A SYSTEMATIC STUDY OF AMYLOLYTIC BACTERIA THAT DECOMPOSE CELLULOSE, ISOLATED FROM QUEBEC SOILS

By

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INTRODUCTION

Under natural conditions, cellulose is biochemically one of the most important compounds to reach the soil by means of plant residues. This is especially true of forest soils and pastures. In agricultural soils, under crops, the normal equilibrium between groups of microorganisms is slightly displaced by the introduction of plant residues and by the addition of manure. There is also a large addition of cellulose and, at the same time, other reserve material from plowing in the roots of annual crops.

In normal soils, cellulose becomes decomposed under the combined action of fungi and aerobic bacteria. Of these two groups of organisms the fungi seem to act more vigorously at first after the addition of cellulose, but their numbers very rapidly drop to normal and it is very likely that the bacteria, which had a slower rise in activity, maintain their action until the excess of the material added has been utilized.

No special mention has ever been made of starch hydrolysing microflora in soils. This carbohydrate is less

refractory to the attack of microorganisms than is cellulose. Many bacteria which are not typical of the majority of those constituting the soil microflora, are reported to be active in hydrolysing starch. It appears to be assumed that such bacteria represent the true amylolytic flora. This may explain why studies of the starch hydrolysing organisms have never been undertaken. But if we consider the different groups of bacteria reported as cellulose decomposers, we come to the proposition of Fuller and Norman (1943a), namely that there exist some more or less obligate cellulose organisms and a greater number of versatile ones. These authors conclude that "the aerobic cellulose organisms in soil are predominantly versatile organisms that individually may not be very vigorous on cellulose but which are likely to be able to maintain themselves in a heterogeneous soil population that is normally utilizing not pure cellulose alone but a complex substrate of which cellulose is only one constituent".

This fact must have been overlooked with the use of cellulose as the only source of carbon in culture media for elective and isolation purposes. Following their proposition, Fuller and Norman (1942) have established cellulose-dextrin media which they claim favor the growth of obligate cellulose decomposers like

Sporocytophaga myxococcoides, as well as that of the versatile species. It will be seen in the literature that the great majority of cellulose decomposing bacteria are also recorded as starch hydrolysers.

As the preparation of cellulose-dextrin is tedious and since that material is not far different in chemical constitution from starch, and since, furthermore, the aim of the work was to isolate as many species as possible, it was decided to use starch instead of cellulose in the enrichment cultures from some Quebec soils.

LITERATURE REVIEWED

CELLULOSE: Its presence in soils, decomposition and effects

The importance of cellulose in plants does not need to be discussed at length as it is well-known that this organic compound is the major structural constituent of plants and probably the most abundant single entity..

Waksman (1932) gives an analysis of some plant materials (Table I).

This table gives an idea of the occurrence of cellulose in widely different plant materials, some of which reach agricultural soils. The natural conditions for a soil under culture are somewhat different in that the organic materials, which are incorporated into it, are mainly composed of grasses and straw in manure. As to the grasses, the composition is influenced by the maturity of the plant, but the soil receives the residues of such plants mainly at the time of their maturity. Since some young plants are often added to the soil as green manure, the complete table given by Waksman and Tenney (1927) is reported (Table II).

The fall of heads, leaves and stems being accidental and being returned either as green manure or stable manure, we will consider those two items later. The main consideration should be placed now on the roots which form

TABLE I - Proximate chemical composition of various plant materials on per cent basis of dry material.

Chemical constituent	Wheat straw	Soybean meal	Mature oak leaves	Dead pine needles	Corn stalks	Alfalfa tops
Ether-soluble fraction	1.10	3.80	3.85	11.37	1.75	2.75
Cold water soluble organic matter	5.57	22.09	8.28	4.42	10.58	12.44
Hot water soluble organic matter	--	--	5.73	2.86	3.56	4.80
Alcohol soluble fraction	--	--	5.82	12.58	4.19	7.66
Hemicelluloses	26.35	11.08	17.97	17.10	21.91	13.14
Cellulose	39.10	28.53	12.78	14.79	28.67	23.65
Lignin	21.60	13.84	24.76	21.89	9.46	8.95
Crude protein	2.10	11.04	4.25	2.12	2.44	12.81
Ash	3.53	9.14	5.10	2.51	7.54	10.30

TABLE II - Composition of rye straw at different stages of growth.
On dry basis. (Seed planted in the fall)

Sample	Moisture content at time of harvesting	Ash	Nitrogen	Cold water soluble fraction	Pento-sans	Cellu-lose	Lignin
April 28	80.0	7.3	2.39	32.6	15.9	17.2	9.9
May 17	78.8	5.7	1.76	22.0	20.5	26.1	13.5
June 3 (Heads)	57.4	4.9	1.01	18.2	22.7	30.6	19.0
June 3 (Stems and leaves)	60.2	5.9	2.20	20.3	22.7	20.1	16.0
June 30 (Heads)	15.0	3.2	1.22	4.7	11.9	4.6	13.4
June 30 (Stems and leaves)	15.0	3.7	0.22	9.5	21.7	34.6	18.8
June 30 (Roots)	?	?	0.55	4.7	26.6	37.7	21.0

an important mass of organic matter left in the soil. The figures give emphasis to the statement that cellulose is the major constituent of plant material, and when we think of the natural cover of agricultural soils, mainly constituted of grasses which are either cut for hay, grazed, or left wild, the importance of the annual additions of cellulose is clear.

These, however, are not the only sources of cellulose, for green manure and stable manures are also added to the soil. From the above table of rye analysis it is seen that young plants contribute more to readily available substances (cold water soluble and hemicellulose fractions) than to cellulose, although the latter constitutes a large part. So it is evident that green manures also contribute to the cellulose increase in the soil.

The case of stable manures is slightly different in that they are composed of animal feces and of straw. Furthermore, some primary decompositions take place in the manure heaps before the spreading and final incorporation into soil. Waksman (1932) gives results which are reported here, showing analysis of fresh and of composted manures (see Table III).

Evidently composting of this manure has reduced the cellulose content to a very low level. Waksman (1932)

TABLE III - Composition of horse manure (litter free) at different stages of decomposition. On per cent basis of dry material

Plant constituents	Fresh	39 days	96 days	157 days	290 days
Ether soluble fraction	1.89	1.89	1.47	0.88	0.95
Cold water soluble fraction	3.19	4.11	4.73	4.36	3.81
Hot water soluble fraction	2.39	3.86	3.37	2.19	1.90
Hemicelluloses	23.52	22.84	15.76	13.36	12.67
Cellulose	27.46	23.18	16.07	6.98	5.97
Lignin	14.23	16.63	17.92	20.54	28.43
Crude protein	6.81	7.00	14.81	18.56	16.38
Ash	9.11	13.64	20.93	22.22	19.32

compares this decomposition with that of straw that has been composted as "artificial" manure (see Table IV).

TABLE IV - Relative composition of "artificial" manure from straw and horse manure composted under exactly the same conditions and for the same period of time (290 days). On the basis of dry matter

Plant constituents	"Artificial" manure %	Composted horse manure %
Ether soluble	0.41	0.95
Soluble in cold and hot water	8.73	5.71
Hemicelluloses	14.77	12.67
Cellulose	13.75	5.97
Lignin	24.39	28.43
Protein	14.56	16.38
Ash	19.44	19.32

Again the results of composting show a very depressive influence on the cellulose.

The common practice in Quebec is to have the manure composted for less time. As a matter of fact stable manure is piled up during winter time and spread over the field as soon as the soil can support the carts in springtime. We have no actual figures showing the percentage of cellulose

of such manure, but we can take it as probable that it amounts to 15-25% cellulose. The rural practice of adding 10 to 15 tons of manure per acre would then contribute to very nearly a ton of cellulose per acre, taking the moisture content of the manure as approximately 80 per cent. This is a considerable amount of added cellulose, about 0.1 per cent.

Cellulose is, after lignin, the more resistant carbohydrate, being readily hydrolysed only by concentrated strong acids or by special reagents. Such hydrolysing agents do not occur in the normal process of cellulose decomposition and, as it constantly proceeds in normal soils, this degradation has to be accounted for by the action of saprophytic microorganisms, either free or living in the stomachs or digestive tracts of higher organisms, such as earthworms.

There is as yet no definite information as to the way cellulose is broken down. Pure culture studies on cellulose point to the fact that the hydrolytic process is the only one effective (Pringsheim, cited by Norman and Fuller in "Advances in Enzymology", 1942) giving cellobiose and glucose. This view has been supported recently by Simola (1931b).

Another theory has attracted interest at one time but seems to be rejected now; that was the oxydative

process postulated by Winogradsky (1929). The theory was that cellulose would be oxidized to oxycellulose; this was based on the fact that cellulose attacked by bacteria gave similar reactions to those of chemical oxycelluloses, except for the reducing power which was absent with biologically attacked cellulose.

