NITROGEN FIXATION AND CELLULOSE DECOMPOSITION BY SOIL MICROORGANISMS. I.

AEROBIC CELLULOSE-DECOMPOSERS IN ASSOCIATION WITH AZOTOBACTER.

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(Two Text-figures.)

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Introduction.

Although the study of the cellulose-decomposing bacteria has made great progress during the two last decades, the biochemistry of these organisms, and especially the aerobes, still remains incompletely understood. An important phase of this problem is the association between cellulose-decomposing and nitrogen-fixing bacteria which may enter into a symbiotic relationship—a phenomenon which has often been credited with great importance in the cycles of nitrogen and carbon in nature, and particularly in the soil, but which has chiefly been studied by means of impure cultures or total soil populations. Most of the literature in question has been reviewed elsewhere (Jensen, 1940); a few additional papers deserve mention.

McBeth (1911) reported fixation of 8 to 11 mgm. N per 500 ml. solution with 0·1% $(NH_4)_2SO_4$ and 0·2% cellulose, inoculated with *Azotobacter* and cellulose-decomposing bacteria. This appears dubious in view of the high initial content of NH_4–N, some of which might also have been lost during incubation by evaporation from the alkaline control medium, which apparently was only analysed at the end of the experiment. (For instance, McBeth's Table 3 shows 93–94 mgm. N in sterile controls, 102–104 mgm. in cultures. The theoretical N-content of the original medium should, according to its composition, be 106·1 mgm.!)

Tuorila (1928) found gains of 7–8 mgm. N per gm. decomposed cellulose in aerated medium inoculated with soil. The gains decreased with increasing additions of NH_4–N.

Vartiovaara (1938) found only small or insignificant gains of N in combined cultures of *Azotobacter* and cellulose-decomposing fungi under aerobic conditions, but considerable gains in cultures intermittently deprived of oxygen. Similar results were found with fungi + *Clostridium pasteurianum*, and still larger fixations were achieved by fungi + impure cultures of clostridia. Unfortunately the gains of N were not expressed in terms of weight of cellulose destroyed.

Nitrogen-fixing bacteria might derive nutrients from cellulose in several ways; for instance: (*a*) by intercepting the intermediate breakdown products of the cellulose, formed by extracellular enzymes of the cellulose-decomposing organisms; (*b*) by utilizing organic end-products of the metabolism of these organisms; and (*c*) by utilizing their products of cell autolysis. (If the products of nitrogen fixation were in their turn utilized for cellulose decomposition, the association would be an example of real "symbiosis", otherwise of "metabiosis".)

Obviously the metabolism of the cellulose-decomposers is the crucial point of the whole problem. Leaving aside, for the present, the small separate group of more or less strictly anaerobic and often thermophilic spore-forming bacilli which produce large amounts of organic acids and alcohols from cellulose, we shall briefly survey the main

groups of cellulose-decomposing microorganisms and their metabolic products. (For general references, see Winogradsky, 1929, and Waksman, 1932.)

1. *The genus Cytophaga* (Winogradsky, 1929): strictly aerobic organisms of uncertain systematic position and a characteristic morphology and life cycle (Hutchinson and Clayton, 1919; Stapp and Bortels, 1934). They attack no other carbon compound than cellulose, which is partly transformed into an alkali-soluble mucilage and appears to be directly oxidized without previous cleavage of the molecules. Hutchinson and Clayton (1919) stated that organic acids (butyric?) were formed in amounts of about 7% of the decomposed cellulose, but Winogradsky (1929) and Imshenetskij and Solntseva (1936) were unable to detect any organic by-products other than the mucilage. Imshenetskij (1938) found "traces" of reducing sugars in old cultures deprived of oxygen (autolysis products?). Walker and Warren (1938) found about 20% of the decomposed cellulose transformed into mucilage, which gave xylose on acid-hydrolysis and appeared resistant to bacterial but not to fungal attack; small amounts of a non-volatile acid and a non-reducing carbohydrate were found besides the mucilage. Bucksteeg (1936) found the metabolic products of *Cytophaga* unserviceable for *Azotobacter*.

2. *The genus Cellvibrio* (Winogradsky, 1929).—This group is closely related to the familiar genus *Vibrio*, only differing by the capacity of attacking cellulose and the often poor growth in routine media. It appears doubtful whether the "cellvibrios" of Winogradsky (1929), Stapp and Bortels (1934), and others, belong to a genus different from the "vibrios" of Kalnins (1930), Snieszko (1934), and others. The little known genus *Cellfalcicula* Winogradsky (1929) appears very similar except for a slight difference in morphology. The bacteria of this group are obligate aerobes and produce a cellulose-splitting ectoenzyme as shown by the formation of clear zones on cellulose-agar. Winogradsky (1929), Kalnins (1930), and Imshenetskij and Solntseva (1936) found no organic by-products in normal cultures, but Kalnins (1930), Itano and Arakawa (1931) and Imshenetskij (1938) detected the formation of reducing sugars in cultures deprived of oxygen. Snieszko (1934) found no reducing sugars, but "small amounts" of acetic and "traces" of lactic acid (his Table 2, indeed, shows as much as 0·11 gm. acetic and 0·20 gm. lactic acid from 0·376 gm. cellulose; misprint?). Gray (1939) found production of glucose from starch, but not from cellulose.

3. *Aerobic spore-forming bacteria.*—Organisms of this group, which also frequently grow badly in ordinary media, were first found by McBeth et al. (McBeth, 1916). *Bac. latvianus* Kalnins (1930) was stated to produce small amounts of volatile acid. Simola (1931) found that his *Cellulobacillus myxogenes* transformed about 10% of the decomposed cellulose into formic and acetic acid, besides producing traces of alcohol and lactic acid, some unidentifiable non-volatile acid, and certain protein-like excretion compounds. Cellobiose and glucose were identified as intermediate products. Zarembska (1936) found a similar production of volatile acids and no reducing sugars in a closely related organism. Another species (Horowitz-Wlassowa, 1936) produced neither aldehydes, oxy-acids nor reducing sugars.

