Aerobic Heterotrophic Bacterial Populations of Sewage and Activated Sludge

I. Enumeration

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Agar plating media containing solely activated sludge extracts yielded, in general, higher viable counts of activated sludge bacteria than any other culture medium tested. Activated sludge extracts made from different treatment plants varied in efficacy in evoking maximal viable counts. Frequently, homologous plating, i.e., plating inocula of activated sludges on extracts made from the same activated sludges, tended to yield lower counts than the heterologous platings tried in this investigation. The counts obtained by homologous plating of activated sludge were not significantly lower and sometimes were even significantly higher than the counts obtained on standard Nutrient Agar, which had been found by previous workers to be a good medium for counting activated sludge bacteria. The higher counts obtained with activated sludge extracts set objectives for formulating reproducible or defined culture media for the enumeration of activated sludge bacteria.

Although it is accepted that microorganisms are responsible for a large share of the effectiveness of biological treatment of waste waters, studies of the bacteria involved in the normal functioning of the processes are relatively few in number. There are several extensive studies of the flora of sewage treatment plants (1-7, 11, 13, 14, 15, 16, 21, 22), but, because of the differences in the systems studied and techniques employed and the obvious complexity of the flora and of its chemical transformations, there is still much more to be learned.

Most of the attention has been directed toward the aerobic heterotrophs, since this group seems most likely to be of the greatest importance in the degradation and final stabilization of organic matter. One of the major problems, however, in the study of this group has been how to enumerate total and estimate relative numbers of the different physiological or taxonomic types included within it. It is impossible to cultivate many of the bacteria on presently known culture media, and it is seemingly impossible to formulate a suitable culture medium to support all the heterotrophs that might be expected to be present in waste under treatment. It is possible, however, to evaluate and then to improve culture media to bring about greater productivity of total numbers and greater heterogeneity of bacterial types.

In a study of the microorganisms of the soil, Lochhead and Taylor (19) recognized that, to isolate a wide variety of organisms, the medium must be as nonselective as possible. Because media with added energy sources were considered more selective, these workers used as the plating medium a soil extract without added energy sources. Foot and Taylor (10) found that counts of the bacteria in freshwater were higher when obtained on a poor rather than on a nutritionally rich medium. Hayes and Anthony (12) reported that extract media made from sediments of productive lakes were adequate to produce colony growth, but that supplemented sodium caseinate agar produced colonies larger than extracts made from sediments of unproductive lakes. Skerman (24) developed a relatively nonselective medium for the cultivation of marine organisms by enriching seawater extracts of marine muds with phosphate and iron.

In the waste treatment field, apparently fewer efforts have been made to isolate bacteria on a nonselective basis. Because of its high numerical productivity, Allen (1) recommended the use of Nutrient Agar in studying the bacteriology of activated sludge. In studying the bacteriology of an activated sludge developed on a milk waste, Jasewicz and Porges (17) isolated the micro-
organisms on a medium containing Nutrient Agar and skim milk. Van Gils (28) obtained higher counts by plating untreated activated sludge on tryptone-glucose medium rather than on sewage agar. In contrast, Dias (5) considered sewage agar to be the most satisfactory nonselective medium for the cultivation of the dominant bacteria in activated sludge.

This paper describes an approach similar to that of Lochhead and his associates (18, 19) for the cultivation and enumeration of a variety of aerobic, heterotrophic bacteria of activated sludge on a nonselective basis. Comparative evaluations of the productivity of various culture media of known composition as well as of those developed in our laboratory were made, by use of inocula of activated sludge and sewage from different treatment plants.

Activated sludge extract agar gave consistently higher productivity. For routine field work, activated sludge extracts leave much to be desired; however, the counts on activated sludge extract set objectives for formulating more reproducible and more defined culture media for the cultivation of bacteria found in waters and in biological waste treatment processes.

**MATERIALS AND METHODS**

**Samples and collection.** Most of the samples of settled sewage and of activated sludge (mixed liquor from the aeration tank) were obtained from the Marlboro, N.J., State Hospital treatment plant. Other such samples were collected from Bernardsville and Morristown, N.J., treatment plants for purposes of comparison and to minimize bias in sampling. The Marlboro plant treats sewage which is predominantly domestic in nature, except for one discharge in spring of cattle fodder silo liquor. The plant in Bernardsville receives mainly domestic sewage from a residential community, and the Morristown plant treats domestic sewage as well as some industrial waste.

