

STUDIES ON AEROBIC BACTERIA COMMONLY CONCERNED IN THE DECOMPOSITION OF CELLULOSE

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I. INTRODUCTION

The decomposition of cellulose by microorganisms offers a field for investigation which has, for the past sixty years, been of interest from both purely scientific and practical standpoints. The natural decomposition of this resistant compound is not brought about by chemical processes alone, but is participated in by various biological agents and forces.

Cellulose is of almost universal distribution in the various tissues of plants, and forms a very large part of organic wastes. Unfortunately no way has yet been found for utilizing this ever-abundant product, except for a few limited purposes, as for example the manufacture of paper, nitrocellulose, etc. In nature the decomposition of cellulose goes on uncontrolled, and the products formed are immediately acted upon by microorganisms as sources of energy and food. In this way countless tons of decaying vegetable matter go to waste yearly. It might well be said that cellulose is almost an economic loss. If the biological process of decomposition could be carried on by pure culture methods and under scientific control, it should be possible to redeem the economically important cleavage products and convert them to every day use for man. Such mastery of nature is as yet only a dream, but from an analytical viewpoint it seems entirely possible.

Organisms varying widely in morphological, cultural and physiological properties have been associated by various workers with the natural decomposition of cellulose, namely certain

anaerobic and aerobic forms of bacteria, filamentous fungi, and some species of Actinomyces.

The painstaking investigations of van Iterson (1904), and of Kellerman, McBeth, Scales and Smith (1912-1916) show that aerobic bacilli play an important part in the decomposition. The present investigation deals with organisms of this group, some of which are transfers of the original Kellerman strains, while others are bacteria of similar type which have been isolated more recently, some of them by the authors.

In the present investigation attempts were made to simplify the isolation and cultural technique as a means of carrying out further studies on the activities of this interesting group of organisms.

II. GENERAL HISTORICAL REVIEW*

The first experimental evidence of cellulose decomposition by biological agents was advanced by E. Mitscherlich in 1850. He demonstrated that the cellulose parts of potato tissue were destroyed under certain definite laboratory conditions, and ascribed the change in structure to the action of certain vibrios found in the "substrate" of the potatoes.

Trecul (1865) published two papers on the microflora of macerated plant tissues. In these reports he discusses the activities of a group of organisms to which he applied the generic name *Amylobacter*. Van Tieghem (1877) later made an extensive study of the life history, cellulose-decomposing properties and physiological requirements of this group.

Popoff (1875) was the first worker to associate methane fermentation with the fermentation of cellulose. His experimental studies brought out the optimum temperature for the reaction and the effect of antiseptics, with some knowledge of the gaseous decomposition products.

Tappeiner (1881), Gayon (1883), and Deherain (1884), studied the various cleavage products of cellulose fermentation, observ-

* For a more complete historical review the reader is referred to the doctorate dissertation of the senior author, on deposit in the Yale University Library.

ing the formation of organic acids and numerous gases. The effects of oxygen supply, moisture, and composition of the medium on the nature of the by-products were also emphasized.

Hoppe-Seyler (1881), in an interesting series of experiments, came to the conclusion that cellulose decomposition goes through the following steps:

1. The hydration of the cellulose with the formation of a hexose.

2. The destruction of the monosaccharide with the formation of equal quantities of carbon-dioxide and methane.

Van Senus (1890) first suggested the phenomenon of symbiosis as entering into the decomposition process, and Herbert (1902) demonstrated that the speed of the fermentation was dependent to a large extent upon the reaction of the medium.

Schloessing (1889), Omelianski (1894), and Khanvine (1923) have contributed considerable data on anaerobic cellulose decomposition. The intensive studies of Omelianski cover a period of ten years and are especially interesting, since he was the first investigator to attempt work with pure cultures. He believed that cellulose could be decomposed by either of two fermentations, a hydrogen or a methane producing process, and that each process was influenced by a specific organism. In 1904 he published a method for separating the "hydrogen bacillus" from the "methane bacillus." His pure culture technique has been questioned, however, by later workers, since contaminating forms were isolated from his so-called pure cultures.

Meusel (1871), showed that certain organisms in the presence of cellulose are able to reduce nitrate to nitrite. Van Iterson (1904), in an extremely interesting study demonstrated that aerobic decomposition of cellulose is very vigorous, and that the oxygen requirements of the bacteria could be obtained from nitrates as well as from the atmosphere.

Pringsheim (1912), on the basis of experimental evidence, classified cellulose fermenting organisms as aerobes, denitrifiers, methane producers, hydrogen producers and thermophiles. His experiments with the cellulose-splitting enzyme are also striking.

