THE MICROBIAL DECOMPOSITION OF CELLULOSE

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THE MICROBIAL DECOMPOSITION OF CELLULOSE

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ABSTRACT

Despite its economic importance, the process of cellulose decomposition by microorganisms is not well understood. Contributing to this lack of understanding are the structural complexity of the cellulose macromolecule and its natural variability. Nevertheless, the importance of certain factors is clear. These include the degree of crystallinity of the cellulose fiber, its average degree of polymerization, the extent to which cellulose is associated with other materials in the plant cell wall, the amount of moisture available, and the extent of organism-substrate interaction. Laboratory experiments and an analytical evaluation of the role of diffusion in cellulose decomposition have emphasized the fundamental importance of the last of these factors, particularly in the ease with which decomposition is initiated.

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TABLE OF CONTENTS

I.	The structure of cellulose	1
II.	The mechanism of degradation	3
III.	Factors affecting cellulose decomposition	5
IV.	Laboratory observations	8
V.	Cellulolysis in well-mixed liquid cultures	19
VI.	Cellulolysis in natural situations	31
VII.	Summary	35
VIII.	Appendix	37
IX.	Literature cited	41

LIST OF TABLES

I.	Population levels of mixed cultures of soil bacteria in cellulose isolation medium, T = 10C	10
II.	Population levels of pure cultures of soil bacteria in a cellulose plus vitamins medium with citrate, T = 4C.	11
III.	Population levels of pure cultures of soil bacteria in a cellulose plus vitamins medium without citrate, T = 4C.	12
IV.	Population levels of pure cultures of soil bacteria in a cellulose plus vitamins medium without citrate, T = 10C.	12
V.	A pure culture of LC-1 in a cellulose plus vitamins medium, T = 10C.	14
VI.	Population levels of pure cultures of LC-1 and LC-5 in a cellulose plus vitamins medium, T = 20C.	16
VII.	Population levels of pure cultures of known cellulolytic bacteria in cellulose isolation medium with vitamins, T = 25C	17
VIII.	Summary of enzyme synthesis rates	24
IX.	Expected rates of hydrolysis and glucose production for various initial cell populations	29

I. The structure of cellulose

Being the major structural component of plant cell walls, cellulose is the most abundant organic compound in nature. In the form of grasses, cereals, and straws it is an important food source; in the form of cotton, flax, and ramie it is the basis of the textile industry; and in the form of wood it is the foundation of the pulp and paper and lumber industries. Yet despite its obvious economic importance, the process by which cellulose is decomposed by microorganisms is not well understood. One reason for this is the structural complexity of natural cellulose itself.

In textbooks cellulose is depicted as a polymer of glucose molecules in β-1,4' linkage. The number of glucose molecules per chain can vary from about 100 to 10,000;[36-46] the average degree of polymerization (DP) is about 3000 glucose units.[46] But this formula is incomplete. In nature about one hundred of these linear molecules are found packed closely together in a bundle termed a microfibril.[40] The molecules are oriented longitudinally with respect to one another, and adjacent strands are held together by hydrogen bonds, van der Waals' forces, and probably some primary linkages.[46] The resulting structure is only partially crystalline; that is, regions of randomly oriented molecules alternate with regions of highly ordered molecules. These amorphous and crystalline regions are interspersed with one another and do not seem to be uniform in size.[8] Values for the proportion of crystallinity in native celluloses range from 50 to 94%.[46]

In the plant cell wall the microfibrils are intimately associated

with hemicelluloses, lignins, pectins, waxes, oils, fats, proteins, pigments minerals, and lesser amounts of extraneous substances. The nature of this association seems to be primarily physical, but some chemical bonding may be involved. The structures of the cellulose molecules of different plants are similar; it is mainly in the nature of and the degree to which the cellulose is associated with other substances that the plants differ. The cellulose content of cotton is typically 94%.[46] In woods it varies from 41 to 53% and in cereal straws from 30 to 43%.[17] Cotton cellulose is associated with only small amounts of hemicelluloses and no lignin, whereas wood fibers contain considerable quantities of both.[8]

Hemicelluloses are short polymers, (DP 15 to 200), of sugars other than glucose, as well as their uronic acids, in β-glycosidic linkage.[8] Representative sugars are xylose and mannose. In both wood and cotton fibers the hemicelluloses are found predominantly in the outermost layer of the cell wall. Although shorter than the cellulose molecules, the hemicelluloses are probably oriented in the same direction and intermingled with them.[36]

The structure of lignin has not been defined, but it is thought to be a polymer of substituted phenyl-propane units.[8,40] It is found predominantly in the middle lamella of wood cells and makes up 20 to 30% of most woods.[36]

Thus, the term cellulose denotes no single, homogeneous chemical compound; the chemical formula which we assign to it incompletely describes the macromolecular structure found in nature.

II. The mechanism of degradation

The large size of the cellulose molecule and its essential insolubility in water require that a microorganism secrete extracellular enzymes capable of hydrolyzing both the β-1,4'-glucosidic linkages and the cross-linkages between cellulose molecules. The solubilization of a molecule many times larger than the microorganism itself probably takes place in this way: the enzymes secreted by the organism diffuse into the network of cellulose molecules where they free the cellulose molecule from its matrix and at the same time break it into shorter chains. The shorter polymers are further hydrolyzed to small molecules permeable to the cell wall of the organism. These soluble molecules then diffuse back to the organism and are assimilated by it. Obviously such a scheme requires that the cellulolytic organism manufacture either a single, particulate multi-enzyme complex or a whole family of enzymes with different specificities in order to use cellulose as an energy source. Some microorganisms have the capacity to hydrolyze the β-1,4'-glucosidic bond and are thus able to solubilize the shorter anhydroglucose chains, but because they are unable to initiate the solubilization of the native cellulose molecule, they are considered to be non-cellulolytic, although they may assist in the decomposition of cellulose.

By what mechanism the cellulolytic enzymes dissolve the cellulose molecule is uncertain, precisely because most cellulase preparations seem to be made up of several components with different activities toward various substrates. Two modes of hydrolysis are conceivable: successive glucose or cellobiose units may be removed from the end of the

molecule in an endwise-splitting mechanism, or intramolecular bonds may be hydrolyzed in a random fashion. There is evidence for the endwise mechanism of hydrolysis by the cellulases of *Cellvibrio gilvus*,[23,35,47,48] but most cellulases studied seem to be the random-splitting type.[6,13,14,22,35,50,51,52] It should be mentioned, however, that most of the work done so far to determine enzyme specificity and mode of action has been done with non-homogeneous cellulase preparations, and that preparations from the same organism have given somewhat varied results in the hands of different investigators. There is only one instance of extensive purification of a cellulase, that from *Myrothecium verrucaria*, a fungus, and in this case a random mechanism of hydrolysis was observed.[15,52]

The evidence in favor of either mechanism is not conclusive. A random-cleaving enzyme would seem to be more advantageous to the organism in the initial stages of decomposition, simply because of the long length of the cellulose molecule. The rate of hydrolysis by an endwise-cleaving enzyme would be inversely proportional to the degree of polymerization, whereas the rate of random hydrolysis would be independent of it. In the later stages of decomposition, when the cellulose has essentially been reduced to cellodextrins (DP 10 to 200), the endwise-splitting enzyme might be more advantageous since each cleavage would release an assimiable product.