**Culture media.** Unless otherwise indicated, all media were prepared with distilled water, sterilized at 121°C for 15 min, and solidified with Ionagar No. 2 (0.85%, w/v; Colab Laboratories, Inc., Chicago Heights, Ill.).

*Activated sludge extract (ASE), single strength.* One liter of mixed liquor collected from the effluent end of the aeration tank was autoclaved at 121°C for 30 min. The suspension was then filtered through glass wool and the sediment was discarded. The filtrate was made up to 1 liter with distilled water. Agar was added to the filtrate and autoclaved again for 30 min.

*ASE, double strength.* After settling 2 liters of mixed liquor for 30 min and discarding 1 liter of the supernatant fluid, the remainder of the supernatant fluid was treated as was ASE (single strength).

*Soil extract.* Soil extract was prepared according to Löhnis (20).

*ASE plus soil extract.* ASE (single strength) was prepared as above and mixed with 25% (v/v) soil extract. The mixture was autoclaved after addition of agar at 121°C for 30 min.

*Sewage agar.* One liter of settled sewage was filtered through glass wool and sterilized for 30 min at 121°C with the agar.

*Iron-peptone-agar.* Iron-peptone-agar was prepared according to Ferrer, Stapert, and Sokolski (9).

*Basal medium.* Basal medium and basal medium plus yeast extract were prepared according to Lochhead and Chase (18).

*Taylor's medium.* A medium containing soil extract was prepared according to Taylor (27). Modified Taylor's medium was prepared as above except that 250 ml of ASE (single strength) was added instead of soil extract.

*Casitone-yeast autolyse medium.* This medium consisted of 5 g of Casitone, 1.5 g of yeast autolyzate, and 1,000 ml of water; pH 7.2.

*Nutrient Agar.* Nutrient agar was made up from dehydrated Nutrient Agar (Difco).

*Plating method.* Homogenized samples of sewage and activated sludge were serially diluted in distilled water and plated on various media by spreading 0.1 ml of the diluted suspension on the surface of the agar with bent glass rods.

**Statistical treatment of results.** In all plating runs, the mean plate counts were derived from the counts of a minimum of four replicates. Since the bacterial counts followed the Poisson distribution, the plate count data were first subjected to the square root transformation so that procedures designed for normally distributed populations could be used. This transformation involved taking the positive square root of each plate count (x). The means of plate counts reported in the results of plating experiments were obtained by squaring the arithmetic mean of all square roots of x, not by averaging the raw plate counts as such. Analysis of variance (8) was calculated on the square-root transformed data. The least significant difference (LSD; 25) derived from the transformed data was used to determine which differences between means were significant, and to provide a basis for grouping those means which cannot be shown to be significantly different. In the tables, these similar means are grouped by brackets or ranking.

**RESULTS**

Viable counts of settled sewages and of activated sludges from the three different treatment plants were compared on several agar culture media. Nutrient Agar was used in all plating experiments to provide common elements for comparison between different experiments. Other culture media were used when it seemed desirable as the testing progressed. Since the plating experiments were conducted on different days with different samples, the results obtained in the various experiments were presented and analyzed statistically under separate headings to take into account the effects of day-to-day variations in
Table 1. Plating experiment I, effectiveness of culture media for supporting high viable counts of activated sludge (Marlboro)

<table>
<thead>
<tr>
<th>Mediuma</th>
<th>Medium no.</th>
<th>Mean count (10^6/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Agar</td>
<td>8</td>
<td>43.3</td>
</tr>
<tr>
<td>Basal medium</td>
<td>6</td>
<td>46.7</td>
</tr>
<tr>
<td>Basal medium + yeast extract</td>
<td>7</td>
<td>61.9</td>
</tr>
<tr>
<td>ASE + soil extract</td>
<td>2</td>
<td>68.4</td>
</tr>
<tr>
<td>Sewage agar</td>
<td>1</td>
<td>78.3</td>
</tr>
<tr>
<td>Taylor's medium</td>
<td>5</td>
<td>139.2</td>
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<tr>
<td>ASE (single strength)</td>
<td>3</td>
<td>152.5</td>
</tr>
<tr>
<td>ASE (double strength)</td>
<td>4</td>
<td>175.6</td>
</tr>
</tbody>
</table>

a Sewage agar and activated sludge media were made from sewage and activated sludge of Marlboro treatment plant.