Norman and Bartholomew (1940) have shown that this latter process was most improbable since the oxycelluloses, being insoluble in water, should accumulate; that the insoluble residues of cellulose decomposition had no reducing properties; and that the analysis of such residues point to the fact that the substances are microbial gums of polyuronide nature containing both uronic and pentose groupings. This conclusion supports the views expressed by Schreiner and Dawson (1927); these authors assume that after the breakdown of complex carbohydrates such as starch, gums and cellulose into their simpler monose derivatives by hydrolytic action, a very reactive compound is formed, the furan aldehyde hydroxymethylfurfural. This substance is in turn decomposed further, yielding equal parts of levulinic and formic acids on one hand and humus-like material on the other. The former compound involves the breakdown of the furan ring while the latter is either due to condensation of the intact furan aldehyde or to the condensation of highly reactive transition products formed on rupture of the ring and recondensing before the acids are formed. The building up of material

of lesser atomic weight to the level of humus-like material is so rapid that it has proved impossible to isolate any specific homogeneous intermediate product.

The above assumption points to the importance for the soil of the by products obtained in the decomposition of cellulose. A part of such products is turned back to the atmosphere as carbon dioxide while the other is intimately incorporated in the soil under the form of humus and contributes to the base exchange capacity of those soils. Immediately after the application of cellulose the effect is contrary to the plant growth in that the microorganisms compete with the roots for the available nitrogen in order to decompose the excess of carbonaceous material. This is, however, only temporary, and beneficial effects finally arise when the very readily available nitrogen is combined in the form of non-leaching proteins. The competitive activity may well be avoided if the cellulosic material is composted before application to the soil.

STARCH: Its presence in soils, decomposition and effects

The presence of this carbohydrate is more difficult to establish than is cellulose, and is certainly met with in much lower concentrations. It is known that starch is the plant's reserve material; it is present in the grains of cereals, seeds and tubers; in potatoes it forms 80 per cent of the dry matter.

There is at present no means of analysing starch with a very high degree of certitude from plant materials or organic matter. Waksman (1939) established the fraction in which this carbohydrate should be placed, namely, in the "dilute acid hydrolyzable constituents", but making it clear, as Norman (1939) indicated cautiously, that this fraction is neither homogeneous nor very clearly defined. It does not accurately represent the hemicellulose fraction of plants and will be of a different structure in decayed residues and parent materials.

We thus know that starch reaches the soil by way of a few plant residues, but we have no means of separating it from the hemicelluloses and polyuronides also present in the same fraction.

Reference to Table I shows the importance of the "hemicelluloses" fraction. As it must be assumed that part of the dilute acid hydrolysate is starch, we can conclude that some starch comes to the soil. The same assumption can be applied to the figures of Table II in which the substance under consideration is included under the heading "Pentosans".

This applies as well to the green manures, but is not true for stable manures. Starch being readily hydrolysed in the digestive tract of animals, it is not expected to be found of much importance in the manure. Furthermore, if some

starch happens to be left undigested, it will provide immediate food for very numerous microorganisms in the composting heaps.

Thus it may be assumed that starch certainly reaches the soil in quantities which cannot be evaluated on the single basis of proximate analysis.

There is no doubt about the hydrolytic breakdown of starch to maltose by means of the enzyme amylase and finally to glucose by maltase. From there on the further breakdown may be compared to that of cellulose, yielding either levulinic and formic acids or humus-like materials, through microbial protein.

The great majority of genera of heterotrophic bacteria include a few species that hydrolyse starch. Many non-specialized fungi too are strong amylolytic agents.

The particular group of microorganisms with which we are interested, the aerobic mesophilic cellulose decomposing bacteria, are for the most part starch hydrolysers. A more searching review of them will be done in a subsequent section.

CULTURE MEDIA USED FOR THE ISOLATION OF CELLULOSE DECOMPOSING BACTERIA

The very simplest concept originally controlled the selection of cellulose as carbon source for the isolation of bacteria decomposing this polysaccharide. Knowledge was needed about cellulose decomposition, the organisms were therefore grown on this substratum. As decomposition proceeded, the substance was used in elective anaerobic culture (Omeliansky 1902). Filter paper in a mineral salt solution was inoculated with mud or soil. Decomposition was active in two cultures called the hydrogen and the methane cultures because they respectively produced hydrogen and methane besides carbon dioxide. But it was very difficult to obtain pure cultures although seven or eight successive transfers were made to fresh media.

Omeliansky though, after microscopic observation, that the contaminations were so slight as to be disregarded. As a matter of fact, Kellerman and McBeth (1912) could isolate two species of cellulose destroying bacteria and five contaminating forms from the hydrogen culture; the methane culture gave one species of cellulose destroying bacteria and two contaminating forms. The three cellulose decomposing bacteria were more active under aerobic conditions.

van Iterson (1904) introduced the use of filter paper in Erlenmeyer flasks with a shallow layer of mineral solution and with successive transfers of pieces of the attacked paper to fresh culture media in flasks. This certainly was the first step in the isolation of aerobic forms and van Iterson probably came very close to obtaining a pure culture; there persisted, however, some bacteria which were not cellulose decomposers, as was revealed by plating on ordinary agar.

Christensen (1910) then devised a process of enrichment cultures in which the paper came in close contact with soil, in which it was moistened by the capillary absorption, in Erlenmeyer flasks. This showed evidence of cellulose decomposition but gave no help in isolating pure cultures.

Such was the state of affairs when Kellerman and McBeth (1912) set in an innovation which made easier the isolation of these organisms. These authors used cellulose precipitated by means of the cuproammonium reagent and subsequently incorporated in agar with a mineral salt solution. No other source of carbon than cellulose was present, except those found in the peptone or meat extract which have occasionally been used as source of nitrogen (Kellerman, McBeth, Scales and Smith, 1913). After enrichment cultures in flasks with filter paper circles bent in four folds, and arranged in such a way as to be completely buried in liquid to check the fungi, and transferred to

fresh identical culture media three or four times (McBeth and Scales 1913), the organisms were plated with dilutions on cellulose agar plates; the colonies growing on this substrate produced an enzymic clear zone on hydrolysing the cellulose. In carrying these bacteria on ordinary media some of the organisms lost their power of hydrolysing the cellulose. Omeliansky (1913) and Pringsheim (1920) raised the objection that the solubilizing of the calcium carbonate was really the cause of the assumed enzymic zone, but Löhnis and Lochhead (1923) found the answer in flooding the plates with hydrochloric acid to dissolve the carbonate. The cleared zone persisting after the treatment, they concluded that the cellulose was really attacked by the bacteria.

Kellerman et al., (1913) still had difficulties with the isolation on cellulose agar and they corrected this situation by plating on starch agar, claiming that only two organisms were not starch hydrolysers. Kellerman and McBeth (1912) already had isolated, on starch agar plates, two of the three forms found to decompose cellulose in Omeliansky cultures.

Hutchinson and Clayton (1919) used elective cultures similar to that of van Iterson without obtaining pure cultures. They then devised a method by which a sheet of filter paper was incorporated between two layers of agar, the top one being very shallow and carrying the inoculum. As this also

failed to produce pure cultures, they devised a method in which filter paper strips were set in tubes with a mineral salt solution in such a fashion as to have the strip of paper projecting over the surface of the liquid. With this last method they succeeded to isolate the first pure culture of Sporocytophaga myxococcoides, (Spirochaeta cytophaga).

Groenewege (1920) enriched his cultures with cellulose and isolated them on bouillon agar. Von Gescher (1922) used Kellerman and McBeth's cellulose agar plates and silica gel plates; he gives no details for the composition of the latter medium.

Gray and Chalmers (1924), using Hutchinson and Clayton's method, isolated in pure culture the cellulose hydrolysing and agar liquefying Vibrio agar-liquefaciens.

Bojanowsky (1925) perfected the silica jelly method for plates and used it for the isolation of cellulose bacteria. Silicic acid was permitted to harden in plates and then the mineral nutrients were added with finely precipitated cellulose evenly distributed on the surface of the plate. This method of culture was used also by Winogradsky (1929) and Imsenecki and Solntzeva (1936) for studies on cellulose bacteria.

Bojanowsky supported the view of Löhnis and Lochhead

in stating that the bacteria forming an enzymic ring were real cellulose hydrolysers because they could not grow with carbon dioxide and that the only carbon source in the silica gel plates was cellulose.

Dubos (1928), attempting to estimate the numbers of cellulose destroying bacteria in soil, established that the cellulose agar used by Kellerman et al. (1913) and others was not of advantage for his special purpose and also not in accordance with the aerobic character of the cellulose decomposing bacteria. The objections he made to this cultural method were that:

1. It is tedious to prepare.
2. It needs elective cultures which ruled out the process of enumeration.
3. It affords nearly anaerobic conditions in elective and plate cultures.

Against the silica gel he states that "the numbers of organisms developing on the plates do not bear any relation to the number of organisms present in the inoculum". He then utilized Hutchinson and Clayton's method of culture on pure cellulose, in tubes, with a strip of filter paper part of which projected over the surface of the liquid. Isolation of pure cultures was obtained by repeated transfers in tubes of fresh media, or occasionally by plating on starch agar. The mineral salts solution had a pH value of 7.3, thus checking the growth of fungi.

Kalnins (1930) used Hutchinson and Clayton's culture tubes for enrichment and Kellerman and others cellulose plates for isolation, with the difference that the cellulose was precipitated by treatment with sulphuric acid instead of the cuproammonium reagent.

Stapp and Bortels (1934) tried Christensen's method with no greater success than with the same combined with the Rossi-Cholodny method of buried slides to observe the soil microflora. Fungi developed so intensely as to mask the growth of bacteria, except in soils rich in bases. After comparative trials they used the cellulose agar plates for their isolations.