III. Factors affecting cellulose decomposition

Factors other than the structural complexity and variability of natural cellulose hinder our understanding of the way in which cellulose is decomposed by microorganisms. For example, the extent to which hydrogen bonding and van der Waals' forces draw neighboring cellulose molecules together affects both the susceptibility of the substrate to degradation and the activity of the cellulase it induces. Several investigators have observed that the amorphous regions of cellulose are more readily degraded that the crystalline regions. [8,38,52] Consequently, the greater the degree of crystallinity of the native cellulose, the more resistant to enzymatic attack it will be. Recently Rautela and King[38] used celluloses of different degrees of crystallinity to induce cellulase synthesis by the fungus *Trichoderma viride*. Because one could expect the reaction rate to increase with an increase in the surface area of the substrate, and because King[24] had earlier observed that the measurement of the rate of hydrolysis of cellulose was biased by the particle size, Rautela and King took precautions to use crystalline substrates of the same particle size. They found that not only did the crystal structure affect the extent of growth of the fungus, it also affected the enzyme yield. The fact that the activation energies and reaction rates of enzymes induced by different cellulose substrates differed when tested against the same substrate indicates that the cellulases themselves differed. This finding may explain how different investigators can obtain a cellulase with different properties from the same organism.

The initial degree of polymerization of the cellulose is likely to affect the observed rate of decomposition, particularly when the enzyme involved cleaves in an endwise manner. In most experimental work the degree of polymerization is not determined. This only adds to the uncertainty in the interpretation of the results.

The degree to which cellulose is associated with other substances in the plant cell wall affects its susceptibility to enzymatic attack. Since physical contact between an enzyme and its substrate is necessary for reaction, substances such as lignin which encrust the microfibrils represent a physical barrier to hydrolysis. Lignin itself is extremely resistant to biological degradation, and therefore it drastically hinders cellulose utilization. Apparently the combination of lignin with cellulose found in wood prevents any bacterial attack of the cellulose until essentially all the lignin has been removed by fungi.[37]

Any substance which obstructs the capillaries between the cellulose molecules will limit diffusion of the enzyme into the microfibrils. However, if this substance can be metabolized by the microorganism, then the obstacle is not insurmountable. In fact, it may even promote cellulose decomposition by enabling a microbial population to develop. Such appears to be the case with the hemicelluloses,[2,9,36] whose sugar moieties provide a sustaining energy source for microorganisms with extracellular carbohydrases other than cellulase. Similarly, the amount of growth-promoting substances, such as minerals, vitamins, proteinaceous material, and soluble carbohydrates, found in association with the microfibrils also affects the readiness with which cellulose is attacked.

These extraneous nutrients have a marked positive effect on the production of cellulases by various microorganisms.[2-3-29]

In addition, one would expect there to be some materials that inhibit enzyme activity and possibly cell growth.

Another factor that influences the decomposition process is the extent of association of the organism with its substrate. Direct contact is probably necessary for stimulating enzyme synthesis, and close proximity to the decomposing fiber better enables the microorganism to benefit from the activity of its enzymes. The importance of this factor to the initiation of cellulose decomposition will be brought out later in an analytical look at cellulose decomposition in different environments.

Finally, some moisture is needed for the hydrolytic reaction. It is also needed to provide a medium for diffusion of the enzymes into the fiber network and for migration of the end-products of degradation to the cell. A thin film of water coating the fiber surfaces is all that is required. As will be shown later, too much water can adversely affect cellulose decomposition.

IV. Laboratory observations

It has been frequently recorded that a mixed culture of microorganisms decomposes cellulose more rapidly and more extensively than a single species of organism acting alone.[4-36] The conditions and organisms with which this observation was made varied, but in general the cellulosic substrates used were of woody origin. They therefore probably contained lignin, hemicelluloses, and other plant materials in addition to cellulose. A mixed culture of microorganisms, representing a variety of metabolic capabilities, could utilize the hemicelluloses and lignin and thus strip away the physical barriers encrusting the cellulose and make it more susceptible to hydrolysis. A pure culture of a cellulolytic organism lacking the ability to degrade lignin or hemicelluloses would have greater difficulty in gaining access to the cellulose fibers. One would therefore expect cellulose decomposition to be more limited under these conditions.

If lignin and hemicelluloses are merely mechanical hindrances for the cellulolytic microorganisms, then in using a 100% cellulose substrate, one would not expect to observe different rates of hydrolysis for pure and mixed cultures of equal cellulolytic capacity, provided that organism-organism interactions such as commensalism or mutualism are prevented by suitable nutrient additions to the reaction media. To test this hypothesis it was planned to compare rates of cellulose hydrolysis by pure and mixed cultures of known composition using a cellulose powder prepared from cotton.

The enrichment culture technique of Winogradsky was used to isolate

cellulolytic bacteria from the soil. Three 500-ml Erlenmeyer flasks containing 100 mls of a sterile cellulose isolation medium* were inoculated with about 0.5 g of soil. This medium contained 2 g/l of cellulose powder as the sole carbon source, 1 g/l of ammonium sulfate as a nitrogen source, and mineral salts; it was buffered at pH 7.0. Three soil samples, collected near Lake Chandalar, Alaska, were used. The flask cultures were incubated at 10C and aerated by continuous shaking on a New Brunswick rotary shaker. An incubation temperature of 10C was used so that an estimate of the rate of cellulose decomposition at a low temperature could be obtained simultaneously for another purpose. After two days one ml of culture fluid from each flask was aseptically transferred to a second flask of sterile medium, and these flasks were incubated at 10C with shaking.

To isolate individual species, on the fourth and seventh days a loopful of each culture fluid was streaked onto the surface of an agar plate prepared from the isolation medium. The plates were incubated at 10C. After two weeks incubation, pinpoint, translucent and white colonies were visible on the agar, but these did not reach an appreciable size even after several months. The low temperature was apparently not the limiting factor, for at 20C as well only very small colonies appeared.

After twelve days the shake flasks were removed from the shaker and left in the 10C incubator. Because after two months the culture medium in flask #1 had turned orange, it was examined microscopically. The bacterial population was still viable and had even increased;

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* The details of the analytical procedures and media used can be found in the Appendix.

Table 1. Population levels of mixed cultures of soil bacteria in cellulose isolation medium

Cellulose: 2 g/l	pH: 7.0	Temperature: 10C
shake flask cultures		
Time (days)	Number of bacteria per ml	
	Flask #1	Flask #2	Flask #3
7	1.0×10^7	1×10^7	7.5×10^6
12	1.2×10^7	7.8×10^6	8.1×10^6
63	5×10^8	7×10^5	8×10^6
147	1.6×10^8	---	---

rod-shaped bacteria were swarming around the cellulose particles. See Table 1. No change was noted in the other two flasks. Three months later the culture fluid in flask #1 had cleared. Microscopic examination revealed a varied, viable bacterial culture. Moreover, most of the cellulose had been dissolved.

Five isolates were later obtained from this culture and another shake flask culture of soil #2; these were labeled LC-1 through 5. Subsequent morphological and biochemical tests indicated that these were four different species; LC-1 and LC-2 apparently were the same organism. All were rod-shaped, Gram-negative, polarly flagellated bacteria, whose sizes varied from 0.5-1μ by 1-8μ, depending on the age of the culture.

To enhance the growth of the organisms on agar plates and in liquid culture, the cellulose content of the medium was increased to 5 g/l. The nitrogen and mineral salts contents were proportionately increased. Vitamins known to be beneficial or necessary to cellulose-

decomposing rumen bacteria[42] were also added. Unfortunately, 1 g/l of sodium citrate, to chelate heavy metals, was added at the same time. Much later it was discovered that all five isolates were able to use citrate as the sole carbon source. Thus, the data on the growth rates of these organisms in this improved medium do not reflect growth on cellulose alone. Nevertheless, the results of one experiment are reported here for comparison with the data subsequently obtained when citrate was eliminated from the medium. All five organisms grew rapidly at 4C, as indicated by the generation times, θ, shown in Table II.

