THE DECOMPOSITION OF CELLULOSE IN NATURE*

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The breaking down of cellulose in nature is apparently a function of the lower forms of life. Protozoa, fungi and bacteria are the most active organisms in this respect. To be sure the higher forms of animal life use cellulose for food. This is especially true of the ruminants. This utilization, however, seems to be dependant on the presence of some bacteria or protozoa in the alimentary tract. According to Yonge (1925), cellulose has never been demonstrated in the vertebrates. On the other hand, many organisms capable of digesting cellulose have been found in both higher and lower forms of life.

Khouvine (1922) claimed to have isolated an anaerobic bacterium from the intestinal tract of man. Zuntz (1892) stated that in the case of the horse the cellulose passed to the caecum and was there digested by microorganisms. Hopffe (1919) made a bacteriological analysis of the rumen of cattle and isolated 33 organisms. Of this number only a few of the common soil forms had the ability to digest cellulose. This ability was soon lost after transferring to a new medium. She believed that protozoa were necessary for cellulose digestion in cattle. Tappeiner (1880) said that cellulose was digested in the first three stomachs of ruminants. He compared this digestion with that of sugars and starches and pointed out that if such carbohydrates were present the cellulose would not be attacked. The presence of large amounts of albuminoids seemed to favor the digestion of cellulose even in the presence of sugars. Shirokikh (1900) said that in sheep one-third of the cellulose was digested in the paunch. The percentage of cellulose to the total bulk was very low in the small intestines but increased before the cellulose was finally excreted. This same author stated that in the case of the rabbit, the digestion took place in the blind intestine. Like sheep, goats are able to digest large amounts of cellulose. Edin (1920) reported between 83 and 98 per cent of the cellulose ingested as food by these animals as digested.

Unlike the mammals the birds do not seem to have the ability to digest cellulose. The rapidity with which digestion takes place would preclude any possibility of bacterial action. Radeff (1928) reported on his experiment with chickens. He found that raw fiber as found in barely could not be digested, while five per cent of wheat fiber, seven per cent of oat fiber and seventeen per cent of cracked corn could be. As for the mammals, this digestion occurred in the caecum.

Cleveland (1925), in a series of papers covering his researches with termites, brought to light a very interesting type of symbiosis. These insects were able to utilize pure cellulose for food provided certain protozoa were present in the intestinal tract. By starvation, oxidation and change of temperature, the author was able to free the intestinal tract of all protozoa. Under these conditions, the termites could live for only a short time on cellulose alone as food. If fed fungous-digested cellulose they carried on a normal existence. With the protozoa present the insects were able to survive eighteen months or more on a diet of pure cellulose. No bacteria were mentioned. Where the termites got their nitrogen

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was not explained. The author, however, did mention the possibility of nitrogen fixation although he was not able to demonstrate the process.

There are a few crustacea that can digest cellulose. Biederman and Moritz (1898) showed that the fresh water cray-fish was able to dissolve reserve cellulose from the endosperm of seeds. They further stated that the alimentary tract of *Helix* contained a fluid which would attach hemicelluloses but not celluloses. Seillière (1906) showed that the digestive fluid of *Helix* acts on cotton fibers to a very slight extent; much more rapidly if the cotton was finely divided. Yonge (1925) found that the Teredinidae could digest cellulose. The material was stored in the caecum and was there slowly digested. Eighty per cent of the cellulose was changed in passing through *Teredo*.

The fungi are very active in decomposing cellulose. Kellerman and associates (1913), Daszewska (1912) and others stated that they were more active than bacteria. The higher fungi do not lend themselves well to experimentation. Some doubt is expressed as to whether they have the property of digesting cellulose that the lower forms have. Schmitz (1919) concluded that the rate of decomposition of wood by *Fomes*, *Polystictus* and *Lenzites* was greatly increased by the presence of saprophytes. He was, however, unable to isolate any cellulose decomposing bacteria.

Kellerman (1913) demonstrated the presence of an active cytase in *Penicillium pinophilum*. The author, no doubt, would now term his enzyme cellulase to conform with present usage. His method was to inoculate either spores or pieces of mycelium on the surface of a tube of cellulose agar. After from 20 to 40 days the fungus had developed an enzyme that decomposed the cellulose in the tube below the growth. To demonstrate the presence of the enzyme, he transferred slices of this agar, removed from the tube aseptically, to plates of cellulose agar. The slice removed from below the cleared zone showed no decomposition of cellulose when transferred to agar while the three taken in the zone, but below the growth, showed decomposition but no growth. Scales (1915) did the same with the species of *Aspergillus*.