The literature covering the years 1912–1916 inclusive contains a series of papers published by Kellerman and several co-workers (McBeth, Scales and Smith) as a result of experiments inaugurated by the Bureau of Plant Industry of the United States Department of Agriculture. The collective scope of these papers deals with the following phases of aerobic cellulose decomposition:

1. A discussion of the earlier work in the field by Omelianski and van Iterson, and the isolation of three strains of aerobic cellulose-fermenting organisms from the cultures of Omelianski.
2. The development of suitable mediums and technique for the isolation of aerobic cellulose-destroying organisms in pure cultures.
3. The collection of soil samples from various parts of the country, and subsequent isolation of over twenty strains of aerobic cellulose-decomposing bacteria and many filamentous fungi.
4. A description of the morphological, cultural and biochemical characteristics of these organisms.
5. A tentative classification of the group based on their cultural and biochemical properties.

Mutterlein (1913) developed a cellulose-agar medium which enabled him to isolate from soil cultures twenty strains of *Actinomyces* and one *Bacterium* which could destroy cellulose under aerobic conditions.

Kronlik (1913), using an ingenious technique, studied the activity of thermophilic cellulose-fermenting organisms under aerobic and anaerobic conditions. He concluded that the aerobic process is more rapid than the anaerobic, but that it is only through anaerobic conditions that complete destruction takes place. He also noted a difference in the gaseous end-products of the two types of fermentation.

Groenewege (1920), in an extensive study of the flora of aerobic cellulose fermentation, classified the organisms in three groups on the basis of nitrate reduction in the presence of cellulose. In a series of experiments he demonstrated the beneficial effects of nitrate-reducing organisms upon the action of the cellulose fermenters. Data are also given on the metabolic requirements of the cellulose-decomposing group.

The metabolic requirements of aerobic cellulose-decomposing bacteria were further studied by Löhnis and Lochhead (1922). They found that the organisms could utilize widely diversified forms of nitrogen, although there was evidence in favor of 0.1 per cent beef-extract as a nitrogenous source. Potassium nitrate seemed the best adapted source of inorganic nitrogen.

In the study of cellulose decomposition in soils by Waksman and Heukelekjan (1924), three methods for the quantitative measurement of fermentation were discussed, Charpentier's method being adopted as the most simple and the most accurate. This work also demonstrated the stimulative effect of sodium nitrate upon the decomposition of cellulose in soil.

III. SOURCES OF STRAINS EMPLOYED

Transplants of the following strains, originally isolated by Kellerman, McBeth, and Scales, were obtained from the Bureau of Plant Industry: *Bact. flavigenum*, *Bact. fimi*, *Bact. liquidum*, *Bact. udum*, *B. bibulus*, *B. biazoteus*, *B. gelidus*, *B. subalbus*.

Culture *B. x* is also an isolation of Kellerman and his associates, obtained directly from the Krall Laboratory. The label on the tube was not legible upon arrival of the culture, and to date the identity of the strain has not been determined.

Strains *8B*, *8G*, *5A*, *15*, *15N*, *16*, *Riv. 3* were isolated during the present investigation. Strains *8B* and *8G* were obtained from cow manure, *5A*, from compost, *15* and *15N* from decaying vegetable matter, and *16* and *Riv. 3* from soil.

IV. EXPERIMENTAL

According to the various publications cited the biological decomposition of cellulose is brought about by several morphologically distinct types of microorganisms; but it has been necessary to limit the present investigation to a study of aerobic cellulose-destroying bacteria. These organisms are able to attack cellulose under strictly aerobic conditions, or may carry out the oxidation with limited supplies of atmospheric oxygen if they are provided with nitrate or nitrite.

1. *The collection and study of field specimens*

In a preliminary experiment numerous samples of soil, decomposing vegetable matter, and sewage were collected for the purpose of studying the distribution of cellulose-destroying organisms in nature. It is obviously impossible to carry on an investigation of this kind unless sterile apparatus is used for the collection of each individual sample.

A very simple, inexpensive and convenient sampling outfit may be prepared from old cigar boxes and large test tubes in the following manner:

The boxes are split into one inch strips which are shaped into spatulas for collecting the soil. These are sterilized in the hot air oven along with large test tubes which serve as receptacles for the sample. Soil samples may be kept in good condition over a period of time by the frequent addition of sterile distilled water.