4. *Aerobic non-spore-forming bacteria* (*Cellulomonas* Bergey et al., 1939).—A large number of more or less strictly aerobic bacteria may decompose cellulose, as first shown by Kellerman, McBeth et al. (summarized by McBeth, 1916). They form a heterogeneous collection, being mostly not specialized in their action on cellulose, and differing widely in morphology and cultural requirements. The justification for a separate genus *Cellulomonas* seems disputable. Their mode of action on cellulose is incompletely known. The many organisms described by McBeth (1916) were stated to grow feebly under anaerobic conditions and to produce acid from several sugars; unfortunately this test did not include cellulose. Bradley and Rettger (1927) and Kalnins (1930) found that some of them produced acid from cellulose in solutions of peptone or digested casein. The latter author isolated two species, *Bact. bosporum* and *Bact. protozoides*, that produced glucose from cellulose, especially when deprived of oxygen, and to a lesser extent at supraoptimal temperatures. Groenewege (1920) found glucose as intermediate product in a somewhat inadequately described aerobic organism. Rubentschick

(1928) mentions two unidentified bacteria that produced no organic acid or reducing sugars. A typical facultative anaerobe was isolated by Dubos (1928), but its metabolic products were not studied. Horowitz-Wlassowa (1936) described a somewhat similar organism (*Bact. cellulosolyticum flavum*) which produced no oxy-acids, aldehydes or reducing sugars, but certain other soluble organic compounds oxidizable by chromic acid.

5. *Myxobacteria.*—Members of this group (to which *Cytophaga* is possibly related) have only in very recent years been known to decompose cellulose. Krzemieniewski (1937) and Imshenetskij and Solntseva (1937) found no acids, alcohols or reducing sugars, but the first author noticed certain pectin-like compounds that gave reducing sugars on hydrolysis with acid.

6. *Actinomycetes.*—Many species of this group are capable of cellulose decomposition, but the biochemistry of this process has been almost completely neglected. Rubentschick (1928) found no organic acids or sugars in the one species studied by him.

7. *Fungi.*—A vast number of fungal species are known to decompose cellulose, many of them very actively, but here, too, our knowledge of their final and intermediate products is remarkably limited. *Chaetomella horrida* produces oxy-acids from cellulose, according to Söhngen (1913), who also mentions this phenomenon in many other cellulose-decomposing organisms, without specifying their identity. Heukelikian and Waksman (1924) found that *Penicillium* and *Trichoderma* transform cellulose quantitatively into carbon dioxide, water, and cell substance. Skinner (1930) observed, by qualitative tests only, that a sterile mycelium, but not *Trichoderma*, produced substances that support growth of *Azotobacter*. *Sporotrichum carnis*, according to Vartiovaara (1935), produces no sugar from cellulose in normal cultures, but does so under the influence of antiseptics or oxygen-starvation. In a *Sterigmatocystis*, Horowitz-Wlassowa (1936) found no aldehydes, oxy-acids or reducing sugars, but large amounts of certain other soluble carbon compounds; the cultures were quite old when analysed, and it is possible that these compounds represent autolysis products. According to Bucherer (1933), fungal mycelium itself may contain certain constituents utilizable by *Azotobacter*.

Symbiosis between cellulose-decomposers and nitrogen-fixers obviously depends not merely on the organic by-products of the former organisms, but also on their ability to use the nitrogen fixation products as sources of nitrogen. This problem has not been studied in much detail. Sanborn and Hamilton (1929) stated that cellulose decomposition by an *Actinomyces* and two *Cellulomonas* was stimulated by *Azotobacter*. Skinner (1930) largely failed to confirm this with *Cytophaga*, *Cellvibrio*, and two fungi. Kalnins (1930) found various unspecified bacteria unable to utilize the nitrogen fixed by *Azotobacter* (by qualitative tests only), and Bucksteeg (1936) made the same observation with *Cytophaga*. Bucherer (1933) found *Azotobacter*-substance well utilized by *Aspergillus* and *Penicillium*, of which particularly the former is not a very active cellulose-decomposer.

From our survey it appears that indisputable proof of nitrogen fixation in combined cultures of one cellulose-decomposing and one nitrogen-fixing organism has been given only by Vartiovaara (1938), whose work was confined to fungi, and possibly by Krishna (1928), who also had pure cultures of fungi only, and in whose data the gains of N look suspiciously small in comparison with the original N-content, especially of the sand media. Secondly it appears that formation of organic acids from cellulose is widespread but inconstant and often slight, and that reducing sugars generally tend to accumulate when the normal course of the metabolism is disturbed by lack of oxygen, high temperature, or antiseptics. Partial nitrogen starvation, which might obtain where the cellulose-decomposers have to depend on the activity of associated nitrogen-fixers, might have a similar effect, with the consequence that larger amounts of breakdown products would be available for interception by the latter group of organisms. Under such conditions it is also possible that by-products like acids or alcohols would be formed more copiously than in normally-fed cultures, or that such compounds might be formed by organisms which do not normally produce them.

With the exception of the important contributions of Bucksteeg (1936) and Vartiovaara (1938), both dealing with only a few specific types of organisms, most work on the problem has been either tentative or incidental to other studies on cellulose decomposition or nitrogen fixation. A systematic investigation of the main groups of cellulose-decomposing microorganisms in their relation to nitrogen fixation has therefore been carried out. The general results obtained with the aerobic types of organisms are presented here. Experiments with anaerobes will follow.

Methods.