A number of workers report the isolation of fungi capable of destroying cellulose. Scales (1915) found 32 out of 40 species of *Aspergillus* and *Penicillium* studied, capable of destroying cellulose. Hopffe (1919) isolated an aspergillus from the digestive tract of cattle which could digest cellulose at 35 to 37°C. Mutterlein (1913) did a very extensive piece of work on cellulose decomposition in soils and manures. He reported the isolation of 21 different organisms capable of destroying this material. Of this number 20 were fungi and actinomyces. Heukelekian (1928), Waksman (1926) and Dubos (1928) all reported the isolation of fungi active in cellulose destruction.

The actinomyces are less able to use cellulose than either the fungi or the bacteria. Nevertheless, in dry soils, according to Dubos (1928) they were quite active. Actinomyces capable of digesting cellulose have been isolated by Mutterlein (1913), Krainsky (1913), Dubos (1928) and many others. Waksman and Skinner (1926) claimed that these organisms were limited in their action to the by-products of the cellulose fermentation.

Cellulose Decomposition by Bacteria. The bacterial decompostion of cellulose seems to have occupied the attention of workers much more than that brought about by either actinomyces or fungi. As early as 1850 Mitscherlich reported on the decomposition of cellulose by bacteria. In a potato culture he noticed that the walls of the parenchyma cells were decomposed. He filtered his

culture and introduced new slices of potato. These were decomposed more readily than the first. Many vibrios were found in the culture and to these he attributed the digestion. Trecul (1865) described rod forms which appeared in decomposing plant cells. These had the property of staining blue with iodine. To these he gave the name "Amylobacter." It is this group of organisms that played the leading rôle in the early history of the study of cellulose decomposition.

Popoff (1875) was the first to study the fermentation of cellulose from a bio-chemical angle. He claimed that the destruction of Swedish filter paper took place with the evolution of CO_2, CH_4 and H_2. The carbon dioxide and methane were evolved during the fermentation of cellulose while the H_2 resulted from the secondary fermentation; that of butyric acid.

Van Tieghem published the results of his studies of cellulose fermentation in 1879. He claimed that *B. amylobacter* was the true causative agent. His organism could use sugar as readily as cellulose, but if the two were present, cellulose was not attacked. The enzyme bringing about the change was not extra-cellular and was formed only when the organism was stimulated by the cellulose. This seems to be the first mention of the nature of the enzyme. Unfortunately Van Tieghem worked with plant cells rather than with pure cellulose. Further his cultures were not pure, as is shown by the work of Omeliansky in 1895.

Hoppe-Seyler (1886) observed anaerobic fermentation of cellulose, using sewage mud as inoculum. His experiments lasted for four years at room temperature. The bacteria described cannot be distinguished from Van Tieghem's amylobacter. The fermentaion according to this author, was divided into two phases: *first*, the hydrolytic phase where cellulose was broken down to a monosaccharide,

$$C_6H_{10}O_5+H_2O=C_6H_{12}O_6;$$

second, the oxidation-reduction phase which took into account the breaking down of the C_6 sugar to methane and carbon dioxide,

$$C_6H_{12}O_6=3CO_2+3CH_4.$$

He was unable to detect any volatile acids and therefore, claimed that the views of van Tieghem were incorrect. He stated, however, that there was a possibility that such acids might be formed as intermediate products.

Symbiosis as a factor in tearing down cellulose was first worked out by Van Senus in 1890. He worked with organisms from the blind intestine of rabbits. This part of the alimentary tract of the rabbit, he described as being filled to about half its capacity with organisms resembling *B. amylobacter* of van Tieghem. This large bacillus and a small one which he was able to isolate from the same source, caused the decomposition of cellulose. Each one separately had no effect on the carbohydrate. One organism made the harmful product of fermentation harmless for the other organism; a statement difficult to understand if neither has any effect by itself. The process he classified as anaerobic. His views on the mechanism of fermentation were at least novel. The products he gave as H_2, CO_2, acetic and butyric acids. Butyric acid came from the protein decomposition. He did not believe the production of CH_4 to be an independant process. Acetic acid was reduced by the hydrogen present successively to aldehyde, alcohol, ethane and methane. Where no other easily reducible substance was present CH_4 and CO_2 only were found. Thus he explained Hoppe-Seyler's results.

