THE USE OF THE SILICA GEL PLATE FOR DEMONSTRATING THE OCCURRENCE AND ABUNDANCE OF CELLULOSE-DECOMPOSING BACTERIA

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The question of the identity of the organisms concerned in the decomposition of cellulose in nature has long attracted the attention of the microbiologist, due to the great predominance of this group of polysaccharides in nature and to the important rôle that microorganisms play in its transformation into simpler compounds. The ability of fungi to decompose celluloses and the part played by actinomycetes has been pointed out elsewhere (Waksman and Skinner, 1926). It is the question of cellulose decomposition by bacteria which has been the subject of most active controversy; and, even at present, while the bacteria capable of breaking down celluloses are classified into at least five groups (Pringsheim, 1923), a great deal of confusion still exists concerning their identity and interrelationship.

Without going into a detailed review of the literature, attention may only be called to the following facts: 1. There is no definite proof that anaerobic bacteria are capable of decomposing cellulose, at least in normal soils. Cellulose can no doubt be decomposed very actively under anaerobic conditions, but it still remains to be proven whether this process is carried out by purely anaerobic forms or by an association of aerobic cellulose-decomposing bacteria and anaerobic forms which utilize the products of the former. Khouvine (1923) is the only worker who has undoubtedly succeeded in isolating an anaerobic organism, Bact. cellulosae-dissolvens, capable of decomposing cellulose actively (much less vigorously, however, in pure than in crude culture).

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This organism was isolated from the human intestine and grew only in the presence of fecal extract; it still remains to be proven to what extent it is capable of breaking down celluloses in nature, as in soil, manure, sewage purification, etc. 2. There is no doubt that a large number of aerobic bacteria, including spore-forming and non-spore-forming organisms, rods and cocci, are capable of attacking cellulose, but experimental proof concerning the rapidity of their action and the biochemical processes involved is still insufficient. This is largely due to the slow growth of these organisms and the comparatively slow decomposition of cellulose upon the agar plate (McBeth, 1916); the presence of zone formation and even of maceration of cellulose in liquid media is hardly a sufficient index of these processes as they may take place in nature. 3. Although there is no doubt that certain thermophilic bacteria are capable of decomposing celluloses and although these organisms are abundantly distributed in nature, it is doubtful whether, outside of composting of manures, they are active in normal natural processes, such as in the soil. 4. The same is true of cellulose decomposition in association with nitrate reduction. 5. The isolation of *Sp. cytophaga* by Hutchinson and Clayton (1919), an organism capable of decomposing celluloses using various inorganic salts as sources of nitrogen, was of great interest, not only on account of the specific morphology and physiology of the organism, which place it in a class by itself, but largely because of the fact that this organism is aerobic and decomposes cellulose at 30°C. with great rapidity, forming a mucilage and organic acids, but no gas; the products can be utilized as sources of energy by nitrogen-fixing bacteria. The greatest difficulty experienced in the isolation of cellulose-decomposing bacteria has been the lack of an appropriate simple medium, which would allow a rapid development of the organism and which could also be used for the study of its morphological and physiological activities. Such a medium seemed to have been offered by the cellulose agar of Kellerman and associates (1913 to 14) and McBeth (1916). However, this medium is rather tedious to prepare; the use of agar will allow the development also of other organisms which have nothing to do with
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cellulose decomposition. The fact that the bacteria isolated from cellulose plates and cultivated in common media were greatly reduced in their cellulose-decomposing power led Omelianski (1913) to suggest that the true cellulose bacteria were never isolated by Kellerman and McBeth, but that they were studying only contaminating forms. This is further emphasized by the fact that one of the few bacteria isolated in pure culture, whose identity cannot be doubted, namely the *Sp. cytophaga* H and C, does not grow on media not containing cellulose and is even injured by certain concentrations of sugars and protein degradation products. Löhnis and Lochhead (1923) found that *Sp. cytophaga* plays a predominant rôle in cellulose decomposition, under aerobic conditions, especially when the numerous other aerobic bacteria of a lower cellulose-decomposing efficiency are eliminated, by long continued cultivation and purification of the mixed growth obtained in the enrichment experiments.