All of the workers who isolated cellulose bacteria used the same or slight modifications of the cellulose culture media described above.

Recently Fuller and Norman (1942) gave a description of a new technic in the isolation of cellulose bacteria. With the recent development in the chemistry of plant materials it has been established that pure cellulose does not frequently occur in nature, and that the cellulose fabric in plants is probably interwoven with amorphous systems composed of lignin and polyuronide hemicellulose. In order then to get nearer to natural conditions these two authors tried and succeeded in isolating cellulose

organisms by means of cellulose-dextrin agar. The dextrins, water soluble and insoluble dextrins, were prepared by prolonged cold hydrolysis of cellulose with sulphuric acid. Even the insoluble dextrin gave an enzymic halo and the organisms which caused it were cellulose decomposers. These culture media, the authors claim, are well suitable for isolation and enumeration purposes.

Starch has never been used as enrichment culture media, but a few authors, Kellerman and McBeth (1912), Kellerman et al., (1913), Dubos (1928) and Sniezko (1934) occasionally used it for isolation purposes. Platings from crude enrichment cultures may yield pure colonies of aerobic cellulose bacteria; Gray (1939) isolated Vibrio amylocella by this means.

ORGANISMS PREVIOUSLY FOUND

The object of the present study being only the aerobic mesophilic bacteria, there will be no mention made of the fungi, anaerobic or aerobic thermophilic bacteria, or actinomyces.

The organisms under consideration may be broadly divided in two groups. According to the term used by Winogradsky (1929) there is first the group of obligate cellulose decomposing microorganisms which can thrive only on cellulose as the source of carbon. Fuller and Norman (1943) use the expression "versatile" to designate the organisms of the second group which grow either on cellulose or on a wide variety of other carbohydrates. This latter group, which comprises an important number of the genera forming the cellulose decomposing microflora of the soil, as will be seen subsequently, are thought to grow at the expense of the complex constituents of the soil organic matter and to be able to decompose cellulose facultatively.

Considering the group of obligate cellulose decomposing bacteria we notice that the genera are few, though they are widely distributed in soils. The most important genus is the Cytophaga group. Until recently this group was stated to be composed of very strictly obligate cellulose decomposing

organisms, but Stanier (1942) has succeeded in making them grow on glucose and cellobiose sterilised by filtration. Furthermore he described some marine species which were far from being specialized in their use of carbohydrates. A short while later Fuller and Norman (1943) described species of Cytophaga that not only were not specialized but very rapidly lost the weak cellulose decomposing property which they exhibited at the time of their isolation. One species, Sporocytophaga congregata, which clearly belonged to this group by morphology, even showed no cellulose decomposition.

However, it may be well said that some species of the two genera of the Cytophaga group, Sporocytophaga and Cytophaga, can be considered as specialized organisms, obtainable by the usual process of isolation carried on in the studies of soil organisms decomposing cellulose. Up to the present time they have been isolated by only one means other than by paper either on silica gel plates, tubes or agar plates. This exceptional culture media is the water insoluble cellulose-dextrin as given by Fuller and Norman (1942). The organisms could not develop on the water soluble dextrin, but they produced a good growth and enzymic ring on the former.

Another genus of specialized cellulose-destroying organisms has been given by Winogradsky (1929); the Cell-falcicula of which three species are described. Information on these organisms is very scanty. They are quoted as not

growing on peptone, dextrose, starch, and gum agar. Their systematic position is doubtful; their erection to generic standing may be questioned.

Of the large group of Vibrio only one has been given as an obligate cellulose decomposer: Cellvibrio ochraceus. This organism seems to be identical in biochemical properties to the Cellfalcicula species, except for the pigment.

This closes the group of obligate cellulose decomposing bacteria. Their importance, apart from the Cytophaga, has never been questioned, and they have not been reported by any other worker than by their discoverer. This is not the case for the Cytophaga which seems to occur normally in soils of Europe, North America and Australia.

By far the most important group of cellulose decomposing bacteria is represented by versatile organisms. They attack a wide range of carbon and nitrogen sources, though not necessarily vigorously. In a rapid survey of the literature on these bacteria we will pick out those which did not grow on starch as this carbohydrate is the basic compound of the enrichment and isolation media used in the actual study.

Vibrio species have been described by Gray and Chalmers (1924), Dubos (1928), Kalnins (1930), Itano and Arakawa (1931), Stapp and Bortels (1934) and Gray (1939). From a group of 18 species all showed diastatic activity. Five strains not named by Jensen (1940) showed a similar aptitude.

Of the large group of Cellulomonas (26 species) which compose the studies of Rullmann (1898), Kellerman and McBeth (1912), Kellerman, McBeth, Scales and Smith (1913), McBeth and Scales (1913), McBeth (1916), Sack (1924) and Sanborn (1929), only one species, Cellulomonas acidula Kellerman et al., (1913), did not grow on starch. Those of Rullmann and Sack have not been fully described as regards their diastatic activity. The only Cellulomonas isolated from Quebec soils by Hamilton (1928) has not been thoroughly studied and can not be catalogued here as no species name has been given.

Of the ten species of Bacillus which have been reported in the studies of Kellerman and McBeth (1912), McBeth and Scales (1913), McBeth (1916), Kalnins (1930), Simola (1931), Zarembska (1936) and Fuller and Norman (1943a), one is given as not hydrolysing starch, Bacillus imminutus McBeth (1916). Two of Sack (1924) are not sufficiently described. Jensen (1940) also quoted two unidentified species as hydrolysing starch.

The situation is much similar in the Pseudomonas group. Of the three described by Kellerman et al (1913), McBeth and Scales (1913), McBeth (1916) and classified as such by Bergey 5th ed. (1939), and of the three given by Fuller and Norman (1943a), only the one of the later authors, Ps.erythra does not grow on starch, and seems to be an obligate cellulose and cellulose-dextrin decomposer.

Three species of unidentified Corynebacterium (Jensen 1940) are also starch hydrolysers.

One species of what appears to be a Vibrio and called Cytobacter polonicum by Gutgisser (1936), grew well on starch but the starch hydrolysis is not mentioned. The same applies to Cellulococcus albus given by Sack (1924).

The three strains of Bacterium cellaresolvens α, β and γ of Groenewege (1920), and Jensen's (1940) unidentified Bacterium R and Co are starch hydrolysers. The same applies to Achromobacter picrum recently described by Fuller and Norman (1943a) and which is closely related to Bacterium.

It should be noted that Bergey Manual 5th ed. (1939), reports under the genus Cellulomonas a good number of species originally described as Bacillus or Bacterium, and also that many organisms mentioned here are not to be found in the manual.

This concludes a general review of the bacteria reported to decompose cellulose in soils. The largest group of those organisms is composed of versatile organisms. As to rapidity of action it may be seen in the literature that the many versatile bacteria are not less active than the obligate forms. Both groups are widely distributed in soil and have been reported throughout the world. Some particular species have been repeatedly isolated as was seen

for the Sporocytophaga, and as McBeth and Scales (1913) reported in the case of Bact.flavigena and B.cytaseus.

The diastatic power is also a property of the majority of the versatile organisms above, and with this in mind, the author has undertaken the present study.

Quebec soils have been very little studied as to the microflora they contain. Cellulose decomposing bacteria have been reported only on very scarce occasions. Hamilton (1928) has described an organism active in cellulose decomposition but did not go further than the genus for the purpose of his study. The organism was a Cellulomonas, showing only few differences from Pseudomonas subcreta McBeth and Scales (1913). (Bergey et al., 1923, 1st ed., emended the name to Cellulomonas subcreta, but gives Pseudomonas in the 5th edition, 1939). Gray (1935) made a more searching investigation on the microflora of separate horizons. He ascertained of the presence of Cytophaga Hutchinsonii (now Sporocytophaga myxococcoides Stanier), and of other forms not isolated. In a more recent study Gray (1939) described Vibrio amylocella which had been isolated from a manured garden soil.

The present systematic study aims to report the presence of already known cellulose decomposers as well as to describe any new species which may be met with.

EXPERIMENTAL

METHODS

1. The soils, and methods of sampling

The following soils were sampled:

A. Forest, bush of pines, organic top layer, 3 inches from A^0, Senneville, Que.

B. idem, same profile, leached layer, 4 inches from top, Senneville, Que.

C. idem, same profile, B' horizon, 5 inches from top, Senneville, Que.

E. Clay ridge, eroded, Senneville, Que.

G. Muck soil, Macdonald College Farm, Que.

H. Experimental field, cultivated, Macdonald College Farm, Que.

I. Oats, not fertilized, DeBeaujeu clay loam, Les Cedres, Que.

J. Hay and alfalfa, fertilized, DeBeaujeu clay loam, Les Cedres, Que.

K. Sod, not fertilized, Vaudreuil loamy sand (organic), Cote St. Emmanuel, Que.

L. Potatoes, fertilized, Soulange fine sandy loam, St.Clet, Que.

M. Sod, not fertilized, Soulange fine sandy loam, St.Clet, Que.

N. Potatoes, fertilized, Grenville sandy Loam, St. Telesphore, Que.

O. Sod, not fertilized, Grenville sandy loam, St. Telesphore, Que.

To prepare the profile for sampling, the trowel or shovel was cleaned with paper and rags, washed and flamed with alcohol at each new profile. Samples were generally taken at three to

five inches from the top of the profile and special mention is made when this was not the case. One sample was taken of each profile. Samples were collected in small glass vials (15 x 75 mm.) previously plugged with cotton and sterilized. At each soil station, the requisite number of vials were forced horizontally, with a slight screwing motion, into undercut and well scraped profiles of soils in order to avoid contamination from surface dust. The bottles were then closed with sterile rubber stoppers, wrapped and taken to the laboratory.

