

BIOCHEMICAL OXIDATION OF DAIRY WASTES

VI. Isolation and Study of Sludge Microorganisms *

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Microorganisms are important in the disposal of dairy and other wastes. Treatment methods utilize the activities of microbes, and conditions satisfactory for growth and reproduction must be considered in designing disposal units. For example, a simple treatment of dairy waste by rapid biochemical oxidation (13) was developed from laboratory aeration studies in which the activities of the sludge microorganisms were followed. The reduction in oxygen demand was shown to occur in four phases (14). First there was the removal or purification of soluble substances; concomitantly, there was storage of material in the sludge cells; then there occurred oxidative conversion of stored material to cell material; and finally, endogenous respiration digested the cell material.

Interest concerning the organisms that have the ability to rapidly reduce oxygen demand and to cause these changes in aerated sludge-milk mixtures led to the study presented herein. Investigations were specifically directed toward isolating and identifying the microorganisms most prevalent in an aerated dairy sludge that had been continually used in the laboratory. The original seed, obtained from an aerobic process at a local dairy, was propagated in the laboratory by daily simulated milk-waste feedings.

Although the biology of waste disposal systems has been studied, little is known about the organisms associated with the aerobic disposal of dairy

waste. Hotchkiss (9) made bacteriological studies of raw sewage and of effluents from the Imhoff tank, filter beds, and settling beds of a municipal sewage treatment system and classified the bacteria according to their physiological activity such as proteolytic, sulfur cycle, and nitrogen cycle forms. Gaub (6) enumerated and identified the bacteriological population of a sewage treatment plant and showed the predominance of intestinal forms. Studies of sewage purification conducted by Butterfield (3), Butterfield, Ruchhoff and McNamee (4), and Wattie (16) emphasized the importance of zooglea-forming bacteria in activated sludge. Five floc-producing bacteria were identified by McKinney and Horwood (12) in activated sludge. Calaway, Carroll and Long (5) encountered 14 species of heterotrophic bacteria in an intermittent sand filtration study. Species of *Flavobacterium* and *Bacillus* were present throughout the system. The latter were extremely active in the breakdown of carbohydrates and proteins.

The first systematic study of organisms isolated from artificial creamery wastes maintained both aerobically and anaerobically was made by Levine and Soppeland (10). Although this was not a biological survey of a specific waste disposal system, it was an early elucidation of the role of various species in the purification of milk wastes. Thirty-six species were described, all aerobic or facultative in oxygen requirements. In the absence of air, the growth of acid producers

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was favored, while in an abundance of air, proteolytic forms abounded. Levine and Watkins (11) subsequently studied organisms at different depths in a trickling filter receiving creamery wastes with respect to their abilities to utilize lactic acid. Although many species were strong acid producers or acid destroyers, many other forms present subsisted only on the end-products of lactic acid decomposition. A survey by British workers of organisms in a double-filtration sewage treatment plant indicated the presence of only a few isolated filaments of fungi in the activated sludge; however, 19 species of fungi and 7 species of yeast were found on the filtering medium. A detailed table describes 33 species of bacteria isolated in that study (1), but the organisms are not identified.

Isolations from Laboratory Aerator

Laboratory studies were conducted in an aerator which had been operating for about six months on daily charges of 18 l. of synthetic waste solutions containing 1,000 p.p.m. of dried skim milk. Treatment was effected by vigorous agitation of the sludge-milk mix at 30° C. with forced aeration of one volume of air change per volume of waste per minute. The effluent had been showing a chemical oxygen demand (C.O.D.) reduction of 50 per cent (8) and had a rapidly settling sludge with a very clear supernatant. Dilutions of the aerating mixture were made the third day after feeding stopped. One-milliliter amounts of dilutions to cover the 10⁻⁵ to 10⁻¹⁰ range were plated on a nutrient agar medium containing 0.1 per cent skim milk. After three days incubation at 30° C., all the colonies from the 10⁻⁸ dilution plates were counted, picked and inoculated into nutrient broth containing 0.1 per cent skim milk. Sixty-three bacterial isolations were made from two plates of this dilution giving a count of 3.2 × 10⁹ viable organisms per milliliter.

Each isolate was purified by the loop dilution method prior to initiating morphological and physiological studies. The techniques and methods described in the "Manual of Methods for Pure Culture Study of Bacteria" (15) were followed. Identifications were made by comparing these results with the properties listed in "Bergey's Manual of Determinative Bacteriology" (2).

