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THE ISOLATION AND SOME CULTURAL CHARACTERS OF *BACILLUS CELLULOSAE DISSOLVENS*

BY

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(With one Plate)

Microbiological studies concerning cellulose decomposition have been made by many investigators such as MITSCHERLICH (19), VAN TIEGHEM (35), HOPPE-SEYLER (13), KELLERMAN, MCBETH and Co-operators (14), LÖHNIS and LOCHHEAD (10), SANBORN (25, 29), WAKSMAN (36) and WAKSMAN, CAREY and SEINNER (37), STAPP and BORTELS (43). KELLERMAN and MCBETH have isolated 17 new species of bacteria which are capable of breaking down cellulose aerobically. Isolation of anaerobic cellulose decomposing bacteria have been made by OMELIANSKI (21, 22, 23), PRINGSHEIM (24, 25, 26), HENNEBERG (12), KHOUVINE (15, 16), WERNER (39), CLAUSEN (4), MEYER, R. (42) and MEYER, V. (44). and mostly *Plectridium* forms were detected.

In our country, many bacteriologists have tried to isolate powerful cellulose decomposing bacteria for the aid of rotting green manure and Dr. ITANO (45) at Kurasiki, Okayama has isolated a new thermophilic bacteria of which pure cultures have already been applied to practical use.

In our laboratory also an attempt has been made to study the cellulose decomposition and some aerobic species have already been isolated by OTANI, SAKAMOTO and SASAKI. Recently we have isolated an anaerobic cellulose decomposing bacteria which almost identical to *Bacillus cellulosae dissolvens* KHOUVINE and we have studied its morphological and physiological characters.

This paper deals with the isolation and cultural characters of *Bacillus cellulosae dissolvens* KHOUVINE, the anaerobic cellulose decomposing bacteria newly found in this country.

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EXPERIMENTAL

The Sample

The sample which was used for this experiment was surface soil from a depth of 10 cm. from a vegetable garden in the vicinity of Sapporo. The soil was manured with some sediments of a night-soil bucket. The pH-value, when determined by the quinhydron-electrode method, was 6,2. Organisms identical with *Azotobacter chroococcum* and *Azotob. Beijerinckii*, the representative aerobic atmospheric nitrogen-fixer, were found in it in quite an abundance; also the presence of *Clostridium Pasteurianum*, which is capable of fixing atmospheric nitrogen, and the cellulose decomposing bacteria was detected.

Methods of Anaerobiosis

Among the methods of securing anaerobiosis, the principal one employed in the present experiments was this; the cultures were kept in a evacuated desiccator, the remained oxygen was absorbed with alkalic-pyrogallol. In addition to this, the GRUBER method was also adopted. This method, in spite of some danger of contamination when the tubes are opened, is not so difficult to apply because, when the growth of the anaerobic cellulose decomposing organisms is followed by gas-formation, the partial pressure of gas evolved in the sealed tubes are almost equal or not so strikingly lower when compared with that of atmosphere. When the test tubes sealed are opened, the drawn parts are sterilised with flame, and broken with a glass-rod, whereupon the contents are transferred into a sterilized test tube. This procedure, however, may certainly be criticized both for the inconveniences of the treatments after the growth and also for economical faults. But when the tubes have been once evacuated, there is no fear of leaking that would make the cultural condition bad, and furthermore, each culture is treated individually, so that there is convenience for external observation as compared with the BUCHNER and vacuum-jar methods.

However, in case the pure cultures were preserved, the purity of the cultures was sustained by BUCHNER's alkali-pyrogallol method although the purity was also expected to some extent when GRUBER's method was used.

As a device for anacrobiosis, liquid paraffine was employed to cover the upper layers of culture media about 2,5 cm. in depth with undesirable results.

In case of cultures made upon the solid media, except potato or plate culture, the use of the GRUBER method frequently resulted in the breakages of media, on account of air remaining still. Therefore, BUCHNER's alkali-pyrogallol method was chiefly used. When the agar plate cultures are evacuated in a desiccator, the media may be covered with condenced water following vaporization, so that the formation of colonies of the desired organism is hindered by the over-growth of anaerobic motile contaminants. To overcome this difficulty, CLAUSEN (4) employed calcium chloride. However, in this case, the result of applying too small an amount of calcium chloride may result in the over-growth of the contaminants aforementioned; and on the other hand, if too large amounts are used, the growth of the inoculated organisms may be interrupted by the aridity of the agar. Thus the optimal amounts of calcium chloride applied were quite difficult to determine. For these reasons, plate cultures in the anaerobic jar were not desirable in this study. A simple method of covering inoculated agar with dialyzed agar by pouring to the brim of the Petri-dish, was used.

Enrichment Cultures

To isolate the bacteria capable of breaking down cellulose, from the sample above mentioned, the writers first enriched the organism in accordance with the enrichment-culture-method of OMELIANSKI (21), as others have reported (4) (39). To determine the best one for the cultivation of the organism from this sample, the following culture media were selected, viz.,

A. Inorganic media as reported by OMELIANSKI (21) and KELLERMAN and MCBETH (14).

B. A medium which contains faecal extract, first used by KHOUVINE (15).

C. Organic medium which contains protein and cellulose, to test whether the organism utilizes the protein or not (such as cellulose bouillon[1] and digested bouillon[2]).

Ten cc. portions of the above media were placed in test tubes; to these a strip of filter paper was added, and sterilization of the media A and C[1] was made at 2 atm. pressure for 15 minutes while medium B and C[2] was sterilized 30 minutes for three successive days in KOCH's sterilizer.

In these culture media, about three grams of the sample was then inoculated in each of A and B and incubated by GRUBER's anaerobic method. The incubation temperature, if not specially noted, was 37°C.

First, an attempt was made to determine the medium in which the decomposition of cellulose took place most vigorously. For this purpose, the organisms present were examined microscopically, either when the cellulose decomposition was accompanied with gas formation or without evolution of any gases.

Each microscopic examination was performed using the preparations of cellulosic residue stained with gentian violet, any possible adhering solution of which was removed. Furthermore, if needed, a special preparation of cellulose decomposing organisms stained claret-red or brown-red with iodine was used after HENNEBERG (12) and GRAM's stain was also used.

The results of the enrichment culture made with the culture media already described, will be related in the following sections.

1) Omelianski's solution

Four days after inoculation, the filter paper became brown coloured with soil particles suspended by gas evolution; and after three more days the original form of the filter paper gradually disappeared. In the decomposed cellulose, although all parts were nearly colourless, some orange coloured specks of about 3 mm. diameter were noticed. At this time, the gas formation had already ceased, and the solution was turbid and not clear although the soil particles had sedimented at the bottom of the tube. The orange coloured parts gradually fell off from the central parts. Ten days after inoculation, almost all the filter paper had settled down to the bottom, losing its original form. On fusing the drawn part of the tube, gas gushed out, and burned explosively with a colourless flame, indicating that the hydrogen-like gases were admixed in considerable amounts. The reaction of the solution was slightly acid. On microscoping the uncoloured part of the cellulose, a slender, occasionally curved organism with a spherical spore at one end of its cell attached to the fiber was found which reminds one of OMELIANSKI's methane or hydrogen organisms (22); and an organism bearing a somewhat larger oval spore at the end of a cell was recognized at the orange coloured parts. In addition to this, some Gram-positive rods and cocci were found.

Five days after incubation with the same medium inoculated with the organisms formerly described, the microscopic examinations showed the dominant presence of the spherical spored organism as before, while the growth of the oval spored organisms, which had coloured the filter paper orange, was very scanty. When a third and additional subcultures were made, growth of the latter no longer took place. From the facts above

mentioned, as to the growth of the apparent cellulose decomposing organism with orange pigment upon filter paper, when first inoculated into OMELIANSKI's solution, it seems that some substances needed for its growth may have been transferred from the soil sample, and as to the scanty growth in the second subculture, owing to much inoculum, it seems that the substances may have been introduced from the inoculum. The lack of growth in the third or further cultures, is considered to be due to the absence of needed substances caused by the dilution of high grades or consumed by other organisms. From morphological and physiological characters, the organism resembled *Bac. cellulosae dissolvens*, which was first isolated by KHOUVINE (15).

2) Kellerman and McBeth's solution

In this medium, the decomposition of cellulose tended to be more delayed than in OMELIANSKI's. The cellulose was decomposed after 6–7 days by the cultures after two passages has elapsed, and the results obtained by microscopic examinations were also the same as in the former case. Comparable results between this medium and OMELIANSKI's solution were not found, as CLAUSEN (4) has reported.

3) Khouvine's solution

From the beginning of enrichment culture studies, KHOUVINE's solution was recognized as well suited for the growth of anaerobic cellulose decomposing organism present in the sample. That is to say, gas has evolved within 24 hours after the incubation, and after 48 hours, the soil particles floated in the solution as a result of the gas formed; thus the filter paper used as the source of cellulose was blackened just as in OMELIANSKI's solution. After an additional 24 hours, round orange coloured specks, diameter about 1 mm., were formed upon the filter paper; the specks enlarged in size, and then about a quarter part of the filter paper was covered with orange pigment. The filter paper lost its original form in two days and settled to the bottom of the tube. At this time gas-evolution had already ceased and while soil particles had sunk down, the solution was still turbid and orange coloured. When the tips of these sealed tubes were fused in flame, six days after inoculation, gas gushed out slightly and burned. In the combustible gas, hydrogen and hydrogen-sulfide were qualitatively detected.

When microscopic observation was made on the cellulosic residue of these cultures, the organism chiefly present consisted of rods bearing a

rather large oval spore at one end of an occasionally somewhat curved cell, attached to the filter paper by the other end. From its abundant presence, the organism was considered to be a cellulose decomposer. In addition to this, rods occurring singly, 3,0–6,0 × 0,6 μ in size, not stained with iodine solution was abundantly found in the culture. These were considered to be vegetative cells of the organism aforementioned that had not yet formed its spore. The morphological character of this organism was quite the same as described from the cultures made upon OMELIANSKI's and KELLERMAN and MCBETH's solution. Besides a rod bearing a spherical terminal spore which clung to the filter paper by another end of the cell was recognized. In addition to this, some Gram-positive rods and cocci were found as formerly stated in the case of cultures which were made upon OMELIANSKI's solution. Some Gram-negative rods and a Gram-positive spirillum were also seen.

These organisms were then inoculated upon fresh KHOUVINE's and OMELIANSKI's solution. In the former, 5–6 days after the inoculation, the filter paper was covered with orange-pigment, and the solution was exceedingly turbid; gas evolution commenced within 24 hours and lasted for 96 hours after the incubation; the amounts of gas evolved were, however, very much less than in the former cases, and hydrogen-sulfide was not produced at all. The results of microscopic examination showed the presence of the organisms already described. Of these, the organism considered as cellulose decomposer amounted to about 60 per cent; and the rest were contaminated forms not capable of breaking down cellulose, 30 per cent of which had sporulated.

The growth in OMELIANSKI's solution seemed to be unsuitable. The decomposition of cellulose was first seen 2 weeks after the inoculation, and formation of orange pigment was also not very vigorous. The microscopic examinations of these cultures showed the presence of large quantities of the contaminated forms, and the cultures which were made by inoculating these organisms upon the same medium resulted in scanty cellulose decomposition in some tubes, while in others, it did not occur at all. Therefore, further attempts with this medium to enrich the organism were not tried.

