Improved Enumeration of *Streptomyces* spp. on a Starch Casein Salt Medium

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Well-formed *Streptomyces* colonies were counted more rapidly when a starch casein medium containing antibiotics was supplemented with either magnesium chloride or additional sodium chloride.

Undesirable tastes and odors in drinking water are often caused by the production of odorous metabolites (such as geosmin or 2-methylisoborneol), by blue-green algae, or by *Streptomyces* spp. (2, 7, 8). Since we received complaints from consumers supplied by the Metropolitan Water, Sewerage and Drainage Board, we decided to monitor the presence of *Streptomyces* spp. in the water held in our reservoirs. This led to the development of an improved method for the enumeration of *Streptomyces*.

**MATERIALS AND METHODS**

Eight enumeration media were initially tested: (i) glucose-yeast extract medium (1); (ii) Benedict agar (Porter, 6); (iii) chitin agar (Lingappa and Lockwood, 10); (iv) chitin agar (metropolitan water Board, London; 4); (v) arginine glycerol medium (El Nakeeb and Lechevalier; 10); (vi) Küster and Williams agar (10); (vii) rose bengal malt extract medium (5); and (viii) starch casein agar (3, 11).

Results obtained in triplicate using polluted river water indicated complete overgrowth by fungi and bacteria on all media except those containing chitin and starch casein. The more satisfactory chitin medium was that of Lingappa and Lockwood (10), but colonies were indistinct and small. Agate and Bhat (1) stated that the moisture on the freshly poured glucose-yeast extract plate appeared to be the primary factor concerned in the excessive spreading of both molds and bacteria and general overcrowding over the slowly growing actinomycetes. Use of a stereoscopic microscope for counting colonies on chitin agar was recommended (10), and for other media (9) plates could be dried overnight at 37°C. Both of these techniques can be eliminated by adopting the approach given below.

Effect of salt on the colony count. The starch casein medium (11) was the most promising medium, so further work was undertaken to reduce the growth of rapidly growing gram-negative bacteria by adding sodium chloride or magnesium chloride to the medium. The rationale behind this approach was that actinomycetes are known to be comparatively salt tolerant (9). Thus, a second series of tests was run in a more precise manner. The media tested were: (i) glucose-yeast extract medium (1); (ii) arginine glycerol medium (10); (iii) Küster and Williams medium (10); (iv) rose bengal malt extract medium (5); and (v) starch casein agar (3, 11). Different concentrations of sodium chloride were tested in media (i) to (v), whereas magnesium chloride was only tested in medium (v).

Two series were run, the variant being the concentration of penicillin G (see tables 1 and 2). Unchlorinated polluted water from the Lane Cove River, which was low in salt (about 36 mg of chloride per liter), was used for the tests. Each test was performed in triplicate. Water was collected in a large plastic 20-liter container. It was poured into a plastic can, mixed well, and poured into 500-ml stoppered glass bottles. These were heated for 2 h at 44°C to decrease the number of non-sporeforming bacteria, and then 50 ml was filtered under suction through a sterile 5-cm-diameter membrane filter of 0.45-µm pore size (Oxoid). The membrane was placed face upwards on the medium in a petri dish and incubated for 10 days at 28 ± 0.1°C. The number of colonies was determined by using a hand counter.

**RESULTS**

Glucose-yeast extract medium. The actinomycetes were difficult to count since they were pinpoints, were not discrete entities, or had vague lacey outlines on the ever present yellow, red, and white bacterial background. Generally, the number of colonies increased with increasing salt content, but the counting error could well be 5 to 10%.

Arginine glycerol medium. The overgrowth problem was not solved using arginine glycerol medium.

Küster and Williams medium. At lower concentrations of additive, *Streptomyces* colonies tended to be on the rim of the bacterial growth. As the salt concentration increased, colonies became partially exposed but were atypical, lumpy, and distorted. They appeared as aggregates of grey and white colonies, while the bacteria decreased with an increase in additive.
Table 1. Effect of increasing additive on the number of streptomyces colonies developing on six enumeration media in the presence of penicillin G (1 μg/ml)