Omeliansky in a series of papers extending over a period of years gave the results of his efforts to solve the problem of anaerobic decomposition of cellulose.

According to this author, *B. amylobacter* was not the causative agent. The true agent was a short bacillus that elongated before sporulation. It did not stain blue with iodine and therefore, could not be included in the amylobacter group. The anaerobic destruction of cellulose was of two types, one he called the methane fermentation and the other the hydrogen fermentation. Omeliansky's work may be criticised on the basis of his methods. For transfer he used pieces of filter paper from fermenting flasks. He claimed that in a few transfers, because of the enrichment medium, he soon obtained a pure culture. The purity was judged by microscopic observation only. He made several attempts at isolation. He obtained growth on potato in the form of small yellow colonies. These, however, failed to cause fermentation when transferred to his enrichment medium. Concerning these colonies he says: "cependant, les caractères morphologiques des bacilles ayant poussé sur pomme de terre, aussi bien que le fait d'avoir fait fermenter le papier, ne laissent aucum doute que nous avons bien réussi a obtenir des colonies du ferment*." Other than this potato experiment, all attempts at growth on any solid medium failed.

His organism being a spore former, it was thought that the contaminant could be separated by heat. Boiling temperature, however, failed to give the desired results. Fractional heating was tried but with the same negative results. Dilution methods were attempted but to no avail. The use of carbohydrates other than cellulose gave no results. He explained the failure of his dilution method by saying that a large inoculum is necessary. Failing in all attempts to separate what he believed to be two or more organisms he tried to explain their presence on the basis of symbiosis. He tried various experiments of mixing organisms but he was never able to get the typical fermentation.

Omeliansky's researches, although, leaving much to be desired as to purity of cultures, were accepted as rather conclusive evidence of the presence of two types of cellulose fermentation. It was not until seventeen years after his first report appeared that his conclusions were questioned.

Kellerman and McBeth (1912) isolated from Omeliansky's hydrogen culture, two cellulose destroyers and five contaminants. From his methane culture they isolated one cellulose fermenter and two contaminants. The three cellulose fermenters, they named *B. rossica*, *B. amylolyticus* and *Bact. flavigens*. All of these organisms were morphologically different from Omeliansky's hydrogen and methane organisms. They grew well on ordinary gelatine media, although, continued growth resulted in loss of cellulose fermenting properties. They did not produce gas. For isolations, these workers used agar plates. Their medium consisted of a minerial solution to which was added cellulose, agar and $CaCO_3$. The colonies developed with the characteristic halo. When transferred to liquid cellulose media they failed to digest cellulose. The method used and the results obtained led Omeliansky to criticise the work of the two American workers on the basis of faulty observations. It was claimed that the clear zones were not caused by the digestion of the cellulose but by the action of acids on the $CaCO_3$. The acids might have arisen from the decomposition of organic substances in tap water; from extraction materials from the agar; or from products of partial hydrolysis of agar in the presence of mineral salts. The fact that fermentation was lacking in liquid cultures when a colony was transferred, led Pringsheim (1923) to criticise the work stating that pure cultures could not be obtained by the method employed. The organisms transferred from the agar plate were not

*Archives des Sciences Biologiques, 7:724, 1899

cellulose decomposers but contaminants, he stated; therefore, the failure of the liquid cultures. Löhnis and Lochhead (1913) corroborated the work of Kellerman and McBeth and showed very conclusively that the clear areas on the plate were zones of digested cellulose. Their method was to pour HCl over the plate in question, to dissolve out the $CaCO_3$. With the plate transparent the disappearance of the cellulose could very easily be detected.

Aerobic cellulose decomposition was first studied in 1904. Van Iterson found that when a medium consisting of paper and mineral salts was distributed in shallow layers and inoculated with ditch mud, an energetic fermentation followed. His experiments were carried out at a temperature between 25° and 35°C. In addition to the various contaminating forms encountered in mixed cultures he noticed the presence of two forms; one a small rod and the other a large micrococcus. The rod, he named *B. ferrugineus*. The micrococcus, he regarded as an organism that facilitated the decomposition of cellulose but did not play an active part itself. All attempts to digest cellulose with what he claimed to be pure cultures, failed.