During the course of the experiment 39 samples were collected and examined in the laboratory for the presence of cellulose-decomposing bacteria. Approximately 1 gram of the soil was transferred to a tube containing the following enrichment medium:

Di-potassium phosphate.....	1 gram
Magnesium sulfate.....	1 gram
Sodium chloride.....	1 gram
Calcium carbonate.....	2 grams
Potassium nitrate.....	2 grams
Cellulose.....	strip of filter paper
Water.....	1000 cc.

It was assumed that the cellulose-destroying organisms were present in the inoculum when the strip of filter paper was completely cut at the surface of the liquid ten days after inoculation. In uninoculated control tubes the strips of paper were not broken after prolonged incubation, even after considerable handling and shaking.

Table 1 shows the different kinds of material collected and tested in the above medium, and the numbers of each in which cellulose-destroying organisms were demonstrated.

Although the limited number of samples collected does not allow any definite conclusion to be drawn concerning the distribution of cellulose-destroying organisms in nature, it may be said that this group seems to be closely associated with soils that are rich in decaying organic matter.

2. Special cultural mediums

Preliminary enrichment mediums for impure cultures. Although the destructive power of cellulose-fermenting bacteria was noted and described in 1850, it was not until 1912, or sixty-two years later, that pure cultures were isolated for study.

TABLE 1
Occurrence of cellulose-destroying organisms in nature

SOURCE	NUMBER OF SAMPLES	PRESENCE OF CELLULOSE- DESTROYING ORGANISMS INDI- CATED IN TEN DAYS
Garden soils.....	10	8
Forest soils.....	8	8
Decaying plant tissues.....	9	8
Swampy soils.....	3	3
Sewage.....	2	2
Humus heaps.....	2	2
Cow manure.....	3	3
Horse manure.....	2	2
Total.....	39	36

Various mediums and methods were tried, but all gave negative results. Their only value lay in showing the need of a preliminary enrichment medium for increasing the number of cellulose-destroying organisms before the final application of isolation technique.

Soils containing a large amount of organic matter are seeded with many types of organisms, and unless the chosen medium favors the growth of the delicate, slow-growing cellulose-fermenting bacteria, and at the same time retards the development of other forms, cellulose-destroyers are soon overgrown and isolation becomes impossible.

In 1894 Omelianski, employing Winogradski's "method of elective culture," selected for enrichment purposes a nutrient solution almost void of organic nitrogen, and incubated the cultures anaerobically, over a series of transfers. He found that this method gave almost a pure culture of cellulose-destroying organisms.

Van Iterson (1904), McBeth (1912), Löhnis and Lochhead (1913), and Groenewege (1920) have since suggested various modifications of Omelianski's solution, but the basic principle of the selective medium is essentially the same. No organic matter is introduced into the medium, except that contained in the inoculum. The nitrogen supply is limited to the inorganic salts of nitrogen. The cellulose-containing material (filter paper, cotton, plant tissues or commercial cellulose) comprises the sole source of carbon. Other inorganic salts, mainly those of sodium, magnesium and carbon, in the form of sulfates, phosphates, chlorides and carbonates, are added in varying concentrations to fulfil the metabolic requirements of the group.

After a preliminary study of various synthetic solutions, the Löhnis and Lochhead modification of McBeth's medium was chosen as the most satisfactory for enrichment purposes. The composition of this medium has already been stated on page 326, and it will hereafter be referred to as cellulose-nitrate broth.

This enrichment medium can be used with very satisfactory results under either of the following conditions:

1. Approximately 20 cc. of the nutrient solution and a strip of filter paper are placed in a large test tube. The filter paper is so placed that a portion of it projects about an inch above the surface of the liquid.

After sterilization the tubes are inoculated with 1 gram of soil and incubated at 34°C. until unmistakable signs of decomposition are noticed. Then a small piece of the disintegrating paper is aseptically transferred to a new tube of the same medium. Following a series of similar transfers, the cellulose-decomposing bacteria appear as the predominant type, and at this stage they are ready for transfer to the isolation medium.

2. Filter paper is shredded with dissecting needles and placed

in long-necked flasks. The flasks are then filled to the neck with cellulose-nitrate broth and sterilized. After sterilization, the cotton plugs are replaced by rubber stoppers. The flasks are inoculated with soil and the level of the liquid brought well up into the neck of the flask by the addition of sterile nutrient solution. Incubation is at 34°C. Fermentation appears slowly, but gradually increases in intensity. When the solution fails to give positive tests for either nitrate or nitrite, the old liquid is decanted off and new liquid is added aseptically. Small amounts of disintegrating cellulose are transferred to new flasks and the fermentation process repeated. After several such transfers, the cellulose-decomposing flora out-numbers the contaminating forms. Transfer to a solid medium for isolation requires only a small amount of inoculum.