The third cultures upon KHOUVINE's solution were made with the inoculum preliminarily heated at 100°C. for 15 minutes in order to destroy the organism still in vegetative forms. In these cultures, the appearance of pigment was somewhat delayed on account of the lapse of more time for the germination of the spores, than with unheated inoculum. The pigmentation was first recognized four days after the inoculation. When

microscopic examination was made of five days' culture, the spore formation of the apparent cellulose decomposing organism was recognized. However, contaminated forms were still very abundant, attaining about 30–35 per cent of the whole, mostly consisting of spores of the *Putrificus* group which were identified after isolation. On the other hand, in the cultures which were made by inoculating the unheated materials, pigment formation, that is the decomposition of cellulose, tended to be delayed as compared with the cultures in which inoculation was made with heated inoculum. Yet some showed good growth and in certain instances very faint pigment was produced, indicating the prominent development of contaminated forms. These facts were considered to be controlled by many complex factors, the regulation of which factors was conceived to be more difficult than to employ the heated inoculum. In addition to this, the functions of the cellulose decomposing organism were not interrupted by heat. In further experiments inoculum heated at 100°C. for 15 minutes was employed. Next, cultures, inoculum of which was heated for 20, 30 and 45 minutes were made. These heating periods resulted in some effects on the growth of organisms; namely, in the cultures whose inoculum was heated for 20 minutes or more, the appearance of pigment was somewhat delayed. In cases with the inoculum heated 45 minutes some cultures showed no growth at all, or if growth did take place, the appearance of pigment was fairly retarded. These attempts were not effective, however, as the growth of the contaminated forms was not diminished, due to the non-effect of the heating periods.

As the cellulose organisms, which were enriched by this experiment, clung to the filter paper, an attempt was made to remove the contaminated forms by washing the remaining filter paper several times with sterilized physiological salt solution. By this method, the desired organism was gradually enriched and when 5 passages had elapsed, the purpose of enrichment culture of anaerobic cellulose decomposing organism under the formation of orange pigment was attained, although some contaminated forms remained such as Gram-positive spirillum and *Putrificus* group which could not be removed by heating the cultures in KHOUVINE's solution for 15 minutes. These contaminated the bacteria, because their sporulation took place before or at the same time with that of the cellulose decomposing organism, under the treatments above described. Thus, although the relative numbers of the cellulose decomposing organism and the contaminated bacteria were varied, entire removal was not attained. Accordingly other attempts for their isolation were carried on.

4) Cellulose bouillon

The enrichment cultures upon cellulose bouillon were not made by inoculating the soil sample directly as in the cases of media (1), (2) and (3). The first cultures were made with inoculum which had undergone two passages in KHOUVINE's solution. In the cultures which were inoculated with the cellulose decomposing organism, orange pigment appeared on the filter paper after three days' incubation. Thereafter, the filter paper sank to the bottom of the tubes, losing its original form. In this case, the solution was heavily turbid; gas also evolved and considerable amounts of hydrogen-sulfide were produced. Microscopic examinations showed the more contaminated forms than in KHOUVINE's solution and an organism belonging to the *Putrificus* group was recognized as most vigorous.

Interesting to note, in the aerobic cultures made along with anaerobic ones, the growth of cellulose decomposing organism has also taken place, and the filter paper lost its original form. In this case, thick, rumpled, brown pellicles, easily broken by a slight shake, were formed on the surface; gas bubbles also evolved and filter papers were coloured orange at the surface of the solution, and sometimes even at about 5 mm. above the surface. These phenomena are probably caused by some facultative anaerobes, mingled in the cultures, that, under those circumstances, might bring about such conditions as to make the cellulose organisms grow by consuming the free oxygen dissolved in the solution.

However, when the cultures were inoculated with the cellulosic residue contaminated bacteria of which were removed by washing with sterilized physiological salt solution, as made in the case of enrichment culture upon KHOUVINE's solution, the growth of cellulose organism was usually greatly retarded, not occurring within one week at least. As for the cultures made aerobically, the decomposition of cellulose did not take place; and if cellulose was decomposed, very faint, scarcely recognizable pigment was formed upon the filter paper. The pigmentation was seen on only a small part of the filter paper, not all over, and not uniformly.

Thus in cellulose bouillon, which contains considerable amounts of protein, the growth of anaerobic cellulose decomposing bacteria is probably due to the fact that the contaminated forms remove the injurious materials such as protein or carbohydrates other than cellulose, etc., a phenomenon that is called metabiosis; and furthermore, it is due to the "essential substance" formed by these organisms, as is elucidated by later experiments. Diminution in the numbers and kinds of contaminated bacteria which are

caused by various cultural conditions, causes the retardation or interception of their actions, thus the growth of the organism may probably not take place.

As stated above, in the enrichment culture studies made with cellulose bouillon, although some singularities were found, the organism was not enriched. Although several subcultures were made with the same medium, the relative number of both cellulose and contaminated organism remained unchanged, and brought us no more desirable results than by KHOUVINE's solution, as in the case of the results obtained by CLAUSEN (4) and WERNER (39).

5) Digested bouillon

When the enrichment cultures were made upon the cellulose bouillon, the growth of the cellulose decomposing organism studied in this experiment, took place only in the presence of contaminated forms, while a decrease in numbers or kinds did not cause decomposition. In cellulose bouillon, as compared with other media already described, the contaminated forms were so predominant that the desired organism was not enriched, as has already been related. Thus, since metabiosis took place in cellulose bouillon, some substances needed for the growth of cellulose decomposer are considered to be formed, even if the bacterial synergism did not take place between the cellulose decomposing organism and certain bacteria contaminated. If these substances are left in the solution, the contaminated organisms should, to a certain extent, stop their growth, and the purpose of the enrichment culture should be attained.

Some reports (4) (39) have already been made as to the application of so-called "Verdaute Bouillon" or "Einfache Bouillon". Originally the digested bouillon employed for the cultivation of cellulose decomposing organism was ordinary bouillon from which such materials as proteins or carbohydrates, considered injurious for the growth of cellulose decomposing organism, were removed by a vigorous proteolytic organism, such as *Bact. coli*, *Bact. prodigiosum*, *Bac. luteus* or *Bac. putrificus*. These organisms were afterwards removed by sterilization.

In the present experiments, the digested bouillon was prepared by inoculating the *Bact. coli* upon the ordinary bouillon. One week after incubation at 37°C., the bouillon was filtered, 10 cc. parts were placed in the test tubes, then a strip of filter paper and a small amount of $CaCO_3$ were added, and the tubes were sterilized 30 minutes for three successive days in steam.

In this medium the growth of cellulose decomposing organism was somewhat delayd when containing the contaminated forms inoculated from the culture made upon KHOUVINE's solution. Gas evolution took place after 48 hours, and the appearance of orange pigment, a characteristic of this organism, was first recognized 4 days later. The solution was considerably turbid in this case. A microscopic test has showed almost the same results as obtained with KHOUVINE's solution, but the sporulated organism of cellulose decomposer tended to be rather rare. On the contrary, the vegetative cells were much greater in length; some extended 36 μ. The relative number of the contaminated forms was in fact small, affected by the metabolic products. However, their spores existed in a considerably abundant number. These cultures were then inoculated into fresh digested bouillon and incubated at 37°C., anaerobically. The filter paper was coloured orange after 5 days, in these cultures the number of spores of the contaminated forms was not diminished. Furthermore, in the cultures inoculated with the inoculum which was washed with sterilized physiological salt solution and then heated 15 minutes at 100°C., the organism which belongs to the *Putrificus* group was the only contaminating bacteria existing in the cultures. Several repetitions did not show any appreciable results, just as in the case of the cultures made upon KHOUVINE's solution.

From the fact that the organism belonging to the *Putrificus* group remained without being removed from the cultures, digestion of bouillon was made simply by inoculating the filter paper remaining undecomposed with the cellulose decomposing organism, incubating anaerobically at 37°C. for a week. Then the bouillon was treated as already described. In this medium enrichment cultures were made to retard the growth of contaminated organisms. However, the results obtained were not different from those with the culture made upon the bouillon digested by *Bact. coli*. Eventually, the cultures which were enriched to this degree, showed that the contaminated forms are not removed by the treatments above described. Therefore, isolation of the desired organism was then carried on.

From the results obtained by the enrichment culture studies on the anaerobic cellulose decomposing organisms, the following conclusions may be derived:

When the enrichment cultures of anaerobic cellulose decomposing organisms were made with soil sample, of four culture media employed, KHOUVINE's solution was found to be best suited for the growth of rods bearing oval spores, decomposing cellulose accompanied by the formation

of orange pigment and gas containing hydrogen; subcultures yielded the enriched organism and contaminated forms were decreased, and in the cultures in which four passages had elapsed the cellulose was decomposed within 3–4 days. However, in OMELIANSKI's and KELLERMAN and MCBETH's solution, this organism was not enriched, only a cellulose decomposing organism, bearing spherical spore at one end of the cells, was found.

In cellulose bouillon, the cellulose decomposing organisms which act accompanied by the formation of orange pigment grew well both anaerobically and aerobically so long as contaminating organisms were intermixed. However, when the contaminated organisms were diminished, the growth of the organism did not take place even in anaerobic condition, or if growth did take place, it was very scanty or there was considerable delay in decomposition of cellulose. The preparation is, therefore not fully suited for enrichment cultures. To state it simply, it was found that cellulose is digested, a fact which can be explained by metabiosis.

Growth of the organisms in digested bouillon was somewhat dalayed and moreover, some morphological changes were recognized, though the contaminated bacteria were diminished to a certain degree. Thus it appears that this medium was somewhat suited for the growth of the organism next to KHOUVINE's solution. In this case, no difference was noticed in the enrichment cultures of the organism, whether bouillon was digested by *Bact. coli* or by an organism considered to be *Bac. putrificus*.

By washing the cellulosic residue containing cellulose organism with sterilized physiological salt solution, or by heating 15 minutes at 100°C., or by applying both treatments, the contaminated forms were removed to some extent. However, complete removal of these organisms was not attained by these methods.

The Isolation of the Cellulose Decomposing Bacteria

In general, that the isolation of anaerobic cellulose decomposing organisms, owing to their special characteristics, is exceedingly difficult, and that isolation of the organisms by ordinary bacteriological technics is very difficult or quite impossible, has been shown by past investigations. The isolation of the cellulose decomposing bacteria described in this paper was made by the methods detailed in the following paragraphs.

1. Washing with physiological Salt Solution

KHOUVINE (15) has found that the *Bac. cellulosae dissolvens* clinging

to the filter paper remained without being digested. The contaminated bacteria were removed from the filter paper by washing several times with sterilized physiological salt solution. Cultures were then made with these inocula and when the first sign of cellulose fermentation occurred, these procedures were again repeated. After these treatments, she finally succeeded in obtaining pure cultures of the bacteria.

In the present experiments, when this method was applied with KHOUVINE's solution, the decrease of the contaminated forms was clearly noticed either microscopically or by the interception of turbidity of solution in every culture. When five passages had elapsed, no organisms other than cellulose decomposer being scarcely recognizable, apparently pure cultures were obtained. However when cultures were made upon bouillon, by transferring from the cultures, the solution became slightly turbid after 48 hours, and microscopic examinations showed the abundant presence of Gram-positive rods; so a pure culture has not been obtained.

In this case, as small quantities of inoculum bring about no growth of cellulose decomposing organism, and consequently the growth of contaminated forms took place more vigorously, much inoculum was always used to make the desired organism grow in the shortest possible time. Furthermore incubation for 4 days caused a multiplication of the contaminated bacteria, and the accumulation of decomposition products of cellulose exerts still more favourable influences upon their growth. Difficulties in the complete removal of these organisms are easily conjectured.