2. Isolation of starch and cellulose hydrolysing bacteria

In order to obtain a dense growth of powerful starch hydrolysing organisms, elective cultures were set up by inoculating 50 ml. of a starch solution in 250 ml. Erlenmeyer flasks with approximately one gram of fresh soil. The medium contained starch 0.2%, ammonium chloride 0.2%. The mineral salt solution used throughout the course of the work was composed as follows (Kalnins 1930):

	per cent.
K^2HPO^4	0.1
$MgSO^4$	0.02
NaCl	0.01
$CaCl^2$	0.01
$FeCl^3$	0.002

After twenty-four hours of growth at 28°C, the elective cultures were plated out on starch agar. Peptone was used instead of ammonium chloride in the starch agar with the same mineral salts. The dilutions were made with a 3 mm. loop, transferring a loopful of the elective culture to a tube of melted agar and again a loopful from the latter to a second tube. Three such dilutions were made and the one in the last tube in duplicate. The plates prepared from the tubes were incubated at 28°C. After three to five days, when colonies had developed, those which seemed different were selected and transferred to grid-plates of starch agar. The bottom of a petri plate was divided, by means of wax pencil marks, into 16 squares of even dimensions, and marked for orientation. A sheet of filter paper marked in the same fashion and numbered from 1 to 16 permitted the localization of the different colonies without turning the plate upside down at every inoculation. This arrangement also insured a rapid means of identification of the different colonies. The grid plates were incubated 48 hours at 28°C and then all the colonies that developed were transferred to slants of starch agar of the same composition as that in the plates. After making sure that all the organisms had grown on the slants the grid-plates were flooded with an iodine solution, and the organisms which hydrolysed the starch were kept for tests on cellulose. Most of the organisms isolated in this manner were pure cultures.

The ability of these bacteria to decompose cellulose was ascertained in tubes containing the mineral salt solution, 0.2% peptone and a piece of filter paper which emerged over the surface of the solution.

The test for the hydrolysing power on starch gave 103 strains of which many were morphologically alike as judged by microscopic and macroscopic observations. Only thirteen strains of the large group of starch hydrolysers proved to be also cellulose decomposers.

3. Culture media and cultural methods

Since many of the strains isolated proved to grow very poorly in the presence of meat extract and of nitrate salts, the culture media used throughout the tests were supplied with nitrogen in the form of peptone. The basic mineral salt solution for most of the media was that used by Kalnins (1930). The different salts were kept in separate stock solutions at 100-fold concentrations. A brief description of the different culture media used is given below.

Starch broth and agar

Starch is mixed to a little cold distilled water, 25-30 ml., and then poured into boiling water to dissolve. The solution is boiled for approximately one minute when it becomes opalescent. Peptone and the mineral salt solution

are then added and the medium brought to final volume.

In the case of starch agar, 1.5% of agar is added to the starch broth.

Cellulose salts liquid medium

The nutrient solution is composed of 0.2% peptone and the required quantity of mineral salts in distilled water. It is poured in test tubes in 10 ml. quantities and a strip of filter paper (Whatman's No.41) is placed in the solution. The strips of filter paper are cut at such a length as to emerge over the surface of the liquid.

Cellulose agar

This culture medium consisted of finely precipitated cellulose incorporated into a peptone mineral salt agar.

The cellulose was precipitated with sulphuric acid, according to the method given by Kalnins (1930). Finely cut filter paper is dissolved for 20 seconds in slightly diluted sulphuric acid (100 ml. of concentrated sulphuric acid, sp.gr. 1.81, in 60 ml. of distilled water). Then the cellulose is reprecipitated in bringing the volume rapidly up to two liters. The operation is carried out in a two-liter Erlenmeyer flask. The cellulose is then allowed to settle overnight in a great volume of water, 10 to 15 liters. Continuing the operation, the supernatant liquid is siphoned off and the precipitate is filtered on a large Buchner

funnel with repeated washing, until all traces of acid are removed. The operation can be speeded up by neutralizing with N-10 sodium hydroxide and washing two or three times thereafter. The cellulose mat obtained on filtration is turned back in solution for storage and a very even precipitate can be obtained by active agitation with an egg-beater. Six grams of filter paper were used for the original precipitation and the precipitated cellulose was diluted to 800 ml. This gave an approximate concentration of 0.4% cellulose and also a very convenient density of fibers in the agar plates.

The agar concentration was lowered to 0.8% as this is advised for the studies of the Cytophaga group (Stapp and Bortels 1934, Stanier 1942), and it proved to be very valuable for the study of the strains in hand. It always favored larger colonies and more definite evidence of enzymic action.

Gelatin

Two formulae for gelatin were used. The common nutrient gelatin, with 120 gms of gelatin was first used, but as some strains were refractory to the presence of meat extract, it was decided to test for gelatin liquefaction in a medium containing gelatin 10%, starch 0.2%, peptone 0.2%, and the mineral salt solution.

Litmus milk

The dehydrated medium of the Difco Laboratories was used.

Nutrient broth and agar

The ordinary nutrient broth composed of meat extract 0.3%, and peptone 0.5%. Agar 1.5% was added to nutrient broth in order to obtain the nutrient agar.

Dextrose agar

This culture medium was composed of dextrose 0.5%, peptone 0.2%, and the mineral salt solution.

Carbohydrate broths

The different mono- and polysaccharides, alcohols and glucosides were added in concentrations of 0.5% to the basal mineral salt solution with peptone 0.2%. Phenol red in 0.024% concentrations was used as indicator.

Sterilization under pressure in the autoclave caused very little change in the pH, and it was assumed that the more sensitive sugars, as glucose, were not hydrolysed in presence of the mineral salts at a pH value of about 8.0.

The acid and gas production power of the organisms was tested on all of the following carbohydrates, alcohols and glucosides.

Arabinose	Lactose	Glycerol	Salicin
Rhamnose	Sucrose	Mannitol	Esculin
Xylose	Maltose	Sorbitol	
Glucose	Trehalose	Inositol	
Fructose	Cellobiose	Dulcitol	
Galactose	Raffinose		
Mannose	Melezitose		
	Starch		
	Inulin		
	Dextrin		
	Glycogen		

Medium for ammonia production

The test was carried on two-month old cultures in cellulose solution, and on 3- and 6-day-old cultures in urea broth containing urea 2% and yeastrel 0.3%.

Medium for nitrate reduction

Nitrite formation from nitrate was tested in 3- and 6-day-old cultures on starch mineral salt broth containing sodium nitrate 0.2% instead of peptone.

Medium for H^2S production

This test was carried on the Kligler iron agar produced by the Difco Laboratories.

Medium for indol production

The culture medium used here was a Difco product,

Bacto-Tryptone. The test for indol was done on 6-day-old cultures by means of Erlich's reagent.

Relation to free oxygen

The anaerobic faculty of the strains under observation was studied in 300 ml. bottles with 30 ml. of pyrogallol. The bottles were tightly stoppered with rubber stoppers and a tube of reduced methylene blue was introduced in each bottle. After 8 days incubation at 28°C, the bottles were opened for observation and all the tubes with methylene blue were still colorless. After exposure to atmospheric oxygen they all turned blue within a few minutes. It is assumed that the conditions of anaerobicity were realized in the bottles.

Staining methods

Cell morphology

Form and dimensions of the cells were studied on 48-hour-old (occasionally 24 hours) starch agar slants. The smears were fixed by flame and stained with aqueous crystal violet.

Spores

Spore location, form and dimensions were determined with starch agar cultures of different age because of the different ability of the strains to form spores more or less rapidly. One strain of Bacillus (Strain I7) even had

to be studied on nutrient agar because it produced only very few spores on starch agar, this in comparison with another Bacillus (Strain A2c1) which showed no spores after 3 weeks on nutrient agar.

The Dorner method for spores was tried on a few occasions and gave good results. But since this staining method is based on the acid-fast property of the spores, which are stained with carbol fuchsin and decolorized with acid alcohol, and since there is no counterstain for the vegetative part of the cell but a negative mount with nigrosin, it proved to give a picture far from the fact because of cells with heavy capsular material which was not covered by the nigrosin. Thus, some bacteria which had a terminal spore formation, showed a central arrangement. Only after very careful and lengthy observation could the slightly different shade of the cell, as compared to the capsular substance, be observed.

Following these observations the Ziehl-Neelsen method for acid-fast organisms was used throughout as it affords a counterstain, methylene blue, for the cell itself. Pre-treatment with chromic acid 0.5% (30 seconds) proved to increase the density of the absorption of the stain by the spores.

Gram stain

Hucker's modification was used in this case with Atkins sodium hydroxide iodine solution. The stain was done on 24-hour-old cultures on starch agar slants and repeated after 8 days of growth. In each case a 24-hour-old culture of B.subtilis was used as a control.