Isolation of cellulose-decomposing bacteria.—Crude cultures were mostly obtained by placing strips of filter paper upon a layer of moist soil in a Petri dish and incubating at 28–30°C. When decomposition became visible, a small amount of decayed paper was emulsified in sterile water, and plate cultures on cellulose agar were prepared therefrom. Colonies showing clear zones on cellulose agar were transferred to strips of filter paper in nutrient solutions, and the process repeated until pure cultures were obtained. Some strains were isolated by direct plating from soil with addition of straw.

The agar medium used for isolation contained: precipitated cellulose (prepared by the method of Scales as modified by Kalnins, 1930), 0·5%; $(NH_4)_2SO_4$ 0·1%; K_2HPO_4 0·1%; $MgSO_4$ 0·05%; NaCl 0·02%; $CaCO_3$ 0·2%; agar 1·0%; sometimes also 1% yeast extract prepared by autoclaving dry yeast with 10 times its amount of water. Finely divided cellulose and a low concentration of agar were found essential for successful isolation, especially of *Cytophaga*.

The general basal medium for cultivation was a solution of 0·1% K_2HPO_4, 0·05% $MgSO_4$, and 0·02% NaCl. Various sources of N were added: (*a*) 0·05% $NaNO_3$; (*b*) 0·1% $(NH_4)_2SO_4$ + 0·2% $CaCO_3$; (*c*) 0·1% glycine, asparagine, Na-asparaginate, or peptone; (*d*) 1·0% yeast extract, with or without extra addition of $NaNO_3$, $(NH_4)_2SO_4$, and $CaCO_3$. The media were used in test tubes with strips of filter paper (Whatman No. 1) about two-thirds immersed in the solution. Utilization of sugars, etc., was tested in basal solution with $NaNO_3$ and 1·0% organic compound to be tested. Fermentation tests were made in basal medium with 1% yeast extract and 1% carbohydrate. Diastatic action was tested in starch agar as well as solution. Söhngen's (1913) test for oxy-acids was made on strips of filter paper impregnated with MnO_2 and placed on agar plates with $NaNO_3$ or yeast extract. Growth was also tested on ordinary nutrient agar and broth, potato, and soil extract agar with 1% glucose.

One strain of *Azotobacter chroococcum*, of normal N-fixing capacity (10–14 mgm. N per gm. of glucose), was used throughout the work. A strain of *Az. Vinelandii* was tested occasionally.

All cultures were incubated at 28–30°C. unless otherwise stated.

Nitrogen was determined by the Kjeldahl method, with K_2SO_4 and selenium as catalysts, and digestion for 2 hours after clearing; titration took place in CO_2-free solution, with methyl red and 28/n H_2SO_4 and NaOH.

Descriptions of the Cellulose-decomposing Bacteria.

1. *Cytophaga.*—Six strains were isolated, two (G and F) from soils from the University grounds, the others from wheat soils. Contrary to many statements in the literature, isolation of the cytophagae succeeded readily by repeated plating on cellulose-agar, where they produce no regular colonies, but round transparent plaques gradually covered with yellow mucus (Fig. 1). All strains, the chief characters of which are listed in Table 1, conform to the general definition of *Cytophaga*. Morphologically they appear as long, slender, tapering, somewhat flexible rods which gradually (except in one strain) develop into deeply staining "microcysts". No growth takes place in any cellulose-free medium. Cellulose decomposition starts at the level of the solution, where the paper is softened and transformed into yellow mucus. Strains G, R, and 25 seem to conform to the type species, *Cyt. Hutchinsoni* (syn. *Spirochaeta cytophaga* Hutchinson and Clayton, 1919). Strain F differs mainly by its very pale pigment, and strain 8 by its small size and lack of microcysts. Strain 81 is somewhat similar

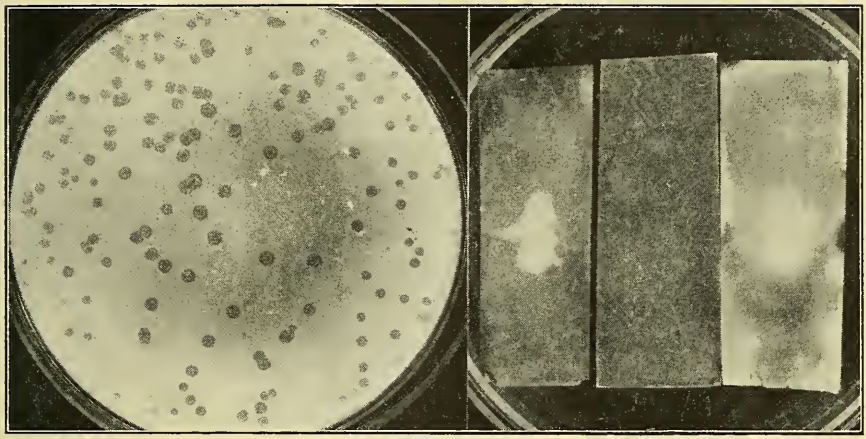

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Fig. 1.—Plate culture of *Cytophaga* R on cellulose-agar (10 days, 28-30°C.).
Fig. 2.—Söhngen's reaction: reduction of MnO_2 by oxy-acids, 6 days, 28-30°C. Left, *Corynebact.* 3; centre, Control, sterile; right, *Corynebact.* Va.

to *Cyt. ellipsospora* Imshenetskij and Solntseva (1936), but utilizes no other N-compounds than ammonia and aspartic acid; it also produces less mucilage than the others. Upon the whole, however, the differences between the strains are not so great that it seems advisable to separate them into definite "species".

TABLE 1.
Description of strains of Cytophaga.