Bacteria in Aerated Endogenous Sludge

Table I itemizes some characteristics of the bacteria isolated from the endogenous sludge at the 10⁻⁸ dilution. The physiological properties listed were chosen for their application to dairy waste decomposition studies. Other extensive identifying tests included observation of colony morphology, characteristic agar slant growth patterns, motility and spore formation; utilization of sucrose, glycerol, mannitol and xylose; liquefaction of gelatin; hydrolysis of starch; and production of hydrogen sulfide, color, indole and acetyl-methyl-carbinol. These results are not reported because of their relative unimportance once identifications have been made.

After the data in Table I were compiled and considered it was obvious that the isolated organisms could not be those primarily responsible for the removal and oxidation of solids from milk wastes. Of the 16 species found, only one specie grew on casein as evidenced by halo formation on milk agar plates. Yet casein is a major constituent of skim milk and, under vigorous aeration, is oxidized as rapidly as lactose (13). The three organisms that produce an alkaline reaction in milk probably produce ammoniacal substances by breakdown of proteins other than casein. The few that produce acidity in milk also produce acid from lactose. However, such acid production is not observed or is masked during oxidation by aerated sludge. It is interesting to note that the bacteria

that use lactose can also use glucose. On the other hand, all glucose utilizers cannot assimilate lactose. Information on starch hydrolysis is also presented as this carbohydrate may find its way into some dairy wastes. Various bacilli have this ability. The oxygen requirements of the isolates are as expected. All but three organisms are highly aerobic; two are able to grow with less oxygen and are called microaerophiles; one, able to grow either in the presence or absence of oxygen, is a facultative anaerobe.

It appeared possible that the conditions of growth of the aerated endogenous sludge favored only those organisms that were isolated at this high dilution. At lower dilutions, many other organisms may have been found, and, possibly, their growth could be favored by the conditions that exist during rapid assimilation of milk.

Bacteria in Actively Assimilating Aerated Sludge

In preparation for the isolation of bacteria from the actively assimilating milk-sludge mix, the endogenous sludge was propagated by daily additions of skim milk solution. In the interval of time required for the identifications, other types of experiments were conducted, but temperature, aeration and agitation remained essentially the same. Several weeks later studies of the nature of this bacterial phase were begun. A sample of sludge was removed about 4 hr. after a one-dose feeding of a one-tenth per cent dried skim milk solution. It was at this point that maximum purification had been observed in previous studies (7). Plate isolations were made on skim milk nutrient agar followed by the extensive tests necessary for identifica-

TABLE I.—Identification and Properties of Bacteria Isolated from Aerated Endogenous Sludge

Genus and Species	Gram Stain	Oxygen Requirements	Action on Litmus Milk	Growth on Casein Agar	Acid Production		Starch Hydrolysis
					Lactose Broth	Glucose Broth	
<i>Achromobacter liquefaciens</i>	—	Aerobic	None	—	—	+	+
<i>Alcaligenes faecalis</i>	—	Aerobic	Alkaline	—	—	—	—
<i>faecalis</i> var. <i>mariense</i>	—	Aerobic	None	—	—	+	—
<i>viscosus</i> var. <i>dissimilis</i>	—	Aerobic	None	—	—	—	—
<i>Bacillus circulans</i>	+	Aerobic	Acid, reduction	—	—	+	+
<i>firmus</i>	+	Aerobic	Acid, reduction, peptonization	+	+	+	+
<i>lentus</i>	+	Aerobic	Acid	—	+	+	+
<i>rubricus</i>	+	Microaerophilic	Alkaline	—	—	—	+
<i>Flavobacterium invisible</i>	—	Aerobic	None	—	—	+	—
<i>Micrococcus candidus</i>	+	Aerobic	None	—	+	+	—
<i>cinnabareus</i>	+	Microaerophilic	Alkaline	—	—	+	—
<i>flavus</i>	+	Aerobic	Acid	—	+	+	—
<i>pyogenes</i> var. <i>albus</i>	+	Facultative	Acid, coagulation	—	+	+	—
<i>varians</i>	+	Aerobic	Acid	—	+	+	—
<i>Pseudomonas eisenbergii</i>	—	Aerobic	None	—	—	+	—
<i>oleovorans</i>	—	Aerobic	None	—	—	+	+

TABLE II.—Identification and Properties of Bacteria Isolated from Actively Assimilating Sludge

