Abstract:**

Earlier studies on the decomposition of cellulose, especially by aerobic microorganisms, are discussed. Several methods for the isolation of aerobic cellulolytic true bacteria were attempted and three cultures were finally obtained by following the technique of McBeth and Scales. A fourth culture was found as a fortuitous plate contaminant.

The morphology and physiology of the four pure cultures were studied. Their characteristics were not found to agree with those of any previously described organisms. No attempt was made to describe adequately or to name the new isolates. The effects of variations in nitrogen source, pH, temperature, type of cellulose, and associated microflora upon the rate of cellulose digestion were studied and are discussed.

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CELLULOSE-DECOMPOSING BACTERIA

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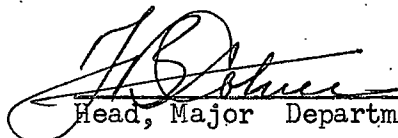
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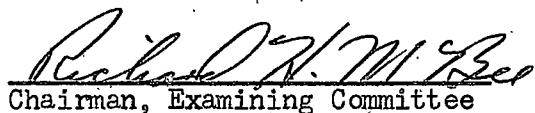
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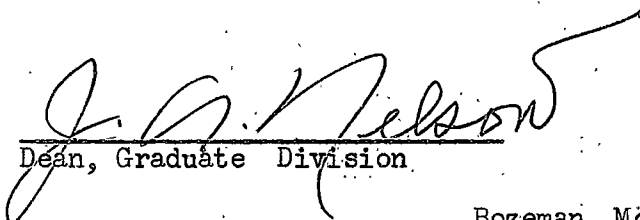
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ABSTRACT

Earlier studies on the decomposition of cellulose, especially by aerobic microorganisms, are discussed. Several methods for the isolation of aerobic cellulolytic true bacteria were attempted and three cultures were finally obtained by following the technique of McBeth and Scales. A fourth culture was found as a fortuitous plate contaminant. The morphology and physiology of the four pure cultures were studied. Their characteristics were not found to agree with those of any previously described organisms. No attempt was made to describe adequately or to name the new isolates. The effects of variations in nitrogen source, pH, temperature, type of cellulose, and associated microflora upon the rate of cellulose digestion were studied and are discussed.

## STUDIES ON AEROBIC CELLULOSE-DECOMPOSING BACTERIA

### INTRODUCTION

Although much has been written concerning cellulose decomposition by microbial action, a survey of recent publications reveals that a controversial state still exists, not only as to how cellulose is decomposed but also as to what organisms can attack it and the conditions which favor their cellulolytic activity. There is a need for more thorough investigations of pure cultures of cellulolytic bacteria under carefully controlled conditions before the mechanism of decomposition can be successfully determined. At present the study of cellulose breakdown is becoming more popular, not only from the standpoint of possible industrial utilization but also from the points of view of the preservation of cellulosic materials such as fabrics, the utilization of cellulose by ruminant animals, and the purely academic investigation with its desire for a better understanding of naturally occurring activities. As a chief component of plant residues, cellulose is one of the most abundant of organic materials. It is a highly insoluble substance and must be put into a soluble form for its utilization by living organisms. This action has been found to be accomplished by aerobic and anaerobic bacteria, by fungi, by actinomycetes, and by some animal species. The work to date concerning aerobic cellulose decomposition has been carried out primarily with fungi and organisms of the order Myxobacteriales, which are not included in the true bacteria. Investigations of these organisms have progressed in a fairly orderly fashion with no great differences of opinion existing among the results of various investigators. In contrast with this, the results of studies on

the cellulolytic true bacteria (organisms belonging to the order Eubacteriales) are conflicting. The available conclusions, when examined as a whole, lead only to confusion.

It was the purpose of this investigation to isolate pure cultures of aerobic cellulose-digesting representatives of the true bacteria and to study, in the laboratory, conditions under which they would show the most rapid decomposition of cellulose. The cultures and information thus obtained could then be used in further studies aimed at understanding the cellulolytic process.

#### REVIEW OF LITERATURE

Because of the inaccessibility of certain very early papers, some of the historical background was obtained from McBeth and Scales (1913), who have presented a good comprehensive review of the early literature on cellulose digestion.

According to them, Mitscherlich in 1850 was the first to arrive at the idea that cellulose decomposition, noted in the destruction of potato, could be carried on by microorganisms. Examination of the material showed a mass of vibrios which he considered to be the active agents of the destruction.

In 1854 Haubner, in the study of ruminant animals, demonstrated that it was impossible to recover from the faeces more than 50 per cent of the cellulosic material fed to sheep. This observation was confirmed by Henneberg and Stohmann, and similar results were obtained by other workers with horses, goats, and rabbits. Their work probably helped to arouse later investigations which attempted to discover the agents of cellulose

decomposition.

Popoff in 1875 considered the methane formed in sewers and marshes as being derived from cellulose fermentation, and made studies on the fermentation of filter paper by action of sewer slime.

In 1890 Van Senus published his observations on the destruction of cotton, pieces of bean, and other materials by an associative action of two organisms, neither of which would do the job alone.

Omelianski in 1894 first used Winogradski's "method of elective culture" in attempting to isolate the agents of cellulose breakdown. His elective culture consisted of an inorganic mineral nutrient solution containing filter paper as a carbon source and river slime as an inoculum. This he incubated anaerobically and observed the changes in flora that accompanied the disappearance of the cellulose and the formation of hydrogen and methane. Although he did not obtain pure cultures, his numerous publications (Omelianski, 1902) established him as an authority on cellulose fermentations and his methods were used by many subsequent investigators.

The first to discover that the destruction of cellulose in soils can be accomplished aerobically as well as anaerobically, and by fungi as well as by bacteria, was van Iterson (1904) who stated the conditions favorable for the development of the various flora as follows:

"Auch bei der aëroben Zersetzung der Cellulose kann man 2 Fälle unterscheiden:

- a) Ist das Medium, worin die Cellulose sich befindet, schwach alkalisch, so spielen bei der Zersetzung gewöhnliche aërobe Bakterien die Hauptrolle.
- b) Ist das Medium jedoch schwach sauer, so sind dabei Pilze und Mycelien von höheren Fungi wirksam."

Others, Waksman and Skinner (1926) and Dubos (1928b), have agreed with the opinion of van Iterson, while McBeth and Scales (1913) and Scales (1915) have shown that the fungi are not confined to acid soils, but are also of importance in alkaline soils. It appeared from his descriptions that van Iterson's aerobic bacteria may have been of the order Myxobacterales, although they were not in pure culture. He obtained them from inorganic nutrient solutions containing filter paper which had been inoculated with sewer slime and incubated aerobically.

Christensen (1910) published his methods of setting up enrichment cultures for cellulose-decomposing abilities of different soils as an index of soil fertility, but did not attempt pure culture studies of the active agents.