	G	R	25	8	F	81
Vegetative cells	0·3–0·5 × 2·5–7·0μ	0·3–0·5 × 2·5–6·0μ	0·3–0·4 × 3·0–6·0μ	0·3–0·4 × 1·8–4·0μ	0·4–0·5 × 2·5–6·0μ	0·2–0·5 × 2·5–6·0μ
Microcysts	Spherical, 1·0–1·2μ	Spherical, 1·0–1·3μ	Spherical, 1·0–1·2μ	Absent	Spherical, 1·0–1·3μ	Oblong, 0·7–0·8× 1·0–1·4μ
Pigment	Ochre-yellow	Ochre-yellow	Light yellow	Ochre-yellow	Pale yellow	Dull orange
Cellulose decomposition with:						
$NaNO_3$	good	good	good	good	good	nil
$(NH_4)_2SO_4$	good	good	good	good	good	good
Glycine	nil	nil	nil	nil	nil	nil
Asparagine	scant	scant	fair	fair	fair	nil
Na-asparaginate	fair	fair	scant	good	fair	scant
Peptone	fair	fair	fair	fair	nil	nil
Cellulose decomposition at:						
6–8° C.	nil	nil	nil	nil	nil	nil
15° C.	slow	slow	nil	slow	nil	slow
18–20° C.	fair	fair	slow	fair	nil	fair
28–30° C.	rapid	rapid	rapid	rapid	rapid	rapid
37° C.	rapid*	rapid*	nil	nil	rapid	rapid

* Variable.

All strains are strictly aerobic; I have not been able to confirm the statement of Imshenetskij and Solntseva (1936) that decomposition may take place under restricted access of oxygen (paper totally immersed in solution). Under these conditions the attack does not start until the paper has reached the surface owing to evaporation. Reducing substances were found neither in normal cultures nor in tubes sealed with paraffin wax and incubated for 6–8 weeks, and no acid reaction developed in cultures with nitrate or peptone. Söhngen's test for oxy-acids was negative.

2. *Cellvibrio.*—Five strains were isolated, one (17) from a wheat soil, the others from soil of the University grounds. On cellulose-agar they produce small, opaque, white colonies surrounded by clear zones, and in liquid media they show a characteristic growth (cf. Kalnins, 1930, et al.), causing first a faint turbidity and then, after 2–3 days, rupture of the paper strip at the level of solution. Morphologically they are much alike: small, non-spore-forming, Gram-negative rods, 0·4–0·6 × 1·0–2·5μ, very actively motile, except strain G3 which showed only a few motile individuals. Curved cells of the typical *Vibrio*-shape were most prominent in strain 17; the others appeared mainly as straight rods with pointed ends, reminiscent of *Cellfalcicula* Winogradsky (1929). It is really disputable whether these two groups should be regarded as separate "genera". Growth is very scant and uncharacteristic on nutrient agar and broth, absent on potato. All grow fairly well on glucose-soil extract-agar, strains G1, G2, and 17 producing a moist, whitish growth, and G3 and G4 a semi-transparent growth of gum-like consistency. On starch-agar the growth of the first three is heavy and yellowish-white, of the two last very scant. Various other characters are seen in Table 2.

TABLE 2.
Description of Cellvibrio-*like organisms.*

	G1	G2	17	G3	G4
Cellulose decomposition with:					
$NaNO_3$	rapid	rapid	rapid	rapid	rapid
$(NH_4)_2SO_4$	rapid	rapid	rapid	rapid	rapid
Glycine	nil	nil	nil	nil	?
Asparagine	slow	nil	nil	slow	nil
Na-asparaginate	fair	fair	slow	fair	fair
Peptone	slow	slow	?	slow	nil
Yeast extract	fair	fair	fair	fair	slow
Utilization of:					
Glycerine, Mannite, Xylose, Arabinose	nil	nil	nil	nil	nil
Glucose	good	good	good	fair	good
Saccharose	good	good	fair	fair	fair
Lactose	nil	fair	fair	nil	nil
Inulin	good	nil	nil	nil	nil
Starch	fair	good	good	scant	scant
Diastatic action, plate	strong	strong	strong	nil	nil
Do., solution	strong	strong	strong	fair	fair
Reduction of NO_3 to NO_2	strong	weak	nil	strong	strong
Growth at:					
5° C.	slow	slow	slow	slow	slow
15° C.	fair	fair	fair	fair	fair
28° C.	rapid	rapid	rapid	rapid	rapid
37° C.	nil	nil	nil	fair	fair

Unlike *Cytophaga*, the cellvibrios utilize glucose, saccharose and starch more or less readily, sometimes also lactose or inulin. They are also better adapted to low temperatures than *Cytophaga*. Strains G1, G2 and 17 are very similar to *Cellv. vulgaris* Stapp and Bortels (1934), except that they form a very faint (often invisible) yellow pigment on the paper exposed to the air. Strains G3 and G4 differ slightly by their weak diastatic effect, tolerance of higher temperature, and more copious formation of polysaccharide, as shown by their very slimy growth in sugar solutions and soil-extract agar. No growth takes place in the anaerobic jar, but paper submerged in nutrient solution is attacked, although very slowly. No acid is produced from any carbohydrate in nitrate-solution or from cellulose in any medium, except, of course, in $CaCO_3$-free solution with $(NH_4)_2SO_4$, where an acid reaction (pH 5·6–6·0) develops owing to selective absorption of the ammonium. In this medium strain G1 and G2 produced after

3 weeks small amounts of substances that reduced Fehling's solution. Otherwise no reducing sugars were found in normal cultures, even after 4 months, but all strains produced such compounds in paraffin-sealed tubes after 5–6 weeks (cf. Kalnins, 1930). Some polysaccharide seems to be produced in cellulose-cultures, where the solution after 2–3 months became slightly viscous and gave a small flocculent precipitate with HCl-alcohol. Söhngen's reaction is negative.