Table II. Population levels of pure cultures of soil bacteria in a cellulose plus vitamins medium with citrate

Cellulose: 5 g/l pH: 7.0 Temperature: 4C

shake flask cultures

Time (days)	Number of bacteria per ml				
	LC-1	LC-2	LC-3	LC-4	LC-5
0.17	8×10^5	2.8×10^6	1.8×10^6	1.6×10^6	3.8×10^6
1.14	4.8×10^6	3.2×10^6	1×10^6	8×10^5	1.6×10^7
2.25	2.2×10^7	2.0×10^7	6.8×10^6	6.5×10^6	2.4×10^8
3.95	4.4×10^8	4.0×10^8	6.2×10^8	3.1×10^8	9.2×10^8
5.90	7.6×10^8	5.6×10^8	6.6×10^8	5.0×10^8	7.2×10^8
8.11	8.4×10^8	6.8×10^8	6.8×10^8	6.9×10^8	4.8×10^8
θ, hours	9.95	7.85	6.25	9.69	6.72

Table III. Population levels of pure cultures of soil bacteria in a cellulose plus vitamins medium without citrate

Cellulose: 5 g/l pH: 7.0 Temperature: 4C

shake flask cultures

Time (days)	Number of bacteria per ml				
	LC-1	LC-2	LC-3	LC-4	LC-5
0.05	1×10^6	2×10^5	1×10^6	2×10^5	1×10^6
0.96	2×10^5	1×10^6	5×10^6	2×10^5	2×10^6
2.06	1×10^6	3.7×10^6	3.8×10^6	4.2×10^6	1.4×10^7
3.75	3.8×10^6	4.6×10^6	5.6×10^6	2.6×10^6	8.4×10^6
6.78	3.0×10^6	4.0×10^6	8.8×10^6	2.8×10^6	1.1×10^7

Table IV. Population levels of pure cultures of soil bacteria in a cellulose plus vitamins medium without citrate

Cellulose: 5 g/l pH: 7.0 Temperature: 10C

shake flask cultures

Time (days)	Number of bacteria per ml				
	LC-1	LC-2	LC-3	LC-4	LC-5
0.10	7×10^5	8×10^5	1×10^6	1×10^6	1×10^6
1.01	2×10^6	3×10^6	8×10^6	5×10^5	7×10^6
2.01	3.2×10^6	1.1×10^7	1.2×10^7	1.7×10^5	6.2×10^6
2.99	6.0×10^6	1.0×10^7	1.1×10^7	2.6×10^6	1.2×10^7
4.06	8.2×10^6	1.2×10^7	4.3×10^7	2.8×10^6	1.9×10^7
8.02	1.8×10^8	1.8×10^8	7.0×10^8	2.8×10^6	2.8×10^8

When the citrate was eliminated and the shake flask experiment repeated, (Table III), there was little evidence of growth in the medium at 4C. When the experiment was conducted at 10C, the bacterial levels had increased only slightly over their initial levels when on the fifth day the incubator and shaker were shut off. Consequently, the cultures had been stationary and at room temperature for three days when they were examined on the eighth day of the experiment and it was found that the populations in all but one of the flasks had increased at least one order of magnitude. See Table IV.

Because it appeared that at least four of the isolates were capable of growth on cellulose alone, a large-scale experiment was set up to measure the rates at which cellulose was utilized and end-products were formed as well as the growth rates of the organisms in a cellulose plus vitamins medium without citrate. The reaction vessel was a 2-liter cylindrical jar fitted with a stainless steel lid and central unit through which the coolant was passed to keep the medium at 10C. Air was passed through a sterile cotton filter and introduced into the medium through an orifice near the bottom of the jar. The air bubbles were dispersed and the medium was mixed with a variable-speed drive assembly located just above the air outlet. The vessel and medium were autoclaved for 15 minutes at 121C. When the desired incubation temperature had been reached and the liquid had been aerated long enough to be saturated with oxygen and carbon dioxide, the medium was inoculated with a sterile suspension of LC-1 in phosphate-buffered distilled water to give an initial cell concentration of about 4×10^5 per ml. The pH,

Table V. A pure culture of LC-1 in a cellulose plus vitamins medium

Cellulose: 5 g/l pH: 6.9 Temperature: 10C

2-liter reaction vessel

Time (days)	pH	Bacteria (# per ml)	Cellulose (mg/ml)	Soluble Carbohydrate (µg/ml)	Glucose (µg/ml)
0	6.88	(4×10^5)	5.18	7.5	0.86
0.88	--	2.3×10^6	--	--	--
1.85	6.95	2.3×10^6	4.82	6.5	0.55
3.64	6.90	1.9×10^6	4.89	6.0	0.53
5.88	--	1.3×10^6	--	--	--
6.69	6.90	1.1×10^6	4.54	5.9	0.52
8.67	6.90	1.1×10^6	4.99	--	--
10.64	--	1.7×10^6	--	--	--
11.94	6.90	1.4×10^6	3.93	6.6	0.60
13.65	--	1.5×10^6	--	--	--

number of bacteria per ml, concentration of cellulose remaining, and the concentrations of glucose and total soluble carbohydrate released into the medium were monitored for fourteen days. The bacterial population was determined microscopically using a volumetric counting chamber. Glucose was determined by the glucose oxidase method of Worthington.[53] Soluble carbohydrate was measured colorimetrically by the phenol-sulfuric acid method of DuBois *et al.*[11] Cellulose was determined by weight. (See Appendix). The results of this experiment are shown in Table V.

No change in any of the variables, except the cellulose concentration, was observed in 14 days. The apparent loss of cellulose was due to its adsorption onto the stainless steel parts of the reaction vessel. That the organisms used to inoculate the medium were viable and were not subsequently inhibited by heavy metals from the reaction vessel is attested to by the fact that a similar experiment with glucose as the sole carbon source instead of cellulose was performed simultaneously in an identical apparatus and the organisms grew readily.

After reconsideration of the data of Table IV, it was thought that perhaps the rise in temperature had stimulated the population increase; thereafter warmer incubation temperatures were used. Table VI shows the results of attempting to grow isolates LC-1 and LC-5 at 20C in shake flasks containing the cellulose plus vitamins medium without citrate. This experiment was done in triplicate. The results were all the same; namely, no change in any of the bacterial population levels for 16 days.

Similar results were obtained when flasks of sterile cellulose isolation medium with vitamins were inoculated with bacteria known to be cellulolytic and incubated at 25C with continuous shaking. The organisms were *Cellulomonas fimi* (ATCC 484) and *Cellulomonas biazotea* (ATCC 486). As shown in Table VII, there was no change in the number of bacteria per ml in 13.5 days.