These considerations suggest that only specific and selective media are desirable for the demonstration, isolation, and study of bacteria decomposing celluloses. Silica gel media which are free from any traces of impurities and contain only the specific nutrients could, therefore, prove ideal for this purpose. This was the medium which was utilized by Winogradsky in his brilliant contributions on the isolation of nitrite and nitrate forming bacteria. Winogradsky (1925) has demonstrated recently that silica gel plates containing the specific nutrients can also be used for the direct isolation from the soil of Azotobacter, “humivorous” and other specific soil bacteria.

While the following investigations were in progress, a paper appeared by Bojanovsky (1925) in which he used silica gel media for the isolation of cellulose-decomposing bacteria. An aerobic organism was isolated upon this medium consisting of two forms, a rod and coccus; since these two forms could not be separated from one another, the author suggested that he had here a symbiotic relationship of two organisms, which participate both in the decomposition of cellulose. This, as well as the nature of growth and the results of our own investigations, suggest that he was probably dealing with the *Sp. cytophaga*.
EXPERIMENTAL

Anyone who has had experience with the preparation of silica gel will appreciate the difficulties encountered in this connection. Of the various dialyzed, semi-dialyzed and undialyzed preparations, the original one of Winogradsky (1925) can still claim first place in simplicity and ease of preparation. It consists in pouring a solution of potassium silicate, sp. gr. 1.06 (B 6 to 8), into an equal volume of hydrochloric acid, sp. gr. 1.10 (B. 13), mixing well, distributing into dishes and allowing the gel to solidify; then placing the dishes in running water, until the wash water is free from chlorides and is neutral to brom-cresol purple. The dishes are then washed several times with boiled distilled water. This method has been somewhat modified, changing the concentration of silicate and acid so as to obtain a solidified gel within a few minutes rather than days as in the method of Winogradsky. After several modifications, the following method was found to give the best results:

A normal solution of hydrochloric acid and an equivalent solution of C. P. potassium silicate solution (about 10 per cent silicate) are prepared. Several 5 cc. portions of the hydrochloric acid are then placed into a series of test-tubes and varying amounts of the silicate solution (4 2/3, 5 1/3, 5 1/3, 5 1/3, 6 cc.) are added to the numbered tubes. The contents of the tubes are immediately mixed and poured into a series of dishes. The dish where gel formation takes place within two to five minutes is selected. The silicate solution is then diluted with distilled water so that equal volumes of the silicate and acid give a gel in the desired period of time. To prevent solidification during the process of gel formation, small quantities are mixed at a time. One hundred to 200 cc. of the silicate solution is poured into an equal volume of the acid solution and well mixed. The mixture is then poured into the lower halves of deep Petri dishes of ordinary bacteriological size; enough of the mixture is added to form a layer of about 3 to 4 mm. (requiring about 30 cc. per dish). The dishes are allowed to rest on a level surface until the gel is well formed. They are then placed in deep flat vessels and dialyzed.
in running tap water until free from chlorides. About twenty-four hours is required for this purpose. The dishes are then removed and transferred to a sterile vessel containing boiled distilled water. This is replaced several times. After they have been properly washed, the dishes are drained and treated with the nutrient medium. The cellulose used for the preparation of the plate consists of thoroughly ground filter paper, suspended in a medium of the following composition:

\[
\begin{align*}
(\text{NH}_4)_2\text{HPO}_4 & \quad \ldots \quad 5.0 \text{ gram} \\
\text{MgSO}_4 & \quad \ldots \quad 1.0 \text{ gram} \\
\text{FeSO}_4 & \quad \ldots \quad 0.02 \text{ gram} \\
\text{KCl} & \quad \ldots \quad 1.0 \text{ gram} \\
\text{Distilled water} & \quad \ldots \quad 100 \text{ cc.}
\end{align*}
\]

Five grams of the paper suspended in 100 cc. of this medium will suffice for the preparation of some 50 plates. About 2 cc. of the suspension of cellulose in the medium is poured upon the surface of each plate, in such a manner as to have the cellulose uniformly distributed over the surface; some CaCO₃ is then powdered on over the whole surface of each plate. The uncovered plates are placed in a thermostat at 65°C., until the excess of water has evaporated, without allowing the plates to become dry. The dishes are covered with sterile tops and are kept until needed for use.

