Isolation of Floc-Producing Bacteria from Activated Sludge[1]

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Although the activated sludge process for stabilizing liquid organic wastes is primarily a biological process, very little research has been conducted on the pure biological phases of this process. Until the work of Butterfield (1935), activated sludge was considered by some as a physio-chemical process and by others as a bio-chemical process. Butterfield isolated a bacterium, *Zoogloea ramigera*, from activated sludge which was capable of stabilizing a liquid organic substrate and of producing floc which was characteristic of activated sludge. Wattie (1942) and Heukelekian and Littman (1939) were also successful in isolating *Z. ramigera* from activated sludge. Although efforts were made to isolate other floc-producing bacteria, none were found. As a result of these studies, *Z. ramigera* was accepted as the predominant bacteria in activated sludge being responsible for both the stabilization of the organic matter and the production of floc.

Isolation of floc-producing bacteria other than *Z. ramigera* was made by McKinney and Horwood (1952). They isolated five additional floc-producing bacteria from activated sludge. These organisms were not only capable of floc production but also of stabilizing the organic matter. It is the purpose of this paper to report on further work in the isolation of floc-producing bacteria from activated sludge.

EXPERIMENTAL METHODS

Four sources of activated sludge were used in the isolation studies: San Antonio Activated Sludge, Austin Biosorption Sludge, Synthetic Activated Sludge and Celanese Activated Sludge. The San Antonio Activated Sludge was obtained from the San Antonio, Texas, Municipal Sewage Treatment Plant, and represented conventional activated sludge formed from sewage. Five isolation runs were made on separate samples of San Antonio Activated Sludge. The Austin Biosorption Sludge was representative of the new high-rate activated sludge processes and was obtained from the Austin, Texas, Municipal Sewage Treatment Plant. Only one sample of Biosorption Sludge was used. The Synthetic Activated Sludge was produced in the laboratory using a completely soluble organic substrate and a sewage seed. Nine different isolation runs were made on the Synthetic sludge. The Celanese Activated Sludge was produced from a biologically toxic industrial waste of the Celanese Corporation of America Plant at Bishop, Texas. The sludge was formed by carefully acclimating normal activated sludge to increasingly stronger concentrations of the waste over a long period. One isolation run was made on this sludge.

The isolation of bacteria from the various sludges was accomplished by the following process. One ml of the activated sludge was pipetted with a sterile pipette into 9 ml sterile dilution water in a 25 ml weighing bottle containing sterile glass beads. The sludge was dispersed by shaking the bottle vigorously for one minute. Serial dilutions were then made into sterile aeration units. The sterile aeration units were made from 150 ml bottles and contained 75 ml sterile substrate. The units were aerated aseptically by passing the air through 500 ml water and 2 ft of cotton. It was found that the units could be aerated in this manner for over a week without becoming contaminated. After 24-hr aeration streaked nutrient agar plates were made from the unit of highest dilution showing floc. The nutrient agar plates were incubated for 24 hr and then examined for growth. If sufficient growth had occurred, representative colonies were picked from the plates and were placed into culture tubes containing 10 ml of the nutrient substrate. If growth was not sufficient for isolation, incubation was continued for an additional 24 hr and then the isolations were made. The culture tubes were incubated at room temperature, approximately 25 C, for 24 hr and then examined microscopically for purity. If the culture appeared morphologically pure, nutrient agar slants were made. The slants were used as stocks for all further work and were maintained by storage in a cold room at 4 C and by monthly transfer to fresh slants.

The nutrient substrate in the aeration units and in the culture tubes varied with the source of sludge being used. A 1:9 dilution of nutrient broth was used with the sludges obtained from the San Antonio Sewage Plant and the Austin Sewage Plant. A 1:19 dilution of the waste from the Celanese Plant was used with the Celanese sludge. The synthetic substrates had a base of 230 ppm dextrose broth and 20 ppm potassium phosphate, monobasic.

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After the bacteria had been isolated, they were identified according to the procedures set forth by the Society of American Bacteriologists (1951). All identifications were based on the descriptions in *Bergey's Manual of Determinative Bacteriology* (1948).

TABLE 1. *Bacteria isolated from activated sludge*

SOURCE	SPECIES	NUMBER OF ISOLATES
San Antonio Activated Sludge	*Escherichia coli*	4
	Escherichia freundii	2
	Aerobacter aerogenes	2
	Klebsiella pneumoniae	1
	Pseudomonas segnis	1
	Pseudomonas turcosa	1
	Pseudomonas perlurida	1
	Pseudomonas tralucida	1
	Pseudomonas salopium	1
	Flavobacterium solare	1
	Flavobacterium breve	1
	Alcaligenes faecalis	4
	Achromobacter liquefaciens	1
	Zoogloea ramigera	3
	Bacillus cereus	1
	Bacillus megatherium	1
	Neisseria catarrahlis	2
	Lactobacillus casei	1
Austin Biosorption Sludge	*Pseudomonas pavonacea*	1
	Alcaligenes faecalis	2
	Bacterium methylicum	1
	Bacillus lentus	1
Synthetic Activated Sludge	*Escherichia freundii*	4
	Escherichia coli	1
	Pseudomonas ovalis	4
	Pseudomonas segnis	1
	Pseudomonas fragi	1
	Pseudomonas perlurida	1
	Pseudomonas solaniolens	1
	Alcaligenes faecalis	4
	Alcaligenes metalcaligenes	1
	Alcaligenes marshallii	1
	Achromobacter butyri	2
	Bacterium linens	4
	Micrococcus conglomeratus	1
	Micrococcus varians	1
	Zoogloea ramigera	1
Celanese Activated Sludge	*Paracolobactrum aerogenoides*	1
	Pseudomonas putida	1
	Pseudomonas segnis	2
	Alcaligenes faecalis	3
	Micrococcus conglomeratus	3