The distinguishing feature of these experiments is the inability of a pure bacterial culture to develop in a well-mixed, aerated, liquid medium in which a particulate, cotton cellulose substrate serves as the only source of carbon, even though nitrogen, vitamins, and mineral salts, including trace elements, are provided. The fact that the same organ-

Table VI. Population levels of pure cultures of LC-1 and LC-5 in a cellulose plus vitamins medium

Cellulose: 5 g/l	pH: 7.0	Temperature: 20C
	shake flask cultures	
Time (days)	Number of bacteria per ml LC-1	LC-5
0.75	1.72×10^7	1.88×10^7
1.04	1.80×10^7	2.08×10^7
1.75	6.11×10^7	3.08×10^7
2.75	2.80×10^7	1.75×10^7
3.82	1.78×10^7	1.97×10^7
4.91	1.26×10^7	2.50×10^7
5.04	The flasks were re-inoculated with 24-hour old cells. The number added per ml was:	
	9.2×10^5	7.2×10^5
8.20	1.91×10^7	2.75×10^7
10.02	2.92×10^7	4.48×10^7
11.30	3.00×10^7	4.73×10^7
12.32	3.43×10^7	6.15×10^7
13.55	2.60×10^7	9.3×10^7
16.20	2.80×10^7	4.75×10^7

isms are able to grow in pure culture at a rapid rate, even at the low temperature of 4C, in the same medium to which a soluble carbon source, citrate, has been added indicates that additional growth factors are

Table VII. Population levels of pure cultures of known cellulolytic bacteria in cellulose isolation medium with vitamins

Cellulose: 2 g/l pH: 7.0 Temperature: 25C
shake flask cultures

Time (days)	Number of bacteria per ml	
	Cellulomonas fimi	*Cellulomonas biazotea*
1.57	4.2×10^6	1.8×10^6
1.84	2.0×10^6	6.8×10^6
2.50	1.2×10^6	1.5×10^6
2.94	4.0×10^6	2×10^6
3.48	7.6×10^6	1.9×10^6
3.67	4.6×10^6	2×10^6
6.50	5.0×10^6	1.8×10^6
8.61	6.0×10^6	3.2×10^6
13.50	7.5×10^6	3.9×10^6

not required and that the medium itself is not inhibitory. The fact that the original cellulose isolation medium supported the mixed culture decomposition of the cellulose substrate indicates that the cellulose powder is susceptible to enzymatic hydrolysis. An important point to mention is that in both instances where any activity was observed; that is, in the mixed culture of Table I and the pure cultures of Table IV, the flasks had been stationary prior to the observation. This fact suggests that a well-mixed liquid culture prevents the bacteria and the cellulose particles from having the intimate contact needed for the

organism to initiate and continue cellulose decomposition. Lignin may indeed be partly responsible for the frequently reported slow decomposition of cellulose in pure culture. However, the data presented here suggest that other factors, physical in nature, may be equally important. The situation in which a bacterium finds itself when faced with a particulate substrate in a well-mixed liquid environment will be considered in more detail in the next section.

V. Cellulolysis in well-mixed liquid cultures

The importance of diffusion in the cellulolytic process is obvious. The enzyme must diffuse from the cell to its substrate, and assimiable reaction end-products must diffuse back to the cell. If the catalytic capacity of the enzyme and the ability of the cell to synthesize enzyme are optimized, then a maximum rate of hydrolysis of β-1,4'-glucosidic bonds can be calculated from a phenomenological equation based on Fick's laws of diffusion.[1] From this rate the rate of release of glucose into the liquid medium can be estimated and compared with the rate required to maintain the cell population at its initial level. Such a calculation was made for a wide range of initial cell populations inoculated into the cellulose plus vitamins medium containing 5 g/l of cellulose powder.

To determine the maximum rate of hydrolysis it was assumed that every time an enzyme molecule and a cellulose particle collided, a reaction occurred. Furthermore, to simplify the calculation the colliding molecules were treated as spheres and steady-state concentrations of reactants were assumed. The expression for the diffusion-controlled reaction rate is:

$$R_h = \kappa\, 4\pi\, a(D_A + D_B)\, f\, C_{A_o}\, C_{B_o},$$

where κ is a symmetry number, equal to 1 for collisions between unlike molecules, a is the sum of the radii of the colliding spheres, in centimeters, D_A and D_B are the diffusion constants of molecules A and B, and C_{A_o} and C_{B_o} are the initial concentrations of these molecules, in particles per ml. The factor f is a function of the electrostatic potential.

For simplicity the molecules are taken to be neutral, whence f equals 1.

Before the phenomenological equation could be used to calculate a maximum rate of hydrolysis, some idea was needed of the enzyme concentrations that could be expected. Surprisingly, there is little decisive information on enzyme synthesis rates to be found in the literature. But enough data are available to enable rates to be calculated for two enzyme systems if certain assumptions are made. One of these systems involves the extracellular enzyme, α-amylase; the other, the intracellular enzyme β-galactosidase.

Markovitz and Klein[31] measured rates of biosynthesis of α-amylase by resting cells of *Pseudomonas saccharophila* and found that, with maltose as the inducer, about 5 enzyme units per hour (per ml, presumably, since the assay volume equaled 1 ml) were synthesized for 3 hours, after which time the rate declined. Presumably the cells were initially grown in a medium containing 0.2% sodium lactate as the carbon and energy source. If a cell yield of 0.5 g of cells (dry weight) per g of lactic acid is assumed, the final resting cell suspension, concentrated fourfold, contained 0.32 g of cells (dry weight) per 100 mls.

Markovitz and Klein defined one unit of α-amylase as that amount of enzyme which caused a loss in color with iodine at 660 mμ equivalent to 0.5 mg starch in 30 minutes at 37C and pH 5.5. Since the average degree of polymerization of soluble starch is about 300 glucose residues,[49] 0.5 mg starch corresponds to 5.6×10^{15} starch molecules. Starch molecules with a DP less than 20 units do not give a color with iodine that would be detected at 660 mμ.[49] To reduce a starch with a DP of 300 to one with a DP less than 20 would require breaking at

least 15 bonds per molecule, and most likely more than 15 bonds would be broken in the actual process. If the turnover number and molecular weight of bacterial α-amylase are presumed to be similar to the values for pancreatic α-amylase, 25,000[27] and 45,000,[10] respectively, and if only one active center per enzyme molecule is assumed, then one unit of enzyme corresponds to at least 1.11×10^{11} enzyme molecules or 8.3×10^{-6} mg enzyme. Thus, the enzyme synthesis rate observed by Markovitz and Klein, 5 enzyme units per hour per ml, is equivalent to at least 1.74×10^{2} enzyme molecules per cell per hour or 2.16×10^{-7} mg enzyme per mg cells per minute.

When starch was used as the inducer, α-amylase was synthesized at the rate of 0.22 units per hour per ml of cell suspension for the first three hours, and then the rate declined. In this case one unit of α-amylase was defined as that amount of enzyme which released 0.021 mg of reducing sugar (as maltose) in 3 minutes at 37C and pH 5.5. One unit thus corresponds to 4.68×10^{11} enzyme molecules or 3.50×10^{-5} mg enzyme, and the observed rate of enzyme synthesis equals 32.1 enzyme molecules per cell per hour or 4.01×10^{-8} mg enzyme per mg cells per minute.

Rates of α-amylase synthesis by *P. saccharophila* were also calculated from the data of Schiff, Eisenstadt, and Klein[41] using the following assumptions: (1) that 70% of dry cell material is protein;[43] (2) that the dry weight of a bacterium is about 10^{-9} mg;[45] and (3) that the molecular weight of α-amylase is 45,000.[10] Resting cells synthesized the enzyme at an average rate of 1.7×10^{3} molecules per cell per hour or 2.1×10^{-6} mg per mg cells per minute, whereas growing cells synthe-

sized 6.0×10^2 molecules per cell per hour or 1.0×10^{-6} mg enzyme per mg cells per minute. Note that α-amylase was manufactured more rapidly by resting cells than by cells growing on starch.

May and Elliott[33] have described a somewhat different biphasic production of α-amylase by 24-hour old cells of *Bacillus subtilis*. These cells were washed and incubated at 30C in a medium supplemented with a mixture of 16 amino acids. Initially α-amylase was synthesized at a rate of 9.2 units per ml of culture per hour, but after 75 minutes the rate increased to 26.6 units per ml of culture per hour. As no estimate of the cell population was given by the authors, this value was taken to be 10^9 cells, or 1 mg dry weight, per ml. In a previous paper[7] it was stated that 1 mg of enzyme nitrogen was equivalent to 38,800 enzyme units. If the enzyme protein is 16% nitrogen, then α-amylase was initially formed at the rate of 1.83×10^4 molecules per cell per hour or 2.47×10^{-5} mg per mg cells per minute. During the second phase it was produced at the rate of 5.32×10^4 molecules per cell per hour or 7.16×10^{-5} mg per mg cells per minute. The fact that these values are an order of magnitude higher than those previously calculated is not unreasonable in view of the supplemental amino acids with which the cells were provided.