Löhnis and Lochhead (1913) isolated aerobic cellulose fermenters using the Kellerman method. Contrary to Kellerman's conclusions, however, the authors were able to get an active fermentation in liquid media inoculated with colonies from the plate.* They raised the question of purity of their cultures as they observed spherical as well as rod shaped cells. They hinted at symbiosis but carried out no experiments to prove or disprove this. Löhnis suggested that the organism might have had a life cycle and that these different morphological forms might have been stages in this cycle. Their findings, according to the authors, check up pretty closely with those of Hutchinson and Clayton (1919).

McBeth and Scales (1913) isolated 15 cellulose destroying bacteria from soils. All were facultative but destroyed more cellulose under aerobic conditions. Transferred from the agar plate to liquid media, they all lost the property of using this material. All of the organisms grew on gelatin, although, this was not a good medium for isolation. No gas was given off during the process. They explained the presence of CO_2 and CH_4 on the basis of a secondary fermentation of by-products. Many organisms that could use these by-products were present in the soil, according to the authors, and a mixed culture would give gas while the pure culture would not; if their contention were correct. They carried out experiments to show this. Two flasks that had undergone a typical fermentation were used. One was inoculated with soil while the other was not. The first showed gas formation in 48 hours and continued to produce gas for two to four weeks. One hundred c.c. of gas were collected and analysed. It proved to be CO_2 and H_2. The second flask never showed any gassing. In no case did they find CH_4 in any of the filter paper fermentations. Only when large amounts of nitrogenous matter were present were they able to show the presence of this gas. Attempts were made to isolate organisms capable of giving off gas when associated with cellulose fermenters. In no case were they successful, although, they did get organisms capable of fermenting sugars with gas formation. They stated that the chief by-products were acetic and formic acids with no trace of aldehydes, ketones, alcohols or carbohydrates capable of reducing Fehling's solution.

Hutchinson and Clayton (1919) reported the presence of an organism capable of breaking down cellulose in Rothamstead soil. Morphologically it

*Private correspondence.

presented many characteristics of the Spirochaetoideae and the name *Sp. cytophaga* was suggested. In many respects their organism answers to the description given by van Iterson for his so-called mixed culture. According to the authors, the organism passes through a number of phases which terminate with the production of a "sporoid"—the micrococcus of van Iterson. This "sporoid" differs materially from the true spore of the bacterial cell. On germination it gives rise to a flexible form, therefore, the genus *Spirochaeta*. The organism is aerobic; its optimum temperature around 30°C. All phases are killed by a temperature of 60°C. in 10 minutes. It is very limited in its energy source, being able to use only cellulose. It does not grow well on agar. The authors used mineral salts in tubes to which they added the filter paper in strips, allowing the ends to protrude above the liquid, thus making for better aerobic conditions. Unfortunately, according to Löhnis, the authors' cultures were lost during the war as were the cultures of Löhnis and Lochhead*. From the descriptions it seems that the two organisms belong to the same group if they are not identical. Löhnis' suggestion and Hutchinson and Clayton's statement that the organism goes through a life cycle are probably correct. Further work, however, will be necessary to prove this point.

A number of workers have attempted to show that the process is a symbiotic one. Groenewege (1920) claimed that the process was one of denitrification involving the interaction of two groups of organisms. One group decomposed cellulose while the other reduced the nitrates. He isolated five organisms: *Bact. cella-resolvens alpha, beta* and *gamma* belonging to the cellulose decomposing group and *Bact. opalescens* and *Bact. viscosum* belonging to the denitrifying group. C. M. Hutchinson (1923) reported the isolation of a specific aerobic cellulose dissolving organism and added that the decomposition could be accomplished only through symbiosis with other soil forms, which he does not name. Aoi and Orikura (1928) isolated what they termed the contaminating organism but were unsuccessful in isolating the cellulose decomposer.

Dubos published his results on aerobic cellulose decomposition in 1928. He isolated a number of pure cultures which he placed in three physiological groups as follows: (a) strict aerobes, using only cellulose for carbon; (b) strict aerobes, cellulose decomposers, able to grow on starch but not on nutrient agar; (c) facultative anaerobes, cellulose decomposers which grow well on ordinary media.