Homologous ASE and other media, and the results (Table 3) indicate that ASE (single strength) had higher counts than other media; ASE (double strength), however, gave a lower value.

The suppression of total counts on these double-strength plates resulted from antagonistic effects of several large colonies of bacteria that suppressed the growth of other colonies. These antagonistic colonies did not attain sufficiently large size on single-strength medium to exert suppressive effects. These observations, though not encountered in several other plating experiments, indicate that the concentration of nutrients in the activated sludge extract itself can be critical, the higher concentrations not necessarily promoting higher counts.

Plating experiment IV. To test whether the sewage or the activated sludge of a particular plant was a better substrate for making viable counts, agars were prepared from the sewage and the ASE of the Bernardsville plant. Two sets of homologous plating experiments were conducted with inocula of sewage and activated sludge from the Bernardsville, N.J., treatment plant. Table 4 gives the results and LSD ranks in order of productivity.

Analysis of the data indicated that the mean counts on several different media were signifi-
TABLE 4. Plating experiment IV, comparison of sewage and activated sludge extract and other culture media as substrates for viable counts of bacteria of sewage and activated sludge

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Sewage</th>
<th>Activated sludge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium no.</td>
<td>Rank b</td>
<td>Mean count (10^6/ml)</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>3.3</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>4.3</td>
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<tr>
<td>5</td>
<td>2</td>
<td>5.9</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>11.0</td>
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<td>2</td>
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<td>4</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>12.2</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>13.2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>13.5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>14.7</td>
</tr>
</tbody>
</table>

1 = Sewage agar (Bernardsville); 2 = soil extract; 3 = ASE (Bernardsville); 4 = Taylor’s medium; 5 = Nutrient Agar.

* Rank 1 = highest productivity.

Significantly different. Sewage was not a particularly good substrate for sewage bacteria or activated sludge organisms. In any case, there seemed to be no value over Nutrient Agar. The Taylor’s medium yielded higher viable counts than the ASE for activated sludge. Although this was true in this experiment, our experience indicated that ASE agar gave appreciably and consistently better results than Taylor’s medium in many other experimental runs.

Nevertheless, because the ASE agar from Bernardsville seemed to give different results from that of Marlboro, a series of heterologous plating studies (experiments V, VI, and VII) were undertaken to see how the extracts compared with one another in ability to support high viable counts. An extract not particularly rich in growth factors could conceivably support a high count on homologous plating, whereas it would then be expected to support smaller numbers from a more heterogeneous inoculum from another plant.

**Plating experiment V.** Bernardsville activated sludge was plated on Marlboro ASE agar as well as on other media. Results are summarized in Table 5.

This heterologous plating of Bernardsville activated sludge on Marlboro ASE yielded a significantly higher count. The increase could have been due to: (i) Marlboro sludge containing nutrients absent from other substrates, (ii) Bernardsville sludge extract containing inhibitors not present in Marlboro sludge extract, or (iii) other unrecognized effects that resulted from differences in received waste or in plant operation.

**Plating experiment VI.** Results of homologous and heterologous plating of sewage and activated sludge from the Morristown plant are shown in Table 6, in which Marlboro ASE medium is shown to be superior in productivity to other plating media.

**Plating experiment VII.** An expanded experiment in heterologous plating involved the activated sludges and sewages of three treatment plants. Nutrient Agar was retained for comparison with other experiments, and homologous platings were also performed. In this experiment, however, the Taylor’s medium was modified by substituting ASE for soil extract. The modification was prompted by the possibility that a combination of ASE with the other nutrients would elevate the count. To avoid bias, samples of sewage and activated sludge were subjected to homologous as well as heterologous plating on the same day (each sample was plated on all the media under consideration). In plating sewage, sewage agar was used in addition to other media, but, in plating of activated sludge, sewage agar...
was omitted since it had not previously given higher counts. All samples of sewage and activated sludge were collected from the three treatment plants on the same day, and plates of ASE as well as of sewage agar were made from these samples. Small quantities of each of the sewages and activated sludges were saved for plating. Plating of activated sludges and sewages was done on the day of sample collection to avoid effects incidental to storage. The degree and order of productivity of the various media as determined by LSD are indicated for sewage in Table 7 and for activated sludge in Table 8.

As in other experiments, Nutrient Agar gave consistently higher counts for sewage. Other plating media gave counts of similar value to those obtained with Nutrient Agar for a particular sewage sample, but they did not rank in the same order of productivity as with other sewages previously tested. Supplementation of Taylor's medium with Bernardsville activated sludge extract did not improve the productivity ranking above that of Nutrient Agar.