Until 1912 work had been greatly hampered by the use of impure cultures, although many of the earlier workers had believed their cultures to be pure. Kellerman and McBeth (1912) demonstrated the need for a solid selective medium for the isolation of pure cultures. No better proof can be given than the fact that with the use of cellulose agar and starch agar, they were able to isolate from the very anaerobic cultures of Omelianski, three cellulose-decomposing organisms and seven contaminating forms. The three cellulose decomposers were noted to be more efficient when incubated aerobically. The results obtained were criticized by Omelianski (1912), who was supposed to have obtained "practically pure" cultures through rapid transfer and dilution in liquid media. The cellulose in the agar used by Kellerman and McBeth and later by McBeth and Scales (1913), was precipitated from Schweitzer's reagent. With the use of this uniformly

opaque medium, colonies of cellulose-digesting bacteria could be detected by the gradual formation of clear zones surrounding them. Until this time it is very likely that no clear-cut colonies of cellulose-decomposing bacteria had ever been seen.

Using agar containing dispersed particulate cellulose, prepared either by precipitation from Schweitzer's reagent or by the hot sulfuric acid-cold water method of Scales (1916), Kellerman and his associates (1912, 1913; McBeth and Scales, 1913; McBeth, 1916) did most of the pioner work on the aerobic cellulose-digesting bacteria. They isolated and described a number of species. However, their studies on the decomposition of cellulose by the aerobic true bacteria have been subject to a great deal of criticism, much of which seems to be due to the fact that their work has not often been duplicated. Thaysen and Bunker (1927) criticized the work and expressed the belief that the cultures were mixed. They cite that Pringsheim and Lichtenstein studied strains of some of Kellerman's organisms and were unable to confirm their findings.

Omelianski and Pringsheim, as quoted by Waksman and Skinner (1926), were very critical of the fact that the cellulose-decomposing abilities of the organisms isolated by Kellerman et al. were based on the formation of dissolved zones in the cellulose agar surrounding the colonies. It was even claimed by Omelianski that the zones were caused by the reaction between the calcium carbonate in the medium and acid formed in the tap water constituent. However, the results of Kellerman and co-workers were confirmed by Löhnis and Lochhead (1913), and Bradley and Rettger (1927) proved that the zones on cellulose agar were due to enzymatic action on the

cellulose, resulting in its solution.

Dubos (1928a) stated that the cellulose agar method was not good in that it did not reveal strictly aerobic species, and that all organisms isolated by Kellerman and McBeth were "more or less anaerobic." This does not seem to be a valid objection because a great majority of the aerobic species of bacteria are facultatively anaerobic.

Others, Hutchinson and Clayton (1919), Bradley and Rettger (1927), followed more recently by Stanier (1942), by Fahraeus (1944, 1947, 1949), and by Reese (1947), have conducted extensive research on aerobic cellulose digestion by organisms of the genus Cytophaga. As a result of their work our knowledge of these organisms is more nearly complete. The cellulolytic true bacteria, on the other hand, have not received as much attention.

Norman and Fuller (1942) have presented an extensive review of the general picture of microbial decomposition of cellulose and the work that had been done up to that time. They emphasized the chaotic condition of the classification of cellulose-decomposing bacteria, and on the basis of their work, reached the following conclusion (Fuller and Norman, 1943):

"The taxonomy of the cellulose-decomposing bacteria has been confused by the policy of creating for these organisms special genera within the families to which they belong on morphological grounds. A physiological property has no place in a genus description unless the characteristic in question is obligate or so outstanding as to outweigh most other considerations. Almost all of the cellulose bacteria are versatile organisms, capable of utilizing other polysaccharides and carbohydrates to various degrees. In most cases the cellulose-decomposing ability does not outweigh the other characteristics. This property, therefore, is best relegated to the key, where it may well be conveniently used for separating species within the genus as morphologically described. The only exception to

this might be the case of specific cellulose organisms. A few species which develop poorly on substrates other than cellulose are known but until their physiology has been more fully studied, it is probably unwise even to set these apart from other organisms closely related morphologically."

What appeared to be the best work done to date was that of Harmsen (1946), who in his thesis gave the results of his studies with all manner of agents of aerobic cellulose decomposition in the soil. He pointed out that the different investigators arrived at various conclusions as a result of using only one method of isolation. Consequently each one was inclined to be skeptical of the findings of others. By combining and using as many different isolation methods and materials as possible, and by varying conditions, Harmsen was able to obtain representatives of nearly all the aerobic cellulolytic organisms mentioned in the literature. In addition he discovered representatives in taxonomic groups that had not been previously recorded as being cellulolytic; for example, the proactinomycetes and mycobacteria. He listed the cellvibrio, the cytophaga, the polyangides, the bacilli, the actinomycetes and the proactinomycetes as the chief groups containing aerobic cellulose-decomposing bacteria. Harmsen's work was more extensive than that of Fuller and Norman and led to substantially the same conclusion; namely, that the ability to decompose cellulose should not be regarded as an unique accomplishment that should set these organisms apart from those which are similar morphologically and biochemically. For example, as a result of Harmsen's work, it can be said that the ability to digest cellulose is more common among bacteria than the ability to ferment lactose with the production of acid and gas. In support of his experimen-

tal data, Harmsen presented excellent photographs of his organisms, both of colony growth in cellulose agar and of cell morphology. He was of the opinion that the difficulties experienced by other users of cellulose agar (perfected by Kellerman and co-workers) were very likely due to improper preparation of the dispersed cellulose.

Perlin and his associates (1947) still regard cellulose decomposition as a mystical property. They conducted their studies on digestion of cellulose with an impure culture of Vibrio perimastrix. This fact illustrates the belief that is still maintained by many bacteriologists that cellulose decomposition is unique and that the organisms involved will not grow well in pure culture.

Although pure cultures of cellulose-decomposing bacteria have been obtained by several persons, they have not been used to determine the mechanism of cellulose digestion. The workers interested in the biochemistry of these bacteria have too frequently used mixed cultures. Therefore, it appeared to be worth-while to re-examine the whole field starting with the isolation of pure cultures, progressing to the study of their characteristics, especially of the factors related to cellulose digestion, and eventually using the organisms and information so obtained for the study of the cellulolytic process.

## MATERIALS AND METHODS

### Cultures

Aerobic cellulose-decomposing bacteria were isolated from samples of soil from the Montana State College area. These samples came from enriched garden soils as well as from uncultivated areas. However, not

until a number of attempts had been made were the aerobic cellulolytic true bacteria isolated in pure culture.