Winogradsky (1928) states that anaerobic cellulose decomposition is of minor importance in cultivated soils. He describes three types of aerobic cellulose destroyers:

1. Thin vibrio, spreads rapidly on paper and produces a pale yellow tint. The fibers are lined both externally and along the walls of the lumen with vibrios. The yellowing increases with age. The process seems to stop half way, as many of the fibers are untouched. After three or four weeks the vibrios are autolysed.

2. Decomposition proceeds further, up to 80 per cent. The group is not described.

3. Cytophaga group. These produce colonies and transform the paper to a thick jelly. The pigment is egg yolk yellow, orange or rose according to species. The action is slower than that of the first two groups but much more complete.

In a later publication (1929) Winogradsky goes into further detail regarding

*In a private correspondence, Bunker states that Hutchinson and Clayton's cultures still exist.

the study and classification of the aerobic cellulose decomposers. His method involved the use of silica gel and filter paper. Liquid cultures were never used. Particles of soil were placed on the sterile piece of filter paper on the gel and the plates were incubated at 30°C. After a few days the characteristic color developed and transfers were made either with a straight platinum wire or with a piece of glass tubing drawn out to a very fine point. The colors developed were so characteristic that he claimed they were sufficient to identify the organism. The colors included the various shades of yellow, brown, rose and green. He does not believe in the dilution method for isolation purposes for these groups, due to the fact that the organisms adhere to the fibers and are not dislodged. He describes his method of transfer as follows: "on ne faisait que toucher aussi légèrement et rapidement que possible avec le bout d'un fil de verre capillaire un point choisi et soumis a l'examen microscopique, puis de la même manière le rond neuf*." The question might well be asked whether the author considers that the method described would dislodge more organisms than the vigorous shaking in a dilution bottle? It would seem that if the organisms are so loosely attached as to be removed by a light touch of the wire they would most certainly be removed by shaking. In the case of the *Cytophagas* he does not agree with Hutchinson and Clayton as regards the relationship of the "sporoids." He believes they are a different organism. Still, he says that when they do appear, he is not able to separate the two. He is uncertain about the motility of this group, suggesting that if there is any motility it is of the crawling kind; and he mentions seeing haustoria like appendages, but so minute that he is not ready to affirm their presence. The culture soon autolyses and nothing but round bodies remain which do not stain well and cannot develop into the rod again. He has observed small round bodies which he likens to arthrospores and supposes that reproduction may be carried on by them.

The three groups are classified as:

1. *Cytophaga*, long, flexible filaments, pointed at each end. Motility questionable. Very specific in that they can use nothing but cellulose for carbon. Colors the paper egg yolk yellow, orange, rose, brick red, etc. No spores. He studied four species.

2. *Cellvibrio*, long rods, rounded ends. Actively motile, having only one flagellum. Not as specific as the cytophagas. Produces yellow to cream colored pigment, very diffuse and spreading rapidly over the gel. No spores. Two species studied.

3. *Cellfalcicula*, sickle shaped with pointed ends. Very motile, only one flagellum. Appears to be specific as regards cellulose. Produces greenish tints. No spores. Three species studied.

The author claims that in all cases the cellulose is changed to oxycellulose. He was never able to detect any volatile products.

Thermophilic Decomposition. The first mention of cellulose decomposition at temperatures above 40°C was made by MacFayden and Blaxall in 1899. Soil was added to a nutrient solution and incubated at 60°C. The paper contained in the medium was destroyed in 10 to 14 days. Bacilli as well as many spores were detected in the culture. Zoögloea enclosing many small bacteria were likewise observed. No reducing sugars were found. Their products were

*Ann. Inst. Pasteur, 43:584, 1929.

acetic acid, butyric acid and furfurol. They stated that the action seemed to be symbiotic.

Primm (1913) reported the isolation of pure cultures of thermophilic cellulose fermenters. Unfortunately there is nothing left by which his claim may be proved or disproved. Other than a note and two photographs in a bulletin issued by the Wisconsin Experiment Station, no record of his work seems to exist. In this same year, Kroulik (1913) isolated four cellulose decomposers, two aerobic and two anaerobic, all thermophiles. Three of these were plectridia while the fourth produced equatorial spores. The aerobic organisms did not ferment cellulose after growing on agar while the anaerobes did not grow on solid media. He believed, with Omeliansky, that heavy transfers were necessary. He also considered symbiosis an important factor in the process but was unable to demonstrate it. Grieg-Smith (1921) described a large bacillus with terminal spores as responsible for the thermophilic fermentation of tan-bark.

