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:
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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. Mo 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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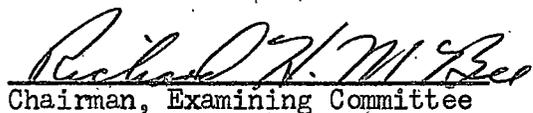
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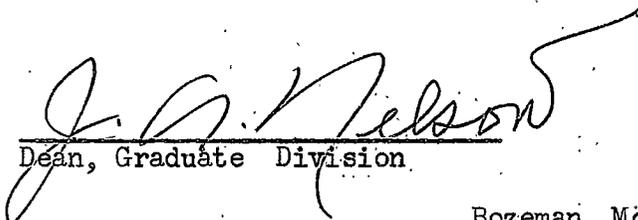Master of Science in Bacteriology

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TABLE OF CONTENTS

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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 Eubac-
teriales) 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 Myxobacteriales, 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 con-
> siderations. Almost all of the cellulose bacteria are versatile
> organisms, capable of utilizing other polysaccharides and carbo-
> hydrates 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 subtrates 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 con-

ditions, 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 previ-

ously 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 phos-

phate, 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 decom-
> position, 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 dis-

secting microscope (27x) to slants of cellulose agar. About three weeks

later, a check for purity was made from those slants showing excellent di-

gestion. 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, depres-

sions in the agar accompanied the cellulose digestion, and also grew in

size with age. However, in those areas, no definite colony was ever de-

tected. 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 incuba-

tion 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 creat a humid atmosphere. Moisture was a factor which seemed im-
portant 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 car-
bonate, 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 quar-
ters 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 con-
trol 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 con-

sidered to be pure. Since the type of spreading growth indicated that the

bacteria were motile, it was obvious that the only alternative was to pre-

pare 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 disinte-

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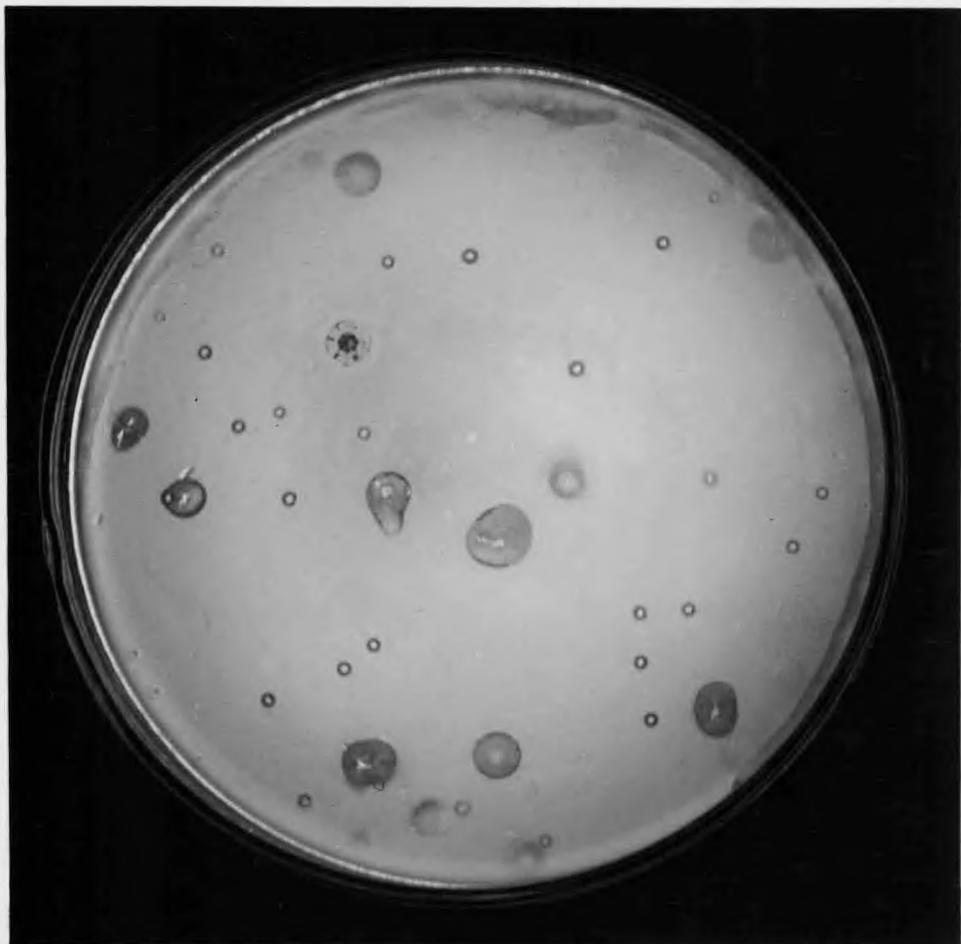
Colonies of culture 2-R (figure 2) in cellulose agar characteristically had a flower-like appearance with parts of the colony projecting like petals. Chromogenesis was never observed. Clear areas of cellulose digestion first appeared in about two days increasing in size as the colonies became older.