Mediums for isolation. In a series of experiments the cellulose agar of Kellerman and McBeth was employed. Colonies of cellulose-dissolving bacteria could be easily detected on this medium by the appearance of a clear zone around the growing colonies. These areas have been referred to as the "enzymatic rings." Kellerman and others believe that they were formed by the action of a cellulose-decomposing enzyme produced by the bacteria within the colonies. Microscopic examination showed the areas to be free from cellulose fibers.

While using this medium considerable difficulty was experienced with molds which overcrowded the plates during the prolonged period of incubation. In an endeavor to overcome this difficulty by shortening the incubation period, casein-digest was substituted for the mineral constituents of Kellerman's medium. The results obtained with the modified medium were strikingly satisfactory. The incubation period was shortened from three to five days and the size of the colonies materially increased. Calcium carbonate was not added to the medium; yet the characteristic halo appeared around the colonies of cellulose-destroying organisms. This fact supports Kellerman's original claim that the clear zones around the colonies appear as a result of enzyme action, and not from the solution of calcium carbonate by acids produced during the fermentation.

Mediums for the cultivation of pure cultures. The problem of growing pure cultures of cellulose-fermenting bacteria is much more complex than the cultivation of the unpurified organisms. Cellulose-fermenting organisms will grow on ordinary laboratory mediums such as nutrient agar, gelatin, pepton meat-extract broth, and milk, but under these conditions some strains gradually lose their cellulose-fermenting powers or allow them to become dormant. Although Kellerman and others have devised means of rejuvenating this power from time to time, such a process solves the difficulty only temporarily. Several types of mediums that are useful in the study of these organisms have been devised, but they do not overcome the difficulties of continued cultivation.

A preliminary study made with the older methods revealed the following:

1. A diminution of the cellulose-fermenting power of the organisms on certain artificial mediums.
2. The production of delicate growths by these bacteria.
3. The necessity of long periods of incubation for obtaining maximum growth.
4. The irregularity of growth in cellulose-nitrate broth.

The results obtained in *cellulose-nitrate broth* were very irregular. In a few cases growth appeared in the initial transfer when large amounts of inoculum were used, but died out when a second subculture was attempted. This seemed to indicate that an inorganic medium was not favorable. The following experiment was carried out to determine the effect of the presence of organic matter upon growth.

A quantity of cellulose-nitrate broth was prepared in thoroughly washed glassware. The medium was divided into three equal portions; one of these was tubed and used as a control free from organic matter. Organic matter in the form of soluble casein-digest (Kulp and Rettger, 1924) was added to the other portions before tubing in final concentrations of 0.1 and 0.25 per cent respectively.

The three mediums were inoculated with suspensions of organisms which had been washed with saline solution to remove all

traces of organic matter. The inoculum was made up to a definite turbidity corresponding to 1.0 on the McFarland Nephelometer scale and added to the tubes in a 2 per cent concentration. Growth and change in hydrogen ion concentration of the various mediums is shown in table 2.

In a subsequent experiment no evidence of growth or decomposition could be obtained when the concentration of the inoculum was increased to 5 and 10 per cent.

TABLE 2
Growth of pure cultures with and without organic matter

ORGANISM	CELLULOSE-NITRATE (NO ORGANIC MATTER)		CELLULOSE-NITRATE (0.1 PER CENT ORGANIC MATTER)		CELLULOSE-NITRATE (0.25 PER CENT ORGANIC MATTER)	
	Paper cut after 8 days	pH after 8 days	Paper cut after 8 days	pH after 8 days	Paper cut after 8 days	pH after 8 days
<i>Bact. udum</i>	0	7.9	+	6.0	+	5.6
<i>Bact. flavigenum</i>	0	7.9	+	6.8	+	6.8
<i>B. gelidus</i>	0	8.1	0	7.4	x	7.0
<i>B. subalbus</i>	0	8.1	+	6.1	+	5.4
No. 15.....	0	8.1	+	7.4	+	7.0
<i>Ps. perlurida</i>	0	8.1	0	7.2	+	6.8
No. 8B.....	0	8.0	+	7.2	+	5.6
<i>B. biazoteus</i>	0	8.0	0	7.4	+	7.4
Riv. 3.....	0	7.9	+	6.0	+	6.0
B. x.....	0	8.1	+	6.4	+	5.6

0—Paper not cut after eight days incubation.

x—paper incompletely cut after eight days incubation.