Flagella stain

The bacteria from a 24-hour-old culture on starch agar slant were allowed to swarm in weak suspension, for 15 minutes, in a few drops of distilled water. A loopful of the suspension was then delicately placed on a well roasted and cooled slide. From this loopful a smaller one (1 mm. loop) was transferred to a drop of distilled water placed immediately below. By this means many films may be prepared at once on one slide. Gray's (1926) method of staining flagella was used.

RESULTS

1. Starch hydrolysing bacteria

One hundred and three strains were isolated. Many of the organisms were alike on the basis of their colony formation and morphology.

Most of the cultures could be assigned to the genus Bacillus, some to Bacterium. A few Pseudomonas, two strains of Proactinomyces, and one Micrococcus were also isolated.

Only thirteen strains proved to be cellulose hydrolysers as well. These only were studied more in detail.

2. Cellulose and starch hydrolysing bacteria

The thirteen strains of starch and cellulose hydrolysing bacteria isolated, and which will be described at length below, can be placed in nine groups which present greatly different morphological and physiological characteristics.

The species concept adopted by Bergey's Manual (1939), has been followed in the course of identification and classification of these bacteria, considering more particularly the morphological characteristics, the form of colonies, and some biochemical properties, such as gelatin

liquefaction, nitrate reduction and preferential utilisation of some carbohydrates. Such characters are used in Bergey's Manual for subdividing the genera of organisms related to those here described.

On this basis nine species are described tentatively and distributed in three genera, namely, six Bacillus, two Vibrio, and one Bacterium. The latter genus has been adopted in preference to Cellulomonas as advocated by Bergey et al., since it is believed that a biochemical character should not be the base of genus differentiation. In presence of the increasing number of species of cellulose hydrolysing bacteria in the genera Vibrio, Pseudomonas and Bacillus, and with the delimitations of groups of bacteria with peculiar biochemical activities, the concept put forward by Bergey et al., would lead to an excessive formation of genera which may well add more confusion in the systematizing of bacteria. Even Bergey's Manual shows lack of constancy in holding that point of view since it does not give the genus status to Simola's (1931) Cellulobacillus, under which heading might have been classified the other Bacillus species reported as cellulose decomposers by Kellerman and McBeth (1912), McBeth and Scales (1913) and Kalnins (1930). This new genus would have been represented by at least five species to compare to Winogradsky's Cellvibrio and Cellfalcicula, adopted by Bergey's Manual, and

which have respectively four and three species.

a. Description of the strains

Vibrio perimastix, n. sp. (Plate I)

(from Greek, peri, around, and mastix, whip)

Motility: weak at 24 hours.

Flagella: 8 - 20, peritrichous.

Spores: no endospores.

Cell form: curved rods. 0.4 - 0.7μ X 1.4 - 5.6μ. Arranged singly.

Gram stain: negative.

Gelatin stab: no liquefaction; scanty plumose growth along the stab.

Agar colony: spreading, irregular, thin, bluish, flat, barely visible, butyrous. 8 - 12 mm. at 24 hours. Similar on starch and dextrose agar (Plate XVIII).

Agar slant: thin, colourless to grayish, scanty, curly, barely visible, shining, butyrous.

Nutrient broth: no apparent growth.

Potato: good growth, filiform, light yellow, shining.

Litmus milk: no change.

Indol: negative.

Nitrite: reduction of nitrate.

Ammonia: negative.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: slight acid from: arabinose, xylose, glucose fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, melezitose, starch, dextrin, glycogen, salicin and esculin.
No acid from: rhamnose, inulin, glycerol, mennitol, sorbitol, dulcitol and inositol.

Cellulose decomposition: paper is broken in 3 or 5 days with peptone nitrogen in culture tubes. With nitrate the action is delayed to 8 or 9 days.
On cellulose agar the hydrolytic power is only superficial and spreading. The fibrils are not lysed in the thickness of the agar (Plate XIV -2).

Vibrio hyperion n. sp. (Plate II)

(from the Greek Hyperion, God of the Sun. The allusion is to the flame-shaped arms projecting from the colonies.)

Motility: active.

Flagella: one, polar.

Spores: no endospores.

Cell form: short curved rods, with rounded ends. 0.4 - 0.7μ x 1.4 - 2.4μ. Some few long cells (4.2μ)

Gram stain: negative.

Gelatine stab: no liquefaction; scanty plumose growth along the stab.

Agar colony: on starch agar plates it forms colonies with the following characteristics: spreading, curly, irregularly raised, bluish gray, butyrous, shining. It shows motility of colonies by means of projecting arms; this is less visible on dextrose agar plates where only slight curling occurs. The colonies are yellowish gray.

Agar slant: growth good, filiform, shining, semi-opaque, yellowish gray, some few curls; yellowing of the agar in old cultures.

Nutrient broth: no apparent growth.

Potato: growth good, filiform, buff, shining.

Litmus milk: no change.

Indol: negative.

Nitrite: no reduction of nitrate.

Ammonia: negative.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: slight acid from: arabinose, xylose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, melezitose, starch, inulin, dextrin, glycogen, glycerol, salicin, esculin.

No acid from: rhamnose, mannitol, sorbitol, dulcitol, inositol.

Cellulose decomposition: the filter paper is broken in 5 or 7 days with peptone and a slight yellow pigment is produced on the paper above the liquid. The liquid is turned yellow in old cultures. With nitrate nitrogen, the paper is not broken before three weeks or even longer.
It produces well defined enzymic zones on cellulose agar plates (Plate XIII - 1).

Bacterium infirme n. sp. (Plate III)

(from Latin: infirmus, weak; the allusion is to its action on cellulose).

Motility: active.

Flagella: 5 - 15, peritrichous..

Spores: no endospores.

Cell form: straight rods with rounded ends. Some few curved rods. 0.5 - 0.7 μ x 1.7 - 2.8 μ. Some long cells up to 6.3 μ. Single or paired.

Gram stain: negative.

Gelatin stab: no liquefaction; gas formation in starch peptone gelatin.

Agar colony: 3 - 4 mm. whitish, circular, regular, umbonate to slightly convex, shining, translucent, butyrous, embedded colonies are lens-shaped. The same applies to the dextrose agar colonies, but these are smaller.

Agar slant: growth good, filiform, translucent, grayish, butyrous; occasionally with gas in the butt.

Nutrient broth: turbid, with white slimy sediment.

Potato: growth scanty, filiform, buff, shining, dull white border.

Litmus milk: acid with sediment.

Indol: negative.

Nitrite: no reduction of nitrate.

Ammonia: negative.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: Acid and gas from: inulin, mannose, starch, glycogen and melezitose.

Acid, no gas from: arabinose, xylose, glucose, fructose, galactose, lactose, sucrose, maltose, trehalose, raffinose, dextrin, glycerol, mannitol, salicin, and esculin.

Slight acid and no gas from: rhamnose, cellobiose, sorbitol, dulcitol and inositol.

Cellulose decomposition: filter paper strips broken after 17 - 19 days with peptone and 30 days with nitrate in tubes with mineral salt solution. No perceptible decomposition of cellulose in cellulose agar plates.

Bacillus paulatim n. sp. (Plate IV)

(from Latin: paulatim, little by little; the allusion is to its relatively slow action on cellulose).

Motility: very active.

Flagella: 5 - 15, peritrichous..

Spores: present, 0.8 - 1.0μ x 1.0 - 1.7μ; central to subcentral.

Sporangia: swollen medially.

Cell form: straight rods with rounded ends. 0.7 - 0.8μx 1.4 - 3.5μ; single or in pairs.

Gram stain: negative.

Gelatin stab: liquefaction infundibuliform.

Agar colony: 2 - 4 mm., circular, white, center denser, entire, convex, shining, viscid, opaque. Embedded colonies are lens-shaped.

Agar slant: growth good, filiform, border translucent, center yellowish-white, shining, butyrous.

Nutrient broth: slightly turbid, scanty viscid white sediment.

Potato: growth good, filiform, light yellow, shining.

Litmus milk: slightly acid.

Indol: negative.

Nitrite: no reduction of nitrate.

Ammonia: slight from old cultures (two months) in cellulose peptone solution; none from urea broth.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: Acid from: arabinose, xylose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, starch, inulin, dextrin, glycogen, glycerol, mannitol, salicin and esculin.

Slight acid from: melezitose, dulcitol and inositol.

No acid from: rhamnose, and sorbitol.

Cellulose decomposition: filter paper is broken in 9 - 12 days in cellulose peptone mineral salt medium. It produces no perceptible decomposition on cellulose agar plates (Plate XV - 1).

Bacillus vagans n. sp. (Plate V)

(From Latin: vagans, wandering; the allusion is to the spreading colonies).

Motility: very active.

Flagella: 5 - 15, peritrichous.

Spores: present, 0.8 - 0.9μx 1.4μ, central to subcentral.

Sporangia: slightly swollen medially.

Cell form: straight rods, with rounded ends. 0.7 - 0.8μx 1.4 - 4.2μ, single or in pairs.

Gram stain: negative.

Gelatin stab: liquefaction infundibuliform.

Agar colony: 2-3 mm. circular, regular, bluish, translucent, shining, slightly convex, butyrous. Embedded colonies are lens-shaped.