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;
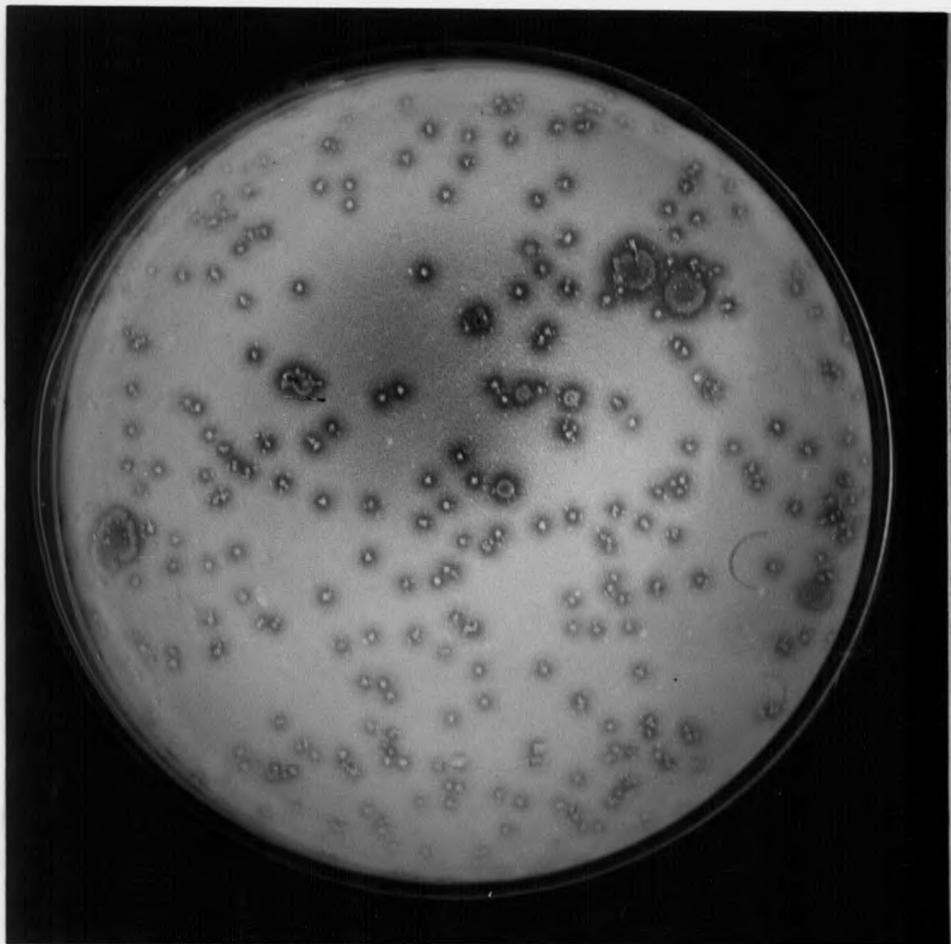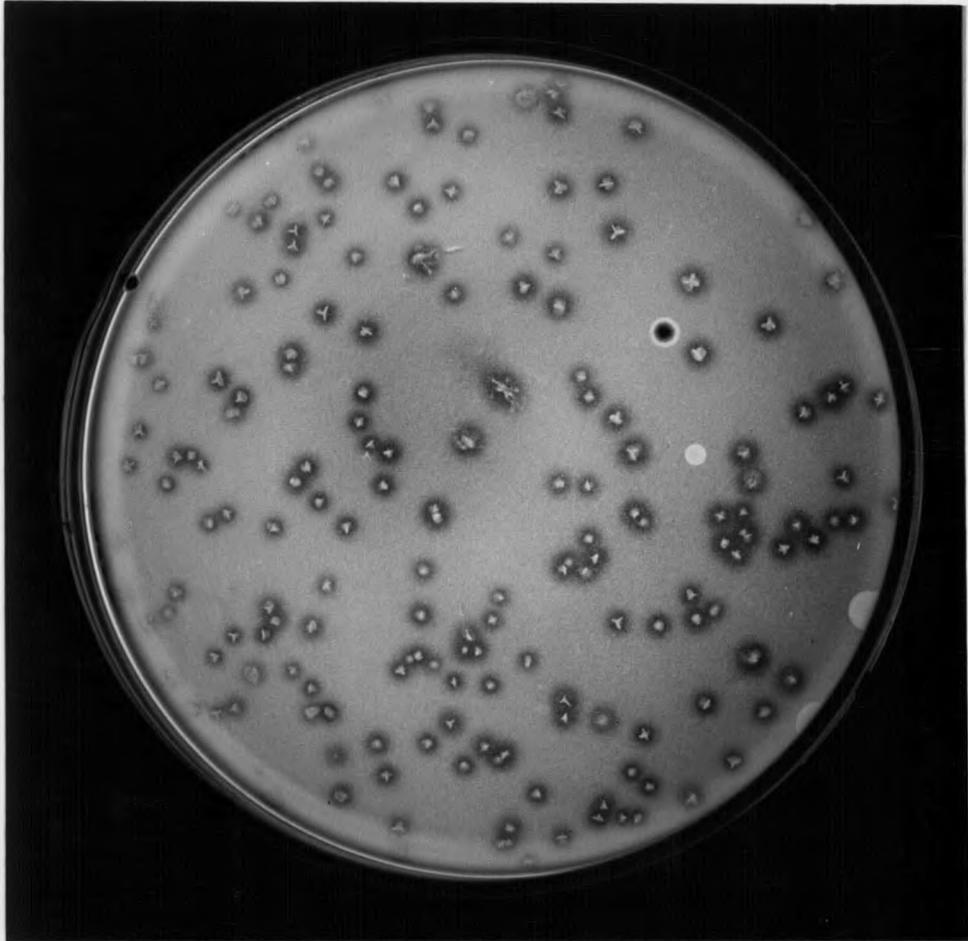   the small colonies are subsurface.

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

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 re-

action and the diphtheroid arrangement of the cells. The only cellulo-

lytic 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 differ-

ences 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 ascer-

tained. 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 ex-

tremely 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 decom-

position by the four strains of aerobic cellulolytic bacteria isolated, a

series of tests were performed to find the effects of various modifica-

tions 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 aspara-

gine, 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 magnifi-

cation, the results were recorded as negative. As a result of this experi-

ment, 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 ex-

tract 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 ad-

justed 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

Nitrogen source and type of cellulose

Culture	Temp. (C)	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.

-32-

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	26	35	40	55
		Temperature (C)			
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 demonstra-
tion 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.