Genus and Species	Gram Stain	Oxygen Requirements	Action on Litmus Milk	Growth on Casein Agar	Acid Production		Starch Hydrolysis
					Lactose Broth	Glucose Broth	
<i>Bacillus brevis</i>	+	Aerobic	Alk.; pept.	+	-	+	+
<i>Bacillus cereus</i>	+	Microaerophilic	Alk.; pept.	+	-	+	+
<i>Bacillus firmus</i>	+	Aerobic	Alk.; pept.	+	+	+	+
<i>Bacillus laterosporus</i>	+	Facultative	Alk.; pept.	Weak	-	+	-
<i>Bacillus lentus</i>	+	Aerobic	Slightly acid	-	+	+	+
<i>Bacillus pasteurii</i>	+	Aerobic	Slightly alk.	-	-	-	-
<i>Bacterium heali</i>	+	Microaerophilic	Acid, alk.; pept.	+	-	+	-
<i>Bacterium linens</i>	+	Aerobic	Alk.; pept.	+	-	-	+
<i>Corynebacterium bovis</i>	+	Aerobic	Slightly alk.	Slow	-	-	-
<i>Flavobacterium aquatile</i>	-	Facultative	Slightly alk.	-	-	-	-
<i>Flavobacterium breve</i>	-	Aerobic	Slightly alk.	-	-	-	-
<i>Flavobacterium suaveolens</i>	-	Aerobic	Alk.; pept.	+	-	-	+
<i>Microbacterium liquefaciens</i>	+	Aerobic	Alk.; pept.	+	-	-	-
<i>Micrococcus aurantiacus</i>	+	Facultative	Slightly acid	-	+	+	-
<i>Pseudomonas aeruginosa</i>	-	Aerobic	Alk.; pept.	+	-	+	-

tion. Fifty-two isolations were obtained from two plates giving a count of 2.6×10^8 viable organisms per milliliter.

Table II summarizes pertinent information. All of the 16 species into which the 52 isolates were classified acted on milk. The majority of these species grew on casein, but only three produced acid from lactose. *Bacillus firmus* was the only organism present to utilize both casein and lactose. Most of the starch digesters were found in the genus *Bacillus*. Of those organisms isolated all were rods except for a single micrococcus. A complete change in the flora occurred from that found in the endogenous study reported in Table I.

Discussion

It must be emphasized that these studies, though made on the same sludge, were not made on consecutive days. An interval of several weeks separated the first study from the sec-

ond, a period of time during which the changes obviously were taking place gradually. Furthermore, since sterile conditions were not maintained, the change in population may have resulted from contamination. Yet, there are points of interest.

Contrary to expectations, the total number of organisms found during assimilation was somewhat less than the number found in the endogenous phase (2.6×10^8 for the former as compared to 3.2×10^8 for the latter). However, as shown in Table III, there was a change in the type of organisms. Of the total bacteria counted during the assimilative phase, 74 per cent were members of the genera *Bacillus* and *Bacterium*. These genera accounted for only 8 per cent in the endogenous period. It may be speculated that these are the organisms responsible for the high purification rate and the ability of the sludge to remove and store oxygen-demanding substances (14). *Bacterium linens* alone accounted for

TABLE III.—Bacteria in Aerated Skim Milk Sludges

Genera	Endogenous (%)	Assimilative (%)
<i>Achromobacter</i>	2	—
<i>Alcaligenes</i>	26	—
<i>Bacillus</i>	8	31
<i>Bacterium</i>	—	43
<i>Corynebacterium</i>	—	6
<i>Flavobacterium</i>	34	8
<i>Microbacterium</i>	—	6
<i>Micrococcus</i>	14	2
<i>Pseudomonas</i>	16	4

40 per cent of the total number. *Bacillus cereus* was also present in large numbers. Only three organisms appear to have the ability of breaking down lactose, while the others utilize the breakdown products of lactose, glucose and galactose.

During the endogenous phase, other organisms seem to predominate. Of the organisms isolated, 42 per cent were *Alcaligenes* and *Pseudomonas*, most of which are strongly proteolytic; 48 per cent were saccharolytic, either *Flavobacterium* or *Micrococcus*.

The only organisms found common to both phases of this laboratory waste treatment were *Bacillus firmus* and *Bacillus lentus*. Both these aerobes

actively ferment milk and lactose. *Flavobacterium* and *Pseudomonas* species were represented in both phases but not by the same species.