From previously induced cells of *Escherichia coli*, Kameyama and Novelli[21] isolated a cell-free system that catalyzed the synthesis of β-galactosidase. The isolated system represented 5.71% by weight of the original cell protein, if one assumes that the cells were originally 70% protein on a dry weight basis. Kameyama and Novelli defined one unit of β-galactosidase activity as that amount of enzyme required to hydrolyze

one μmole of o-nitrophenyl-β-D-galactoside in one hour at 37C. According to Jacob and Monod[20] this hexameric enzyme has a molecular weight of 810,000 and a turnover number of 240,000 at 28C and pH 7.0. If at 37C the turnover is 1.8 times faster than at 28C, then one Kameyama and Novelli enzyme unit is equivalent to 2.32×10^{10} hexameric enzyme molecules. At 37C it was observed that 2.51 mg of the cell-free protein formed 215 enzyme units in 60 minutes; this rate corresponds to 84 molecules per cell per hour or 1.78×10^{-6} mg enzyme per mg cells per minute.

In another experiment with cell-free extracts from pre-induced cells of *E. coli*, Eisenstadt, Kameyama, and Novelli[12] measured an enzyme synthesis rate of 156 units per hour per 2.76 mg of cell-free protein. This rate is equal to 52.5 hexameric enzyme molecules per cell per hour or 1.18×10^{-6} mg enzyme per mg cells per minute. When particulate protein from non-induced cells was mixed with supernatant fluid from an induced cell preparation, an enzyme synthesis rate equivalent to 16.2 molecules per cell per hour or 3.63×10^{-7} mg enzyme per mg cells per minute was observed. And when particulate protein from induced cells was mixed with supernatant fluid from non-induced cells, 42.9 hexameric molecules were formed per cell per hour or 9.64×10^{-7} mg enzyme per mg cells per minute.

Mandelstam[30] measured the rate at which methyl-thiogalactoside induced β-galactosidase synthesis in leucine-starved cells of *E. coli*. His results were in terms of μmoles of o-nitrophenyl-β-D-galactoside hydrolyzed per hour per mg dry weight of cells at 30C and pH 7.0, so that the turnover number for β-galactosidase reported by Jacob and Monod[20] could be used to calculate the rate of enzyme synthesis.

Table VIII. Summary of enzyme synthesis rates

Enzyme	Organism	Temperature C	Enzyme molecules per cell per hr	mg enzyme per mg cells per min	Ref.
α-amylase	*Pseudomonas saccharophila*				
	resting cells	25	1.74×10^{2}	2.16×10^{-7}	31
	" "	25	32.1	4.01×10^{-8}	31
	" "	30	1.7×10^{3}	2.1×10^{-6}	25-41
	growing cells	30	6.0×10^{2}	1.0×10^{-6}	25-41
	Bacillus subtilis				
	resting cells	30	1.83×10^{4}	2.47×10^{-5}	33
	" "	30	5.32×10^{4}	7.16×10^{-5}	33
β-galactosidase	*Escherichia coli*				
	cell-free extracts	37	84	1.78×10^{-6}	22
	" "	37	52.5	1.18×10^{-6}	12
	" "	37	16.2	3.63×10^{-7}	12
	" "	37	42.9	9.64×10^{-7}	12
	leucine-starved cells		2.08×10^{2}	4.68×10^{-6}	30

Although the rate was not constant throughout the incubation period, 1 mg of cells formed 6.27×10^{11} molecules in three hours. Thus, the average rate of synthesis was 2.08×10^{2} hexameric molecules per cell per hour or 4.68×10^{-6} mg enzyme per mg cells per minute.

Table VIII summarizes the enzyme synthesis rates calculated. The fact that data from different sources yielded similar values invites confidence in the results. The most rapid rate of synthesis was that at which *Bacillus subtilis* manufactured α-amylase; namely, 7.16×10^{-5} mg enzyme per mg cells per minute. But because the medium in which these cells were incubated was supplemented with amino acids, and because the estimate of the number of cells per ml used in this calculation may be an order of magnitude or two in error, 5×10^{-6} mg enzyme per mg cells per minute is probably a more reliable estimate to use. This rate is equivalent to about 3×10^{3} cellulase molecules per cell per hour, a liberal value but one not out of line with observed rates of enzyme synthesis by bacteria.

Supposing that a resting cell can maintain this rate for three hours, and that at this time it will have exhausted about half its synthetic capacity, then from an initial inoculum of 1×10^{3} cells per ml, one could expect there to be an eventual cellulase concentration of 1.8×10^{7} molecules per ml, provided the cells remain viable long enough to synthesize this amount.

The cellulase of *Myrothecium verrucaria* is a cigar-shaped molecule with a molecular weight of 63,000.[8] The volume of water which a protein of this size displaces was calculated from the equation:

$$m_w/\rho = m\bar{v},$$

where m_w is the mass of water displaced, ρ is the density of water (taken equal to 1), m is the mass of the protein, and $\bar{v}$ is the partial specific volume of the protein (taken equal to 0.7).[28] The volume was estimated to be 7.32×10^{-20} cm^3 per enzyme molecule. Were the enzyme spherical, it would have a radius of 2.60×10^{-7} cm.

By analogy with diffusion constants for molecules of similar size,[28-44] cellulase was assigned a diffusion constant of 6×10^{-7} cm^2 per second and cellulose one of 0.05×10^{-7} cm^2 per second.

The highest concentration of cellulose powder used in the laboratory experiments was 5 g/l. From an average particle volume of 1.10×10^{-8} cm^3 and density of 1.6,[16] this concentration was calculated to equal 2.79×10^5 particles per ml.

The phenomenological equation could now be applied to the situation in which 5 g/l of cellulose powder in a liquid medium containing all the necessary mineral salts, trace elements, and growth factors is inoculated with a pure bacterial culture. For an initial organism population of 1×10^3 cells per ml, the maximum rate of hydrolysis to be expected is 7.92×10^4 reactions per ml per second, or about 2 reactions per cellulose particle per minute:

$$\kappa = 1$$

$$a = r_A + r_B = 2.09 \times 10^{-3} \text{ cm} + 2.60 \times 10^{-7} \text{ cm}$$

$$= 2.09 \times 10^{-3} \text{ cm}$$

$$D_A = 0.05 \times 10^{-7} \text{ cm}^2/\text{sec}$$

$$D_B = 6 \times 10^{-7} \text{ cm}^2/\text{sec}$$

$$f = 1$$

$$C_{A_o} = 2.79 \times 10^5 \text{ particles/ml}$$

$$C_{B_o} = 1.8 \times 10^7 \text{ particles/ml}$$

$$R_h = \kappa\, 4\pi\, a(D_A + D_B)\, f\, C_{A_o}\, C_{B_o} = 7.92 \times 10^4 \text{ reactions/ml·sec.}$$

The rate of release of glucose into the medium is equal to the product of the frequency of hydrolysis and the probability of hydrolyzing an end linkage:

$$R_G = R_h \cdot P(G_1).$$

For a cellulose molecule of n glucose residues, this probability equals 2/(n-1), if it is assumed that hydrolysis is completely random. For cellulose with an average DP of 3000, the initial probability of freeing a glucose molecule would be 2/2999. Thus, the initial rate of release of glucose for the situation being considered here would be 52.9 glucose molecules per ml per sec or 1.90×10^2 glucose molecules per cell per hour. The rate at which glucose is required to be released to maintain 10^3 cells per ml can be estimated from the endogenous maintenance requirement determined by Marr *et al.*[32] for *E. coli* at 30C; namely, 0.028 mg glucose per mg cells per hour or 9.35×10^7 glucose molecules per cell per hour. Thus, the initial rate of production of glucose is not sufficient to satisfy the endogenous respiration requirement of even one cell per ml. Moreover, the probability of releasing glucose from the cellulose molecule remains substantially unchanged for a very long time at a hydrolysis rate of 7.92×10^4 reactions per ml per second. A cellulose molecule with a DP of 3000 is about 8 Å wide and 15,400 Å long,[46] so that there are at least four million such molecules exposed on the surface of each cellulose particle. At the rate of 2 hydrolyses per parti-

cle per minute, it would take over 1700 days to improve the probability by a factor of 10, if an average of 10 collisions per molecule will reduce the DP to 300.