3. *Aerobic spore-forming and non-spore-forming bacteria* ("*Cellulobacillus*" and "*Cytobacter*").—Two non-spore-forming cellulose-decomposers, "R" and "Co", were isolated from wheat soils, and two spore-formers, "G" and "43", from University grounds soil and pasture soil, respectively. (Two other spore-formers were isolated, but died out after a few transfers). Their chief characters are seen in Table 3.

TABLE 3.

Description of non-spore-forming and spore-forming cellulose-decomposing bacteria.

	Bact. R.	*Bact.* Co.	*Bacillus* 43.	*Bacillus* G.
Vegetative cells	Rods often curved, 0·5–1·0 × 2·5–5·0μ	Straight rods, 0·5–0·6 × 1·5–2·5μ	Slightly curved rods, at sporulation spindle-shaped, 0·4–0·6 × 1·5–2·6μ	Straight rods, spindle-shaped at sporulation, 0·6–1·2 × 2·4–4·5μ
Spores	absent.	absent.	Subterminal, or central, oval, 0·5–0·8 × 1·2–1·6μ	Subterminal, oval, 0·7–1·0 × 1·2–2·0μ
Motility	none	none	none	?
Gram	–	–	–	variable
Cellulose decomposition with:				
$NaNO_3$	nil	nil	nil	nil
$(NH_4)_2SO_4$	nil	nil	nil	scant
Glycine	nil	nil	nil	nil
Asparagine	scant	nil	nil	scant
Na-asparaginate	nil	nil	nil	nil
Peptone	scant	scant	nil	nil
Yeast extract	fair	fair	fair	fair
Acid in:				
Glycerine	–	–	–	–
Mannite	–	–	–	–
Xylose	–	–	–	(+)
Arabinose	–	–	(+)	(+)
Glucose	–	+	–	?
Saccharose	–	–	–	–
Lactose	–	–	?	?
Inulin	–	–	–	–
Starch	–	+	–	–
Cellulose	–	+	–	–
Diastatic action, plate	+	–	+	+
Do., solution	+	+	+	–
Söhngen's reaction	–	+	–	–
Reduction of nitrate	+	+	+	+
Cellulose decomposition at:				
9–10° C.	nil	nil	nil	nil
15° C.	nil	very slow	very slow	very slow
28–30° C.	fair	fair	fair	fair
37° C.	fair	fair	fair	nil

They are strictly aerobic, do not grow in the anaerobic jar, and attack submerged paper very slowly. Otherwise the paper is attacked in the same manner as by *Cellvibrio*, but less rapidly. The growth on nutrient agar and broth, soil-extract agar and potato is very scant or absent. *Bac.* G alone produces a heavy, colourless, semi-transparent, gummy growth on starch agar with $NaNO_3$ or yeast extract. Reducing sugars are formed from starch, and from cellulose in sealed but not in open tubes. *Bact.* Co produces acid from cellulose (oxy-acid as shown by Söhngen's reaction), and from glucose and starch, while the fermentative powers of the other isolates are only weak. *Bact.* R bears some resemblance to *Cytobacter polonicum* Gutgisser (1936), but unlike this it is unable to utilize nitrate, ammonia, or simple amino-compounds; this is common to all except *Bac.* G, which strongly resembles *Cellulobacillus myxogenes* Simola (1931) and *Cell. varsaviensis* Zarembska (1936), except that it is almost non-motile (only a few cells show vibratory movement), has less fermentative power, and does not produce acid from the cellulose. The remarkably small *Bac.* 43 does not seem identifiable with any hitherto described species.

4. *Facultative aerobic, non-spore-forming bacteria.*—This group, which largely conforms to the genus *Cellulomonas*, includes an authentic strain of *Cell. biazotea* (McBeth, 1916), received from the Biological Branch, Dept. of Agriculture, N.S.W., and three *Corynebacterium*-like organisms. One of these (*Cor.* 3) was isolated from wheat soil, the others (*Cor.* Va and Vb) from a crude culture of cellulose-decomposers from leaf compost (kindly supplied by Mr. J. M. Vincent, School of Agriculture, University of Sydney).

Morphologically, *Cell. biazotea* appeared as short, straight rods, non-motile and of unstable Gram-reaction. Strains 3 and Vb were typical small corynebacteria: in young cultures somewhat irregularly shaped, slender, non-motile, Gram-positive rods in angular arrangement (strain 3: 0·5–0·6 × 1·0–2·0μ; strain Vb: 0·4–0·5 × 2·0–4·0μ; later the cells become very short to coccoid). *Cor.* Va was similar to *Cor.* 3, but actively motile in broth culture (cf. Topping, 1937, on motile soil corynebacteria). Cellulose is attacked as by the cellvibrios, but in suitable media even more intensely, especially below the surface of the liquid; in accordance herewith, submerged paper is readily attacked, and a slow but definite cellulose decomposition takes place in the anaerobic jar. Other characters are given in Table 4.

TABLE 4.

Description of cellulose-decomposing Corynebacteria, and Cell. biazotea.

	Cell. biazotea.	*Cor.* 3.	*Cor.* Va.	*Cor.* Vb.
Motility	none	none	motile in broth	none
Gram	variable	positive	positive	positive
Growth on:				
Nutrient agar	abundant, slimy, light yellow	abundant, slimy, yellow	abundant, pasty, yellow	fair, white to pale yellow, sticky
Nutrient broth	turbid, white sediment	turbid, white sediment	turbid, white sediment	turbid, white sediment
Potato	good, yellow	good, yellow	good, yellow	very scant, yellow
Growth on cellulose with:				
$NaNO_3$	nil	nil	nil	nil
$(NH_4)_2SO_4$	nil	nil	nil	nil
Glycine	nil	scant	scant	scant
Asparagine	nil	nil	nil	nil
Na-asparaginate	nil	nil	scant	scant
Peptone	fair	fair	fair	fair
Yeast extract	good	good	good	good
Acid in:				
Glycerine	+	+	+	+
Mannite	−	−	−	−
Arabinose	+	+	+	+
Xylose	−	+	+	+

TABLE 4.—*Continued.*
Description of cellulose-decomposing Corynebacteria, and Cell. biazotea.—Continued.