Fungi and Yeasts

Fungi and yeasts were not present on the agar plates from the assimilative phase at the high dilutions used. On the other hand 9 fungi and yeasts were isolated from the endogenous phase. Since no attempt was made to prevent contamination of the fermentor, it may be possible that these are chance contaminants. Six of the isolated organisms were the same fungus, two were red-pigmented yeasts and one was a white yeast.

Protozoa and Other Living Organisms

Protozoa are generally assumed to be important members of waste disposal biota. The nonbacterial forms existing in dairy waste sludge cultivated under laboratory conditions of vigorous agitation and aeration were examined. This sludge had been fed a skim milk solution daily for about a week. A 200-ml. sample (No. 1) was removed prior to feeding; 4 hr. later another sample was taken (No. 2); 24 hr. after feeding the third sample was

TABLE IV.—Biota in Laboratory Aerated Dairy Waste Sludge

Organisms	Size (microns)	Sample Number	Total Count (per 0.1 ml.)
Blue-green algae (<i>Myxophyceae</i>)			
<i>Chroococcales</i>			
<i>Gloeocapsa</i>	3.5 to 7	1, 2, 3	13
<i>Microcystis</i>	to 60	1, 2, 3	15
<i>Hormogonales</i>			
<i>Phormidium</i>	30 (10 cells)	1, 2, 3	1 Filament
Diatom (<i>Bacillariaceae</i>)			
<i>Pennales</i>			
<i>Cymbellaceae</i>	18	3	1
Protozoan (<i>Ciliata</i>)			
<i>Spirotricha</i>	24 × 26	1	2
Nematode (<i>Aschelminthes</i>)			
<i>Nematoda</i>	432 × 24	1	1
	360 × 24	3	1
Rotifera			
<i>Lecane</i>	150 to 180	1	188
		2	174
		3	174
Unidentified (2 species)	—	—	Sparse

taken (No. 3). Each 200-ml. sample was preserved by adding 10 ml. of commercial formalin.

Table IV gives a compilation of the results obtained. There were no marked qualitative differences in the populations composing each sample. All samples were characterized by the same predominant forms. The blue-green algae were equally distributed in each of the three samples. Likewise, a rotifer of the genus *Lecane* was found in practically equal numbers in all samples and was the most predominant of the species detected. Protozoa were present in limited numbers in sample No. 1, but were not observed in the other samples. Formalin may have destroyed the structure of other protozoa present but undetected. A diatom and a nematode were also reported.

Apparently, the highly aerobic environment of the laboratory aerator simulated the healthy conditions of clean streams. With the exception of the blue-green algae which can thrive under conditions of pollution, most of the organisms found were those usually associated with a clean stream. In fact, the presence of 50 per cent or more of the forms not tolerant to pollution indicates a clean stream (17). Thus, a properly aerated tank precludes the growth of biota associated with pollution.

Summary

Investigations into the microbial nature of laboratory-aerated dairy waste sludge resulted in several interesting observations. A quantitative comparison of the numbers of bacteria present in a well aerated three-day endogenous sludge *versus* a thriving, recently-fed sludge actually showed more bacteria present under starving conditions. Expectations were for a greater population under the ideal growth and multiplication conditions offered within the first 4 hr. after feeding.

Qualitatively, a complete change in

the bacterial flora was observed in the same sludges. At the dilution studied, endogenous material yielded primarily those forms associated with air, water and sewage bacteriological studies. There were no evidences of the typical water pollutants, *E. coli* or *A. aerogenes*, but members of the genera *Alcaligenes*, *Flavobacterium*, *Pseudomonas* and *Micrococcus* were present.

Analysis of the actively assimilating sludge organisms gave a totally different picture. Members of the genera *Bacillus* and *Bacterium* predominated, and many of the species were those natural to dairy product habitats. The radical differences in bacterial flora of the two samples, one taken at the peak of nourishment and the other long after the onset of starvation, seem to constitute a study in extremes. Samples taken at more frequent intervals would probably have shown that the bacterial flora changes gradually. As suitable conditions and substrate become available, dormant species flourish and become predominant.

Microscopic studies of the nonbacterial biota were made on three samples of the laboratory sludge. There were negligible differences in the total counts of samples taken at 0, 4 and 24 hr. after feeding. Protozoa deemed essential in aerated sludges as devourers of excess bacteria were not predominantly evident. On the other hand, a multicellular animal, the rotifer *Lecane*, was the single specie found most numerous.

The great majority of highly aerobic bacterial and other biota forms detected as related to the comparatively few algal and protozoal forms was a good indication of the desirable conditions of aeration existing in the laboratory dairy waste disposal system examined.

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