Pringsheim (1912) established the fact that the fermentation of cellulose passed through the cellobiose and dextrose stages. By using toluol he was able to arrest the fermentation and detect the presence of these carbohydrates. His work was carried out at 60°C. The author further demonstrated that the enzymes did not pass through a Berkefeld filter. The filtrate of an active culture failed to show any decomposition of cellulose when added to this material. He made the statement that the enzyme was intracellular and the organism producing it could be stimulated to its elaboration only by contact with a piece of cellulose. This is essentially what was voiced some years before by van Tieghem. Löhnis points to the clear zone around the colony as proof of the solubility of the enzyme. Kellerman showed the enzyme, in the case of fungi, to be extracellular. Grownwege did the same with the enzyme of bacteria.

Khouvine (1923) reported on her investigations of the digestion of cellulose by the intestinal flora of man. After testing a number of media she limited herself to the use of a substrate consisting of potassium phosphate, sodium chloride, peptone and tap water. A culture was finally enriched that attacked filter paper rather slowly and in spots. Microscopically the spots appeared to contain a large rod, a small rod and a coccus. All methods of dilution or plating failed to separate these, so she picked out a few fibers of the paper and after several washings in physiological salt solution, she claimed that the culture was pure. The cellulose organisms, she reasoned, would adhere to the fiber while the contaminants would be washed away. Fermentation in pure culture was very slow. Her best and most rapid decomposition was obtained when an extract of fecal matter was added. Large inoculations were apparently needed to start the fermentation. She describes the organism, *B. cellulose dissolvens* as a single rod, 2 x 0.4 microns, elongating to 12 microns just before sporulation. The spores are oval and terminal. The organism is gram negative and stains well with anilin dyes. It is a strict anaerobe and does not grow where there is an atmosphere with an O_2 tension of more than 12 mm. of mercury. It grows well in an atmosphere of nitrogen, CO_2, or hydrogen. The spores resist a temperature of 100°C. for 45 to 50 minutes. The optimum for the fermentation is between 35 and 51°C. The organism will survive a temperature of 67°C. indefinitely but will not ferment cellulose. The minimum for fermentation is 33°C. It does not grow on ordinary culture media, and does not ferment carbohydrates other than cellulose. The products of fermentation are CO_2, H_2, acetic and lactic acids, traces of alcohol and pigment. The composition of the pigment is not known. Like Pringsheim and others, she stated that the enzyme concerned was an endo-enzyme.

The work of Viljoen, Fred and Peterson on the isolation of *Cl. thermocellum* and the fermentation of cellulose by this organism was reported in 1926. They used horse manure as the source of inoculating material, and a temperature of 65°C. H_2S was very abundant in cultures made from the first five enrichment transfers. After this the gas was no longer noticeable. The characteristic fermentation with the formation of a "head" and pigment, resulted. When manure was used for inoculum the fermentation lasted for 10 to 14 days. The fermentation in the later transfers lasted only 6 to 8 days. The culture was subjected to a temperature of 118°C. for 25 minutes. At this point the culture was considered pure but growth on solid media was resorted to as a final proof. All attempts to grow the organism on a plate resulted in failures. The culture finally called pure was taken from a shake tube in cellulose agar of a dilution of 1-10,000. A gas bubble formed in the agar and this was removed aseptically and placed in a tube of cellulose medium. The characteristic fermentation resulted. Here again, the failure of earlier workers with the plate method was cited and encouragement was apparently taken from this failure for "it is suggested that some highly oxidizable component is lost during the plating process" and the relation of the organism to solid media was forgotten with that statement. The organism described is a long rod with oval spores and peritrichous flagella. It is gram negative, grows well in nutrient broth, on agar and potato. Many of the sugars are fermented. The products of fermentation are CO_2, H_2, acetic acid, butyric acid and ethyl alcohol.