Therefore, these cultures were then inoculated upon KHOUVINE's solution and incubated at 37°C., anaerobically. Gas evolved after 24 hours, so, the filter paper in the solution was washed three times with sterilized physiological salt solution and then transferred into fresh KHOUVINE's solution and incubation was made as usual. Exceedingly delayed, the decompotion of cellulose first took place 10 days after the inoculation, and in some tubes no growth of the organism was observed. In such delayed cultures, the contaminated bacteria did not decrease, but rather tended to increase. As already described, favourable results were obtained in the cultures, the inoculum of which contained large quantities of cellulose decomposing organisms. In order to avoid the predominated growth of contaminated organisms, the method must be applied using cultures, upon which the cellulose organism has grown more vigorously. Consequently, isolation was tried with cultures which were incubated for 36, 48 and 72 hours after inoculation.

In this case, the cultures incubated for 36 hours were somewhat more

satisfactory than those of 24 hours', but the organism incubated for 72 hours had already entered the resting stage increasing the length of its cells and also was undesirable. On the other hand, the growth of cultures incubated for 48 hours was vigorous. In the cultures which were made three times repeatedly after the above treatment, the presence of contaminated bacteria was not recognized microscopically. However, when these cultures were transferred into bouillon, after 3 days at 37°C. the solution became slightly turbid in which some Gram-positive rods were found, and when plate cultures were made with nutrient agar by inoculating the filter paper, almost equal quantities with that of KHOUVINE's solution, 10–30 colonies, were formed. These numbers of contaminated organisms remained unchanged in the cultures which were made several times under the same treatments. Pure cultures of the cellulose decomposing organism were not obtained.

2. Prevention of Growth of Contaminating Organisms by Means of Cultural Conditions

It is already an obvious fact that the organisms which form spores, under some cultural conditions may gradually proceed into asporogenesis, and it is also known that such organisms as *Bac. putrificus*, contaminated into the cultures of anaerobic cellulose decomposing organism and not easily removed, lose their spore-forming capacity by repeated cultivation in media which contain abundant protein. Therefore, when the enriched cellulose organism is transferred into the medium rich in protein by which its spores are not affected, if the spores of the contaminated form have germinated and remained in their vegetative forms, by heating the solution, the former spores only may be allowed to survive. Before attempts were made to carry on these treatments, ascertainment was made:

(1) that asporogenesis may be caused at 42°, 45° and 50°C., rather higher temperatures than the optimal, the growth of the cellulose decomposing organism, and,

(2) of the thermal death point of the cellulose decomposing organism.

Experiment I

Inoculation was made upon KHOUVINE's solutions with cellulosic residue to which cellulose decomposing organism clung, and then the tubes were incubated anaerobically at 37°C. as control, and at 42°, 45° and 50°C., respectively. The growth was tested by the pigment formation on filter paper in the solution and whether the spore formation took place or not

was determined microscopically. Each culture was duplicated.

TABLE I. Growth of the Cellulose Decomposing Organism at 42°, 45° and 50° C. and their Spore Formation.

Temperature °C.	Pigment formed after (days) (1)	(2)	Spore formation (1)	(2)
37 (Control)	5	4	+	+
42	4	4	+	+
45	4	4	+	+
50	4	5	+	+

+ denotes that spore formation took place.

As is evident from the table, the growth of cellulose decomposing organism at 42°, 45° and 50° C. did not strikingly differ from that at 37° C., and also, spore formation was found in every case.

Experiment II

In order to determine how long the spores of the cellulose decomposing organism resist heating, the organism was inoculated upon KHOUVINE's solution as usual, and after the solution was heated for 5, 10, 15, 20, 30, 40, 50 and 60 minutes respectively, in boiling water, the tubes were cooled quickly and incubation was made at 37° C. by GRUBER's method; then the survival of spores was established by means of pigment which formed upon filter paper. In this case, the authors could not ascertain whether the variation in time required for pigmentation was due to the oxygen tension and quantities of inoculum used or to other causes. However, in cultures heated for 40 minutes or less, growth was found; on the other hand, in tubes heated for 50 and 60 minutes, no growth took place after a month, thus indicating the decease of spores. In those cultures where growth took place, no abnormalities of spore formation were recognizable.

From the experiments above described, it was learned that with the enriched cellulose decomposing bacteria, the cellulose is decomposed at 37°–50° C. without injuring its activities. The spores of the bacteria resist the heat of boiling temperature for 40 minutes. Next, filter paper re-

mainders which contained the cellulose organisms and other contaminated organisms were inoculated into the bouillon, and after the medium was heated 5 minutes at 100° C., the tubes were incubated at 37°, 42° and 50° C., both anaerobically and aerobically. The bouillon thus treated, after 24 hours was turbid in either case. These cultures were heated again 5 minutes at 100° C., and after the filter paper remainders were inoculated into fresh bouillon, they were repeatedly cultured. Thus after 24 hours' incubation, slight turbidity was again recognized in all the cultures. These tubes were heated 5 minutes at 100° C., and the same procedures were repeated. These cultures were made under various combinations of conditions within the scope of temperature and relation to free oxygen. Thus the bouillon which was cultivated for 72 hours, heated for 5 minutes at 100° C. every 24 hours, did not show any turbidity or very slight if recognized. Consequently, these bouillons were again heated to 100° C. for 5 minutes and inoculations were made with the filter paper remainders from the bouillon and incubated anaerobically at 37° C. In some tubes growth took place within 8–14 days after the incubation. The cellulose decomposing bacteria grown by the method above mentioned, were considered microscopically to be pure, but as to the bouillon cultures which were made with the filter paper remainders both anaerobically and aerobically, the former was not turbid while the latter was sightly turbid and Gram-positive rods were found under the microscope, indicating a not pure culture.

On the other hand, the cultures in which the pigment formation did not take place upon the procedures above mentioned, included about 50 % of the total number. Microscopically, it was seen that spores of cellulose decomposing organisms easily decolourized dye by MÖLLER's spore staining method, and also that they were easily stained with anilin dyes, and furthermore, that the spore-membrane was deranged more than ordinarily. It is considered that these things were caused by the fact that the spores, probably because of the metabolic products accumulated by contaminated forms, may have commenced to germinate, under the favourable conditions, as in KHOUVINE's solution, and by the fact that the resistance of the spores had been lost by heating. In addition to these, too long heating was considered to be a cause.

No doubt, the contaminated forms that could not be removed from bouillon, may be introduced into KHOUVINE's solution in their resting forms; then, these spores may not germinate in bouillon or if transformed into vegetative forms, these cells may have sporulated before heat is applied.

So, isolation was attempted with the same procedures using bouillon having double concentrations. In this case, the duration of the heating period was 3 minutes at 100° C., and heating was done 9 times in 5 days after incubating them 7-9 hours and 15-17 hours alternately. As to temperature and relation to free oxygen, the same procedures were employed as in the former cases. Under this treatment the bouillon did not become turbid except in cases of the first two times incubated for 7-9 hours, but in bouillon, incubated for 15-17 hours, at first solution became turbid, and it was barely after 3 days, that the solution remained clear. In the bouillon which was not turbid after incubation, after the removal of filter paper, the presence of the organisms capable of growing upon the bouillon was proved by their causing turbidity of the solution. Under these treatments, no organisms, able to grow in bouillon were recognized after 4 days; however, spore germination of contaminated organisms was encouraged in bouillon for more than 24 hours, and after the medium was heated, filter paper was inoculated upon fresh KHOUVINE's solution, and cultures were made as usual. At the same time, with about the same volumes of inoculum as were used for KHOUVINE's solution, plate cultures were made and incubated at 37° C. anaerobically and aerobically. All the plates saving one intance on which only one colony was recognized, were quite sterile.

The growth of the cellulose decomposing organism was abnormally delayed in these treatments, and growth did not take place in any of the tubes, or only partly after 13-30 days at 37° C. When microscopic examinations of the cultures were made, the organism was considered to have grown in pure culture, but on inoculating with the cultures upon bouillon, Gram-positive rods which bore spores at one end of cells were found in the solution indicating that the cultures were not quite pure.

In these experiments also, there were many tubes in which pigment formation did not take place even after a month, the cause of which was considered to be the fact that the spores remained without being germinated as shown by microscopic tests.

By trying with solid media, the same results were also obtained as WERNER (39) has already reported.

3. Isolation by the Use of Solid Media

If bacteria grow well upon certain liquid media, then the organisms must be capable of growing upon the same media, with agar or gelatin added to them; attempts have been made, with failures, to cultivate anaerobic cellulose decomposing organisms upon solid media, but it was

at last achieved by WERNER (39), who cultured *Bac. cellulosam fermentans* upon the OMELIANSKI's solution containing agar. However, the growth of the organism in that medium was so gradual that he decided that it was unsuitable for the isolation of the organism in pure culture. After that, by cultivating upon various solid media, CLAUSEN (4) succeeded in obtaining pure cultures of *Bac. fossicularum*, *Bac. methanigenes* and *Amylobacter navicula*. Now, the organisms which the present authors have enriched are, both morphologically and physiologically, closely similar to *Bac. cellulosae dissolvens*. As KHOUVINE (15) has alreedy stated, if the organism does not grow upon any solid media, then the addition of solidifying agents, such as agar or gelatin, may play a partially hindering rôle. The authors prepared portion of KHOUVINE's solution containing 0,05, 0,1, 0,2, 0,4, 0,6, 0,8, 1,0, 1,2 and 2 per cent agar and gelatin respectively, and as for gelatin, 5 and 10 per cent portions were also prepared.

For the cultivation of amoeba which are rather difficult to grow upon the solid media, BEIJERINCK (1) employed washed agar, soluble matters of which were removed. In the experiment to determine whether the soluble maters of ager intercept the growth of the cellulose decomposing organism or not, untreated agar and one which had been treated as described below were used. Twenty grams of agar were added to 1 liter of water and filtered after the agar had been melted in autoclave. Each 300 cc. of melted agar was then divided into 1-liter ERLENMEYER's flasks. After solidification, distilled water was poured into the agar, and it was kept in thermostat at 37°C. for a week, the distilled water of each flask being changed two times a day. The dialyzed agar thus obtained was then dried at 70° C. As cellulose source, the filter paper which was dissolved with SCHWEIZER's reagent, precipitated with hydrochloric acid and then washed with water, was added to the amount of nearly 2 per cent to KHOUVINE's solution containing agar or gelatin. Ten cc. portions of these media were divided into tubes, and then sterilized. Of these media thus prepared, solid media were obtained at room temperature only by addition of more than 0,8 per cent of agar and over 5 per cent of gelatin.

These media were heated at 100° C., before the inoculation and cooled to 40°–50° C., then inoculated with the filter paper remainders to which cellulose organism clung. Before the agar or gelatin had solidified, these tubes were evacuated by GRUBER's method, then cooled and incubated at 37° C. Observation were made to determine the time required for the formation of orange pigment, a characteristic of the organism. Each culture was duplicated. However, when hydro-cellulose alone was used, the growth

of the organism was not satisfactory, even in the control itself which contained no agar or gelatin, although precipitated cellulose is reported to be better than raw. In some papers (5) (11) hydro-cellulose prepared by SCHWEIZER's reagent which has been employed for bocteriological purposes in the past, was occasionally reported as unsuitable. BOKOR (3) has indicated later, that this is due to acid which is used for reprecipitation of cellulose dissolved with the reagent. In the next experiment, hydro-cellulose prepared as BOKOR (3) did was used, and also a strip of filter paper was added to each tube. The results thus obtained are as follows:—

TABLE II. Growth of the Cellulose Decomposing Organism in KHOUVINE's Solution to which Agar or Gelatin was added.