#### Culture media

The basic nutrient solution of Kellerman and McBeth (1912) was found to be satisfactory for good growth and was used throughout this work unless otherwise stated. It was composed of 0.05 per cent dibasic potassium phosphate, 0.05 per cent magnesium sulfate, 0.05 per cent sodium chloride, 0.1 per cent ammonium sulfate, and 0.1 per cent calcium carbonate. Cellulose agar was prepared by the addition of 0.5 per cent cellulose and 1.0-1.5 per cent agar to the inorganic base medium. For the purpose of the initial work it was found advantageous to add 0.05 per cent yeast extract to the cellulose agar. Glucose and cellobiose agar were prepared by adding 0.5 per cent concentrations of the sugar desired, 0.1 per cent yeast extract, and agar to the basal medium.

Except for filter paper in the enrichment cultures, the cellulose used in the experiments was of a finely dispersed type and was usually present in a concentration of 0.5 per cent. This type of cellulose was used because, as Boswell (1941) stated:

"Whatever biological agent is used for cellulose decomposition, it is universally recorded that the greater the degree of dispersion of cellulose the higher is the rate of attack."

With the use of agar containing this type of cellulose, decomposition was very clear-cut and manifested itself by the formation of clear zones in the medium surrounding the bacterial colonies.

The cellulose designated as "ball-milled cellulose" was made according

to the directions given by Hungate (1950). They are as follows:

"Finely dispersed cellulose is obtained by packing absorbent cotton into a liter Erlenmeyer flask containing HCl (270 ml conc. HCl diluted to 300 ml). Sufficient cotton is added to absorb all the acid solution. After standing at room temperature for 24 hours, the fibers break up and a suspension of small particles is obtained. These are collected by filtration at reduced pressure, washed and air-dried. This material (24 gm) is ground in a pebble mill with 600 ml water for 72 hours, giving approximately a 4 per cent concentration of very finely divided cellulose."

This cotton cellulose prepared in the above manner is said to be very highly hydrated, but not so much altered in composition as the other types of dispersed celluloses (Farr and Eckerson, 1934).

Cellulose prepared according to the method of Scales (1916) is designated as "Scales cellulose." It was prepared by dissolving filter paper in dilute sulfuric acid (100 ml conc. sulfuric acid with 60 ml distilled water) at a temperature of 60-65 C, and immediately precipitating it again in small flocs by the rapid addition of ice-cold water. The resulting precipitate was then washed free of acid with distilled water by filtration. It was found that the washing process could not be completed in three hours as Scales stated in his paper. The dissolution in hot sulfuric acid forms sulfo-esters of cellulose which are soluble. However, the addition of cold water hydrolyzes the esters back to cellulose in a different form. This process was also used, substituting "Rayocord F", a highly purified wood cellulose produced by Rayonier Inc., for filter paper. It tended to give a lumpier precipitate.

The third type of dispersed cellulose used and designated as "cuprammonium cellulose", was prepared according to the directions of

Kellerman and McBeth (1912). Filter paper was dissolved in Schweitzer's reagent (ammoniacal solution of copper oxide) and precipitated out in flocs by the addition of dilute hydrochloric acid. This then had to be washed repeatedly with water in order to remove the chloride ions. The washing process was best carried out by allowing the precipitate to settle out in a glass cylinder of several liters capacity. After settling, the supernatant water was siphoned off and fresh water added and mixed with the flocculent mass. In this way after many washings, the supernatant liquid would give a negative chloride test with silver nitrate and the cuprammonium cellulose was ready for use. It was concentrated by settling for several days, after which time the clear supernatant was removed. The percentage of cellulose was determined by drying and weighing 100 ml aliquots of the resulting preparation. The final product had a concentration of 0.5 to 0.6 per cent cellulose.

#### Preliminary studies

In order to become acquainted with the aerobic cellulose-decomposing bacteria, the first investigation was an attempt to study five cultures of bacteria which at one time were known to be cellulolytic. These bacteria were very versatile and would grow on ordinary laboratory media. To check the purity of the cultures and to determine whether or not they were truly cellulolytic, they were plated and streaked on a mineral base cellulose agar. Their failure to grow on this minimal medium prompted the addition of a small concentration of yeast extract to the medium to provide growth factors. On this medium there was growth but no cellulose digestion, indicating that the organisms were growing by utilizing the

yeast extract. Repeated attempts to demonstrate cellulose digestion by these organisms were carried out over a period of two months. All of the results were negative. It was therefore concluded that the original cultures were contaminated and that the contaminants had overgrown the cellulose-decomposing bacteria during the several months that the cultures had been maintained on stock culture agar. It is very likely that at the time these cultures were obtained there were no cellulose-digesting bacteria present. Therefore, these cultures were discarded and the attempts to isolate fresh strains of aerobic cellulose-decomposing bacteria were initiated.

#### Isolation techniques

An attempt was made to obtain enriched cultures of cellulolytic bacteria by partially burying pieces of filter paper in small dishes of soil collected from various sites. The soil sample and the partially buried filter paper were kept moist by the daily addition of water or a dilute solution of potassium nitrate. Other enrichments were tried by mixing ball-milled cellulose with soil which was kept moist. These, however, were discarded because the addition of that type of cellulose altered the soil structure in such a way that it tended to dry to a hard impenetrable mass. The filter paper-soil mixtures were left standing in the laboratory and were examined daily for signs of disintegration of the paper. According to Waksman and Skinner (1926), the addition of cellulose to a soil made relatively little difference in the numbers of bacteria present in the sample, but brought about an appreciable increase in the numbers of fungi. Apparently that was the case with the above-mentioned filter paper-

soil mixtures, because the paper observed to be disintegrating was covered with mold growth. It may have been that the soil samples used were acid in reaction, in which case the filamentous fungi would have been very active (Waksman and Skinner, 1926; Dubos, 1928b). The pH of the soil was not determined. All attempts to isolate cellulolytic bacteria from these enrichments were unsuccessful. They were abandoned because of the difficulties involved in separating aerobic bacteria from enrichments overgrown with filamentous fungi.