The hydrolysis rates and rates of release of glucose were calculated for initial inocula of 10^3 to 10^{10} cells per ml. These values are shown in Table IX. It can be seen that in no case is the initial glucose production rate sufficient to sustain the original cell population. Considering only the amount of glucose released and ignoring the nutrients released by dead, lysed cells, one can see that the amount of glucose produced is only enough to support a population five to six orders of magnitude smaller than that originally present. Even if the probability of hydrolyzing an end linkage were taken equal to 1, the calculated initial rate of release of glucose would not be enough to meet the maintenance requirement. Only in the highly improbable situation that a glucose molecule was released with each hydrolysis and that all the cell material was converted to enzyme would this requirement be met. Obviously, some part of the original cell population must die. Those cells which lyse will release nutrients into the medium which will enable a larger cell population to be supported than that calculated from the rate of glucose release alone. And eventually, if some part of the population survives and cellulolysis begins, the average DP of the cellulose molecules will decrease, and the probability of hydrolyzing an end linkage will increase. Consequently, the rate of release of glucose into solution will be gradually increasing, and more and more cells will be able to survive.

Table IX. Expected rates of hydrolysis and glucose production for various initial cell populations

Initial number of cells per ml	Enzyme concentration (#/ml)	Hydrolysis rate R_h (rxs/ml/sec)	Initial rate of glucose production R_G (molecules/ml/sec)	Rate of glucose prod'n required R_G' (molecules/ml/sec)	Time required to improve R_G by 100X
1×10^3	1.8×10^7	7.92×10^4	5.29×10^1	2.59×10^7	1.74×10^4 days
1×10^4	1.8×10^8	7.92×10^5	5.29×10^2	2.59×10^8	1.74×10^3 days
1×10^5	1.8×10^9	7.92×10^6	5.29×10^3	2.59×10^9	1.74×10^2 days
1×10^6	1.8×10^{10}	7.92×10^7	5.29×10^4	2.59×10^{10}	17.4 days
1×10^7	1.8×10^{11}	7.92×10^8	5.29×10^5	2.59×10^{11}	41.7 hours
1×10^8	1.8×10^{12}	7.92×10^9	5.29×10^6	2.59×10^{12}	4.2 hours
1×10^9	1.8×10^{13}	7.92×10^{10}	5.29×10^7	2.59×10^{13}	25 minutes
1×10^{10}	1.8×10^{14}	7.92×10^{11}	5.29×10^8	2.59×10^{14}	2.5 minutes

The times required to improve the rates of glucose release by two orders of magnitude are also shown in Table IX. Only with very large inocula will the initial collision frequency between the cellulose and enzyme molecules be high enough to support a substantial number of cells -- and then only if the cells can survive long enough on their own energy reserves to produce the postulated amount of enzyme. From inspection of the times required to increase the glucose production rates by a factor of 100, it would appear that at least 10^8 cells per ml are required initially for cellulolysis to begin within a time practical for laboratory observation. The collision frequency could be increased by increasing the number of cellulose particles per ml, but a more noticeable effect could probably be had by including small amounts of soluble nutrients in the medium to enable the cells to survive until cellulose decomposition is well under way and they can maintain themselves. Because of the limitations which diffusion imposes on the process, it appears that cellulose decomposition is difficult to initiate in a well-mixed liquid environment in which no carbon and energy source other than cellulose is immediately available to sustain the cells.

VI. Cellulolysis in natural situations

Circumstances in nature are more favorable for cellulose decomposition than those employed in the laboratory experiments. In the natural environment one finds a variety of species of microorganisms capable of utilizing the food sources such as sugars, organic acids, proteins, hemicelluloses, and lignins which are available in addition to cellulose. Through a complex symbiosis a microorganism population is established and supported. Hence, cellulose decomposition should be initiated more readily in nature.

Aerobic cellulose decomposition is probably maximum in a terrestrial environment such as the upper layer of soil, a rotting log, or the leaf litter on the forest floor. Provided the humidity is suitable, diffusion limitations should be minimal, because the organisms remain in intimate contact with the substrate. The fungi generally dominate the cellulolytic process in the soil,[5] unless the soil is very alkaline or has been fertilized,[19] for they require less nitrogen than the bacteria to metabolize an equivalent amount of carbohydrate. The fungi penetrate the cellulose fiber by releasing cellulase at the tips of their extending hyphae.[39] Because the cellulolytic enzymes do not have to diffuse far to reach the substrate and digest it, the local concentrations of both the enzyme and the hydrolysis products are effectively greater, in relation to the substrate and the cell, than in an aquatic environment. A similar description applies to cellulose decomposition by bacteria in this situation. In addition, the protoplasmic contents of dead fungal cells help support the bacterial population, both cellulolytic and non-

cellulolytic.

Although a variety of microorganisms and food sources are available in natural aquatic environments as well, the large volume of water and lower cellulose concentrations essentially decrease organism-substrate interactions, so that diffusion hinders the progress of cellulolysis here just as it does in the shake flask. One would expect the benthic regions of a body of water to be the site of cellulolytic activity rather than the water itself. Insoluble cellulosic materials settle to the bottom and sink into the upper layer of mud where close contact between the cellulose and anaerobic cellulolytic bacteria is possible. Few fungi exist in muds, so that their contribution to cellulolysis in this situation is negligible.[26] Cellulose decomposition is probably initiated readily by anaerobic bacteria but may not be prolonged, for these organisms produce organic acids in the process of assimilating the cellulose. Unless the cellulolytic bacteria are accompanied by species capable of metabolizing the acids, the pH of their environment will become so low as to prevent them from functioning. In the absence of organic acid-metabolizing organisms, cellulose decomposition probably occurs sporadically. Data substantiating this picture of cellulose decomposition have been reported by Laurent,[26] who found that cellulolytic activity in the water of a pond was very weak compared with that in the underlying mud. Maximum cellulolytic activity occurred in the mud immediately below the mud-water interface and declined in the deeper mud layers. As would be expected for a process limited by diffusion, increasing the temperature from 5C to 25C had no influence on cellulolytic activity in

the water but did have a distinct positive effect in the mud.

In waste treatment practice cellulose is treated primarily by anaerobic methods. The major portion of the cellulose found in domestic sewage is separated from the soluble waste matter in an initial sedimentation step. The settled waste, or primary sludge as it is called, is then digested anaerobically. Cellulose decomposition is promoted by the close organism-substrate contact that is possible and by the variety of microorganisms and food sources present in the sludge. The cellulose is largely converted to organic acids by acid-forming bacteria in the first stage of digestion. The acids are subsequently metabolized to methane and carbon dioxide by another group of bacteria. In the process these bacteria raise the pH and permit further degradation of cellulose by the first group. Successful operation of an anaerobic digestor obviously requires a balance between the activities of the acid-formers and the methane-formers.