—paper completely cut after eight days incubation.

The results of these experiments seem to indicate the need of an organic medium for the continued growth of cellulose-decomposing organisms. All further attempts to cultivate them in inorganic medium were abandoned.

The tryptic digest of commercially pure casein, prepared by the method described by Kulp and Rettger (1924) has been shown in this laboratory to be superior to commercial pepton for the culture of certain organisms. Preliminary studies with the casein-digest showed that it was superior to commercial

pepton for the cultivation of cellulose-destroying bacteria. A *cellulose casein-digest medium* of the following composition was prepared:

Casein-digest.....	100 cc.
Tap water.....	900 cc.
Beef-extract.....	1 gram

The hydrogen ion concentration of the medium was adjusted to pH 7.4. A strip of filter paper was added to furnish the cellulose factor.

The cultural results obtained with this medium were very striking. When inoculated with either old or newly isolated strains, the organisms always grew. Definite changes in pH and turbidity could be demonstrated after twenty-four hours incubation. The strips of filter paper were attacked vigorously. The time required for a complete cutting of the paper varied from twenty-four to ninety-six hours.

The following experiment was carried out to determine whether the cellulose-fermenting power of the organisms would be altered by a series of successive transfers in this medium.

Six strains of cellulose-decomposing bacteria were employed over a series of nine transfers. The time necessary for complete cutting of the paper by each subculture, and the total number of days required to cut the nine strips of filter paper are given in table 3.

The length of the different trials varied from seventeen to twenty-seven days. This is probably explained by variation in the attacking powers of the different strains employed. At the end of the ninth transfer the cultures appeared to be as active in their growth and cellulose-fermenting properties as they were in the first subculture. Continual use of this medium during the past two years has furnished repeated verification of these results.

The advantages offered by the casein-digest medium for the cultivation of pure strains of these organisms may be summed up as follows:

1. It stimulates a rapid and luxuriant growth.

2. Cellulose is readily attacked and vigorously digested.
3. A very small amount of inoculum is required to stimulate growth.
4. The members of the group can utilize casein-digest without the presence of cellulose.
5. There has been no evidence that the different strains lose their cellulose-destroying activities.

Attempts to use this medium for enrichment purposes were without success, for it was non-selective and encouraged the growth of all organisms in a mixed culture.

TABLE 3
Cellulose-decomposition in cellulose casein-digest broth

ORGANISM	DAYS REQUIRED FOR CUTTING PAPER IN INDIVIDUAL SUBCULTURES									TOTAL DAYS RE- QUIRED
	1	2	3	4	5	6	7	8	9	
<i>Bact. flavigenum</i>	2	2	2	2	1	2	2	2	2	17
<i>B. x.</i>	2	2	2	2	1	2	2	2	2	17
No. 8B.....	2	2	2	2	2	2	2	2	2	18
<i>Bact. udum</i>	2	2	2	2	1	2	2	2	2	17
<i>B. gelidus</i>	3	4	3	3	2	4	3	3	2	27
No. 15.....	3	3	3	3	2	2	2	2	2	22

3. *The isolation of pure cultures*

The isolation technique of Kellerman, McBeth and Groene-ge was tested with varying degrees of success. The methods of the above workers were then combined and modified in the preparation of a new technique that has worked satisfactorily during this investigation.

A small piece of fermenting cellulose (filter paper) was removed from the enrichment medium (cellulose nitrate broth) and carefully washed in sterile physiological saline solution. The decomposing material was then shaken vigorously in a tube containing sterile broken glass until it was completely disintegrated. At this point dilutions were made from the suspensions and plates poured, or the undiluted suspension was streaked over a series of plates containing cellulose casein-digest agar.

This modified method is believed to be an improvement over the others, for the following reasons:

1. The use of casein-digest as a nutrient solution, in the place of an inorganic medium, shortens the incubation period by stimulating the growth of the cellulose-fermenter.

2. The use of broken glass results in a more complete disintegration of the cellulose fibers, and likewise a more efficient separation of the bacterial cells attached to these fibers.

4. *Morphological and cultural characteristics*

Morphology. In the present investigation the strains under observation appeared as small, slender, non-spore-forming rods. Branching or chain formation was not observed. All strains stained readily with the ordinary basic dyes. When stained by Gram's Method they did not retain the stain after treatment with alcohol. All strains isolated during the investigation, with the exception of 8B and 8G, were motile at the time of isolation. Flagella stains were attempted, but did not give satisfactory results.