A good growth of the bacteria capable of decomposing cellulose can best be obtained by inoculating particles of soil directly upon the properly prepared plates. This method has been suggested by Winogradsky (1925) for his auxiliary cultures. Crude cultures may also be obtained by inoculating liquid cultures, containing paper as the only source of energy, with soil. By using the first method, small particles of soil are pressed into the gel, in several places over the plate. Ordinary fertile field soil or greenhouse soils can be used; a soil, in which 1 per cent of ground filter paper and 0.1 per cent ammonium phosphate or nitrate have been incorporated a month previously and kept at optimum moisture and temperature, is, however, best suited for this purpose. The presence of cellulose decomposing bacteria will be manifested within forty-eight to seventy-two hours, by the appearance of a
yellow or orange colored growth around the soil particles rapidly spreading over the plate. When examined under the microscope, the growth is found to consist of various bacteria and protozoa, later also accompanied by actinomycetes. When the growth has proceeded for four to five days, transfer is made from the edge of the growth into flasks containing about 1 gm. of filter paper and 25 cc. of medium of the above composition (without CaCO₃), the flasks being previously sterilized. Various dilutions of the culture can thereby be made. The flasks are incubated at 28° to 30°C. Growth in the flasks will take place within two to four days, the paper becoming covered with a yellow or orange growth and showing rapid maceration. The culture is now transferred again to fresh silica plates, making a number of various dilutions from each flask. The highest dilution giving growth on the plate is then used for making further transfers. By transferring back to the flask and again to the plate, pure cultures may be finally obtained.

The yellow organism is very readily recognized as the Spirochaeta cytophaga of Hutchinson and Clayton. It decomposes the paper very rapidly, it does not grow on ordinary nutrient media and develops readily and abundantly on the silica gel plate. The identity of the orange-colored organism, which is a short rod, has not been established as yet. Both of these organisms are distinctly aerobic.

It can be demonstrated that, in addition to these aerobic organisms, there are also various other bacteria in nature which are capable of decomposing cellulose under aerobic and anaerobic conditions. Two grams of ground filter paper is added to about 100 gram portions of soil placed in tumblers, enough water is added to saturate the soil with an excess of about one inch of free water over the surface; the tumblers are placed in the incubator and kept at about 30°C. A considerable period of time will elapse before any manifestations of cellulose decomposition occur; only after one to two months incubation will gas begin to evolve accompanied by a sharp odor. This gas is found to consist of methane and CO₂. Cellulose decomposition which was at a standstill until this time will now begin rapidly. After all the
cellulose has become decomposed, as indicated by the cessation of active "fermentation" and by actual quantitative cellulose determination, fresh portions of ground filter paper are added. Decomposition or "fermentation" will now set in immediately. This is due to the fact that normal soils do not contain the anaerobic bacteria or rather "the proper population required for anaerobic decomposition of cellulose," such as would no doubt be found in the case of sewage purification plants, and in peat or cranberry soils, always saturated with water. This population has to be developed in normal soils; a considerable period of time is required for this; once the population has become established,