The extent to which the cellulose remaining in the liquid portion of the waste after primary sedimentation is subsequently decomposed will depend on the process by which the waste is treated. The activated sludge process is the conventional biological method of treating soluble wastes. It essentially consists of aerating the waste in the presence of bacteria until the bacteria have stabilized the organic matter.[34] The "activated sludge" thus formed is a conglomeration of bacteria, protozoa, possibly some fungi, and particulate organic matter which is in the process of being degraded or is not biologically degradable. If particles of cellulose become attached to this active mass, it is conceivable that their decomposition would be easily initiated if cellulo-

lytic bacteria were present. It is unlikely that the decomposition would be extensive by the time the activated sludge is settled out and the treated effluent is disposed of, for contact times between the activated sludge and the waste are typically a matter of hours. But if the cellulose remains attached to the sludge when it is settled and returned to the aeration tank to be mixed with untreated waste, it would probably be degraded eventually. However, the extent to which this attachment occurs is not known.

The cellulose which does not become attached to the active mass but remains in suspension as the waste is aerated probably passes out of the treatment plant unaffected. Although the bacterial population in the liquid portion of the activated sludge-waste mixture is higher than in most natural aquatic environments, the proportion which is cellulolytic is not known and would be a function of the composition of the waste. Also, the concentration of cellulose found in domestic sewage which has undergone primary sedimentation is about one-thousandth of that used in the laboratory experiments,[18] so that the opportunity for interaction between cellulose and cellulolytic organisms is likely to be substantially less.

VII. Summary

Despite its economic importance, the microbial decomposition of cellulose is not well understood. Contributing to our lack of understanding are the natural variability and structural complexity of the cellulose molecule itself; the chemical formula which we assign to it incompletely describes the macromolecular structure found in nature. Furthermore, the mode of action of cellulolytic enzymes cannot as yet be described definitively.

The importance of certain factors to the process has become clear. Among these are the degree of polymerization of the single cellulose strand and the proportion of crystalline regions within the fiber. The extent to which neighboring cellulose molecules are drawn together into a crystalline structure affects both the susceptibility of the substrate to degradation and the activity of the cellulase which it induces. The greater the degree of crystallinity of the cellulose, the more resistant to enzymatic attack it is. The amount of other substances associated with cellulose in the plant cell wall can also affect its susceptibility to enzymatic attack. The effect may be negative, as in the case of lignins and other materials which encrust the cellulose microfibrils and hinder contact between the cellulolytic enzyme and its substrate, or it may be positive, as in the case of growth-promoting susbstances such as vitamins, minerals, proteins, and soluble carbohydrates. The availability of extraneous nutrients is understandably beneficial. It is probably due to their presence that cellulose decomposition has been observed to occur more rapidly in a mixed culture of microorganisms

than with a single cellulolytic species; the organisms accompanying the cellulolytic species can provide it with energy sources which it could not obtain by itself. Another factor affecting cellulose decomposition is the amount of moisture present. Some moisture is required to coat the fiber surfaces and provide a medium for diffusion, but too much water tends to defeat the process. The closer the association of the organism with its substrate, the more rapidly its enzymes will be able to penetrate the cellulose fiber and digest it, and the more readily it will be able to obtain assimiable end-products for self-maintenance. As emphasized by laboratory experiments and an analytical evaluation of the role of diffusion in cellulolysis, the key factor in the process of cellulose decomposition appears to be the extent to which organism-substrate interaction is possible.

VIII. Appendix

Media:

Cellulose powder was prepared by mild acid hydrolysis of non-absorbent cotton. 10.0 g of cotton was suspended in one liter of 3N HCl in a 1500-ml beaker and heated to 80-90C. The mixture was stirred continuously until the fibers broke and formed a loose, fairly homogeneous floc. The beaker was removed from the heat and allowed to cool to about 60C. The floc was separated from the liquid by filtration through a Whatman 41H filter paper in a Büchner funnel with vacuum applied. It was then washed with distilled water until the final washing was of neutral pH. To do this rapidly the floc had to be resuspended in distilled water and filtered several times. After most of the moisture had been removed by suction, the floc was dried overnight in a 103C oven. It was then pulverized in a mortar and pestle. Microscopic examination revealed that the powder was made up of flat, rectangular particles with an average size of 100μ x 20μ x 5μ.

Cellulose isolation medium contained, per liter of distilled water, 2.0 g cellulose powder, 1.0 g $(NH_4)_2SO_4$, 2.5 g Na_2HPO_4, 1.26 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 15 mg $CaCl_2$, 0.4 mg $FeSO_4 \cdot 7H_2O$, and 0.2 mg of each of the following trace elements: $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The medium was adjusted to pH 7.2 with NaOH and autoclaved for 15 minutes at 121C. Its final pH was 7.0.

Cellulose isolation medium with vitamins. Prior to sterilization, each of the following six vitamins was added to the cellulose isolation medium so that the final concentration of each was 10^{-9} molar: biotin,

pyridoxine hydrochloride, folic acid, riboflavin, paraminobenzoic acid, and cyanocobalamine (B_{12}).

Cellulose plus vitamins medium contained, per liter of distilled water, 5.0 g cellulose powder, 3.0 g $(NH_4)_2SO_4$, 5.0 g Na_2HPO_4, 2.75 g KH_2PO_4, 0.10 g $MgSO_4 \cdot 7H_2O$, 7.5 mg $CaCl_2$, 0.2 mg $FeSO_4 \cdot 7H_2O$, and 0.1 mg of each of the trace elements added to the cellulose isolation medium. In addition, each of the six vitamins was added to give a final concentration of each of 10^{-9} molar.

Analytical procedures:

Cell counts were made microscopically using a Zeiss phase-contrast microscope at 1200X magnification. A measured volume of samplewas mixed with at least an equal volume of a 36% formaldehyde solution containing methylene blue. This solution kills the cells and stains them at the same time. About 0.1 ml of the mixturewas transferred to a Petroff-Hausser bacteria counting chamber. This device consists of a glass base with a grid of squares of known size and a cover slip which provides for a known depth of liquid over the grid. One can thus count the number of bacteria found in the liquid over x number of squares, determine the volume which this represents, and calculate the number of bacteria per ml. This value is then multiplied by the dilution factor to give the number of bacteria, living and dead, per ml of culture fluid.

Cellulose was measured by filtering a 20.00 ml sample of culture medium through a 5μ porous-bottom crucible which had been previously dried and tared to constant weight at 103C. Although a volumetric pipet was used for sampling, for consistency it was necessary to rinse

it out with distilled water in order to flush out the cellulose particles adhering to the walls of the pipet. (This was done after a portion of the filtrate had been collected for the glucose and soluble carbohydrate determinations). After most of the moisture had been drawn off by suction, the crucible was again dried to constant weight at 103C. The difference in weights gave the cellulose remaining in suspension in the medium.

Soluble carbohydrate was determined by the colorimetric method of DuBois *et al.*[11] Because this method can be used for polysaccharides as well as oligosaccharides and simple sugars, the filtrate from the cellulose determination was used. To a 2.00 ml sample, in a 1/2" B & L colorimetric test tube, was added 1.0 ml of a 5% (w/w) phenol solution. Then 5.0 mls of concentrated reagent grade sulfuric acid was added rapidly, in 10-20 seconds. The acid stream was directed against the liquid surface rather than the side of the tube. After 10 minutes the sample was shaken for 10 to 20 minutes in a water bath at 25-30C. The absorbance of the resulting orange solution was measured at 490 mμ in a Bausch and Lomb Spectronic 20 against a blank prepared by treating 2.00 mls of distilled water in the same manner as the sample. Standard glucose solutions, containing between 5 and 50 μg of sugar per ml, were used to prepare a standard curve which was linear in this concentration range.