Cultural characters. These were studied in nutrient agar plates and slants, nutrient broth, litmus milk, potato slants, cellulose meat-extract broth, cellulose casein-digest broth, and maltose casein-digest agar plates. All cultures were incubated at 30° to 34°C.

Agar plates: The colonies formed in this medium varied from small (less than 1 mm. in diameter) to large (5 to 10 mm.) after fifteen days incubation. The surface colonies were round or almost round with well defined edges. The presence of a granular nucleus was not uncommon. Subsurface colonies were often granular, and lenticular in shape. A yellow chromogenesis was present in some strains and absent in others.

Nutrient broth: There was no surface film and only a slight amount of sediment formed. Slight to moderate turbidity appeared after from two to five days incubation. Odor was absent.

Agar slants: A moderate amount of filiform growth appeared after forty-eight to seventy-two hours. Chromogenesis appeared

in some strains. No discoloration of the surrounding medium was noted.

Litmus milk: Growth is slow. After from three to five days incubation, a slight reddening is noticeable in most of the cultures. Acid curds were not formed, but in one case a rennet curd appeared and was followed by peptonization. Litmus was not reduced. The cultures were incubated for fifteen days.

Potato slants: Eight of the cultures gave a sparse greyish-white growth along the line of inoculation; the scantiness of growth would indicate that the medium was not favorable for their cultivation.

Cellulose meat-extract broth: The type of growth in this medium was similar to that in nutrient broth. Cellulose was attacked after from three to five days.

Cellulose casein-digest broth: Cultivation of the various strains on this medium gave satisfactory results, both as to the amount of turbidity formed and the ability of the strains to attack cellulose. Twenty-four-hour cultures showed a distinct turbidity throughout the tube. In some cases a settling out of the organisms was noticed after from five to seven days.

Maltose casein-digest agar: This medium was found very useful for routine platings of the different strains. The amount of growth and the size of the colonies were greatly increased by the addition of the carbohydrate. The organisms were not allowed to remain in contact with this medium for long periods for fear of its altering their cellulose-fermenting properties.

5. Biochemical properties

Gelatinolytic action. The gelatinolytic action of the various strains was tested in ordinary gelatin stab cultures. These were incubated at 20°C. for thirty days. All of the strains tested grew in the medium, both on the surface and along the track of the inoculating needle. Liquifaction of the gelatin took place slowly and was generally infundibular in shape. After thirty days incubation all of the strains with the exception of no. 16 had produced liquifaction. Culture no. 16 gave a doubtful reaction after sixty days incubation.

Nitrate reduction. The organisms were cultivated in casein-

digest broth containing 0.2 per cent potassium nitrate. All gave a positive reaction for nitrite.

Indol production. None of the strains produced indol.

Fermentation studies. No reference could be found in the literature to changes in hydrogen ion concentration produced by aerobic cellulose-decomposing organisms. A series of experiments was carried out with the hope that fermentation reactions might show strain variations and thus furnish a basis of classification.

The basic medium employed was casein-digest broth. To this were added fermentable substances in the concentration of either 1.0 or 0.25 per cent. The majority of carbohydrates were sterilized by filtration through a sterile Berkefeld candle and then added aseptically to the basic medium. The detailed technique has been previously published by Kulp and Rettger (1924). The slightly soluble materials were prepared under aseptic conditions and subjected to a shortened sterilization period.

The determinations were carried out in triplicate. Uninoculated controls were frequently employed. The purity of the inoculum was tested by plating on maltose casein-digest agar.

The following test substances of the highest purity were used in the experiment:

<i>Triose</i>	<i>Poly-saccharides</i>
Erythrose	Dextrin
	Soluble starch
<i>Pentoses</i>	Inulin
Xylose	Cellulose
Arabinose	
	<i>Glucosides</i>
<i>Hexoses</i>	Salicin
Glucose	Aesculin
Levulose	
	<i>Alcohols</i>
<i>Di-saccharides</i>	Glycerol
Maltose	Mannitol
Lactose	Dulcitol
Sucrose	
<i>Tri-saccharides</i>	
Melizitose	
Raffinose	

The results obtained may be summed up as follows:

1. The lowest pH produced by any of the strains during the experiment was 4.6. A hydrogen ion concentration of from 4.6 to 4.8 seemed to be a maximum for all strains. The reactions varied both with the strains and the fermentable substances employed.

2. In several cases where no fermentation accompanied the growth of the organisms, alkali production could be noted by an increase in pH.