Solidifying agents	Agar				Gelatin	
	Washed agar		Untreated agar			
%	Cellulose decomposed after (days) (1)	(2)	Cellulose decomposed after (days) (1)	(2)	Cellulose decomposed after (days) (1)	(2)
Control	4	4	4	4	4	4
0,05	4	5	4	5	7	6
0,1	4	4	5	5	7	7
0,2	4	4	5	9	7	6
0,4	4	5	8	10	12	13
0,6	4	5	12	No digest.	7	14
0,8	5	6	No digest.	"	12	13
1,0	4	6	"	"	16	21
1,2	5	6	"	"	18	21
2,0	5	8	"	"	23	No digest.
5,0					No digest.	"
10,0					"	"

In the above experiments, the portions of KHOUVINE's solution, to which 0,8–2,0 per cent washed agar or untreated agar was added, always remained in solid state, and in those where growth took place, gas-bubbles

were recognized in the medium, before the pigment had formed. On the other hand, the solution to which gelatin was added, did not again solidify after cooling, but assumed a gluey state.

From the above table, the growth of the organism is seen to be most vigorous upon KHOUVINE's solution containing agar, with soluble matters removed; growth was merely retarded upon the medium which contains 1,2 per cent agar when compared with that of control, and was not entirely intercepted by addition of 2 per cent agar. However, upon the media containing agar ordinarily used in bacteriological work in our laboratory without any treatments, the growth of the organism was retarded even by the addition of 0,1 per cent, and when higher contents of agar were added, a greater retardation of growth was evident. When 2 per cent agar was added to the medium the growth was intercepted.

On the other hand, when gelatin was added to KHOUVINE's solution, the growth of cellulose decomposing organism was far more conspicuously retarded. Cellulose decomposition took place upon the media containing 2,0 per cent gelatin, but very tardily, and not at all upon the media containing 5 per cent or more. In addition to this, gelatin liquefies at 37° C. even when there is a 10 per cent content, therefore contents of 1,2–2,0 per cent are not quite suited for use as solidifying agent. Such use of gelatin was not made in later experiments.

Thus, upon KHOUVINE's solution, to which 2 per cent washed agar was added, the growth of cellulose decomposing organism took place, so attempts to obtain the pure culture of the organism by causing growth in colony-formation upon solid media were made as CLAUSEN (4) has already tried. That is, spores of the cellulose decomposing organism were heated at 80°C. for 15 minutes and inoculated into the medium aforementioned, then poured into Petri-dishes of 6,5 cm. diameter. After solidification, the agar was cut crosswise with a sterilized knife, in order to avoid sucking up to the cover of the Petri-dish by evacuation. Then cultures were made in an evacuated jar. In this case condensed water was absorbed by means of calcium chloride of suitable amounts. Thus over-growth of the organisms on the surface of media was checked.

Under these circumstances, gas formation was recognized in agar after 6 days; and 2 days later, small colonies having a diameter of about 1,5 mm. were found near the gas-bubbles. The size of the colonies became gradually larger, attaining 2,5-4,0 mm. after 10 days' incubation. The form of the colonies on the medium surface was first circular, but afterwards became irregular, and those which were grown in the medium, were disk-

formed, and later also became irregular. When spots near the colonies were touched with a platinum needle, the agar became somewhat brittle, but no clear zones were observed. The results of microscopic examinations showed the presence of more abundant spores of cellulose decomposing organism than in liquid cultures. These colonies, however, were not formed by the cellulose decomposing organism only, but were contaminated with many Gram-positive rods which bore a spore. When less inoculum were used, growth did not take place as it did in liquid media. Pure cultures of the organism upon a solid medium were not attained.

4. Isolation by the Use of Dye Stuff

EISENBERG (7) studied the action of dyes upon microorganisms and found that in general the Gram-positive bacteria are from 3 to 10,000 times as sensitive as the Gram-negative. GAY and BECKWITH (8) confirmed and extended the observations that the Gram-positive organisms are more readily destroyed by dye stuffs in general than are Gram-negative forms. For the cultivation or isolations of soil bacteria, attempts to retard or intercept the undesired organism by dye stuffs have been made by CLAUSEN (4), GRÄF (9), NELSON (20), RUEHLE (27), SMITH (31) and others, and it was proved that the growth of Gram-positive organisms is retarded. TETRAULT (33) has applied crystal violet for the isolation of his cellulose decomposing bacteria.

It has been repeatedly stated that the Gram-positive organisms contaminated in the culture of the cellulose decomposing organism which the authors enriched, can not be removed readily. Now, if the Gram-positive bacteria are impeded in their growth by dye stuff which does not intercept Gram-negative, then from the mixed culture, though the Gram-positive organisms can not be removed completely, the quantitative ratio of both organisms may be enlarged, and by repeated culturing the Gram-positive organisms may finally be removed from the culture. Undoubtedly, there must exist differences in sensibilities to dye stuff among Gram-positive bacteria, so care must be taken as to the kinds of dye stuffs, their concentrations, and the time they are allowed to act. As dye, crystal violet of concentration of 1/25,000 was used and allowed to act for 2, 5, 10 and 20 minutes. Filter paper remainders containing Gram-positive bacteria were dipped into the dye solution for the times above given. Immediately, those filter paper remainders were washed with sterilized physiological salt solution several times and the small bits of filter paper thus treated were then introduced into bouillon and incubation was made at 37° C. for 2 days. The

presence or absence of Gram-positive bacteria was determined both microscopically and by the turbidity of the bouillon. Before the treatments were made with dye-solution, a test was also made with inoculum, heated for 15 minutes at boiling temperature. The following table indicates the relative numbers of Gram-positive bacteria.

TABLE III. Influence of Dye-Solution upon the Growth of Gram-positive Bacteria contaminated in Cultures of Cellulose Decomposing Organism.

Time treated (min.)	Growth in bouillon	
	Inoculum unheated	Inoculum heated for 15 min. at 100°C.
Control	++++	++++
2	++++	+++
5	++++	+++
10	+++	++
20	++	+

From the table, it will be seen that although Gram-positive bacteria were not destroyed completely, yet in inoculum which was heated at 100°C. for 15 minutes before the dye was acted on, the growth of the bacteria was considerably retarded. In order to remove the Gram-positive bacteria which appeared in these experiments, the organism should, therefore, be heated at 100°C. for 15 minutes before exposure to the action of dye-solution concentration of 1/25,000 for 20 minutes. In case heat is not applied, the concentration of the dye-solution, or the time of exposure to its action should be increased; but when the inoculum is heated, undesired organisms other than spores are removed.

Anaerobic cultures were made upon KHOUVINE's solution with filter paper remainders, in somewhat larger quantities than usual, which were treated as described above. Certainly, it seemed that the functions of the cellulose decomposing organism were injured. The appearance of orange pigment upon filter paper was seen after 6–9 days' incubation. In these cultures Gram-positive bacteria were also recognized, but not in very large numbers; they tended to be considerably decreased in comparison with

former cultures. Morphologically, the cellulose decomposing organism remained unchanged. These procedures were repeated several times, but complete removal of contaminated organisms was impossible, the only result being to foster the enrichment of the cellulose decomposing organism.

5. Isolation by "Ausschneide Verfahren"

When bacteria, not capable of growing upon a solid medium and other bacteria which are able to grow upon the same medium, are mixed together, these mixed organisms are smeared upon these substrates, by removing the grown organism, not fully grown organism only may be left upon the medium. When the latter organism is inoculated into another suitable medium by the proper method, pure cultures may be obtained. This procedure was first applied by WINOGRADSKY (41) for the studies of nitrifying organisms and afterwards, WERNER (39), COWLES and RETTGER (5) employed it for the isolation of cellulose decomposing organisms. By this method alone, they succeeded in obtaining pure cultures of the organisms they studied. The present authors, too, tried this method for the isolation of the cellulose decomposing orgamism under consideration.

Filter paper remainders, any vegetative cells of which had been previously removed by heat, were smeared thoroughly upon the nutrient agar plates, incubated at 37° C. aerobically and anaerobically, and the parts in which bacterial growth took place were taken away with a sterilized knife. Then the parts of the agar where no colonies appeared (apparently sterile) were divided into small pieces and transferred into KHOUVINE's solution by means of a sterilized forceps and incubated anaerobically. When these procedures were carried out, after 22 hours, the surface of the agar plates which were incubated anaerobically, were already covered with numerous colonies. After an additional 24 hours, notwithstanding removal of the colonies, the agar was again crowded with so many colonies that it was impossible to make any treatments. No doubt, this was due to the abundance of bacteria which are capable of growing upon nutrient agar. Therefore, the next trials were made by thinly smearing over the agar the filter paper remainders, contaminated organisms in which were removed by repeated washing with sterilized physiological salt solution. The surface of the apparently sterile agar thus obtained, was wiped with sterilized cotton. The spores of cellulose decomposing organism were then inoculated into KHOUVINE's solution. On these cultures which were made as usual, no growth took place either in consequence of want of inoculum nor for any other reason. Completely sterile tubes were obtained by these treatments.

It is already an obvious fact that, when cellulose decomposing organism is isolated in pure culture, no growth will take place if there is insufficient inoculum in the medium (5). It was suggested (5) that this is caused either by shortage of food supply for cellulose organism or by lack of bacterial association which is absolutely necessary for their growth. Reports have been made also that *Bact. coli*, *Bact. aerogenes* and *Proteus vulgaris* fulfil these requirements.

The cultures were made as already described, associating with *Bact. coli*, in which orange pigment on filter paper was recognized after 7–10 days' incubation. In this case to obtain as much inoculum as possible, "Ausschneide Verfahren" was carried out with filter paper rests from which the contaminated organisms had been previously removed by treating with crystal violet solution. When microscopic examinations were made of the mixed culture, *Bact. coli*, was recognized as the only contaminated organism. Then the cultures were heated at 85°C. for 15 minutes, and inoculations were made upon various media, under aerobic or anaerobic conditions. None of these media showed any appreciable growth of contaminated organisms. Thus was obtained the culture of the cellulose decomposing organism which was regarded as pure. The cellulose decomposing organism thus secured grows well successively upon KHOUVINE's solution and at 37°C. decomposes cellulose anaerobically within 5–7 days.

In short, isolation of the cellulose decomposing organism studied is pretty difficult.

In case of washing the filter paper remainders to which cellulose organism clings, with sterilized physiological salt solution, although the treatments are repeated, isolation of the organism is not attained successfully if the spores of the cellulose decomposing organism are already being formed; some contaminated organism has also entered their resting stages, multiplying rapidly. Or, if the treatments are made when the growth of contaminated forms is not vigorous, then the growth of the cellulose organism does not take place fully; accordingly if the inoculum is lacking, then growth does not take place; much inoculum gives opportunities for contamination.

To remove the contaminated bacteria by heat through causing asporogenesis of the organism, or through germinating their spores in bouillon in which growth of the cellulose organism does not take place, either through improper conditions for their germination or through having remained without germination, the former spores are transferred into KHOUVINE's

solution; thus pure cultures of the latter organism can not be made. Moreover, in this case, the resistance of spores of the cellulose decomposing bacteria seems to be changed either by growth of contaminated bacteria or by other causes. Thus, under these circumstances, although much inoculum is used, no growth of the desired organism took place either with or without contaminated organisms.