At the same time that the filter paper was buried in soil, similar soil samples were placed into liquid enrichment media containing cellulose in the form of filter paper strips. These enrichment media were proposed by Bradley and Rettger (1927) and Dubos (1928a) and were essentially the same. They consisted of inorganic mineral nutrient solutions in which the strips of filter paper were partially immersed. According to users of this technique, the aerobic bacteria were supposed to grow on the filter paper at the liquid-air juncture, and to cut the paper cleanly across as they decomposed it. In some cases the filamentous fungi were dominant and were seen growing on the moist filter paper just above the liquid. These cultures were discarded because of the difficulties previously encountered. From those flasks which were apparently free of mold, bits of disintegrating paper were transferred to fresh flasks of similar sterile media. From these cultures dilutions of the decomposing paper were made in an attempt to isolate the cellulolytic bacteria in pure culture. This was done by plating in cellulose agar and also by adding some of the diluted material directly to the surface of nutrient agar plates which had been first

covered with rounds of sterile filter paper. After four days incubation at 35 C, the cellulose agar showed signs of digestion. Small areas of the plates showed clearing of the opaque medium without the formation of a well-defined colony. Accompanying the dissolution of the cellulose were small indentations in the agar, indicating that the agar, too, was being digested. Picking from these areas an attempt was made to purify the organisms causing the breakdown by again plating on cellulose agar. Again similar zones of clearing appeared and these were picked under the dissecting microscope (27x) to slants of cellulose agar. About three weeks later, a check for purity was made from those slants showing excellent digestion. Microscopic examination showed mixtures of cocci, spore-forming rods and weakly-staining filaments. The mixed cultures were purified with the use of cellulose, glucose, and starch agars. None of the spore-formers were cellulolytic. Digestion started on the cellulose agar plates with small areas which increased in size with age. At the same time, depressions in the agar accompanied the cellulose digestion, and also grew in size with age. However, in those areas, no definite colony was ever detected. Gram stains made from smears of the areas showed gram negative weakly-staining filaments and large yeast-like cocci. These were considered to be members of the Myxobacteriales, with which the investigation was not concerned and the cultures were abandoned. The agar plates which had been covered with filter paper became overgrown with molds and were discarded.

In order to prevent drying out of the medium during prolonged incubation periods, it was necessary to use moist chambers as containers for the petri plates. For this purpose wet absorbent cotton was placed in a

coffee can or similar container which had a lid. When a larger container was used for incubation purposes, small beakers of water were placed within it to create a humid atmosphere. Moisture was a factor which seemed important in the decomposition, for when plates were incubated without added moisture, the digestion of cellulose was prolonged or did not occur at all. At intervals the moist chambers had to be sterilized to kill the mold spores which became abundant.

The enrichment culture method, used and described by McBeth and Scales (1913) for the isolation of their cellulose-digesting organisms, was tried next. The nutrient enrichment medium consisted of 0.1 per cent di-basic potassium phosphate, 0.1 per cent magnesium sulfate, 0.1 per cent sodium carbonate, 0.2 per cent ammonium sulfate, 0.2 per cent calcium carbonate, and tap water. 100 ml portions of this inorganic nutrient solution were placed in Erlenmeyer flasks containing rounds of filter paper which had been folded to quarters. The paper was entirely immersed in the liquid. One gram soil samples were used as inocula, and the flasks were incubated at 35 C. In a few days there was a noticeable fraying of the upper quarters of the folded filter papers in the flasks. In some cases this was preceded by a clouding of the solution and accompanied by a yellow or brown discoloration of the paper. One flask, however, showed none of this preliminary turbidity and no yellowing. As soon as the frayed appearance became apparent, bits of the disintegrating filter paper were transferred to a fresh flask of the same medium. The paper readily fell apart when it was shaken in a tube of sterile water in contrast to the uninoculated control paper which retained its structure. Three or four successive trans-

fers were made as soon as decomposition of the filter paper became evident. This tended to increase the numbers of bacteria which would attack the cellulose without allowing the development of the bacteria which might grow on the decomposition products of the cellulose. From the latest transfers, attempts were then made to isolate the agents causing the destruction of the filter paper by streaking cellulose agar plates. Here there was a digression from the original method advocated by McBeth and Scales, who recommended pour-plates instead of streaked plates. The cellulose agar streaked-plates were incubated at 35 C and in four days, showed abundant growth with vague patches of digestion of the cellulose. Some of the growth over the area of digestion was suspended in sterile water and re-streaked. Further streaking from the digested areas, no matter how dilute the inoculum, gave rise to a uniformly spreading growth which showed digestion but no isolated colonies. This type of growth could not be considered to be pure. Since the type of spreading growth indicated that the bacteria were motile, it was obvious that the only alternative was to prepare dilutions from the bacterial growth and also from frayed paper in the enrichment cultures, and to make pour-plates as McBeth and Scales directed. These were made with agar containing ball-milled cellulose and also with that containing Scales cellulose. Because quantitative studies were not involved here, dilutions were made by suspending small pieces of disintegrating paper or bacterial growth in a tube of sterile water, mixing well and pouring a small amount of the mixture to a second tube of sterile water, and so on to about eight dilutions. About one ml of each dilution was placed in a petri dish and mixed with cellulose agar. After three or four

days incubation at 35 C, isolated colonies surrounded by small clear zones were observed on plates of the higher dilutions. Such isolated colonies were picked by means of micro-pipettes under a dissecting microscope. In this way one colony could be picked carefully with less chance of contamination. The colonies picked in this manner were replated in cellulose agar by the dilution method just described. It was soon found to be more advantageous to make pour-plates from test tubes containing 10-12 ml of sterile cellulose agar, instead of first making the dilutions in sterile water. Part of a cellulose-digesting colony was suspended in the first tube and the tube closed with a sterile rubber stopper. The tube was then inverted several times to insure thorough mixing. One large loopful was transferred from the first tube to a second which was similarly stoppered and inverted. Two loopfuls of the second dilution were transferred to a third and final tube. At first five dilutions were made in this manner, but it was soon found that three were sufficient. The media in the tubes were then poured into petri plates and allowed to harden. Usually the first plate was too thickly seeded but the second had well-isolated colonies, while the third had only a few colonies or none at all. This was especially useful in conserving time and media.

After several transfers, all colonies on the cellulose agar plates of any one culture appeared to be the same. A critical test for culture purity, however, was then applied in which a single colony was picked from a cellulose agar plate to glucose or cellobiose agar pour-plates and from the subsequent growth, a single well-isolated colony was picked for further study. This passage through cellobiose or glucose agar would allow any

contaminants to grow and indicate any impurity of the cultures. The cellulose-digesting bacteria were also able to grow faster on these media, lessening the danger of contamination of the plates from the air. The colonies picked from the sugar media were then inoculated into cellulose agar to make sure that cellulolytic bacteria instead of contaminating colonies had been picked. This rigorous proof of culture purity appeared to be necessary because of the confusing morphological characteristics of the bacteria and also because of the fact that many bacteriologists are extremely skeptical of the purity of all of the cultures of the cellulose-digesting bacteria.

#### Cultural studies

The pure cultures of cellulolytic bacteria were examined for morphology by studying gram-stained preparations from 20-hour cultures in glucose broth and also from colonies on cellulose agar. Dimensions were determined by means of an ocular micrometer. Motility was ascertained in semi-solid agar and in hanging-drop preparations. Flagella stains using Gray's method were also prepared. Observations of colonial appearance were made from cellulose agar. The type of growth in nutrient broth and also on nutrient agar slants was observed.