Once the bacteria had been identified, their ability to form floc was established. Rapid floc formation was determined by simulating the conditions of the activated sludge process in the small aeration units. Both sterile settled sewage and sterile synthetic sewage were used as substrates. The settled sewage was obtained from the effluent of the primary settling tanks at the San Antonio Sewage Plant during the afternoon, at which time the strongest sewage was passing from this unit. The sewage was filtered through coarse filter paper to remove any large non-settling particles. The synthetic sewage consisted of 230 ppm dextrose broth and 20 ppm potassium phosphate, monobasic, in distilled water. The synthetic sewage was completely soluble. Both substrates had an approximate 5 day BOD of 180 ppm. Each pure culture was aerated for 48 hr in the substrate and then was allowed to settle for one hr, at which time it was examined for floc formation.

Since the aeration procedure required extensive equipment, a study was made to determine whether it would be possible to demonstrate floc formation in standard bacteriological culture tubes. A 1:99 dilution of dextrose broth and 20 ppm potassium phosphate, monobasic, were used as substrate. Ten ml of media were placed in 125 mm culture tubes, sterilized and inoculated with the pure cultures of bacteria. Daily examinations for floc were made over a 21-day period.

TABLE 2. *Bacteria isolated from activated sludge capable of forming floc within 48-Hr aeration*

SOURCE	SPECIES	NUMBER OF ISOLATES
Sterile Sewage	*Escherichia coli*	1
	Escherichia freundii	1
	Pseudomonas perlurida	1
	Pseudomonas ovalis	1
	Alcaligenes faecalis	5
	Alcaligenes metalcaligenes	1
	Zoogloea ramigera	1
	Bacillus cereus	1
Synthetic Sewage	*Escherichia coli*	1
	Escherichia freundii	4
	Pseudomonas ovalis	1
	Pseudomonas segnis	1
	Pseudomonas solaniolens	1
	Pseudomonas fragi	1
	Alcaligenes faecalis	3
	Alcaligenes metalcaligenes	1
	Bacillus lentus	1

RESULTS

In all, 72 bacteria were isolated in the 16 isolation runs. Table 1 shows the source, identification and number of bacteria isolated from the various activated sludges. The number of bacteria isolated from a given sludge were not indicative of the total number of bacteria in that sludge or of the predominance of various species. Only one of each type of colony was isolated during a single isolation run, but the isolation of a given species of bacteria from the various sources of activated sludge as well as the repeated isolation from several samples of the same sludge tended to indicate the importance of that species to the activated sludge systems considered in this study.

Very few of the bacteria were capable of forming floc in either settled sewage or synthetic sewage within 48-hr aeration. Table 2 lists the number and species of the bacteria which formed floc under the conditions of normal activated sludge.

The use of media in standard culture tubes, unaerated, proved a simple means for determining floc of all of the bacteria under identical conditions and at the same time. The bacteria which formed floc in the aeration systems formed floc just as readily in the unaerated tubes. Incubation in the synthetic sewage for 17 days was sufficient for all of the bacteria to form floc; whereas, only 14 days' incubation was necessary for all of the bacteria to form floc in settled sewage.

Discussion

The isolation of a large number of different bacterial species common to water and soil substantiates the view that activated sludge is a heterogeneous bacterial system. But despite the large number of different bacterial species, it is doubtful whether the majority of the bacteria are important in stablizing sewage or the synthetic sewage used in this study. One of the chief characteristics of a successful activated sludge system is the ease with which floc is formed. Therefore, it is reasonable to believe that the floc-producing bacteria are the predominating organisms in activated sludge. The bacteria which formed floc so readily in the aeration units after 48-hr aeration are probably the important bacteria to the activated sludge systems studied.

Microscopic examinations were made to determine the time of formation of floc. Floc formation was correlated with the metabolic activities of the bacteria. The bacteria did not form floc as long as they were actively metabolizing organic matter and reproducing. It was only after the bacteria had ceased their metabolic activities that floc could be determined microscopically. The use of the substrates in culture tubes, unaerated, helped to show that if the bacteria were given sufficient time to metabolize the organic matter, they were all capable of forming floc.

Contrary to popular concepts, none of the bacteria isolated herein formed large masses of zoogloea or slime, under the conditions employed in these experiments. Floc was formed by the joining of several bacteria at their capsular interfaces. None of the organisms had thick capsules. As the floc grew, the thickness of the capsular material separating the cells appeared to remain relatively constant. But with aging some of the cells lost their identity as individual cells and the floc appeared to be composed of zoogloeal material. The use of sterile sewage accentuated the pseudo-zoogloeal appearance of the floc through the incorporation of colloidal particles.

The fact that floc formation is not a special property of any particular group of bacteria helps to explain the ease with which activated sludge floc is formed in various industrial wastes. The chemical nature of the wastes determines the bacterial predomination; whereas, floc formation is the resultant of complete metabolism of the organic matter in the wastes by the predominant bacteria.

Summary

Seventy-two bacteria were isolated from 16 different samples of activated sludge from 4 sources. Of these, 14 were capable of floc formation in sterile synthetic sewage, and 12 in sterile settled sewage. All cultures formed floc when incubated, unaerated, in these substrates for 17 days. Microscopic observations indicated that floc was the normal result of complete metabolism of the organic substrate and was not caused by special zoogloeal-producing bacteria.

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