Upon the KHOUVINE's solution containing agar, the studied organism grows in a colony formation, but growth does not occur unless much inoculum is used. In these cases, in the colonies of the cellulose organism, abundant contaminated bacteria were found; and pure culture of the organism was not obtained.

To remove the contaminated Gram-positive bacteria by dye stuff, is not successful. Even Gram-negative cellulose decomposer causes injury to their functions.

Though attempts were made to secure isolation by "Ausschneide Verfahren" in these experiments, the purpose was not attained except when the contaminated organisms were few in number. In this case, also, growth did not take place, in the organism alone, due to the fact that the inoculum was not sufficient or for other reason, and that bacterial association was needed. In "Ausschneide Verfahren" agar surface on which no other bacteria are found was wiped with sterilized cotton, and introduced into KHOUVINE's solution, at the same time, the organism was associated with *Bact. coli.* Thus the pure cultures of the cellulose decomposing organism were obtained after the removal of associated organism by heat when decomposition of cellulose is over.

As stated above, pure culture of the desired organism is not attained by any single method, or if the contaminated bacteria are removed by some of the above described treatments, activities of the organism are affected by some unsatisfactory influences. In addition to these, growth does not take place within the limit of inoculating quantities although the organism is isolated. Therefore, such a method as "Ausschneide Verfahren" that minimizes the influences upon the activities of the cellulose organism and that includes the use of much inoculum, must be employed. By comprehending the characters of organisms that contaminate tenaciously, the difficulties in isolation of cellulose decomposing organism can be eliminated to some extent.

Morphology and Biology of the Isolated Organism

Vegetative cells in KHOUVINE's solution measure 0,5–0,7 μ in diameter and from 2,5–11,2 μ in length. When spores are about to form, the cells increase in length, not rarely reaching 23 μ. Also in digested bouillon, lengths of 36 μ are found. Vegetative cells cling to cellulose by one end, curving irregularly. They usually occur singly, very rarely in pairs, but chains of more than two cells are not seen.

The organism is Gram-negative. It stains well with fuchsin, gentian violet, methylen blue, but not with iodine.

The organism is non-motile when viewed by the usual hanging drop method.

The spores are formed at the end of the cells which is not attached to fiber, oval shaped, and in size 1,6–2,3 (2,0) × 2,2–2,8 (2,5) μ. First the vegetative cells increase in length, then a tiny swelling, which stains deeply is found at the end of the cells unattached to cellulose. This swelling becomes spherical and, increasing in size, is finally oval shaped. The spores when fully ripened, fall off from the mother cells. Therefore in media where fermentation has been completed or on filter paper remainders left without being digested, abundant free spores are seen. The spores germinate from a pole within 10 hours at 37° C. in KHOUVINE's solution, and resist a boiling temperature for 40–45 minutes.

The Process of Cellulose Fermentation

When the isolated organism is inoculated upon KHOUVINE's solution and kept at 37°C. anaerobically, the fermentation of cellulose is commenced after 20 hours at the earlist, usually in 36–48 hours. But when the cultures are made with the inoculum from which vegetative cells have been removed by heating for 30 minutes at 85°C., the fermentation of cellulose is delayed. It takes place after 5–6 days. When the cultures are made with the cellulose organism alone, which the present authors have isolated, the decomposition of cellulose takes place less vigorously than in cultures which were made by admixing intestinal bacteria such as *Cellulomonas fecalis* HANZAWA et SASAKI, *Bact. coli*, *Proteus vulgaris*, etc. This has already been recognized by KHOUVINE, WERNER (39) and others, who suggested that this phenomenon might be caused by the associated organisms which are facultative anaerobe consuming the remaining free oxygen or by special catalytic actions introducing anaerobic conditions more promptly and thus

shortening the incubation period. Furthermore, decomposed or metabolic products of such organisms supply some suitable organic nitrogen for the cellulose decomposing organism, thus probably accelerating its growth. The first signs of cellulose decomposition are recognized by gas evolution, later, faint yellow pigment is formed on filter paper, then increasing its depth of colour, finally becomes deep orange. The filter paper is decomposed gradually losing its original form, and sinks to the bottom of the tube. The fermentation lasts about 5–14 days upon KHOUVINE's solution. The solution does not became turbid in this case. After 3–4 days, rods begin to sporulate and abundant spores are found in the remaining filter paper fiber, being attached densely thereto.

Relation to free Oxygen

The organism is obligate anaerobe; when kept aerobically at 37°C. in KHOUVINE's solution, no growth took place and filter paper also remained unchanged. On keeping these cultures under anaerobic condition, fermentation occurred after 6–8 days. During the fermentation, if the condition is altered into aerobic, the fermentation ceases; however, dilatory gas evolution takes place for 1–3 days. In our experiments, no attempts were made to determine by what degree of decreased oxygen tension the growth of studied organism is promoted, in other words, the maximum oxygen tension for cellulose decomposition.

Relation to Temperature

To determine the influence of temperature upon the decomposition of cellulose by the isolated organism, portions of KHOUVINE's solution after inoculation, were kept at 18°, 27°, 32°, 37°, 40°, 45°, 50° and 60° C., respectively. The velocity of cellulose fermentation has varied with the temperature. The following table shows the results obtained. Each experiment was duplicated, and the numbers given in the table indicate the required time in days for the appearance of orange pigment upon filter papers.

TABLE IV. Influence of Temperature upon Cellulose Decomposition by the isolated Organism.

Temperature °C.	Pigment formed after (days)
18	No digestion
27	"
32	7
37	5
40	5
45	5
50	5
60	No digestion

As shown in the table, the optimum temperature for the decomposition of cellulose by the organism under study lies between 37° and 50° C.; at 32° C. growth of the organism is gradual, but at 18°, 27° and 60° C. no fermentation of cellulose occurs.

Growth upon various Media

The cellulose decomposing organism with which the present writers experimented was cultured upon KHOUVINE's cellulose faecal infusion solution. Upon this solution filter paper was used as the carbon source. Upon this medium, the growth of the cellulose organism was most favourable. However, when these cultures were continued for 3 years, gradual decrease in fermentation and spore forming capacities were recognized. The organism did not grow upon OMELIANSKI's (21), KELLERMAN and MCBETH's (14), COOLHAAS' (6), PRINGSHEIM's (26) and BOKOR's (3) media. No growth took place upon bouillon with or without glucose nor upon cellulose bouillon. However, the organism grew gradually upon bouillon digested by *Bact. coli* or *Bac. putrificus verr.* to which a strip of filter paper was added, and sterilized. And at 37° C. after 6–8 days, the faint yellow pigment upon the filter paper was seen, and digestion of cellulose occurred. But, when compared with the growth upon KHOUVINE's solution, the activities of the organism were so exceedingly dilatory, that gas evolution continued for 3 weeks or more after the incubation. In addition to this, microscopic exami-

nation showed that the cells increased their length, some extending to 36 μ. Spore formation is also delayed, being rarely recognizable after 2 weeks. Even when fermentation has been completed, the number of spores is very mach smaller than upon KHOUVINE's solution. When the cultures are made with inoculum in which spores are not yet formed, the growth becomes more inactive, and in the cultures in which four passages have elapsed, no growth occurred.

The organism does not grow upon milk, nutrient agar, glucose agar or potato.

It has been already described how the organism, when mixed with some other bacteria, is capable of growing in colonies upon KHOUVINE's solution which precipitated cellulose according to BOKOR's method and to which 2 per cent washed agar was added. Upon this medium, when deep layer cultures were made by inoculating the pure culture of cellulose organism, after 4–6 days at 37° C. small gas bubbles were recognized in the substrate, then pale orange coloured spots were formed; the spots increased in their size and depth of colour as they coalesced with each other. After 2 weeks, the medium was evenly coloured a deep orange. The substrate lost its original form as a result of the small gas-bubbles evolved. However, gas bubbles did not increase greatly in their size. The substrate became brittle. Then the plate cultures were made to in order to cause the organism to grow in colony-formation. Filter paper remainders were inoculated upon the melted medium, poured into the Petri-dish, and after the medium has solidified, anaerobic culture was carried out. After 6–8 days gas-bubbles were formed in the medium, and after 2 more days, yellow spots were recognized near the gas-bubbles. These spots increased in size and became orange coloured. Surface colonies were first circular, not elevated, compact, somewhat granular, moist glistening, undulated margin, 5–8 mm. in diameter after 2 weeks. On the other hand, deep-layer colonies, although accurate observation was not made because of the presence of calcium carbonate and cellulose contained in the substrate, were disk-formed; 2 weeks after the incubation their diameter measured 2–5 mm. but their form became gradually irregular. In these cases, clear zones were not found around the colonies as KELLERMAN and MCBETH (16) and LÖHNIS and LOCHHEAD (18) have already reported. Microscopically, no appreciable differences were recognized from the cultures made upon KHOVINE's solution, and abundant spores were also found. Cultures inoculated from these colonies resulted in normal growth of the organism.

Influence of Carbohydrates

To determine the influences of various carbohydrates upon the studied organism, 0,5 g. respectively of glucose, saccharose, maltose, dextrine, raffinose, laevulose, galactose, rhamnose, mannose, xylose, lactose, mellitose, mannite, methylglcoside, xylan, lichenin, inulin, fucose, arabinose, erythrite, amygdarin, soluble starch and cellulose were each added to 100 cc. portions of KHOUVINE's solution which contained no cellulose. These culture solutions were then sterilized and inoculated with the bacteria which had been cultured upon KHOUVINE's solution, then incubated anaerobically at 37° C. Among these solutions, in the one to which cellulose was added, gas evolution was recognized 36 hours after the inoculation and after 5 days, cellulose was digested losing its original form. On the other hand, no changes were found in the solutions which contained carbohydrates other than cellulose; thus after 2 weeks when the reaction of each solution was compared with the control in which culture was not inoculated, no changes had occurred. Thus it was known that these carbohydrates had not been utilized. However, in KHOUVINE's solution to which cellulose was added, acid formation was recognized; the solution, pH-value of which was first 8,0 became 5,8 after 2 weeks. The above experiment was repeated two times.

Consequently, the organism seems to be not capable of utilizing any carbohydrates other than cellulose. This fact has already been shown by *Bac. fossicularum* and *Bac. methanigenes* (21), *Bac. cellulosae dissolvens* (15) and *Bac. cellulosam fermentans* (39), hence these organisms seem to belong to some specialized group.

Nitrogen Source

To determine the nitrogenous matter which is most suited for the growth of isolated cellulose organism, the presence or velocity of cellulose decomposition was examined by substituting various nitrogen sources for the peptone in KHOUVINE's solution. At the same time, to know what rôles faecal extract plays in the growth of the organism or to learn if it serves as nitrogen sources, identical attempts were made with KHOUVINE's solution faecal extract and peptone in which the various nitrogenous matters were altered. Contents of each nitrogenous matter were 0,1 per cent excepting lecithin 0,05 per cent.

TABLE V. Influence of various Nitrogenous Matters upon Growth of the Organism.