3. The alcohol, dulcitol, and the triose, erythrose, were the only two substances not attacked.

4. Inulin was attacked by only one strain, *Bacterium flavigenum*.

5. Mannitol, raffinose, and melizitose were attacked by only a few strains. Mannitol was fermented by *B. bibulus*, *B. x*, and *Ps. perlurida*; raffinose by *Bact. fimi*, no. 15, *B. biazoteus*, *Bact. liquatum* and no. 15N; and melizitose by *Bact. fimi*, no. 15, *Bact. liquatum*, and no. 15N.

6. The glucosides, aesculin and salicin, were attacked and fermented by all strains except *B. subalbus*.

7. The fermentation of glycerol was characterized by a small change in pH, except for a few strains. This substance did not appear readily fermentable.

8. Twelve of the seventeen strains used severed the filter paper (cellulose) within forty-eight hours incubation. Three more completed the cutting within the next twenty-four hours, and at the end of the fourth day all of the paper strips were completely cut.

9. All of the strains fermented the following:

Pentoses: Xylose and arabinose

Hexoses: Glucose and levulose

Di-saccharides: Maltose, lactose, and sucrose

Polysaccharides: Dextrin, soluble starch, and cellulose

The present investigation, although somewhat limited in its scope, due to the small number of available strains, points out the possibility of using carbohydrate fermentation as a means of differentiating various strains.

6. *The Influence of Hydrogen Ion Concentration upon the Rate Of Cellulose Decomposition*

Several workers have noted that cellulose-decomposing bacteria are very active when the reaction of the surrounding medium is neutral or slightly alkaline, but that the organisms are checked by the presence of small amounts of acid. A study was made of the changes in hydrogen ion concentration brought about by cellulose-destroying organisms in casein-digest broth. The results emphasized the apparent deterrent action of a slightly acid reaction in this medium. In a casein-digest broth having an initial pH of 4.9, growth and cellulose-decomposing powers were entirely inhibited. At pH 6.2 five out of the seventeen strains employed were able to grow and attack cellulose. All strains grew vigorously and attacked the cellulose when the initial pH was 7.4. In the last instance the pH was rapidly lowered during fermentation, and soon reached a point where activities were more or less hampered by the acidity of the medium.

Additional buffer material, in the form of secondary potassium phosphate, was added to the casein-digest medium in an effort to control the hydrogen ion concentration. The results indicate that 0.5 per cent phosphate in the medium exerts a buffer effect that holds the pH of the fermenting cultures above 6.5 for six days. In the control medium (casein-digest broth) and in a medium containing only 0.1 per cent phosphate the pH was considerably lowered during this incubation period.

7. *Quantitative Determination of Cellulose Decomposition*

In the present investigation decomposition was studied only in artificial cultural mediums. The basic medium employed was a casein-digest broth containing cellulose (strip of filter paper). Aside from the filter paper the medium contained no insoluble ingredients and passed readily through an ordinary filter.

The strips of paper used as a cellulose substrate were dried to constant weight before being added to the casein-digest broth. Paper and broth were sterilized together.

The amount of decomposed cellulose was determined by filter-

TABLE 4

Decomposition of cellulose in casein-digest broth containing a strip of filter paper

ORGANISMS	FILTER PAPER USED	FILTER PAPER DESTROYED DURING FIFTEEN DAYS INCUBATION	PER CENT DESTROYED
	<i>mgm.</i>	<i>mgm.</i>	
8B.....	254	30	12*
	255	29	11
	233	31	13
	227	32	14
	246	33	13
<i>Bact. udum</i>	255	35	14
	269	31	12
	259	29	11
	251	34	14
	260	34	13
<i>B. gelidus</i>	249	25	10
	257	30	12
	250	29	12
	252	28	11
	265	26	10
No. 16.....	244	22	9
	246	17	7
	267	28	10
	266	31	12
	262	24	9
<i>Bact. flavigenum</i>	266	22	8
	219	17	8
	246	28	11
	258	31	12
	265	24	9
B. x.....	277	8	3
	285	31	11
	266	38	14
	241	38	16
	246	34	14

* Figured to the nearest unit.

ing through a previously tared filter paper. The residue was thoroughly washed with distilled water to remove any trace of

soluble material. When secondary potassium phosphate had been added to the basic medium, it was necessary, first, to wash the residue with dilute hydrochloric acid (1:20).

Filter papers containing the residue, after washing, were dried to constant weight and the amount of decomposed cellulose readily calculated. Check weighings within three milligrams were required on all cultures. As a general rule four days drying in the oven at 110°C. was sufficient. Uninoculated control tubes were frequently employed and always registered within 5 mgm. of the calculated weight after being subjected to the above technique.