Agar slant: growth good, filiform, translucent all over, grayish, shining, gummy.

Nutrient broth: slightly turbid with scanty white viscid sediment.

Potato: growth restricted, filiform, light yellow, shining.

Litmus milk: slightly acid.

Indol: negative.

Nitrite: no nitrate reduction.

Ammonia: slight production from old cultures on cellulose peptone mineral salt solution; none from urea broth.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: Acid from: arabinose, xylose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, starch, inulin, dextrin, glycogen, glycerol, mannitol,

salicin and esculin.

Slight acid from: melezitose, dulcitol, and inositol.

No acid from: rhamnose, and sorbitol.

Cellulose decomposition: filter paper broken in 8 - 10 days in tubes. Spreading colonies on cellulose agar plates. The cellulose is hydrolysed without clear enzymic zones (Plate XIV - 1).

Bacillus Kellermani n.sp. (Strains O-1 and M-3) (Plate VI)

(Latinised name of Kellerman, in honor of Dr. Karl F.Kellermen's work on this group of bacteria).

Motility: active.

Flagella: 5 - 15, peritrichous.

Spores: present, 0.8 - 1.0μ x 1.7μ, central to subcentral.

Sporangia: slightly swollen medially.

Cell form: straight rods with squared ends. 0.7 - 0.8μ x 1.0 - 3.5μ, single or in pairs.

Gram stain: negative.

Gelatin stab: liquefaction stratiform.

Agar colonies: 2.5 - 3.5 mm. white, irregularly round, entire, umbilicate, shining, gummy. Embedded colonies, lens-shaped.

Agar slant: growth good, filiform, opaque to semi-opaque, undulating area, creamy to whitish, shining, gummy.

Nutrient broth: slightly turbid, white sediment.

Potato: growth good, white, dull, filiform, depression at line of inoculation.

Litmus milk: slightly acid, with sediment.

Indol: negative.

Nitrite: no nitrate reduction.

Ammonia: slight production in old cellulose peptone solution; none from urea broth.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: Acid, no gas from: arabinose, xylose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, starch, inulin, dextrin, glycogen, glycerol, mannitol, salicin, and esculin.

Slight acid from: melezitose, and inositol.

No acid from: rhamnose, sorbitol, dulcitol.

Cellulose decomposition: filter paper broken in 7 days with peptone and in 16 or 21 days with nitrate in cellulose solution. No apparent cellulose hydrolysis on cellulose agar plates (Plate XV-2).

Bacillus soli n. sp. (Plate VII)

(From Latin solum, the soil)

Motility: active.

Flagella: 4 - 12, peritrichous..

Spores: present, 0.8 - 1.0μ x 1.4 - 1.7μ, terminal.

Sporangia: swollen terminally, clavate.

Cell form: straight and a few curved rods, rounded ends. 0.7 - 0.8μ x 1.4 - 3.5μ, single cells.

Gram stain: negative.

Gelatin stab: no liquefaction; the gel is broken with gas bubbles in starch gelatine tubes.

Agar colonies: 2 - 5 mm. irregularly round grayish white to white, convex, shining translucent, butyrous. Embedded colonies are lens-shaped.

Agar slant: growth scanty, filiform, grayish, translucent, butyrous, gas in the butt.

Nutrient broth: turbid with white slimy sediment.

Potato: growth scanty, filiform, buff, shining, dull white border.

Litmus milk: reduced.

Indol: negative.

Nitrite: no reduction of nitrate.

Ammonia: negative.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Butylene glycol: positive, 2 - 3 percent in 15 percent corn meal mash. (Analysis done by Mr. M. Boss of the Chemistry Department.)

Carbohydrates: Acid and gas from: arabinose, xylose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, melezitose, starch, inulin, dextrin, glycogen, glycerol, mannitol, salicin, and esculin. Slight acid and no gas from: rhamnose, sorbitol, dulcitol and inositol.

Cellulose decomposition: filter paper broken in 21 days with peptone and in 30 days with nitrate in cellulose liquid medium. No enzymic ring on cellulose agar plates.

Bacillus torquens n. sp. (Strains M-2, M-10, M-14) (Plates VIII, IX, X)

(From Latin, torquere, to twist; in allusion to the manner of growth on agar plates)

Motility: active.

Flagella: 4 - 16, peritrichous.

Spores: present, 1.4μ x 2.1 - 2.8μ, disposed terminally or subterminally in normal cells in cellulose cultures; in the clostridial forms on starch, they are central to subcentral.

Sporangia: terminally swollen in cellulose cultures; on starch agar it forms either a clostridium or a

greatly swollen cell which immediately produces the spore or shortens to a large coccoid form in which the spore arises.

Cell form: short curved and straight rods, 0.5 - 0.7μ x 1.4 - 2.1μ. On starch agar, the young cells are short and curved; they gradually swell to form either straight or curved clostridial forms with a body staining darkly at one end. These clostridial forms seem to shorten with the gradual increase in size of the body to form finally a very stout clostridium or a spherical sporocyst from which the spores arise. The clostridium measures 0.8 - 1.4μx 2.1 - 4.9μ. The sporocysts measure 1.4 - 2.1μin diameter. The ordinary cells on cellulose are single.

Gram stain: negative.

Gelatine stab: no liquefaction; slight plumose growth along the stab.

Agar colonies: motile by means of narrow threads projected as far as 25 mm. from the mother colony; there, a new colony forms with a curly appearance. Grayish (bluish iridescent on dextrose agar), butyrous, irregularly raised, shining. Embedded colonies are lens-shaped and yellowish white (Plate XVI - 1, 2, and 3).

Agar slant: growth good, filiform, with occasional curling, semi-opaque, yellowish gray, shining, butyrous when young and becoming slimy; the agar is yellowed in old slants.

Nutrient broth: no apparent growth.

Potato: growth good, buff, filiform, shining.

Litmus milk: no change.

Indol: negative.

Nitrite: no reduction of nitrate.

Ammonia: negative.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: Slight acid from: arabinose, rhamnose, xylose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, melezitose, starch, inulin, dextrin, glycogen, glycerol, salicin, and esculin.
No acid from: mannitol, sorbitol, dulcitol and inositol.

Cellulose decomposition: paper broken in 4 or 7 days in cellulose mineral salts solution in tubes with peptone and not with nitrate. Slight yellowing of the paper above the surface of the liquid is occasionally noticed in the peptone

medium and the liquid takes a yellow color when old (one month). In cellulose agar plates, a well defined enzymic zone is produced; the growth does not spread much; the fibers are completely lysed and are replaced by masses of bacterial cells (Plate XIII - 2, 3, and 4).

Bacillus effluens n. sp. (Strains N-3 and N-10) (Plates XI, XII)

(From Latin, effluere, to flow outwards; in allusion to the swarming outgrowths in colonies).

Motility: active.

Flagella: 5 - 16, peritrichous.

Spores: present, 1.1 - 1.4 μ x 2.1 - 2.4 μ, terminal to subterminal in cellulose cultures. On starch agar, it produces clostridial forms with dark stainable end bodies, but the spore formation is either terminal or subterminal.

Sporangia: swollen terminally in normal cells; in the clostridial forms all the cell is swollen. No microcysts have been observed.

Cell form: curved rods, with rounded ends. On starch agar the cells increase gradually to a large curved or straight cell with a polar body more intensely stained. Sporocysts were not formed.

Rods: 0.5 - 0.8μ x 1.4 - 4.2μ; clestridia: 0.9 - 1.4μ x 2.8 - 5.6μ. The cells are arranged singly.

Gram stain: negative.

Gelatin: no liquefaction; scanty plumose growth along the stab.

Agar colonies: <u>on starch agar</u>: 1.0 - 4.0 mm. regular, convex, shining, gummy, with occasional swarming of the colonies. Embedded colonies are lens-shaped and triangular.

<u>On dextrose agar</u>: intense swarming with one strain (N-10), less with the other. Yellowish-white, gummy; arms projecting to 25 mm. from the center of origin (Plate XVII - 1 and 2).

Agar slant: growth good, grayish, light yellow tinge when older, beaded to filiform, margin occasionally curly, opaque, gummy, firmly adherent to the agar.

Nutrient broth: no apparent growth.

Potato: growth scanty, light yellow, shining.

Litmus milk: no change.

Indol: negative.

Nitrite: nitrates are reduced to nitrites.

Ammonia: negative.

H^2S: negative.

Facultative aerobic.

Diastase: positive.

Carbohydrates: Slight acid from: arabinose, xylose, glucose, fructose, galactose, mannose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, melezitose, starch, dextrin, glycogen, salicin and esculin.

No acid from: rhamnose, inulin, glycerol, mannitol, sorbitol, dulcitol, and inositol.

Cellulose decomposition: filter paper is broken in 7 or 10 days with peptone and the action is delayed to two weeks with nitrate, in cellulose mineral salts medium. On cellulose agar plates the growth is spreading and does not produce sharply defined enzymic zones (Plate XIV - 3 and 4).

b. Discussion on the systematic relationship

Comparing the organisms one by one to allied forms it is first seen that Vibrio perimastix is unlike Gray and Chalmer's Vibrio agar-liquefaciens in that it does not liquefy the agar and presents a peritrichous flagellation. One such organism has been reported by Kalnins (1930), V.pericoma, but the colony formation and the growth in nutrient broth are different. Kalnin's organism too has a

peritrichous flagellation in only 10 per cent of cells and produces small quantities of ammonia, while V.perimastix produces no ammonia and the flagella are peritrichous in one hundred percent of the cells.