Nitrogen source	Content %	Cellulose decomposed after (days)			
		With faecal extract		Without faecal extract	
		(1)	(2)	(1)	(2)
No nitrogenous matter		No digestion		No digestion	
Asparagine	0,1	6	7	"	
Casein	0,1	6	6	"	
Glycocoll	0,1	6	7	"	
Lecithin	0,05	No digestion		"	
Nucleic acid	0,1	9	9	"	
Peptone	0,1	5	5	"	
Tyrosin	0,1	6	7	"	
Urea	0,1	No digestion		"	
Uric acid	0,1	6	7	"	
Amm. carbonate	0,1	7	7	"	
Amm. chloride	0,1	7	10	"	
Amm. nitrate	0,1	No digestion		"	
Amm. phosphate	0,1	9	9	"	
Amm. sulfate	0,1	6	6	"	
Pot. nitrate	0,1	No digestion		"	
Sod. nitrate	0,1	"		"	

From the above table, peptone is seen to be most suited for the nitrogen source of the organism; casein, asparagine, glycocoll, tyrosin, uric acid, amm. carbonate, amm. sulfate rank next; nucleic acid, amm. chloride, amm. phosphate are slightly utilized, while lecithin, urea, ammonium nitrate, pot. nitrate and sod. nitrate are not utilized at all.

The above named nitrogenous matters are not utilized by the organism if faecal extract does not accompany them and no growth is caused to take place by faecal extract alone. Whether faecal extract serves for the metabolism of the organism or whether it has other functions, will be described in a later section.

Influence of Sodium Chloride

To determine what degree of concentration of NaCl the isolated organism resists, an attempt was made with KHOUVINE's solution with these varied NaCl content: 0, 0,05, 0,1, 0,2, 0,5, 1, 2, 5, 10 and 15 per cent; the cultures were made at 37° C. anaerobically and the occurrence or velocity of cellulose decomposition was tested.

TABLE VI. Influence of Sodium Chloride upon the Growth of the isolated Organism.

Percentage of NaCl	Cellulose digested after (days)	Pigment
0	6–7	light Yellow
0,05	6	Yellow
0,1	5	"
0,2	5	orange
0,5	5–6	"
1,0	6–7	"
2,0	No digestion	
5,0	"	
10,0	"	
15,0	"	

The data given in the above table show that the optimum contents of NaCl for the growth of the cellulose organism lie between 0,1–0,2 per cent, the pigment formation within this range also nearly agrees with the pigment formation which is the indication of its growth. In the medium which contains no NaCl, growth is recognized although the fermentation is delayed; however, higher concentrations retard action of the organism, and 1 per cent is recognized to be the maximum. From these facts, it is known that the organism is not salt-tolerant or halophilic. In addition to this, no morphological changes are not recognized according to the concentration of NaCl within the limits for the growth of the organism.

Metabolic Products

By the culture of the isolated organism which was kept at 37° C. for 2 weeks upon KHOUVINE's solution, CO_2 and H_2 were detected in the gas evolved. Acetic acid, butyric acid and ethyl alcohol were also proved in the solution, but production of indol or hydrogen sulfide was not recognized.

No substances which are reduced with FEHLING's solution were demonstrable, hence in the process of cellulose fermentation, the organism seems not to form any sugars as intermediate products. Cellulose is decomposed concomitant with formation of orange pigment.

Systematic Position

As stated above, the cellulose decomposing organism under consideration, judging from its characters, belongs to a group which includes, besides itself, *Bac. fossicularum* and *Bac. methanigenes* reported by OMELIANSKI (21), *Bac. cellulosae dissolvens* by KHOUVINE (15) and *Bac. cellulosam fermentans* by WERNER (39). However, the organism differs somewhat from OMELIANSKI's hydrogen-organism both morphologically and physiologically. It also differs from *Bac. cellulosam fermentans*, in respect to form, motility, optimum temperature, and habitat. On the other hand, the characters of the organism mostly resemble *Bac. cellulosae dissolvens* first isolated by KHOUVINE (15). The following is a comparison of the two organisms, between the one which the present authors have isolated and the *Bac. cellulosae dissolvens*.

Characters	KHOUVINE's Bacillus	Authors' Bacillus
Size of rods	2,5–12,5 μ (diameter not given)	2,5–11,2 μ × 0,5–0,7 μ
Size of spores	2,0 × 2,5 μ	2,0 × 2,5 μ
Flagella	absent	absent
Gram's stain	negative	negative
Bouillon	no growth	no growth
Bouillon + glucose	no growth	no growth
Nutient agar	no growth	no growth
Glucose agar	not given	no growth
Milk	no growth	no growth

Characters	KHOUVINE's bacillus	Authors' bacillus
Indol	not given	not formed
H_2S	not given	not formed
Carbohydrates fermented	cellulose only	cellulose only
Optimum temperature	35°–51° C.	37°–50° C.
Relation to free oxygen	obligate anaerobe	obligate anaerobe

As shown above, the two organisms coincide physiologically, though slight differences are recognized in morphological characters. Slight variation of the morphological characters is to be seen from alterations of cultural conditions and also from individual differences, hence the organism used in the present experiments is to be considered identical with *Bac. cellulosae dissolvens* (15).

On the Substances required for the Growth of *Bac. cellulosae dissolvens* in Faecal Extract

The fact that the most favourable growth of *Bac. cellulosae dissolvens* takes place in the medium which contains faecal extract of appreciable amount, has already been reported by KHOUVINE (15). The same results were also obtained by the authors' experiments. That is, the faecal extract contains some substances necessary to the growth of *Bac. cellulosae dissolvens*, and their deficiency results in exceeding retardation or complete inhibition of its growth. KHOUVINE (15) has endeavoured to identify the kinds of substances contained in faecal extract and to determine what rôles these substances play in the activities of the organism. When she substituted the faecal extract in KHOUVINE's solution with 90 % alcohol extract of faecal extract and cultivated the organism she was studying, its growth was fairly intercepted, so that gradually it was led into asporogenesis; furthermore, by animal tests, she has proved that the extract is not vitamin B. On the other hand, it was reported that, by altering the faecal extract with vitamin B prepared from yeast, the growth of the organism was not induced (15). Thus the substances needed for the *Bac. cellulosae dissolvens* have been an unsolved problem for years.

On the occasion of isolating the *Bac. cellulosae dissolvens*, an attempt to determine the substances required for the organism was made.

First, faecal extract was dried at 60°–70° C. under negative pressure. In this case, a colourless distillate was obtained with a strong alkali reaction containing ammonia to considerable amounts. When the residue was extracted with ether, a light brown, hygroscopic substance was obtained. To the residue, after extraction with ether, 95 % alcohol was added and the preparate was extracted 2 times for 24 hours in a cool place shaking several times. Thus light brown substances have resulted. The residue, from which 95 % alcohol soluble matters were removed, was then treated twice with hot 85 % alcohol for 10 hours; dark brown substances were thus obtained. The substances left without being extracted by the above treatments, were next infused with water, and after the removal of some of the resulting precipitates, a brownish black solution was obtained. The solution thus prepared was then dried at 60°-70° C.

Thus, when faecal extract is divided into five fractions, the undesirable odours in the extract are recognized in the distillate and in the ether extraction. Next, to determine the fractions which are utilized by *Bac. cellulosae dissolvens*, the following basal culture solution was prepared.

Water	1000 cc.
Peptone, Witte	1 g.
NaCl	1 "
K_2HPO_4	1 "
$CaCO_3$	2 "

To the above solution each fraction obtained by the methods above stated was added proportionately to 150 cc. of faecal extract. If the substances required for the growth of the bacteria under observation are not contained in a single fraction, then the growth will not take place, or will be exceedingly retarded. Therefore, in order to discover whether the substances are contained in a single fraction, or in two or more, the five fractions were combined with one another, and 15 culture solutions were prepared. The solutions thus prepared, were then placed in 10 cc. portions in sterilized test tubes to each of which a strip of filter paper was added, then sterilized. The reaction of the solutions was not adjusted in these cases. The following are the combinations of the fractions contained in the prepared culture solution.

1. Distillate, colourless, strong alkali, ammoniacal odour ..... A
2. Ether extract, light brown, undesirable odour, water insoluble B*
3. 95 % alcohol extract, light brown, water soluble C

4. Hot 85 % alcohol extract, dark brown, water soluble..... D
5. Brownish black, water soluble matters, alcohol insoluble ... E
6. A + B + C + D + E
7. B + C + D + E
8. C + D + E
9. D + E
10. B + C + D
11. C + D
12. B + C
13. C + E
14. B + D + E
15. A + B + C

* As this fraction does not dissolve in water, its water suspension was used.

In inoculating *Bac. cellulosae dissolvens* upon the prepared solutions, it must be remembered that as the inoculum is required in considerable amounts, it contains not only bacteria but also cellulosic residue and the culture solution, from which the inoculum has originated; these substances which are introduced into new media, may cause the transference of the substance required for the bacterial growth. To overcome this difficulty, these experiments were made by inoculating with filter paper (decomposition of which is not yet vigorous but abundant bacteria are contained) washed with sterilized physiological salt solution under aseptic conditions, following which anaerobic cultures were made. When the cultures were kept at 37°C., the growth of the organism in the media containing the fractions in single or combined states, took place as shown below. Each experiment was duplicated, numbers indicate the period required for the appearance of orange pigment upon the filter papers in days.

1. A	no digest.	9. D+E	no digest.
2. B	no digest.	10. B+C+D	5
3. C	5	11. C+D	5
4. D	no digest.	12. B+C	5
5. E	no digest.	13. C+E	5
6. A+B+C+D+E	5	14. B+D+E	no digest.
7. B+C+D+E	5	15. A+B+C	5
8. C+D+E	5		

Judging from the above results, the substances required for the growth of organism are contained in fraction "C", the 95 % alcohol extract of faecal extract. In case of substituting the faecal extract with 95 % alcohol extract, the size of the organism or its spore formation did not differ at all from those cultivated upon KHOUVINE's solution. Furthermore, no influences were recognized by cultivating the organism upon the same fresh medium or upon KHOUVINE's. The extractions other than 95 % alcohol, as seems obvious from the data above given, play no rôles in the growth of bacteria either singly nor in combined states.

Next, further experiments were carried on with 95 % alcohol extract, and the evaporation of faecal extract was tried. However, to dry up the faecal extract, difficulty was experienced owing to the abundance of colloidal matters present. In addition to this, the dried matters are exceedingly hygroscopic, accordingly, in its treatments, there were many inconveniences. So, in later experiments, the faecal extract was not dried up completely, but kept in a syrupy states. The fats or ether soluble matters were shaken in a separatory funnel, and to the syrup was added absolute alcohol to a contents of 95 %. The precipitates thus caused, were, after filteration, washed several times with 95 % alcohol. Both filtrate and washed alcohol were than mixed together, and the alcohol was removed at 50–60° C. The yield, of course was influenced by the concentration of faecal extract or its composition; about 4,4 g. dried matter was obtained from one liter sample.

To find the effects of substitution of varied quantities of the 95 % alcohol extract for the faecal extract upon the cellulose decomposition of the studied organism, the extraction obtained was dissolved in water, and cultures were tried upon the medium in which the faecal extract was altered by the addition of varied amounts of 95 % alcohol extract. In this case also, attempts to obviate the influence of a former medium were made by washing the inoculum well.

TABLE VII. Influence of 95 % alcohol Extract in varied Quantities upon the Growth and Cellulose Decomposition of the Organism.