Physiological characteristics were determined in duplicate sets of tests. The organisms were tested for reduction of nitrates in nitrate broth, for production of indole in tryptone broth, for liquefaction of nutrient gelatin, for changes produced in milk, and for the fermentation of glucose, lactose, sucrose, starch and cellulose.

Several factors influencing the rate of cellulose digestion were also

examined. These were various organic nitrogen sources, pH, incubation temperature and type of cellulose.

#### EXPERIMENTAL RESULTS

Successful isolations of pure cultures of three strains of aerobic cellulose-digesting bacteria from the soil were made by following the technique of McBeth and Scales (1913). Briefly this consisted of enriching the numbers of cellulose-decomposing bacteria in a liquid medium containing immersed filter paper, followed by isolation of the pure cultures with the use of solid cellulose agar. Definite purity was established when a single colony from glucose agar could be plated on cellulose agar and give rise to cellulose-digesting colonies which were all alike. A fourth culture was encountered as a fortuitous plate contaminant. Cultures 2-R, 2-F, and 11-A were isolated from the soil. Culture 11-C was the plate contaminant.

#### Cellular and colonial morphology

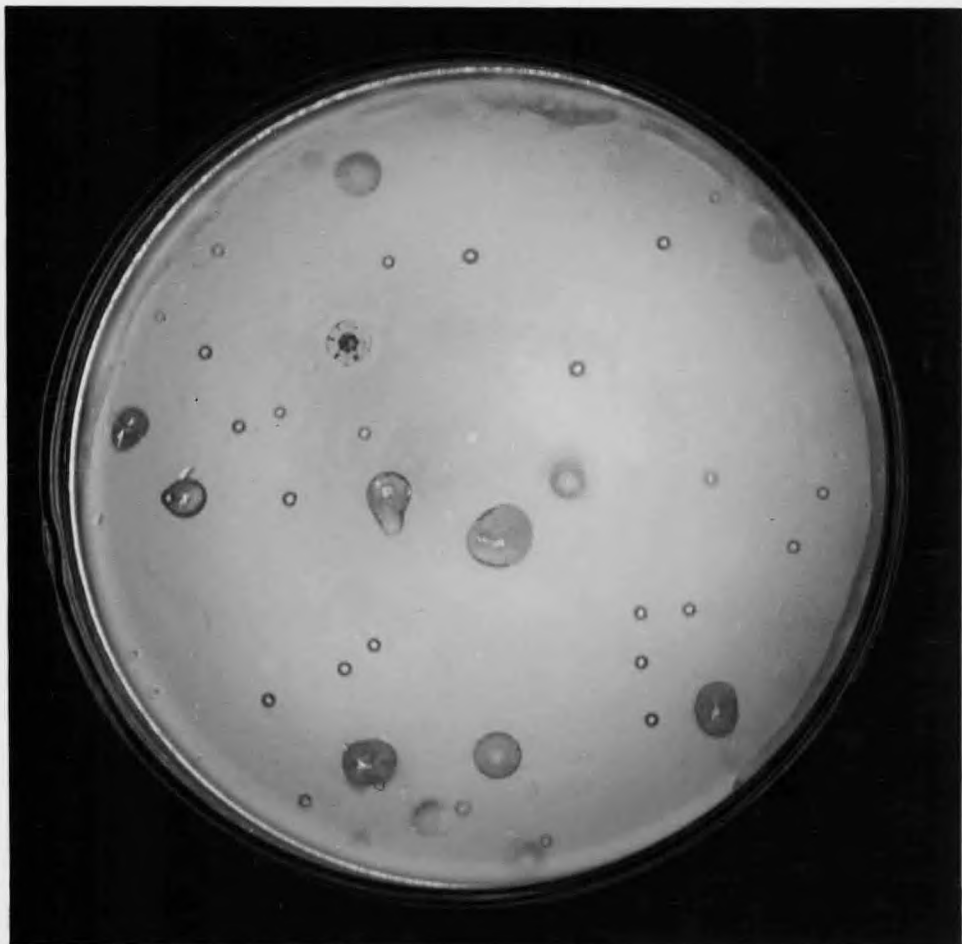
The four strains of cellulolytic bacteria isolated were of two morphological types. Culture 11-A, when stained from a 20-hour glucose broth culture, showed small gram positive rods, some with one end pointed, measuring about 0.4 to 2.0 microns. The cells appeared in typical angular diphtheroid arrangements. Stains made from colonies on cellulose agar showed small gram variable rods of uniform size and with rounded ends. Some of the cells had a granular appearance. No spores have been seen in the course of many examinations. A flagella stain by Gray's method showed the presence of 1 to 3 lateral flagella. The morphological appearance of these organisms was variable with age and variations in media and cultural methods.

Cultures 2-R, 2-F, and 11-C were very similar to each other morphologically. On gram stains made from 20-hour glucose broth cultures, they were gram negative to gram variable straight or curved long rods, with rounded ends and an average size of 0.5-0.7 x 5.6 microns. Flagella stains were not successful, but growth characteristics in semi-solid agar and of surface colonies indicated that the strains were motile. Polymorphism was very noticeable, especially when the bacteria were transferred from cellulose to a new type of medium. This exhibited itself as lengthening of the rods, central swellings, formation of gram positive granules and faintly staining "ghost cells." Spore formation has not been observed but it was interesting to note that culture 2-R, when grown on glucose agar slants, sometimes exhibited subterminal round to oval swellings which looked very much like Tetrault's (1930) drawings of his pigmented thermophilic bacteria. He theorized that the strange morphology might be a stage in the life cycle of the organism.

Colonies of strain 11-A in cellulose agar plates, had a characteristic popcorn ball appearance when viewed under the dissecting microscope (figure 1). Clear zones appeared surrounding them after three or four days and became gradually larger as the culture grew older. The clearing, therefore, appeared to be due to the action of a cellulase which was diffusing outward from the colony. It was also noted that these bacteria appeared to be agar digesters as shown by slight sunken areas above deep colonies after prolonged incubation. In cellulose and nutrient agars, the growth had a slight yellow coloration. Surface colonies were raised, butyrous, and slightly spreading. Growth in broth was uniform.

Figure 1. Colonies of strain 11-A in agar containing ball-milled cellulose, incubated 14 days at 35 C. The large colonies are on the surface of the medium; the small ones are embedded within the agar.

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Colonies of culture 2-R (figure 2) in cellulose agar characteristically had a flower-like appearance with parts of the colony projecting like petals. Chromogenesis was never observed. Clear areas of cellulose digestion first appeared in about two days increasing in size as the colonies became older.