In these plating studies of activated sludge, ASE agars ranked as the most productive media. Contrary to our previous experimental results, Marlboro ASE medium did not rank as the most productive. Only homologous plating of Bernardsville activated sludge gave maximal counts, whereas plating of Morristown sludges yielded maximal counts on heterologous ASE agars.

**Table 8. Plating experiment VIII, summary of ranking of viable plate count media in order of effectiveness for activated sludge by least significant difference**

<table>
<thead>
<tr>
<th>Medium no.</th>
<th>Mean count (10^6/ml)</th>
<th>Medium no.</th>
<th>Mean count (10^6/ml)</th>
<th>Medium no.</th>
<th>Mean count (10^6/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>23.0</td>
<td>1</td>
<td>108.2</td>
<td>4</td>
<td>196</td>
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<td>2</td>
<td>29.2</td>
<td>2</td>
<td>130</td>
<td>3</td>
<td>265.7</td>
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<tr>
<td>4</td>
<td>31.4</td>
<td>7</td>
<td>216.1</td>
<td>6</td>
<td>309.8</td>
</tr>
<tr>
<td>6</td>
<td>37.2</td>
<td>3</td>
<td>231</td>
<td>7</td>
<td>331.2</td>
</tr>
<tr>
<td>1</td>
<td>39.7</td>
<td>5</td>
<td>231</td>
<td>5</td>
<td>309.8</td>
</tr>
<tr>
<td>7</td>
<td>43.6</td>
<td>6</td>
<td>243.4</td>
<td>1</td>
<td>331.2</td>
</tr>
<tr>
<td>3</td>
<td>114.5</td>
<td>2</td>
<td>338.6</td>
<td>2</td>
<td>610</td>
</tr>
</tbody>
</table>

a See footnote "a," Table 7.

b Media of equal effectiveness are grouped together by brackets.
seems possible that the mode of operation of the plant may have influenced the composition of the activated sludge, thus affecting the productivity of the extract medium.

The sewage agars also varied in efficacy, but in general sewage agar was a poor medium for cultivation of aerobic heterotrophs of activated sludge. Although some growth factors are present in sewage, it appears that metabolic activities of activated sludge bacteria produce substances conducive to elevating plate counts above those attainable with sewage.

With respect to culture media necessary for maximizing counts, the counts of each sewage and activated sludge were found to be distinctly different entities. Nutrient Agar was a highly productive medium for the various sewages, although counts from heterologous plating of these sewages on some extract media were not significantly different from those from Nutrient Agar.

It has been recognized for some time that the assortment of bacteria in sewage, which consists of the transient organisms arising from a variety of sources, such as kitchen wastes, soil, fecal matter, and those shed from special habitats of the sewer lines, are quite different from those of activated sludge flocs. Many of the sewage bacteria are there by chance and do not survive long, whereas those of activated sludge flocs have developed as the result of suitable conditions of environment. It thus seems logical that the nutrient media required by the two groups should differ in efficacy.

In the plating experiments, the soil extract medium was a poor substrate for obtaining high numbers of activated sludge bacteria, indicating that the factors contained in a soil extract medium were dissimilar to those of the most productive ASE and were inadequate for supporting the growth of many activated sludge bacteria. It suggests, further, that the flora of activated sludges tested was different from that of soil. A modification of Taylor's soil counting medium used in this study did not increase the productivity to a great degree. Taylor's and modified Taylor's medium yielded larger colonies than activated sludge extract media. A similar observation was made by Stevenson and Rouatt (26) when soil bacteria were plated on original Taylor's medium. In this connection, we also found that low levels of nutrients producing very small colonies were associated with higher counts. The finding that Nutrient Agar was one of the best media for the growth of sewage bacteria supported the view of Prescott, Winslow, and McCrady (23) that meat-peptone media are excellent for the quantitative counting of sewage bacteria in polluted water (under the proper conditions of incubation). On the other hand, Nutrient Agar is not the best for activated sludge bacteria in general. Thus, in waters receiving effluents from activated sludge plants, other media are necessary for the consistent cultivation of bacteria of activated sludge origin.

ACKNOWLEDGMENT

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LITERATURE CITED

BACTERIA IN SEWAGE AND ACTIVATED SLUDGE