Vibrio hyperion is related to only one organism classified in Bergey's Manual: V.cyclosites Gray and Thornton, but the latter differs in that it did not decompose cellulose, caused a turbid growth in nutrient broth, and did not hydrolyse starch. These characters are different from those reported for the previous species. Two bacteria isolated by Kalnins, and not given in Bergey, V.rigensis and V.ranicula seem to be a trifle stouter than V.hyperion. The colony formation on starch agar, the behavior in nutrient broth and the acid production from certain carbohydrates are different for the two organisms of Kalnins as compared to the one described here. One more difference for each of Kalnins bacteria is that V.rigensis is less active in cellulose decomposition with organic sources of nitrogen, which is not the case for V.hyperion as the latter preferentially uses peptone, and shows pleomorphic forms and greatly delayed action with a mineral nitrogen source; V.ranicula produces a yellow pigment which spreads on the paper and gives a yellowish color to the liquid while V.hyperion, which causes a yellowish color in the liquid, do not exhibit a spreading

yellow color on the paper, but just a faint light yellow band (1 or 2 mm.) at the surface of the liquid. This yellow color appears only when peptone is used as source of nitrogen.

The use of Bacterium instead of Cellulomonas as a generic name has already been discussed. Considering the large group of Cellulomonas, Bacterium infirme comes close to McBeth's Cellulomonas albida and Cellulomonas alma, but differs from these in that it produces acid and gas from some carbohydrates, which is not the case for McBeth's organisms. A further difference is the very slow action of Bact.infirme on the cellulose; the breakdown of the paper is delayed to 17 or 19 days with peptone and up to 30 days with nitrate. It produces no enzymic zone in cellulose agar plates. Both organisms of McBeth produce an enzymic ring on cellulose agar and their action on filter paper is more rapid.

The three species Bacillus paulatim, Bacillus vagans, and Bacillus Kellermani can be classified in Bergey's Manual with Bacillus amylolyticus Kellerman and McBeth, which liquefies gelatin but not with Bacillus cytaseus McBeth and Scales, which does not liquefy gelatin. It is apparent that the Bacillus species described above are different from each other on the grounds of morphology, colony formation on starch and cellulose agar, utilization of carbohydrates and

gelatin liquefaction. Bacillus paulatim and Bacillus vagans form spores that are more stout and sometimes nearly spherical; the liquefaction of gelatin is crateriform to infundibuliform. In comparison B.amylolyticus has ovoid spores which are longer and never nearly spherical; furthermore the latter does not grow on potato, while the two former produce a light yellow filiform growth on this medium.

The difference is less pronounced between B.Kellermani and B.amylolyticus. Kellerman and McBeth (1912) report that the sporangium is swollen to twice the breadth of a normal cell; this is not stated in Bergey's Manual. This does not occur with B.Kellermani which is only very slightly swollen. This species forms a whitish slimy growth on potato slants, and the cells from such cultures are swollen and intensely pleomorphic, while B.amylolyticus showed no growth on this culture medium. Another difference is found in their behavior on cellulose agar plates, in which B.amylolyticus produces an enzymic ring, while the two strains of B.Kellermani do not. As a matter of fact, three-old cultures of the latter on cellulose agar plates not show any apparent cellulose hydrolysis while at the time the organisms were just as active in paper in tubes with peptone nitrogen and mineral salt as they were at isolation.

The gas forming Bacillus soli is closely related to

B.macerans Schardinger given by Bergey's Manual in the species of Aerobacillus. Differences from the latter which are taken here to make a species of the former are found in the smaller size of its spores, its restricted growth on potato slants, its inability to reduce nitrates to nitrites and to form gas with rhamnose and sorbitol. B.macerans is reported to be aerobic; B. soli is a facultative aerobe and also shows a high proportion of curved rods on starch agar.

The last group to be considered in this discussion is that of Bacillus torquens and Bacillus effluens; since both are morphologically alike their systematic position may well be considered at the same time. Apparently both are related to Simola's Cellulobacillus. Bergey's Manual, 5th ed., briefly notes that the name given by Simola is a synonym of the generic name Bacillus and does not report the two species in the text. Fuller and Norman (1943a) also reported an organism, Bacillus aporrheus, which shows similar properties to those described by Simola and to the two species isolated in the present study. The dominant characteristic of this particular group of Bacillus species is the formation by all, except one of Simola's, of deeply stained bodies at one or both ends of swollen cells. On morphological grounds a division can be established between Simola's and Fuller and Norman's organisms in that those of Simola have spores placed

medially while the spores of Bacillus aporrheus Fuller and Norman, are terminal. It should be noticed though that Simola followed the spore formation on glucose salt solution where he reports the "clostridium-like" spore-formation. Bacillus torquens, described in the present work, behaves similarly on starch and glucose mineral salt agar plates and shows spore formation in a "clostridium-like" cell which may shorten and resemble a sporocyst. (Plates VIII, IX and X). When grown on cellulose, however, either liquid of solid media, the clostridial cells with deeply stained end-bodies did not appear and the spore formation was terminal or subterminal with the sporangia swollen terminally or subterminally.

Considering the other morphological characteristics, B.torquens was closely related to Simola's Cellulobacillus species. There are no major differences between the strains of B.torquens, though they do show different ability to form sporocysts, strain M-14 being the more able, followed by M-2 and M-10. Bacillus torquens has been differentiated from Cellulobacillus myxogenes in that it grows on potato and is an obligate aerobe. Neither Cellulobacillus species produces acid from rhamnose, inulin, and glycerol, while B.torquens produces slight acidity from all three. Since the latter exhibits only a very restricted growth on nutrient agar, it is still quite different from Cellulo-bacillus mucosus.

The other group, represented by the two strains of Bacillus effluens, is different from B.torquens in that the cells are longer and also in that the spore formation is dominantly terminal and subterminal in the "clostridium-like" cells, with intensely stainable terminal bodies.

No major differences could be noticed that would permit the differentiation of the two strains in two species. Of the two isolates one (N-3) decomposed cellulose a little more actively than the other.

Bacillus effluens is closely related to Fuller and Norman's B.aporrheus. The special morphological characters on starch agar have not been reported by Fuller and Norman (1943a) in the description of their organism, but a personal communication from Dr. Fuller, together with observations made on a strain obtained from him, confirmed the fact that both bacteria were much alike on morphological grounds. Tentative species differentiation is based on the fact that B.effluens produces slight acidity from lactose and that it is facultatively aerobic.

SOURCES OF THE STRAINS

The top organic layer of the soil under forest (Sample A) gave rise to the species B.paulatim and B.vagans, while the lower horizons (samples B and C) of the same profile gave no cellulose decomposers. No strains were isolated from the eroded clay ridge (sample E) in the same locality.

The species V.perimastix and Bact.infirme were found in the muck soil on Macdonald College Farm (sample G). Another sample of mineral soil (sample H) from the College Farm gave no cellulose decomposing organisms.

B.soli was obtained from a sample of DeBeaujeu clay loam (sample I) in a field under oats and not fertilized, while a sample of the same soil fertilized and under hay and alfalfa gave no cellulose decomposing organisms(sample J).

No strains were obtained from a sample in sod, not fertilized, on organic Vaudreuil loamy sand (sample K).

Three species, totalizing five strains, were isolated from a sod on unfertilized Soulange fine sandy loam (sample M). They are, namely, V.hyperion, B.Kellermani (strain M 3), and the three strains of B.torquens. No cellulose organisms could be isolated from a sample of the same soil (sample L), which had grown potatoes and had been fertilized.

The two strains of B.effluens were isolated from a sample of fertilized Grenville sandy loam under potatoes (sample N), B.Kellermani (strain 0) was isolated from the same soil under sod and not fertilized (sample 0). The following are the soil samples yielding species of the bacteria:-

A. Forest, A^0 horizon - B.paulatim, B.vagans.

G. Muck soil - V.perimastix, Bact.infirme.

I. Oats, not fertilized - B.soli.

M. Sod, not fertilized - B.torquens, B.Kellermani and V.hyperion.

N. Potatoes, fertilized - B.effluens.

O. Sod, not fertilized - B.Kellermani.

The remaining soil samples did not yield any strain of cellulose decomposing bacteria.

PHYSIOLOGICAL ACTIVITIES

Starch hydrolysis

To test the power of the organisms described above, to cause starch hydrolysis, starch peptone agar plates were inoculated at five different points by touching delicately to the surface of the agar with a needle charged with organisms from a 24-hour-old culture on starch agar slants. The plates were incubated at 28°C for 16 hours and then an iodine solution was poured on the surface of the agar. Starch hydrolysis was indicated by a colorless area around the colony against the deep blue color assumed by the rest of the plate. The results are given in Table V.