Strain 2-F produced colonies which differed from 2-R in that they were initially disc-shaped (figure 3). In a short time, however, they acquired the appearance of three disc-shapes coming together at a point, similar to the petals of the trillium. These sometimes kept the trillium-shape or gradually added on more discs. Insertion of the fine point of an inoculating needle showed that these discs of growth were hollow. Digestion of the cellulose was evident sometimes as early as 36 hours. Areas of digestion as large as nine millimeters in diameter were sometimes produced in 10 days.

Culture number 11-C was found as an air contaminant on a plate of cellulose agar. Colonies of the strain in cellulose agar were non-chromogenic and were characteristically round with serrated edges. Cellulose digestion usually appeared in four or five days but was not as complete as with the other cultures.

On glucose and cellobiose agar slants, strains 2-R, 2-F, and 11-C had the same appearance. Growth was translucent and had a clear syrupy appearance. It was not luxuriant. In broth media, the growth of these three strains was initially uniform, but in 48 hours it had become a granular sediment.

Figure 2. Culture 2-R in ball-milled cellulose agar, incubated for 14 days at 35 C. The large spreading colonies are growing on the surface of the medium; the small colonies are subsurface.

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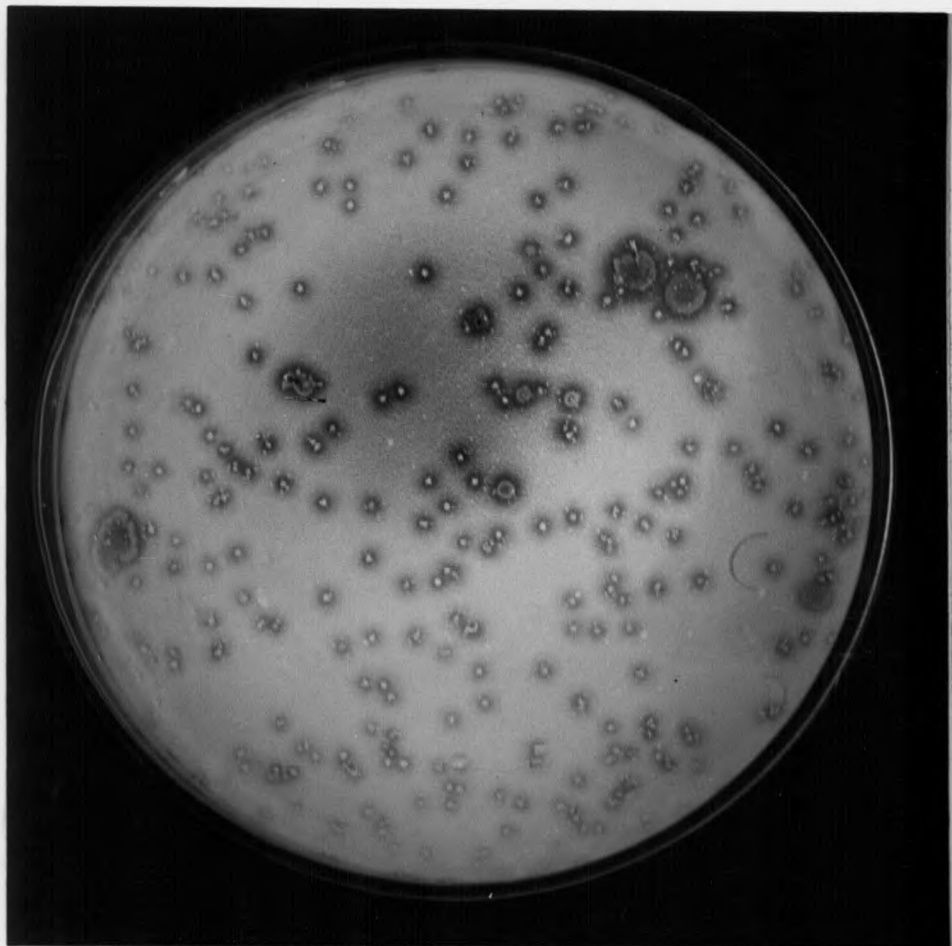
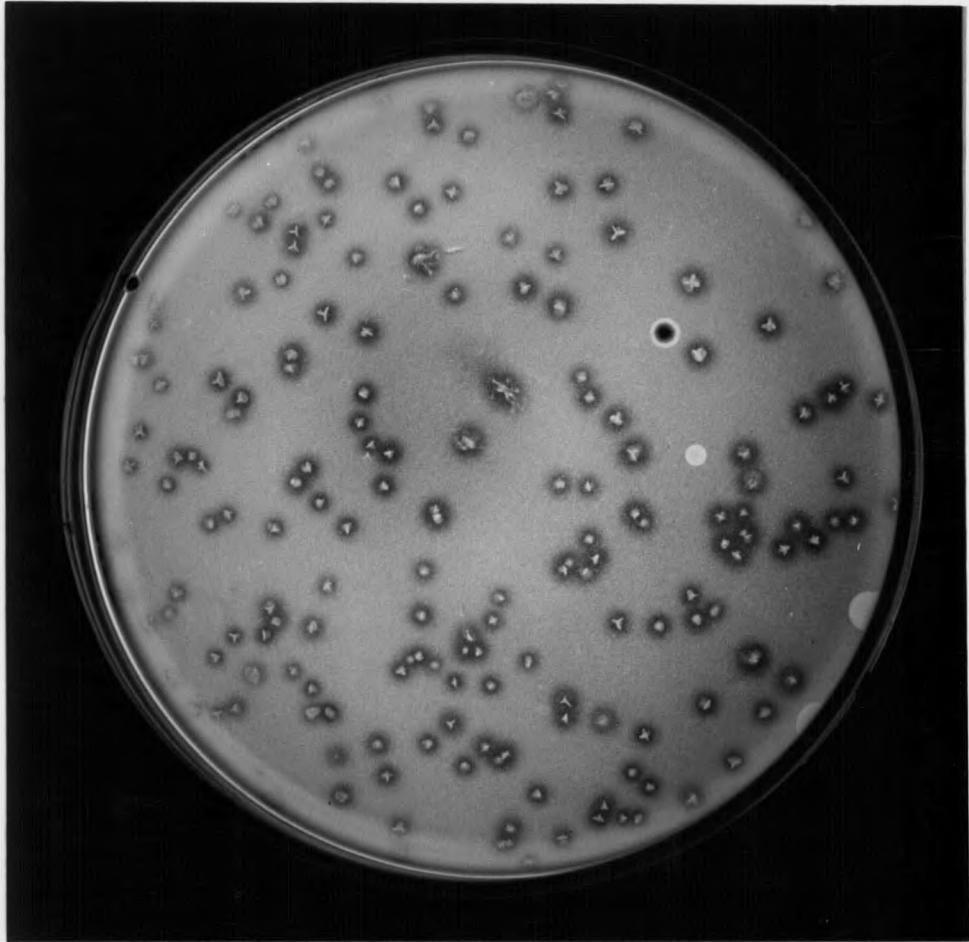


Figure 3. Colonies of culture 2-F growing in cuprammonium cellulose agar, incubated 7 days at 35 C. The characteristic trillium shape is present.