Culture number	95 % alcohol extract added per liter		Peptone g.	Cellulose digested after (days)	
	g.	Nitrogen (mg.)		(1)	(2)
1.	0,1156	13,77	1	No digestion	
2.	0,2312	27,54	1	No digestion	
3.	0,5780	68,88	1	7	6
4.	1,1560	137,77	1	6	6
5.	1,5240	206,65	1	5	5
6.	2,3120	275,54	1	6	6
7.	3,4680	413,32	1	6	6
8.	1,1560	137,77	—	No digestion	
9.	—	—	1	No digestion	
Control	KHOUVINE's solution		1	5	5

As shown in the table, the most vigorous growth of the organism was recognized in the culture made upon the medium which contains about 1,5 g. 95 % alcohol extract of faecal extract per liter. One and five-tenths gram of 95 % alcohol extract is proportionate to 330 cc. of the original solution, but when this was compared with 150 cc. in KHOUVINE's solution, considerable difference was recognized. However, concentration and composition of the faecal extract are not always constant; this difference may probablly be caused by such conditions. If either pepton or faecal extract are removed from the medium no growth of the organism occurs.

Then the 95 % alcohol extract was dissolved in water and as the solution reacted weak acid, neutralization was made by addition of dilute sodium hydroxide solution, and when 10 % neutral lead acetate solution was poured into the solution, voluminous yellowish brown precipitate was caused. The precipitate was then filtered, and washed several times with water containing neutral lead acetate; the filtrate and washed solution were mixed together, the precipitate was suspended in water, the lead in both fractions was removed by introducing hydrogen sulfide. Both the solutions, after filtration, were evaporated at 50°–60° C. under negative pressure, and each syrup thus obtained was dissolved in water, filtrations were made if precipitates were caused.

These fractions were again substituted with faecal extract in KHOUVINE's solution, and cultures were made as usual. The inoculum used in this case was also washed several times with sterilized physiological salt solution. Upon these media, growth of the organism was recognized within 6–8 days in only one, to which had been added the fraction not precipitated with neutral lead acetate. On the other hand, in the medium containing the fraction precipitated by neutral lead acetate, no growth was found even after a month. Further, the organisms grown upon the medium containing filtrate not precipitable by addition of neutral lead acetate solution, did not differ from those which were growth upon KHOUVINE's solution of normal composition. They grew well upon the same medium or upon KHOUVINE's. On the contrary, growth upon the medium containing the fraction which is precipitated by neutral lead acetate, did not take place although several attempts were made. That is, the substances required for the growth of *Bac. cellulosae dissolvens* are not precipitated by neutral lead acetate.

To the filtrate, after removal of the precipitate caused by addition of neutral lead acetate solution, was added basic lead acetate solution. Again brown precipitates have resulted. The precipitates and liquid parts were separated. After the lead of both was removed as formerly described, both solutions were concentrated. These substances were separately substituted with faecal extract in KHOUVINE's solution. Cultures were made upon both media. Thus, pigment formation being found after six days upon the filter paper in the medium to which filtrate was added, it was known that the substance required for the bacterial growth was transferred into the filtrate. The experiments were duplicated, the filtrate was well utilized by the organism, while the precipitate was not suited to its growth; the medium to which the precipitates were added, was always kept sterile.

Attempts were not made to determine the optimum amounts of fractions, which cause the growth of the studied organism, obtained by addition of neutral and basic lead acetate solution to the 95 % alcohol extract of faecal extract.

The fraction not precipitated with basic lead acetate solution, was then dissolved in a little water, sulfuric acid was added to the amount of 5 %, concentrated 5 % sulfuric acid solution of phosphotungstic acid was added, then well stirred, warmed in water bath to cause the precipitate almost to dissolve, and after 48 hours, the precipitate was filtered. To both precipitates and filtrate, barium hydroxide was added, sulfuric acid and phosphotungstic acid were thus removed, then the excess barium hydroxide still remaining was removed qualitatively with sulfuric acid. As barium is

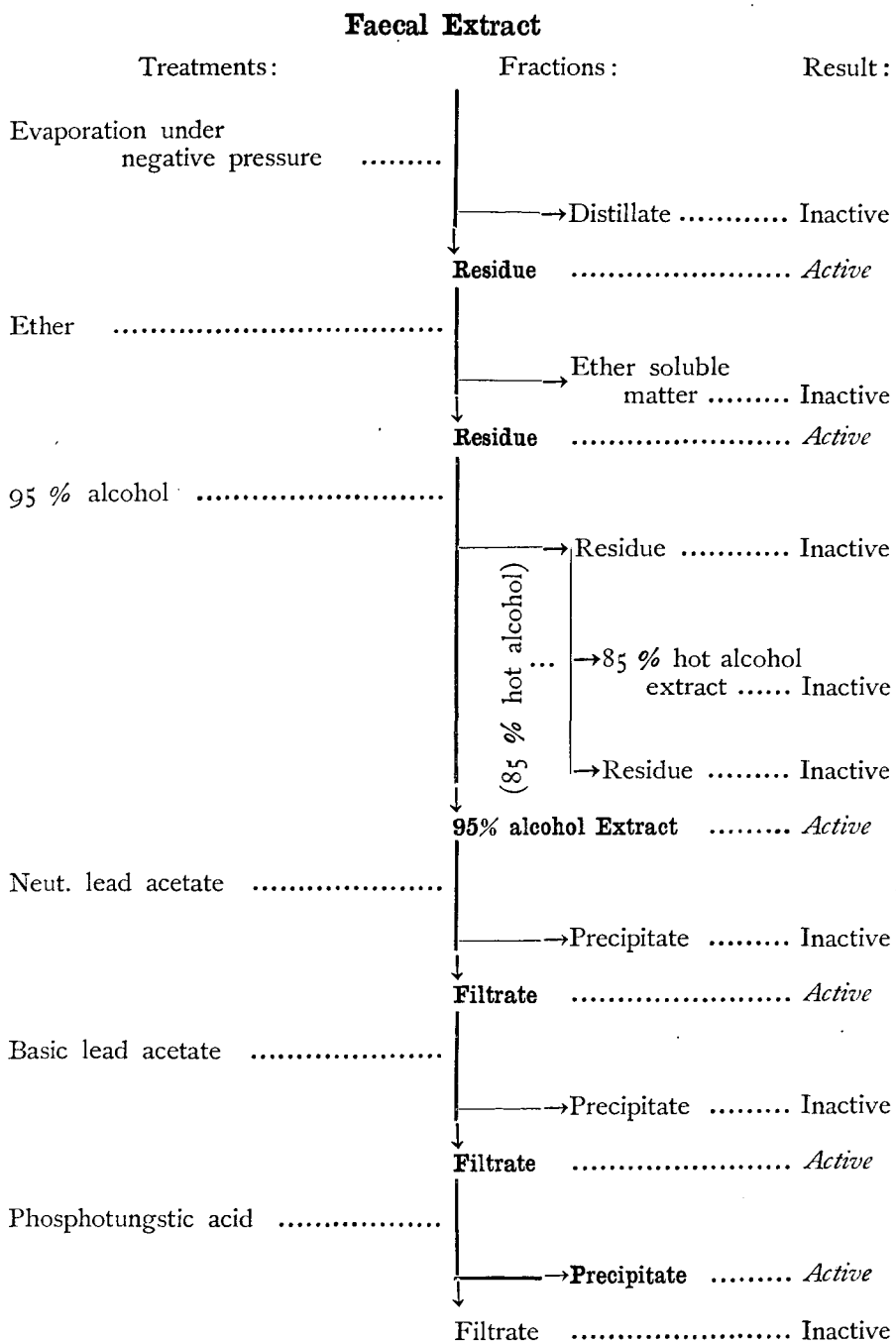
toxic to bacteria, its removal was made with special precautions.

The two fractions obtained were then substituted for faecal extract in KHOUVINE's solution and cultures of *Bac. cellulosae dissolvens* were made as stated above. Under these circumstances, the cultures made upon KHOUVINE's solution, to which the fraction precipitated by phosphotungstic acid had been added in the place of faecal extract, caused the growth of the organism, after 5 days. Also pigmant was found upon the filter papers. However, upon the medium in which the filtrate was substituted for the faecal extract, no growth was found after 21 days' incubation, indicating that it was not utilizable by the organism.

Table VIII shows the modes of cellulose decomposition caused by *Bac. cellulosae dissolvens* upon the medium, faecal extract of which was substituted with the fraction precipitated by phosphotungstic acid in varied amounts.

TABLE VIII. Influence of varied Amounts of Fraction precipitated by Phosphotungstic Acid, upon the Growth and Decomposition of Cellulose by *Bac. cellulosae dissolvens.*

Culture number	Material tested per liter		Peptone (g.)	Cellulose digested after (days)	
	mg.	Nitrogen (mg.)		(1)	(2)
Control	KHOUVINE's solution		1	5	5
1.	6,52	1,728	1	No digestion	
2.	12,34	3,346	1	12	13
3.	32,65	8,164	1	6	6
4.	65,10	16,328	1	5	5
5.	97,66	24 492	1	5	5
6.	123,20	33,456	1	5	6
7.	195,40	50,148	1	5	5
8.	65,10	16.328	—	No digestion	
9.	—	—	1	No digestion	
10.	65.10	16,328	2	5	7
11.	65,10	16,328	4	9	No digestion
12.	65.10	16,328	6	No digestion	
13.	65,10	16,328	10	No digestion	
14.	97.66	24,492	2	6	9
15.	97,66	24,492	4	9	9
16.	97,66	24,492	8	No digestion	


Faecal Extract
Treatments:
Fractions:
Result:
Evaporation under negative pressure .....
→Distillate ..... Inactive
Residue ..... Active
Ether .....
→Ether soluble matter ..... Inactive
Residue ..... Active
95 % alcohol .....
→Residue ..... Inactive
(85 % hot alcohol) ...
→85 % hot alcohol extract ..... Inactive
→Residue ..... Inactive
95% alcohol Extract ..... Active
Neut. lead acetate .....
→Precipitate ..... Inactive
Filtrate ..... Active
Basic lead acetate .....
→Precipitate ..... Inactive
Filtrate ..... Active
Phosphotungstic acid .....
→Precipitate ..... Active
Filtrate ..... Inactive

From the table, it may be seen that when the substance prepared by the treatments above described is contained to the amount of 12,34 mg. in one liter of the medium, growth of the organism is already recognized. When, to the medium, 65 mg. of the substance is added, no appreciable differences were recognized from that cultivated upon KHOUVINE's solution. Furthermore, when the cultures were repeated upon the same medium, no interception was recognizable; hence this fraction was found to be the substance necessary to *Bac. cellulosae dissolvens* for its growth and decomposition of cellulose.

In brief, in faecal extract, the substance required for the growth of *Bac. cellulosae dissolvens* is, as shown in the diagram, soluble in water and 95 % alcohol, not precipitated with neutral or basic lead acetate solution, but only by phosphotungstic acid. From its characters, this substance resembles vitamin B*. KHOUVINE (15) has, however, reported that the substance neccessary to the growth of *Bac. cellulosae dissolvens* is not vitamin B.

Whether the substance above characterized is vitamin B or not, must of course be determined by further investigations. However, regarding the characters of the substance which was determined to be required for the growth of *Bac. cellulosae dissolvens* prepared from faecal extract, further studies were impossible because of shortage of the sample due to the difficulties of its preparation. But if the other substances prepared as described above are utilized for the growth of the organism, as the substance obtained from faecal extract, and if the former are of known characters, then indirectly, the substance which the organism utilizes may be decided. Next, "Beriberol" was used to determine whether the organism utilizes it or not. "Beriberol", which according to the advertisement, was prepared from rice bran, contains vitamin B of appreciable amounts. When employed in place of faecal extract in KHOUVINE's solution, *Bac. cellulosae dissolvens* grew vigorously, and as shown in the table very little quantities brought about its growth.