This test shows that all of the isolated bacteria have the ability to hydrolyse starch. It should be noticed that the stronger cellulose hydrolysers, V.perimastix, V.hyperion, B.torquens and B.effluens exhibit a slower activity on starch. The same observation was made on the carbohydrates tested and reported in the descriptions above. This may indicate that the very active cellulose hydrolysers, which are versatile, exhibit that property only as a means of survival when the cellulosic material is lacking, participating thus in the ultimate breakdown of by-products of their activity on cellulose.

Table V - Comparative starch hydrolysis in starch agar plates

Organisms	Diameter of hydrolysed area	Completeness of hydrolysis
V.perimastix	Restricted to the irregular growth which spreads over 8 - 12 mm.	Clear
V.hyperion	8 - 10 mm.	¾ clear and ¼ light blue
Bact.infirme	10 - 11 mm.	All clear
B.paulatim	7 - 9 mm.	All clear
B.vagans	7 - 10 mm.	All clear
B.Kellermani	10 - 12 mm.	All clear
B.soli	7 - 10 mm.	All clear
B.torquens	10 - 12 mm.	Clear only where there was growth, 4 - 6 mm. The rest with a light blue tinge.
B.effluens	Restricted to the growth 3 mm.	All clear

The reverse may not be true for the less active bacteria, because it has been seen that cellulose is never added in the pure state to the soil when it originates from plant residues or manures, as is the case for agricultural soils. These organisms would then probably be able to maintain life on the substances accompanying the cellulose, namely, the cold water soluble and the cold acid hydrolysable fractions.

Cellulose decomposition

A test has been carried out to judge the preference of the bacteria towards nitrogen sources. Two organic nitrogenous compounds, peptone and asparagine, and two mineral nitrogenous salts, sodium nitrate and ammonium sulphate, were used. 50 ml. portions of culture media containing the mineral salts and 0.2 percent each of the different sources of nitrogen were distributed in 500 ml. Erlenmeyer flasks. A weighed circle of filter paper (Whatman's No.41) was then folded in such a way as to stand upright and introduced into the flasks. These flasks were sterilized by autoclave and then inoculated with the organisms. After incubation for 21 days at 26°C, the cultures were thoroughly shaken with added distilled water and filtered on a Buchner funnel with a weighed filter paper. The amount decomposed was worked out by difference in original and final weight. The figures are reported in Table VI.

Plate VI - Per cent cellulose decomposed with different sources of nitrogen:- Incubated 21 days at 26°C

Organism	Peptone	Asparagine	Sodium nitrate	Ammonium sulphate
V.perimastix	4.4 3.4	5.5 5.3	5.7 7.1	11.0# 10.9#
V.hyperion	8.3 inf. 10.9	1.6 1.4	1.5 inf. 0.8	1.6 0.8
Bacillus vagans	5.6 7.4	6.3 9.4	1.2 1.5	1.1 2.5
Bacillus soli	inf. inf.	1.2 inf.	0.8 2.0	0.6 1.9
Bacillus Kellermani (strain M 3)	11.2 10.1	1.9 inf.	1.5 1.8	2.2 1.6
Bacillus torquens (strain M 10)	10.8 11.7 inf.	0.6 1.1	1.4 1.4	2.1 2.3
Bacillus effluens (strain N 3)	2.9 5.3 inf.	6.1 5.6	4.8 6.7	14.0# 14.7#
Bacillus effluens (strain N 10)	3.6 3.3	4.0 0.5	2.2 3.6 inf.	9.9# 9.9#
Control	2.3 1.0	inf. 0.2	2.5 1.4	1.4 2.9

Note: Inf. = infected by colonies of mold.

\# = presence of reducing substances.

From the above results it is evident that peptone is preferred as the source of nitrogen, even if not used with the greatest efficiency.

Some of the differences and similarities advocated for in the discussion on the species differentiation are clearly visible here. Such is the case of V.ranicula Kalnins which was less active in the cellulose decomposition with organic nitrogen as compared to V.hyperion which causes no cellulose decomposition if grown on other nitrogen source than peptone.

The similarities between Simola's Cellulobacillus species and B.torquens is also well defined here as both groups of organisms need peptone to cause the desintegration of filter paper. Reducing substances were not found in cultures of B.torquens; Simola (1930-II) detected cellobiose and glucose from his organisms.

Only two organisms, V.perimastix and B.effluens were active with all four sources of nitrogen, and their greatest activity was shown with ammonium sulphate. In this solution they lowered the pH value from 8.0 to 5.0, as determined by the colorimetric test with B.D.H. Universal Indicator. Strangely enough, reducing substances were noted only with this source of nitrogen. No further tests were carried on to identify these substances, which were present

only weakly in cultures of V.perimastix and strain N - 10 of Bacillus effluens, but very intensely with strain N - 3 of the same Bacillus, in spite of the dilution of the fraction on which the Fehling's test was made. Microscopic examination of the three cultures giving positive reaction to reducing substances, showed that no bacterial contaminants were present.

CONCLUSIONS

From the results of this study, it is evident that starch is well appropriate for the isolation of cellulose organisms. If not all cultures were pure at the first isolation, it was relatively easy to purify them definitely by means of a second plating with dilution. Difficulties were met with only in the purification of *B.torquens* as it was thought that the slimy cultures would carry in the dilution some cells of *V.hyperion* to which it is morphologically very similar in young cultures, and in physiological characteristics when older. Plating on starch agar of dilutions in distilled water after treatment for spores (10 or 12 minutes heating at 80°C) finally gave a pure culture. No other difficulties were met with in the isolation.

As was expected, no specialized cellulose decomposing organisms were found, but the organisms isolated exhibit a great variety in their activities, some breaking the filter paper strip in mineral salt solution in 3 or 5 days as was the case with *V.perimastix*, and *B.torquens*, while some others like *Bact.infirme* and *Bacillus soli* developed that far only after three weeks. The fact also that the organisms isolated on starch and described as cellulose decomposers grow on many carbohydrates indicates that the versatile cellulose

hydrolysing microflora is well distributed and can be isolated by means of some of the elements on which it thrives in the soils.

It has already been indicated in the discussion on the starch hydrolysing powers of these organisms, that this ability shown by the cellulose decomposing bacteria may well be only one of transition as it has been demonstrated that the most active cellulose decomposers were the weaker starch hydrolysers.

It may be said against the use of starch in the isolation of cellulose decomposing bacteria that the scheme of isolation is long and furthermore that carrying the stock cultures on starch will cause the organisms to loose their ability to decompose cellulose. To the first objection it will be stated that the procedure is not longer than any other since pure cultures are very readily obtained. But what is likely to happen, in the elective cultures, is that the active cellulose decomposing bacteria having a weaker diastatic power, as was shown above, may be ruled out in numbers by the strongly diastatic bacteria of weaker activity in the cellulose breakdown. This may well be the limiting condition of this method. As to the loss of activity of the bacteria when incessantly grown on starch, Simola (1931) attributed this phenomenon to the fact that mineral and not organic nitrogen was fed to the bacteria.

The organisms described in the present work have been grown on starch peptone mineral salt agar slants and apparently they are as active in the cellulose decomposition as they were at the time of their isolation, a year and a half ago.

It may also be advocated in favor of the use of starch that its composition is more uniform than that of the fractions obtained by the hydrolysis of cellulose by strong acids.

This method is not devised for enumeration purposes as such, because it needs an elective culture and because also of the embedded colonies which, though left unconsidered, may be composed of very active organisms.

It may be claimed that the object of the present work has been attained, since it had in view the isolation of cellulose decomposing bacteria from Quebec soils by means of the diastatic activity of these microorganisms. V.amylocella (Gray 1939) is the only described cellulose organism previously isolated from the soils of Quebec.

The Bacillus group is reported officially here for the first time for Quebec soils, though some previous investigations with Quebec soils have shown that species of this genus capable of decomposing cellulose were present; the present work, indeed, has been based upon those findings.

SUMMARY

A new approach to the isolation of cellulose bacteria from soils is described. Soil is inoculated into a starch medium; subsequent plating with starch agar causes the diastatic bacteria to emerge; these are tested for their cellulose hydrolysing power after their diastatic power has been ascertained. Thirteen samples of Quebec soils were studied for this purpose.

Thirteen strains of bacteria have been isolated and allocated to three recognized genera. Nine species have been formed, two belonging to the genus Vibrio, one to the genus Bacterium, and six to the genus Bacillus. All of the strains are shown to be facultative cellulose decomposers as they often grow better on simpler carbohydrates than on cellulose. The organisms are described under the new species names of Vibrio perimastix, Vibrio hyperion, Bacterium infirme, Bacillus paulatim, Bacillus vagans, Bacillus Kellermani, Bacillus soli, Bacillus torquens and Bacillus effluens.

The systematic relationship of the organisms are studied and their elevation to the species advocated on the basis of differentiations used in Bergey's Manual.

The bacteria isolated showed a tendency to the facultative

use of different carbohydrates in a way apparently inversely proportional to their activity in cellulose decomposition.

All of the strains preferred peptone to mineral salts as a source of nitrogen in cellulose decomposition. Two organisms, V.perimastix and Bacillus effluens decomposed cellulose more energetically, with evidence of reducing substances, with ammonium sulphate as the source of nitrogen.

It is realized that the method as used is not suitable for enumeration of cellulose bacteria, since some very active cellulose decomposing organisms may escape notice; it does, however, secure a safe and rapid means of isolating important members of the versatile microflora attacking cellulose.

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PLATE I