<table>
<thead>
<tr>
<th>CULTURE</th>
<th>MEDIUM = SOIL + CELLULOSE, STERILE</th>
<th>GROWTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic* (heated to 70°C. for 10 minutes)</td>
<td>Optimum moisture</td>
<td>No visible growth</td>
</tr>
<tr>
<td>Aerobic, unheated</td>
<td>Optimum moisture</td>
<td>Abundant fungus development</td>
</tr>
<tr>
<td>Aerobic, heated</td>
<td>Excess moisture</td>
<td>No growth</td>
</tr>
<tr>
<td>Aerobic, unheated</td>
<td>Excess moisture</td>
<td>No growth</td>
</tr>
<tr>
<td>Anaerobic† (heated to 70°C. for 10 minutes)</td>
<td>Optimum moisture</td>
<td>No growth</td>
</tr>
<tr>
<td>Anaerobic, unheated</td>
<td>Optimum moisture</td>
<td>Scant fungus development</td>
</tr>
<tr>
<td>Anaerobic, heated</td>
<td>Excess moisture</td>
<td>Rapid cellulose &quot;fermentation&quot;</td>
</tr>
<tr>
<td>Anaerobic, unheated</td>
<td>Excess moisture</td>
<td>Rapid cellulose &quot;fermentation&quot;</td>
</tr>
</tbody>
</table>

* Aerobic = soil in which active cellulose decomposition has taken place under aerobic conditions.
† Anaerobic = soil containing excess water, in which cellulose "fermentation" has become very active.

rapid cellulose decomposition, under anaerobic conditions, will take place. The organisms responsible for the decomposition of cellulose in this case are certain spore-forming bacteria. This can be readily demonstrated by inoculating the culture with sterile soil containing 1 per cent cellulose and 0.1 per cent NH₄NO₃ placed in flasks; some of the soil is brought to an optimum moisture content, adding sufficient water to make up 60 per cent saturation; to some of the soils an excess of water is added.

The results indicate definitely that, under anaerobic conditions, a population has become established in the soil, which is capable of decomposing cellulose and which can resist heating to 70°C.
The population which causes the cellulose decomposition under aerobic conditions consists of fungi (Waksman and Skinner, 1926) and of bacteria, like *Sp. cytophaga*, both of which are destroyed by heating at 70°C. for 10 minutes.

When transfers are made from the soil covered with water, in which active cellulose "fermentation" is taking place, into flasks containing 1 gm. of filter paper, 25 cc. of the above nutrient medium and 150 cc. of water, growth will take place in a few days, manifested first by a uniform turbidity throughout the flask and accompanied by the production of gas and butyric acid, and finally also by the formation of a black precipitate which settles to the bottom. On transferring to aerobic silica plates, *Sp. cytophaga* may develop; in aerobic flasks the latter form may or may not develop; in "anaerobic" flasks, however, growth will take place. This growth is manifested by a rapid disintegration of the filter paper, formation of turbidity and often of a pellicle on the surface. At first gas formation takes place, but, on further transfer, the cultures show only cellulose disintegration without the formation of gas. These results as well as the results of other studies carried on in this laboratory tend to show that possibly both Omelianski (1908) and Kellerman and associates (1913) were correct in their interpretation of the phenomenon of cellulose decomposition by bacteria, namely that cellulose decomposition takes place under anaerobic conditions (Omelianski) and that we may have here a combined action of cellulose-decomposing organisms, which only decompose the paper, without gas formation, and butyric acid or other bacteria, which attack the products formed by the former, with the formation of acids and gases (Kellerman et al.). This does not exclude however, the possibility that there may be in nature organisms which are capable of decomposing celluloses under anaerobic conditions with the formation of acids and gases as shown by Khouvine (1923). In normal soils, however, cellulose decomposition is carried on entirely by filamentous fungi, as shown elsewhere (Waksman and Skinner), and by aerobic bacteria, as can be readily demonstrated by the silica plate method.
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SUMMARY

1. A method is described which is very convenient for demonstrating the presence and nature of bacteria capable of decomposing celluloses.

2. This method, when combined with the proper dilutions, can also be used for determining the abundance of bacteria capable of decomposing celluloses, especially under aerobic conditions.

REFERENCES


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