Acid in:				
Glucose	+	+	+	+
Saccharose	+	+	+	+
Lactose	+	+	−	+
Inulin	−	−	−	−
Starch	+	+	+	+
Cellulose	+	+	+	+
Söhngen's reaction (cellulose–MnO_2)	(+)	+	+	+
Diastatic action	strong	strong	strong	strong
Reduction of NO_3	strong	strong	strong	strong
Cellulose decomposition at pH:		(Paper broken after days:)		
5·2	20–30	13	>30	>30
5·7	9	7	7	7
6·1	9	5	7	7
6·4	5	5	4–5	4–5
6·6	6	4	5	4–5
6·9	6	4–5	4–5	2–3
Cellulose decomposition at:				
5° C.			nil	nil
10° C.	nil	nil	very slow	very slow
15° C.	slow	slow	slow	slow
28° C.	rapid (opt.)	rapid (opt.)	rapid } (opt.)	rapid } (opt.)
37° C.	rapid	rapid	rapid }	rapid }

The organisms of this group differ from all the previous ones in their good growth in routine media, their fermentation of a wide range of carbohydrates, and their facultative-anaerobic nature. High-molecular N-compounds seem required; simple amino-compounds are but slightly utilized, and inorganic N not at all (except in the presence of yeast extract, where small amounts of NH_4–N are assimilated, as shown by a separate experiment). Soil extract can be used instead of yeast extract. The resistance to acidity is considerable, cellulose being still attacked at pH 5·2. The fermentative reactions of the 4 isolates are almost identical, including the production of acid from cellulose; Söhngen's test shows rapid and vigorous formation of oxy-acids (Fig. 2). No reducing sugars are produced from cellulose either in open or in sealed tubes (in the latter case probably because these bacteria, being facultative anaerobes, can further ferment the sugar that might arise). *Cor.* Va gave a faint reduction in sealed tubes with addition of toluene.

Cor. 3 seems closely related to "*Cellulomonas*" *fimi*, which has previously (Jensen, 1934) been recognized as a corynebacterium. The other two isolates cannot with certainty be identified with any other adequately described species.

5. *Actinomycetes and fungi.*—Some representatives of these were included for comparison: two species of *Actinomyces*, a *Micromonospora*, a *Trichoderma* (*koningi*?), and an unknown fungus ("P") isolated from University grounds soil and similar to a *Botryosporium*-like fungus previously studied (Jensen, 1930). They all decompose cellulose vigorously with $(NH_4)_2SO_4$, yeast extract or soil extract as sources of N. Oxy-acids are not produced, except perhaps by *Micromonospora* which showed a trace of reaction. Reducing sugars are formed from cellulose in sealed but not in open tubes; upon the whole this seems to be a general property of obligate aerobic cellulose-decomposers other than *Cytophaga*.

Associations between Azotobacter and Cellulose-decomposing Organisms.

The value of cell-substance of *Azotobacter* as a source of N for the cellulose-decomposers was first tested qualitatively by growing all these organisms in basal solution with filter paper and cell-material of *Az. chroococcum*, grown on mannite-agar 8 days 28°C., and added in a quantity corresponding to 0·1% dry matter. Growth was

also tested on agar corresponding to the basal solution, with 2·5% moist substance of *Azotobacter*.

TABLE 5.

Utilization of cell nitrogen of Azotobacter *for cellulose decomposition, and lysis of* Azotobacter*-cells.*

Organism.	Cellulose decomposition.	Cytolysis.	Organism.	Cellulose Decomposition.	Cytolysis.
Cytophaga (6 strains) ..	nil		*Cell. biazotea*	rapid	fair
			Corynebact. 3	,,	,,
Cellvibrio 1	slow	none	,, Va	,,	none
,, 2	,,	,,	,, Vb ..	,,	,,
,, 3	,,	,,			
,, 4	very slow	,,	*Actinomyces* R	fair	strong
,, 17	,,	,,	,, T	,,	,,
			Micromonospora	,,	,,
Bact. Co.	rapid	strong	*Trichoderma*	rapid	,,
Bact. R.	nil	none	Fungus "P"	,,	,,
Bacillus 43	very slow	,,			
,, G	fair	,,			

Table 5 shows that all except *Cytophaga* and *Bact.* R can utilize *Azotobacter*-N, the bacteria of group 4 even very readily, and several show active lysis of *Azotobacter*-cells. These had indeed been killed by the sterilization; but also the N in untreated *Azotobacter*, added aseptically to basal solution with filter paper, was readily utilized by *Cellv.* G2 and 17, and *Cor.* 3 and Vb.

In another series of qualitative tests *Az. chroococcum* and *Vinelandii* were grown on filter paper in solution with 0·2% glucose and the usual salts including Fe, Mo, and $CaCO_3$. After 6–7 days, when a good growth of *Azotobacter* had appeared, the cultures were superinoculated with the cellulose decomposers and incubated further; in no case did more than a trace of cellulose decomposition result. An active "symbiosis" between pure cultures thus cannot be started with *Azotobacter*-N, at least not in amounts so small as the present (cf. Kalnins, 1930). Theoretically, however, all cellulose-decomposers should be able to derive nitrogen from *Azotobacter*; groups 3–5 readily take up the N of its dead cells, and one of its main vital secretion products is aspartic acid (Virtanen and Laine, 1937; Horner and Burk, 1939), which is quite a favourable source of N for *Cytophaga* and *Cellvibrio*. (Spontaneous formation of NH_4–N by *Azotobacter* is hardly to be expected during symbiosis, since this does not take place when the medium contains more than very small amounts of oxidizable organic matter.)