In the following experiment the cellulose-decomposing power of six strains was studied quantitatively in cellulose casein-digest broth. Five tubes of each strain were incubated simultaneously. The amount of paper destroyed by the various strains during the course of the experiment is shown in milligrams in table 4.

The effect of oxygen supply upon the decomposing powers of the organisms was studied in similar experiments. Reduced oxygen tension was produced by placing the cultures in glass-stoppered jars containing slant agar cultures of *B. cereus*. Increased oxygen supply was attained by gently aerating the cultures during the period of incubation. The results of the experiment indicated that neither of these measures increased the cellulose-decomposing properties of the organisms. Ten to 14 per cent of the cellulose was decomposed in both experiments.

The influence of secondary potassium phosphate upon the amount of cellulose decomposed received further attention here. Previous qualitative studies had shown that the presence of this salt tended to regulate the hydrogen ion concentration of the fermenting culture by its buffering action.

The basic medium employed was again casein-digest broth. The di-potassium phosphate was added in amounts giving concentrations of 0.5, 1.0, and 2.0 per cent respectively in three sets of flasks. The results indicate that the addition of the buffer materially affects the cellulose decomposition. Both one-half and 1 per cent concentrations of the salt favored destruc-

tion in almost every case. *B. gelidus* and *Bact. flavigenum* were the only exceptions, and this may be explained by the relative inactivity of these two strains.

The favorable effects of the phosphate appear to be lost when a 2 per cent concentration is used in the medium. There is approximately 100 per cent more cellulose destroyed in the medium containing 1 per cent phosphate than in one having 2 per cent of the salt. This retarding action is presumably due to growth-inhibiting action of the phosphate in the higher concentration.

V. GENERAL DISCUSSION

The results of the present investigation emphasize the necessity of a suitable culture medium for the artificial cultivation of pure strains of aerobic cellulose-fermenting organisms. In pure culture, as contrasted with the impure, the organisms are very delicate and require certain optimum conditions for growth and maintenance of physiological efficiency.

It was found impossible to obtain satisfactory results in the continued cultivation of the pure strains in nutrient solutions of inorganic salts. In most instances the bacteria refused to grow, and if they grew they rarely survived a second transfer. The addition of small amounts of soluble organic matter increased the growth possibilities.

The apparent beneficial effect of the presence of organic matter led to the development and use of a tryptic digest of chemically pure casein as a nutrient solution. Casein-digest has now been employed in this study for over three years, and it still supports luxuriant growth and permits vigorous cellulose fermentation by the various strains employed.

Isolation of members of the group by the plating method was simplified by the adoption of a modified technique, and the substitution of casein-digest for the inorganic salt solution in McBeth's hydrocellulose agar. The period of incubation is materially shortened, and the size of the colonies increased. Calcium carbonate was not added to the medium; yet the "enzymatic ring" described by Kellerman and McBeth (1913) was clearly visible after incubation of the cultures.

The morphological and physiological properties of the cellulose-decomposing bacteria studied during this investigation were very similar, although the various strains came from widely different sources. The characteristics of the organisms suggest their classification in the genus *Cellulomonas* (Committee on Classification, 1920).

The organisms are very active in their attack on various fermentable substances. Xylose, arabinose, glucose, levulose, maltose, lactose, sucrose, dextrin, soluble starch and cellulose were fermented by all strains. Dulcitol and erythrose remained unattacked. Action on the other fermentable substances showed strain variation.

The results of quantitative studies emphasize the necessity of an optimum reaction for the fermentative process. The addition of buffer material to the basic medium tends to regulate the fermentation and also to increase the amount of cellulose destroyed.

VI. SUMMARY

1. Cellulose fermenting organisms appear well distributed in nature and are closely associated with decaying vegetable matter.
2. Unpurified cultures are most successfully cultivated in a selective inorganic nutrient solution. Pure cultures are best grown in an organic medium. Casein-digest proves very valuable for this purpose.
3. All but one of the strains are gelatinolytic; the one exception produces only slight liquefaction at best after prolonged incubation.
4. All strains reduce nitrate to nitrite.
5. Indol is not formed.
6. The fermentation reactions of the group suggest a possible means for strain differentiation.
7. The cellulose-fermenting powers of the strains may be demonstrated gravimetrically.
8. The addition of buffer in the form of secondary potassium phosphate (0.5 to 1.0 per cent) not only delays the development

of a harmful acid reaction, but also increases the actual amount of cellulose decomposition during fifteen days incubation.

9. The presence of the enzyme cellulase may be shown by the auxanographic method on cellulose casein-digest agar.

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