In case of the substance isolated by means of barium hydroxide after being absorbed from faecal extract by FULLER's earth, or of the substance obtained from faecal extract by decomposing with barium hydroxide the precipitates caused by addition of tannic acid, when the usual treatments were made, these substances were ascertained to exist and to be utilized

* The word vitamin is used in the following discussion to indicate real vitamin, or a vitamin-like substance, or some agent which is chemically or physiologically bound up closely with vitamin.

by the organism.

TABLE IX. Growth of *Bac. cellulosae dissolvens* upon the Medium in which Faecal Extract of KHOUVINE's Solution was substituted by "Beriberol" in varied Amounts.

Culture number	"Beriberol"		Peptone g.	Cellulose digested after (days)	
	mg. per liter	Nitrogen mg.		(1)	(2)
Control	KHOUVINE's solution		1	6	6
1.	10	0,206	1	5	5
2.	20	0,412	1	5	5
3.	50	1,030	1	5	5
4.	70	1,442	1	6	5
5.	100	2,060	1	6	5
6.	250	5,150	1	7	7
7.	500	10,300	1	7	6
8.	750	15,450	1	7	6
9.	1,000	20,600	1	No digestion	
10.	1,500	30,900	1	No digestion	
11.	no "Beriberol"	—	1	No digestion	
12.	100	2.060	1	No digestion	

Furthermore 95 % alcohol extracts of dried yeast, rice bran and butter, and decoctions of potato and soil were utilized in no cases, but 95 % alcohol extracts of digested bouillon and soil on which the studied organism was isolated, were well utilized by the organism. In the above substances, in which bacterial growth did not take place, the absence of the utilizable substance may certainly be a cause, but it is not difficult to conjecture that non-growth was chiefly caused by the presence of injurious substances.

In the writers' experiments, whether or not faecal extract serves as nitrogen source of the organism, as KHOUVINE has reported, was not determined. However, the organism is incapable of utilizing carbohydrates other than cellulose; peptone contained in KHOUVINE's solution seems to play a rôle as nitrogen source. That no growth took place when the peptone or faecal extract was removed from the KHOUVINE's solution, is

obvious from the tables above given. That peptone is well suited for the organism as nitrogen source has already been stated. Although both vitamin B prepared from faecal extract and "Beriberol" contain the nitrogen as seen in the tables, the authors suggest that faecal extract plays rôles in the growth and activities of the organism other than serving as the nitrogen source.

In general, among some bacteria, whose growth takes place with difficulty only by the presence of nutrients as carbohydrates, protein, etc., require the so-called growth accelerants whose fundamental nature is yet not at all well known. To this matter much attention has been paid in recent years. These growth accelerants are divided into two groups, one inorganic and the other, organic. About the former, considerable data have already been gathered, but little is known about the nature of the latter. There are many organic substances which were reported to be capable of accelerating bacterial growth; of which vitamins, auximones, bios, are the principal ones. Above all, it has been several times suggested that vitamins may be either essential to the growth of certain bacteria or that at least they act as accelerants. One of the first of these suggestions was that of LLOYD (17) who concluded that the *meningococcus* could be cultivated only in the presence of vitamin. One of the earlier instances of stimulation of one organism by the growth products of another was brought to light by GRASSBERGER (10) and this phenomenon was called a cultural satellitism. Since this earlier work, many investigations have been made from various directions; it has been well recognized that vitamins are essentially needed for some groups of bacteria when they are cultivated upon synthetic media.

Very few reports have been made on the substances capable of accelerating the growth of cellulose decomposing bacteria when the cultures were made upon synthetic media. SANBORN (29) has found that vitamin B is effectual to the growth and physiological efficiencies of *Cellulomonas folia*; CLAUSEN (4) has recognized that cellulose-liver-bouillon is superior to inorganic media for the growth of *Bac. Omelianskii*; COWLES and RETTGER (5) have found that meat infusion broth is well suited for the cultivation of *Cl. cellulosolvens*, and SNIESZKO (32) has also reported that his thermophilic cellulose decomposing bacteria was stimulated by the addition of some yeast. However, WERNER (39) has found that the juice of turnip rooted cabbage as vitamin source has no effect upon the growth of *Bac. cellulosam fermentans*.

From the fact that the growth of *Bac. cellulosae dissolvens* took place

vigorously in the medium containing vitamin B, while on the contrary no growth took place in its absence, and that vitamin B is not the nitrogen source to the organism, the authors considered that vitamin B exists in faecal extract, and that as an accelerant the substance is absolutely essential for the metabolic activities of the organism. It has already been brought to light that vitamin B is synthetized by many intestinal microflora (2) (30) (40); its existence in the faecal extract is indubitable. Furthermore, so long as intestinal microflora synthetize vitamin B, and the growth of *Bac. cellulosae dissolvens* which does not grow upon cellulose bouillon did take place upon bouillon digested by *Bact. coli*, it is known that the cause is partly due to the fact that vitamin B exists in the medium. However, the nature of the so-called vitamin B is not yet completely known, and in many agents the so-called vitamin B, or substances at least closely connected with it, or substances completely independent, must be contained; what substances in the so-called vitamin B play what rôles in the activities of *Bac. cellulosae dissolvens* must be explained by further investigations.

SUMMARY

1. *Bac. cellulosae dissolvens*, an obligate anaerobic spore bearing bacillus which decomposes cellulose accompanied by the formation of hydrogen, carbon dioxide and orange pigment, was found from the soil.

2. Considerable difficulties were experienced in its cultivation, as the bacillus does not grow upon ordinary culture media. Enrichment cultures of the organism were made in accordance with KHOUVINE, CLAUSEN, WERNER, etc.

3. Pure culture of the organism was obtained in no case, whether by heating the cultures which contain the spores of the subject bacillus for 15 minutes at 100°C. and then washing the filter paper rests, to which the organism clings, with sterilized physiological salt solution, nor by sterilizing the Gram-positive bacteria with dye-solution, nor by attempting to remove by heat the contaminated forms causing their spores to germinated or bringing about their asporogenesis, nor by causing the desired organism to grow under colony formation upon a solid medium.

4. A pure culture of the bacteria was obtained by "Ausschneide Verfahren" alone.

5. In these experiments, attempts were made to determine the rôles

which faecal extract plays in the activities of *Bac. cellulosae dissolvens*. Among the fractions, obtained by various treatments and chemicals from faecal extract, it was proved that the organism utilizes a substance resembling vitamin B, which is soluble in water, 95 % alcohol, not precipitated by the addition of neutral or basic lead acetate solution, and precipitated only with phosphotungstic acid.

6. Whether the substance serves as nitrogen source or not still admits of further discussion. However, from the facts that growth did not occur when either peptone or vitamin B was absent, whereas growth did take place upon the presence of vitamin B in very small quantities, one is led to the belief that faecal extract was considered to be an accelerant essentially necessary to the studied bacillus.

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Explanation of figures

Fig. 1. Photograph showing the decomposition of cellulose by *Bac. cellulosae dissolvens* in KHOUVINE's solution. (2/3 natural size).

1. Control, without bacterial inoculation.
2. Six days' culture at 37° C., showing cellulose partly digested.

3, 4, 5, 6, 7 and 8. Nine days' cultures at 37°C., in which cellulose have lost their original form.

Fig. 2. Microphotograph showing the bacteria attached to cellulose. (Seven days' culture in KHOUVINE's solution at 37°C. Stained with gentian violet solution. × 800).

Fig. 3. Germinating spores of *Bac. cellulosae dissolvens.* (Twelve hours' culture in KHOUVINE's solution. × 1720). (Drawning).

Fig. 4. *Bac. cellulosae dissolvens*, cultured upon KHOUVINE's solution for 6 days at 37° C. × 1720. (Drawning).

Fig. 5. Elongated cells of *Bac. cellulosae dissolvens.* (Culture made upon digested bouillon, 10 days at 37° C. × 1720). (Drawning).

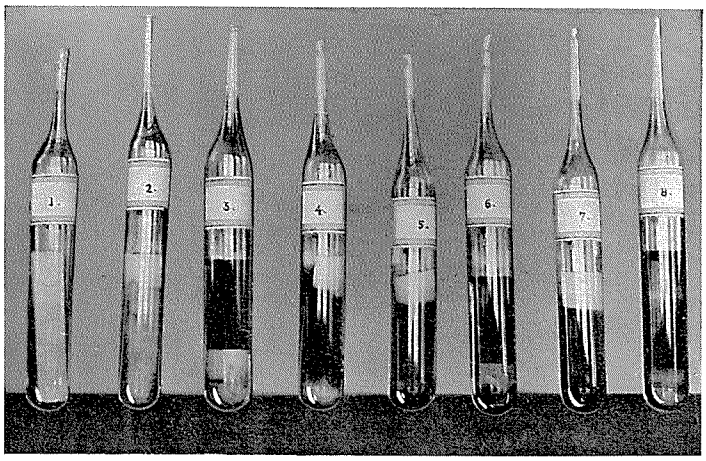


Fig. 1.

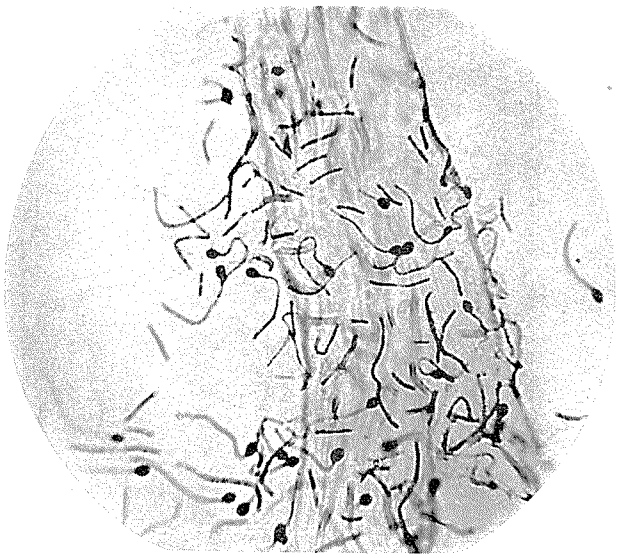
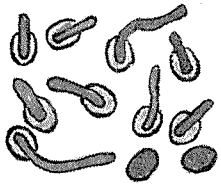


Fig. 2.



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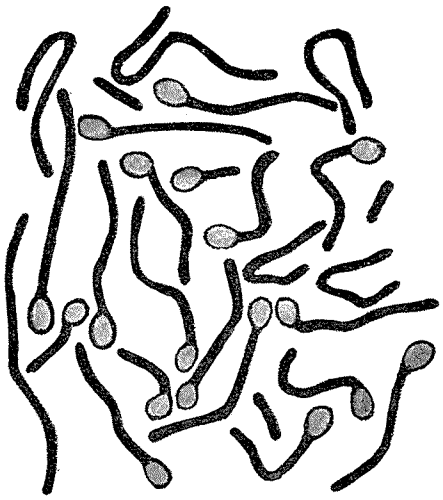

Fig. 4.

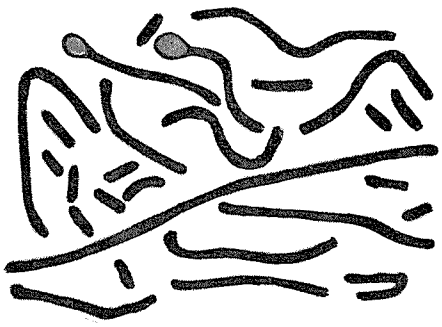

Fig. 5.