Further qualitative tests showed that *Azotobacter* regularly produced some growth (usually sparse) when introduced into filter paper cultures of practically all cellulose decomposers (cf. Kalnins, 1930, and Skinner, 1930). In the main experiment it was therefore attempted to start symbiosis by supplying a quantity of fixed N sufficient to initiate cellulose decomposition, yet small enough to be rapidly consumed and thus ceasing to interfere with the fixation by *Azotobacter*.

This experiment was made with cultures in large test tubes (20 × 3 cm.) containing 40 c.c. nutrient solution and 0·5 or 1·0 gm. filter paper about two-thirds immersed in the solution which contained, besides the usual salts, 0·01% $FeCl_3$, 0·001% Na_2MoO_4, and 0·25% $CaCO_3$. Nitrogen was added in amounts of 2·5 to 3·0 mgm. per culture, as $(NH_4)_2SO_4$, peptone, or yeast extract. After sterilization, tubes were inoculated with various combinations of organisms and incubated for 4 or 5 weeks. Total N was determined both before and after incubation. In all cultures of cellulose decomposers the paper was attacked quickly, although mostly not to a large extent; growth of *Azotobacter* was visible in these cultures, but, except in a few instances, only as faint dark streaks on the paper or thin rings around the surface of the solution.

Table 6 gives the analytical data, as averages of duplicate cultures unless otherwise stated; the agreement between duplicates was such that differences exceeding 0·3 mgm. N may be regarded as significant. These figures show that:

1. Sterile solutions of $(NH_4)_2SO_4$ show a loss of N, whereas yeast extract solutions remain almost unchanged; the initial N-content has therefore been subtracted from that of the cultures to give the N-balance.

2. *Azotobacter* alone fixes no N in the media employed.

3. None of the cellulose-decomposers absorb significant quantities of N from the atmosphere, although they largely prevent the loss of N from the media.

4. No combination of *Azotobacter* with *Cytophaga* (even crude cultures), *Cellvibrio*, bacteria of group 3, actinomycetes or fungi, fixes a significant amount of N.

5. In association with the bacteria of group 4, and especially *Cor.* 3 and *Cor.* Vb, *Azotobacter* fixes considerable amounts of N; in these cultures also the growth and cellulose decomposition appear more vigorous than in others.

TABLE 6.

Nitrogen fixation in combined cultures of Azotobacter *and cellulose-decomposing organisms.*

Series.	Inoculum.	Total N, mgm.	Gain or loss of N, mgm.
I.	Control at start	2·11	
1·0 gm. paper,	Do. incubated	1·48	−0·63
2·5 mgm. N as	*Az. chroococcum* (single)	1·19	−0·92
$(NH_4)_2SO_4$	*Cellvibrio* G2	1·95	−0·16
Inc. 35 d.	Do.+*Azotobacter*	2·30	+0·19
	Do.+do.+crude culture of cellulose-decomposers	2·10	−0·01
II.	Control at start	2·39	
1·0 gm. paper,	Do. incubated	2·15	−0·24
with 5% yeast	*Az. chroococcum*	1·99	−0·40
extract.	*Bacillus* 13* (single)	2·19	−0·20
Inc. 35 d.	Do.+*Azotobacter*	2·27	−0·12
III.	Control at start	2·66	
1·0 gm. paper,	Do. incubated	1·76	−0·90
3·0 mgm. N as	*Trichoderma*	2·47	−0·19
$(NH_4)_2SO_4$.	Do.+*Azotobacter*	2·88	+0·22
Inc. 35 d.	Do.+do.+crude *Cytophaga*-culture	2·52	−0·14
IV.	Control at start	1·94	
1·0 gm. paper,	Do. incubated (triplicate)	1·38	−0·56
2·5 mgm. N as	*Cellvibrio* G3	1·97	+0·03
$(NH_4)_2SO_4$	Do.+*Azotobacter*	2·11	+0·17
Inc. 35 d.	*Cellvibrio* 17	1·93	−0·01
	Do.+*Azotobacter*	1·97	+0·03
	Cytophaga 8+*Azotobacter*	1·94	0·00
V.	Control at start	1·92	
1·0 gm. paper,	Do. incubated	1·57	−0·35
2·5 mgm. N as	*Cytophaga* R (single)	1·86	−0·06
$(NH_4)_2SO_4$.	Do.+*Azotobacter*	1·93	+0·01
Inc. 35 d.	Do.+do.+crude *Cytophaga*	1·90	−0·02
VI.	Control at start	1·88	
1·0 gm. paper,	Fungus "P"	1·90	+0·02
2·5 mgm. N as	Do.+*Azotobacter*	1·98	+0·10
$(NH_4)_2SO_4$	*Micromonospora*	1·84	−0·04
Inc. 35 d.	Do.+*Azotobacter*	2·07	+0·19
VII.	Control at start	1·86	
1·0 gm. paper,	Do. incubated	1·82	−0·04
4% yeast extract.	*Corynebact.* 3 (single)	1·84	−0·02
Inc. 35 d.	Do.+*Azotobacter*	2·51	*+0·65*
	Bacillus G+*Azotobacter*	1·94	+0·08
	Trichoderma+*Azotobacter*	1·91	+0·05

TABLE 6—*Continued.*

Nitrogen fixation in combined cultures of Azotobacter *and cellulose-decomposing organisms.*—Continued.