Glucose was determined enzymatically using Worthington Biochemical Corporation's *Glucostat Special*. This preparation permits the determination of glucose in the presence of poly- and oligosaccharides since it is essentially free of carbohydrases and is specific for glucose.[53]

The glucose concentration in the filtrate from the cellulose determination was measured as follows: To 2.0 mls of sample, in a 16 x 150 mm test tube in a 37C water bath, was added 2.0 mls of the *Glucostat Special* reagent. The sample and the reagent were mixed and incubated for 30 minutes, at which time the reaction was stopped with one drop of 5N HCl. The absorbance of the resulting orange solution was measured at 400 mμ in 1 cm cells in a Beckman DB Spectrophotometer against a blank consisting of 2.0 mls of distilled water treated in the same way as the sample. Standards, containing 10 to 30 μg of glucose per ml, were treated simultaneously, and the concentration of glucose in the sample was calculated from the formula:

$$\text{Concn.}_{\text{sample}} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concn.}_{\text{standard}}$$

Glassware used in the analyses and in the preparation of media was washed in detergent and hot water and rinsed at least three times in tap water and three times in distilled water. It was then treated with acid-dichromate cleaning solution and rinsed thoroughly in distilled water.

IX. Literature cited

1. Amdur, I., and Hammes, G. G. (1966), Chemical Kinetics: Principles and Selected Topics, New York, N. Y., McGraw-Hill, p. 59.

2. Basu, S. N., and Ghose, S. N. (1960), *Can. J. Microbiol. 6*, 265.

3. Bose, R. G., and Basu, S. N. (1965), *Indian J. Exp. Biol. 3*, 42; *Biol. Abstr. 47*, 33874, (1966).

4. Brock, T. D. (1966), Principles of Microbial Ecology, Englewood Cliffs, N. J., Prentice-Hall, p. 122.

5. Brock, T. D. (1966), *ibid.*, p. 247.

6. Charpentier, M. (1965), *Ann. Inst. Pasteur 109*, 771; *Biol. Abstr. 47*, 38176, (1966).

7. Coleman, G., and Elliott, W. H. (1962), *Biochem. J. 83*, 256.

8. Cowling, E. B. (1963), *in* Advances in Enzymic Hydrolysis of Cellulose and Related Materials, Reese, E. T., Ed., New York, N. Y., MacMillan, p. 1.

9. Dehority, B. A. (1965), *J. Bacteriol. 89*, 1515.

10. Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N. Y., Academic, p. 453.

11. DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

12. Eisenstadt, J. M., Kameyama, T., and Novelli, G. D. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 652.

13. Hash, J. H., and King, K. W. (1954), *Science 120*, 1033.

14. Hash, J. H., and King, K. W. (1958), *J. Biol. Chem. 232*, 381.

15. Hashimoto, Y., and Nisizawa, K. (1963), *in* Advances in Enzymic Hydrolysis of Cellulose and Related Materials, Reese, E. T., Ed., New York, N. Y., MacMillan, p. 93.

16. Hermans, P. H. (1949), Physics and Chemistry of Cellulose Fibers, New York, N. Y., Elsevier, p. 197.

17. Heuser, E. (1944), The Chemistry of Cellulose, New York, N. Y., Wiley, p. 3.

18. Hurwitz, E.,Beck, A. J., Sakellariou, E., and Krup, M. (1961), *Jour. W. P. C. F. 33*, 1070.

19. Imshenetsky, A. A. (1968), *in* The Ecology of Soil Bacteria, Gray, T. R. G., and Parkinson, D., Ed., Toronto, Canada, University of Toronto, p. 256.

20. Jacob, F., and Monod, J. (1961), *J. Mol. Biol. 3*, 318.

21. Kameyama, T., and Novelli, G. D. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 659.

22. Ke, Y., and Quinn, L. Y. (1965), *Iowa State J. Sci. 40*, 27; *Biol. Abstr. 47*, 22964, (1966).

23. King, K. W. (1963), *in* Advances in Enzymic Hydrolysis of Cellulose and Related Materials, Reese, E. T., Ed., New York, N. Y., MacMillan, p. 159.

24. King, K. W. (1966), *Biochem. Biophys. Res. Commun. 24*, 295.

25. Klein, H. P. (1963), *Ann. N. Y. Acad. Sci. 102*, 637.

26. Laurent, M., to be presented at the Fourth International Conference on Water Pollution Research, Prague, April, 1969.

27. Long, C. (1961), Biochemists' Handbook, Princeton, N. J., Van Nostrand, p. 232.

28. Mahler, H. R., and Cordes, E. H. (1966), Biological Chemistry, New York, N. Y., Harper & Row, p. 46.

29. Mandels, M., and Reese, E. T. (1960), *J. Bacteriol. 79*, 816.

30. Mandelstam, J. (1957), *Nature 179*, 1179.

31. Markovitz, A., and Klein, H. P. (1955), *J. Bacteriol. 70*, 641.

32. Marr, A. G., Nilson, E. H., and Clark, D. J. (1963), *Ann. N. Y. Acad. Sci. 102*, 536.

33. May, B. K., and Elliott, W. H. (1968), *Biochim. Biophys. Acta, 157*, 607.

34. McKinney, R. E. (1962), Microbiology for Sanitary Engineers, New York, N. Y., McGraw-Hill, p. 213.

35. Nisizawa, K., Hashimoto, Y., and Shibata, Y. (1963), *in* Advances in Enzymic Hydrolysis of Cellulose and Related Materials, Reese, E. T., Ed., New York, N. Y., MacMillan, p. 171.

36. Norman, A. G., and Fuller, W. H. (1942), *Advan. Enzym. 2*, 239.

37. Olson, F. R., Peterson, W. H., and Sherrard, E. C. (1937), *Ind. Eng. Chem. 29*, 1026.

38. Rautela, G. S., and King, K. W. (1968), *Arch. Biochem. Biophys. 123*, 589.

39. Reese, E. T. (1959) *in* Marine Boring and Fouling Organisms, Ray, D. L., Ed., Seattle, Washington, University of Washington, p. 265.

40. Russell, E. W., and Russell, E. J. (1961), Soil Conditions and Plant Growth, New York, N. Y., Longmans, Green, p. 240.

41. Schiff, J. A., Eisenstadt, J. M., and Klein, H. P. (1959), *J. Bacteriol. 78*, 124.

42. Scott, H. W., and Dehority, B. A. (1965), *J. Bacteriol. 89*, 1169.

43. Setlow, R. B., and Pollard, E. C. (1962), Molecular Biophysics, Reading, Mass., Addison-Wesley, p. 16.

44. Setlow, R. B., and Pollard, E. C. (1962), *ibid.*, p. 83.

45. Sistrom, W. R. (1962), Microbial Life, New York, N. Y., Holt, Rinehart, and Winston, p. 23.

46. Siu, R. G. H. (1951), Microbial Decomposition of Cellulose, New York, N. Y., Reinhold, p. 21.

47. Storvick, W. O., Cole, F. E., and King, K. W. (1963), *Biochemistry 2*, 1106.

48. Storvick, W. O., and King, K. W. (1960), *J. Biol. Chem. 235*, 303.

49. Whistler, R. L., and Smart, C. L. (1953), Polysaccharide Chemistry, New York, N. Y., Academic, p. 229.

50. Whitaker, D. R. (1956), *Can. J. Biochem. Physiol. 34*, 488.

51. Whitaker, D. R. (1957), *Can. J. Biochem. Physiol. 35*, 733.

52. Whitaker, D. R. (1963), *in* Advances in Enzymic Hydrolysis of Cellulose and Related Materials, Reese, E. T., Ed., New York, N. Y., MacMillan, p. 51.

53. Worthington Biochemical Corporation (1967), Glucostat, a prepared reagent. Freehold, N. J.