Thaysen and Bunker (1927) commenting on the work of the above authors have the following to say: "Though the authors mention in this second paper that they have succeeded in isolating the type in pure culture, it is very questionable whether independant investigation would confirm this since Viljoen, Fred and Peterson obtained the culture they describe as pure, from a dilution containing as much as 1-10,000 part crude culture and from this dilution in cellulose agar they isolated, not one well circumscribed colony, but were content to employ as inoculant for their pure cultures, a part of this agar medium containing a gas bubble*." Coolhaas (1928) goes a little further. After describing the method used he says "Versuche von Fred, Peterson und Viljoen nichts beweisen" **. Other than the criticisms cited it seems that the fact that results are not consistent; that growth occurs in dilutions beyond the last giving active fermentation; and the fact that it cannot be transferred from solid media and give fermentation; are very good reasons for considering the culture as impure. Peterson, Fred and Marten (1926) found that the by-products of sugar fermentations are quite different from the by-products obtained from cellulose.

Cl. thermocellum and *B. cellulose dissolvens* are the only two thermophilic cellulose decomposers for which data of the chemical analyses of the products are given. A comparison of these products is given in Table 1. The poor recovery of the products in the case of *B. cellulose dissolvens* is due in part to the small amount of cellulose digested. On the other hand, the excess recovered in the case of *Cl. thermocellum* is accounted for by the fact that the process of fermentation is a hydrolytic one. Figured on the basis of percentage recovery for each product, Khouvine shows approximately 50 per cent of Viljoen's figures.

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*The Microbiology of Cellulose, Hemicelluloses, Pectins and Gums. Oxford Univ. Press, ndon 61, 1927.

**Centbl. Bakt. (etc.), 2 Abt., 76:40, 1928.

TABLE I. Comparison of the products obtained from the fermentation of cellulose by *Cl. thermocellum* and *B. cellulose dissolvens*.

	Cl. thermocellum	*B. cellulose dissolvens*
Cellulose digested	35.5 gms.	1.01 gms.
Acetic acid found	18.37 gms.	0.275 gms.
Butyric acid found	not given	0.033 gms.
Ethyl alcohol found	5.5 gms.	0.082 gms.
CO_2	11.51 gms.	0.182 gms.
H_2	not estimated	0.008 gms.
Pigment	not estimated	0.013 gms.
Total recovered	35.38 gms.	0.593 gms.
Per cent recovered	112.3	58.76

Lymn and Langwell first reported the results of their experiments with thermophilic cellulose decomposing bacteria in 1923. They stated that the optimum for the organism was 60°C. to 68°C. but on ordinary media they carried out experiments at 20 to 38°C. Under these conditions the growth was similar to, but not as rapid as, that at higher temperatures. On glucose agar they got good development of colonies in 18 hours. Two types of colonies were distinguished. One was flat, round, semi-transparent with a tendency to spread; yellowish in color and moist. The other type developed more rapidly. It was flat, and irregular in outline, opaque white, with crenate edges. These colonies remained clearer in the center than at the edge. Microscopically, the first type was seen to contain few bacilli, but large numbers of "cocci or spores." The second type contained only bacilli. Glucose shake cultures gave gas bubbles below the surface. On nutrient agar the growth was much the same as on dextrose agar but not as good. No growth in gelatin peptone bouillon occurred. Very slow moist growth on potato appeared after 48 hours. Litmus milk was coagulated in five days, acid in 48 hours. Attempts to differentiate between the two types on media failed. They state that the two types are probably two forms of the same organism growing differently because of environment.

To test the cellulose destroying ability of the colonies, such colonies were transferred to a streak culture on peptone agar and after 48 hours the growth was washed off with sterile water and used for inoculation. This was incubated at 60°C. A vigorous fermentation set in which appeared to be quite normal and seemed to follow the course of the usual cellulose fermentation. From this fermentation the following products were obtained: acetic, butyric and lactic acids; alcohol, CO_2, H_2 and CH_4.

Woodman and Stewart (1928) reported their findings as regards the presence of glucose in the course of digestion of cellulose by thermophilic bacteria. They used filter paper which was torn by hand rather than cut with scissors. The fermentation, they say, occurred much sooner if the paper were not cut. Their medium, which was a synthetic one containing calcium carbonate, sodium phosphate, ammonium sulphate, potassium chloride, tap water and paper, was inoculated with 5 gms. of well rotted dung from an old heap. The cultures were incubated at 65°C. After 24 hours, gas appeared, and a "head" was formed

in 36 hours. Fermentation was complete in from 5 to 7 days. Five c.c. transfers were made into fresh flasks of media and by subculturing every seventh day "a pure culture was soon obtained." Growth at 37°C. was unsuccessful.