Series.	Inoculum.	Total N, mgm.	Gain or loss of N, mgm.
VIII.	Control at start	1·74	
1·0 gm. paper,	Do. incubated	1·29	−0·45
2% yeast extract,	*Cell. biazotea* (single)	1·84	+0·10
1·25 mgm. N as	Do. + *Azotobacter*	1·91	+0·17
$(NH_4)_2SO_4$.	*Bacillus* G (single)	1·73	−0·01
Inc. 35 d.	Do. + *Azotobacter* (single)	1·17	−0·57
	Corynebact. 3 + *Azotobacter*	3·13	*+1·39*
IX.	Control at start	2·70	
1·0 gm. paper,	*Cell. biazotea* (single)	2·61	−0·09
0·04% peptone.	Do. + *Azotobacter*	3·31	*+0·61*
Inc. 28 d.	*Corynebact.* 3 (single)	2·66	−0·04
	Do. + *Azotobacter*	3·81	*+1·11*
	Do. + *Az. Vinelandii*	4·50	*+1·80*
X.	Control at start	1·92	
1·0 gm. paper,	*Actinomyces* R (single)	2·08	+0·16
2·5 mgm. N as	Do. + *Azotobacter*	1·99	+0·07
$(NH_4)_2SO_4$.	*Actinomyces* T (single)	2·08	+0·16
Inc. 35 d.	Do. + *Azotobacter*	2·00	+0·08
	Cytophaga 25 + *Azotobacter*	2·05	+0·13
	Cytophaga F. + *Azotobacter*	2·10	+0·18
XI.	Control at start	3·38	
0·5 gm. paper,	*Corynebacterium* Va (single)	3·25	−0·13
2·4% yeast extract	Do. + *Azotobacter*	3·80	*+0·42*
(new batch).	*Corynebacterium* Vb (single)	3·53	+0·15
Inc. 28 d.	Do. + *Azotobacter*	4·52	*+1·14*
	Bacillus 43 + *Azotobacter*	3·34	−0·04
	Bacterium R + *Azotobacter*	3·27	−0·11
XII.	Control at start	2·38	
0·5 gm. paper,	*Bacterium* Co. (single)	2·58	+0·20
2% yeast extract.	Do. + *Azotobacter*	2·44	+0·06
Inc. 33 d.	*Cellvibrio* G1 + *Azotobacter*	2·27	−0·11
	Micromonospora (single)	2·26	−0·12
	Do. + *Azotobacter*	2·42	+0·04
XIII.	Control at start	2·10	
0·5 gm. straw	*Cellvibrio* G2 + *Azotobacter*	2·07	−0·03
(H_2O-extr.), on agar	*Bacillus* G + do.	2·07	−0·03
medium, 1·0 mgm. N as	*Micromonospora* + do.	2·05	−0·05
$(NH_4)_2SO_4$.	*Actinomyces* R + do.	2·04	−0·06
Inc. 36 d.	*Trichoderma* + do.	2·00	−0·10
24–27° C.			
XIV.	Control at start	2·60	
0·5 gm. paper,	*Cellvibrio* G2 + *Azotobacter*	2·52	−0·08
on agar medium,	*Cytophaga* 25 + do.	2·52	−0·08
2·0 mgm. N as	*Trichoderma* + do.	2·82	+0·22
$(NH_4)_2SO_4$.			
Inc. 36 d.			
24–27° C.			

* This isolate died out after a few transfers.

Two additional series of experiments were made (last section of Table 6). In the first, wheat straw from which the constituents directly available to *Azotobacter* had been removed by extraction with hot water (Jensen, 1940) was used as a cellulosic material possibly more easily attacked by the organisms under investigation (cf. Norman, 1937). Portions of 0·5 gm. dry, finely ground straw and 0·1 gm. $CaCO_3$, sterilized separately, were placed on the surface of 50 c.c. agar medium in 250 c.c. flasks; the agar contained the usual salts and 1·0 mgm. N as $(NH_4)_2SO_4$ per flask. The second series was identical except that filter paper was used instead of straw. *Trichoderma*

made a fair growth in both series, the other organisms almost none, and no nitrogen was fixed.

The whole experiment shows clearly that the more or less highly specialized cellulose-decomposers (*Cytophaga*, *Cellvibrio*, "*Cellulobacillus*", etc.), as well as fungi and actinomycetes, do not form organic metabolic by-products or autolysis-products in such quantities as to serve for a discernible N-fixation by *Azotobacter*. Neither does *Azotobacter* seem to intercept the intermediate breakdown products of cellulose, even in solution cultures with a fairly high layer of liquid which should encourage the production of reducing sugars. The ability to "feed" *Azotobacter* from cellulose seems, apart from the obligate anaerobes, to belong exclusively to the facultative anaerobes of the Kellerman-McBeth group, an outstanding character of which is their ability to utilize *Azotobacter*-N readily and to ferment cellulose under production of organic acids which are favourable sources of energy for *Azotobacter*. (*Bact.* Co, indeed, also forms such compounds, but these seem unsuitable for *Azotobacter*.) In cases where N-fixation has been recorded by *Azotobacter* in combination with impure cultures of cellulose-decomposers, either in solution cultures or in the soil (Jensen, 1940) we must be justified in concluding that either obligate anaerobes or else bacteria of the present type have been active.

The nature of the metabolic products, the quantitative relation between cellulose decomposition and N-fixation, and the influence of environmental factors will be discussed in a subsequent paper.

Summary.

Various groups of aerobic cellulose-decomposing microorganisms, viz., 6 strains of *Cytophaga*, 5 of *Cellvibrio*, 3 of *Corynebacterium*, 4 of unidentified spore-forming and non-spore-forming bacteria, besides fungi and actinomycetes, were isolated and grown in combination with *Azotobacter* in media with cellulose as a source of energy. *Azotobacter* was able to fix nitrogen only in association with the corynebacteria and *Cellulomonas biazotea*. Unlike the "typical" cellulose decomposers, these organisms are facultative anaerobes, grow well in ordinary media, utilize the nitrogen in *Azotobacter*-substance readily, and decompose the cellulose with the formation of organic acids. Organisms of this type may have been the active agents in cases where nitrogen fixation has been reported by *Azotobacter* in combination with impure cultures of cellulose-decomposers.

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