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### Physiology

The physiological characteristics determined were the same for all of the four cultures. Nitrates were reduced to nitrites. Glucose, lactose, sucrose, and starch were fermented with the production of acid but no gas. Tests for the production of indole and for gelatin liquefaction were negative where growth occurred. No change was noted in litmus milk. Differences could be noted only in morphological and colonial characteristics (table I).

Table I

#### Physiological characteristics of cultures

Physiological reaction	Culture number			
	11-A	2-R	2-F	11-C
Nitrate reduction	+	+	+	+
Indole production	-	-	-	-
Gelatin liquefaction	-	-	-	-
Fermentation of:				
Glucose	+	+	+	+
Lactose	+	+	+	+
Sucrose	+	+	+	+
Starch	+	+	+	+
Changes in litmus milk	none	none	none	none

#### Classification

Bergey's Manual of Determinative Bacteriology (Breed et al., 1948) was searched in order to find some clue as to the classification of the

organisms isolated. Strain 11-A is possibly a member of the genus Corynebacterium, but the only bases for this designation are the gram reaction and the diphtheroid arrangement of the cells. The only cellulolytic Corynebacterium listed in Bergey's Manual is C. fimi, which differs from strain 11-A by not being motile and by the production of indole. Comparison with the genus Cellfalcicula was also attempted, but differences were so great that this genus was excluded. It is very possible that this organism has not been previously described.

The description of the genus Cellvibrio suggests that the strains 2-R, 2-F, and 11-C may be members of that genus. The description is as follows:

"Long slender rods, slightly curved, with rounded ends, show deeply staining granules which appear to be concerned in reproduction. Monotrichous. Most species produce a yellow or brown pigment with cellulose. Oxidize cellulose, forming oxycellulose. Growth on ordinary culture media is feeble. Found in soil."

Although the cultures were motile, the type of flagellation was not ascertained. Pigment production was not noticeable after the cultures were purified. The oxidation of cellulose to oxycellulose could not be used as a classification criterion because the description of this compound is extremely vague (Siu, 1951). Again a diligent search of Bergey's Manual has not revealed a possible classification of the organisms isolated and they, too, must be considered as possibly being heretofore undescribed.

#### Effect of various factors on cellulose digestion.

In order to determine the conditions best suited for cellulose decomposition by the four strains of aerobic cellulolytic bacteria isolated, a series of tests were performed to find the effects of various modifications of the medium used for isolation.

Nitrogen source. In addition to the ammonium nitrogen of the basal cellulose medium, single organic nitrogen sources in the form of asparagine, beef extract, human blood serum, peptone, sheep rumen fluid, or yeast extract were added in the concentrations shown in table II. In these media, both cuprammonium cellulose and Scales cellulose were used. Duplicate sets of plates were made of each strain in each nitrogen source and in each cellulose. One set was incubated at room temperature (about 28 C) and the other at 35 C. Plates were observed daily and the reading of the tenth day appears in table II. In this table and in all subsequent tables, "-" indicates either no growth or poor growth with no visible decomposition of the cellulose. "/" indicates slight digestion, "////" signifies good digestion, and the other values are relative to these. In cases in which digestion could not be observed without the use of magnification, the results were recorded as negative. As a result of this experiment, most of the subsequent cultures were grown with the added nitrogen source which gave maximal cellulose decomposition; namely, beef extract, peptone or yeast extract. Increased amounts of beef extract and yeast extract did not increase the rate of cellulose digestion. Culture 11-C was not used in this experiment because it had not been obtained in pure culture at that time.

pH. The cultures were plated in ball-milled cellulose media from which calcium carbonate had been omitted. Portions of the media were adjusted to pH values of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 with sodium hydroxide or phosphoric acid using a Beckman pH meter. It was found necessary to adjust the media after autoclaving because the sterilization

Table II

Effect of various organic nitrogen sources on cellulose digestion

Culture	Temp. (C)	Nitrogen source and type of cellulose					
		1.0% aspara- gine	0.2% beef extract	1.0% blood serum	1.0% pep- tone	1.0% rumen fluid	0.2% yeast extract
Cuprammonium cellulose							
11-A	28	-	+	-	###	#	###
	35	-	+	+	###	#	###
2-R	28	?	###	#	#	###	###
	35	-	###	#	#	###	###
2-F	28	###	###	###	###	###	###
	35	###	###	###	###	###	###
Scales cellulose							
11-A	28	-*	+	-	###**	##	###
	35	-*	+	##	###**	##	###
2-R	28	-*	##	-	-**	#	-
	35	-*	##	##	-**	#	+
2-F	28	-*	+	-	/**	+	+
	35	-*	###	+	###**	##	###

\* 0.5% asparagine used.

\*\* 0.5% peptone used.

process changed the pH. The plates were incubated at 35 C and observed every other day for 10 days. The tenth day reading appears in table III. Good cellulose decomposition was observed at pH values of 7.0 or above in all cases. This is in the pH range maintained by the use of calcium carbonate; therefore, all later experiments were carried out on media containing 0.1 per cent calcium carbonate.

Table III

Effect of pH on cellulose decomposition

Culture	Nitrogen source	pH					
		5.5	6.0	6.5	7.0	7.5	8.0
11-A	0.5 % peptone	-	-	++	++	++	++
2-R	0.2 % beef extract	-	-	++	++	++	++
2-F	0.2 % beef extract	-	-	-	+++	+++	+++
11-C	0.2 % yeast extract	-	-	-	+++	+++	+++

Temperature. In order to determine the optimum temperature at which the cellulolytic activities could take place, the aerobic cellulose-digesting bacteria were plated in quadruplicate in cuprammonium cellulose agar and replicates were incubated at temperatures of about 26 C (room temperature), 35 C, 40 C, and 55 C. These were observed daily for nine days. Maximum differences were shown on the fifth day and the results are recorded in table IV. Except for culture 11-A, which showed maximum digestion at 35 C, there was little difference between the results obtained

at 35 C and 40 C. None of the cultures grew at 55 C.

Table IV

Effect of temperature on cellulose digestion

Culture	Nitrogen source	Temperature (C)			
		26	35	40	55
11-A	0.5 % peptone	+	++	-	-
2-R	0.1 % yeast extract	+	++	+++	-
2-F	0.2 % beef extract	+	+++	++	-
11-C	0.1 % yeast extract	+	++	+++	-

Method of cellulose preparation. Since various investigators have recommended different methods of preparing cellulose for the demonstration of cellulolytic activity and since cellulose prepared by three different methods had been used in these studies, it was thought to be advisable to determine which cellulose was most readily attacked by the organisms isolated. Media containing cellulose in concentrations sufficient to give a good opacity were prepared and used for plating the different cultures. The plates were incubated at 35 C and observed for five days at the end of which time the results given in table V were recorded. It appears that either cuprammonium cellulose or ball-milled cellulose gave about equivalent results. Scales cellulose (precipitated from sulfuric acid), however, was poorly digested by most of the organisms in this experiment. This may have been due to the preparation of this particular batch of cellulose since great variations in the appearance of the several lots were noted.