Their experiments leading to the detection of glucose were as follows: 5 c.c. of culture were added to 100 c.c. of cellulose media in a flask. The flask was incubated at 65°C. for 5 days. The contents were filtered and concentrated at 45°C. in *vacuo*. The residue was tested for sugar with Fehling's solution. Under these conditions no reducing sugar was found. Negative results were also obtained when the culture was tested every 24 hours. Examination of the water in which was suspended a collodion sac culture gave a very weak test for sugar. A flask of inoculated medium was incubated at 65°C. and then 40 c.c. of toluol were added and shaken. The flask was then incubated at 37°C. for 5 days. The filtered solution gave a positive test with Fehling's solution. The osazone was identified as that of glucose. No other osazone could be detected. The authors claimed that the toluol had inhibited the action of the living bacteria and that the enzyme hydrolysing cellulose to glucose continued to function. They stated further that the antiseptic might also function in extracting the enzyme from bacteria. During the life of the organisms any sugar which was formed was immediately changed to lower products, but that after the addition of toluol no such action could have taken place and the sugar accumulated. The fermentation was much less extensive when toluol was present, only about 30 or 40 per cent was fermented. Fermentations carried out at 65°C. and transferred to 37°C. without the addition of toluol gave negative results. Their best results were obtained when the incubator accidently went up to 75°C. for 12 hours.

In common with many investigators they report that once the culture is grown on a medium without cellulose it will never ferment cellulose again. They carried their cultures on plain agar for 13 transfers and in peptone broth for 8 more. After this the cultures would not attack cellulose.

Woodman and Stewart's work cannot be taken too seriously. First, their method of obtaining a pure culture is very crude. In the light of Thaysen and Bunker's as well as Coolhaas's criticisms of Viljoen's work such gross transfers certainly cannot yield a pure culture. Their work with toluol is essentially the same as that done by Pringsheim. The change in temperature to yield an intermediate product has been made with other organisms; the butyric acid bacteria and the butyl alcohol, acetone organism for example*.

Coolhaas (1928) using mud and manure for inocula found that the crude cultures produced CH_4, and CO_2 as gaseous products. The methane fermentation changes over to the hydrogen fermentation after repeated transfers. He regards the former as secondary and as resulting from the decomposition of products of the latter. Attempts were made to get pure cultures from the hydrogen fermentation. Aerobic and anaerobic plates were made using bouillon agar, bouillon agar plus one per cent dextrose, washed agar with inorganic nutrient salts and with one per cent dextrose and also with starch. In some he used NH_4Cl while in others he used asparagin as N source. Colonies of different forms and sizes appeared on the aerobic plates. Few developed on the anaerobic plates. All colonies were inoculated in sterile media with NH_4Cl, asparagin or peptone as N source. No fermentation of paper occurred in any of the cultures. Kellerman's cellulose medium was then used. No growth occurred on anaerobic plates. Many small colonies developed on the aerobic plates after 4 or 5 days

*Thompson W. S. Unpublished M. S. thesis, Univeristy of Wisconsin.

which were surrounded by distinct clear areas. These were transferred to the same medium and cultivated in pure culture. All attempts to ferment cellulose failed. This organism was grown in symbiosis with *Bact. thermobutyricus* but no digestion of cellulose followed.

The bacteria isolated are thin spore bearing rods. The vegetative rods measured 4 to 6 micra, the spore bearing rod, 3.5 to 4 micra by 0.3 micron thick. The bacteria are non-motile and gram positive and do not stain with iodine. Colonies on Kellerman's cellulose agar are round, granulated in the centre, 0.5 to 1 mm. in diameter. The clear space is plainly visible. On bouillon agar plus one per cent sucrose the colonies are somewhat smaller, transparent and glistening. On starch agar the clear zone is larger. Stab cultures show only surface growth. There is no acid produced from sugar, no growth in milk, no nitrate reduction, no gelatin liquifaction, no indol formation nor gas production. The organism is catalase negative. The author suggests the name *B. thermocellulolyticus*.

It seems apparent from the foregoing review that the question of decomposition of cellulose is far from being solved. Much work still needs to be done to show the relationship of the organisms to the process. Then too, if the so-called farm wastes are to be used for industrial purposes, more knowledge of the products of cellulose fermentation is needed. Everything seems to hinge on the isolation of the organisms in pure culture. With the bacteria, this seems to be a difficult problem. No doubt these organisms belong to a different physiological group and new methods will probably have to be developed before much progress is made.

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