Table V

Effect of method of preparation of cellulose upon its digestibility

Culture	Cellulose		
	0.5 % cuprammonium cellulose	0.3 % ball-milled cellulose	0.5 % Scales cellulose
11-A	+	+	+
2-R	+	+	-
2-F	+++	++	-
11-C	++	+++	+

Associated microflora. During the process of purification of the 11-A culture, an interesting phenomenon was observed. The growth and digestion of cellulose were markedly greater beneath and surrounding a large round cream-colored plate contaminant. Believing that this new colony must be producing some growth factor or other substance which enhanced the growth and cellulose-decomposing abilities of strain 11-A, the contaminant was isolated in pure culture and the following preliminary tests were made to determine the nature of the enhancing factor. Cellulose agar plates uniformly seeded with culture 11-A had one drop of each of the following preparations of the contaminant placed on separate quadrants of the plate.

- (1) A broth culture
- (2) A filtrate from the broth culture
- (3) (1) boiled for five minutes
- (4) (2) boiled for five minutes

Visible growth of the contaminating organisms on (1) was present in 24 hours, but no cellulose-digesting bacteria were observed until the third day of incubation at 35 C. At that time, with the aid of a dissecting microscope (27x), colonies under the section marked (1) were noted to be somewhat larger than those on the rest of the plate and there was slight digestion visible. By the fourth day there was definite cellulose digestion under area (1) and slight digestion under (3). The areas (2) and (4) showed no differences from the untreated areas of the plates, and at no time up to a month did any differences appear. However, the area (1) colonies grew to a greater size and had larger areas of digestion than any other part of the plate. Area (3) seemed not to influence the size of the colonies as much as it did the digested areas.

From the results of the above experiment, it may be assumed that whatever was affecting the growth and cellulose-digesting abilities of culture 11-A must have been closely associated with the cells of the contaminant and was not necessarily present in the medium in any large concentrations. Also, the product must have been somewhat thermostabile since it was not entirely inactivated by the action of heat.

Since the contaminating bacteria grew so readily on the cellulose medium, it was thought to be at least weakly cellulolytic in itself. Prolonged incubation of pure cultures of the contaminant revealed that it was weakly cellulolytic. This may have been one of the symbiotic relationships so often mentioned in the literature.

Sanborn (1926a, 1926b) studied associations of Cellulomonas folia with Actinomyces colorata, Azotobacter, Bacillus mycoides, Bacillus

subtilis, and Bacillus cereus, and found there was a stimulation in the growth and physiological efficiency of the cellulose-destroying Cellulomonas folia. Harmsen (1946) did work on the stimulation of cellulolytic activity by the addition of extracts of vegetable and animal matter, soil, and metabolic products of other microorganisms to the culture medium. He, too, observed that accidental infection of the plates by Bacillus species often furthered the growth and decomposing abilities of the cellulose bacteria growing around them.

Perlin, Michaelis, and McFarlane (1947) grew their cultures of Vibrio perimastrix in association with another vibrio because the cellulose decomposition was more active than when V. perimastrix alone was used.

#### DISCUSSION

A variety of culture methods were used in attempting the isolation of aerobic cellulolytic true bacteria from soil.

The first method tried, that of putting filter paper in the moist soil samples, was not satisfactory because the fungi were the first to visibly digest the paper. These, of course, would be difficult to separate from the aerobic bacteria because of their spore formation and their rapidly spreading growth.

The technique of using filter paper strips partly immersed in the nutrient solution was not found to be successful because this method, too, tended to bring forth flora that was not sought in the investigation. Sometimes the moist paper above the liquid surface was suitable for mold growth, but more frequently the method resulted in the enrichment of members of the Myxobacteriales. These grew at the liquid-air junction on

the filter paper.

The method of McBeth and Scales appeared to be the most satisfactory if one followed their directions exactly. Using this method, three cultures of aerobic cellulolytic true bacteria were successfully isolated without difficulty, and it is to be supposed that other cellulolytic bacteria could also have been readily isolated from other soil samples. This confirms the results of Kellerman et al. and Harmsen and makes it appear that much of the criticism aimed at Kellerman and his co-workers was unfounded.

It appears from the above that the method of enrichment and/or isolation is an important factor in the type of organism which is finally isolated. Since there are so many groups which contain cellulose-decomposing representatives, this is not surprising. In view of this fact, one must use an isolation method found most suitable for isolation of the particular group sought.

Although Harmsen has expressed a similar view and has cultivated cellulolytic representatives of many groups of microorganisms, a complete survey of the cellulose-decomposing microorganisms and their classification has not been attempted. The results of this study indicate that the classification of cellulose-digesting organisms is in a sad state.

The four cultures obtained in this study were not found to agree with any organism described in Bergey's Manual, and therefore, may be new previously undescribed species. It is thought unwise to attempt to establish the organisms as new species until classification of cellulose-decomposing bacteria has been revised.

The conditions that were found to favor cellulose digestion were about those that would be expected from the results of earlier workers. On reflection it was realized that these were the conditions that were used for their enrichment and isolation. This information is valuable, however, in planning future experiments with these organisms that may lead to a better understanding of the modes of their attack on cellulose.

#### SUMMARY

Three strains of aerobic cellulolytic bacteria of the order Eubacteriales were successfully isolated from soil samples of the Montana State College area by making use of the technique of McBeth and Scales. A fourth strain came from accidental contamination of a plate of cellulose agar. Physiological studies and cultural characteristics of the four pure cultures failed to give any hint as to their positive identity and they may be considered as possibly yet undescribed species. The flora isolated is dependent upon the method or methods and conditions used for isolation.

Studies made on optimal conditions for growth and cellulose-decomposing abilities of these organisms yielded results comparable to those of other workers in the field. It was found that a pH in the range of 7.0-8.0 was most favorable for cellulose digestion. The addition of small amounts of beef extract, peptone, or yeast extract to the medium increased the rate of growth and cellulolytic activity. In general it was found that a temperature of 35-40 C. was the most favorable for growth. Ball-milled and cuprammonium cellulose were found to be more

uniform than that prepared by Scales method and consequently gave more consistent results when used for measuring the rate of cellulose digestion.

Some cases of increased cellulose-decomposing activity by the presence of associated microflora were observed.

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