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>GYE (NaCl)</th>
<th>AG (NaCl)</th>
<th>KW (NaCl)</th>
<th>RBME (NaCl)</th>
<th>SC (NaCl)</th>
<th>SC (NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
</tr>
<tr>
<td>2.00</td>
<td>30 (43)</td>
<td>256</td>
<td>45</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>47 (57)</td>
<td>160 (187)</td>
<td>102 (76)</td>
<td>81 (24)</td>
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</tr>
<tr>
<td>2.64</td>
<td>87</td>
<td>214</td>
<td>739</td>
<td>20</td>
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</tr>
<tr>
<td></td>
<td>85 (73)</td>
<td>303 (270)</td>
<td>518 (591)</td>
<td>120 (54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.32</td>
<td>176</td>
<td>432</td>
<td>983</td>
<td>768</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>178 (202)</td>
<td>633 (583)</td>
<td>989 (939)</td>
<td>274 (507)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.00</td>
<td>227</td>
<td>463</td>
<td>1,222</td>
<td>710</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>211 (210)</td>
<td>700 (513)</td>
<td>919 (1,038)</td>
<td>406 (589)</td>
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<tr>
<td>4.60</td>
<td>230</td>
<td>766</td>
<td>1,019</td>
<td>386</td>
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<tr>
<td></td>
<td>114 (215)</td>
<td>606 (721)</td>
<td>962 (1,003)</td>
<td>371 (255)</td>
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<tr>
<td>3.01</td>
<td>131</td>
<td>792</td>
<td>1,029</td>
<td>767</td>
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</tr>
</tbody>
</table>

* Additive is given in parentheses. GYE, Glucose-yeast extract; AG, arginine glycerol; KW, Küster and Williams medium; RBME, rose bengal malt extract; SC, starch casein.
* OG, Overgrown.
* Numbers in parentheses are mean number of colonies/plate.
* ND, Not determined.

Table 2. Effect of increasing additive on the number of streptomyces colonies developing on six enumeration media in the presence of penicillin G (3 μg/ml)

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>GYE (NaCl)</th>
<th>AG (NaCl)</th>
<th>KW (NaCl)</th>
<th>RBME (NaCl)</th>
<th>SC (NaCl)</th>
<th>SC (NaCl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
<td>OG</td>
</tr>
<tr>
<td>2.00</td>
<td>34 (48)</td>
<td>216</td>
<td>146</td>
<td>OG</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>34 (48)</td>
<td>121 (198)</td>
<td>10</td>
<td>119 (134)</td>
<td>3 (15)</td>
<td></td>
</tr>
<tr>
<td>2.66</td>
<td>76</td>
<td>259</td>
<td>138</td>
<td>28</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>173</td>
<td>741</td>
<td>107</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>52 (51)</td>
<td>199 (186)</td>
<td>21 (17)</td>
<td>632 (546)</td>
<td>18 (59)</td>
<td></td>
</tr>
<tr>
<td>3.32</td>
<td>58</td>
<td>2 ND*</td>
<td>267</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>58</td>
<td>2 ND*</td>
<td>267</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.00</td>
<td>287</td>
<td>279</td>
<td>1000</td>
<td>180</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>461 (360)</td>
<td>399 (356)</td>
<td>13 (8)</td>
<td>931 (957)</td>
<td>113 (110)</td>
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</tr>
<tr>
<td>4.60</td>
<td>412</td>
<td>390</td>
<td>942</td>
<td>39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>694</td>
<td>529</td>
<td>975</td>
<td>539</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>650 (699)</td>
<td>737 (670)</td>
<td>907 (1037)</td>
<td>823 (681)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>753</td>
<td>746</td>
<td>1229</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>524</td>
<td>311</td>
<td>907</td>
<td>292</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>473 (560)</td>
<td>667 (453)</td>
<td>929 (916)</td>
<td>860 (943)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.60</td>
<td>684</td>
<td>383</td>
<td>913</td>
<td>1047</td>
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<td></td>
</tr>
</tbody>
</table>

* Additive is given in parentheses. See Table 1 for abbreviations of media.
* OG, Overgrown.
* Numbers in parentheses are mean number of colonies/plate.
* ND, Not determined.

Rose bengal malt extract medium. All plates were overgrown with red and yellow bacteria to such a degree that counting of Streptomyces colonies was impossible.

Starch casein medium (NaCl). As the salt concentration increased to 4.6% the bacteria decreased due to suppression. At 2.0% additive, overgrowth occurred, whereas at 3.3% colonies
were mainly on the rim, the center ones being very small. At 4.0%, some bacteria remained and *Streptomyces* colonies were quite large and unobscured. With a salt level of 4.6%, the membrane was free of bacteria, and the grey colonies were typically well-formed units, which were easy to count. They emitted the characteristic musty odor.

**Starch casein medium** (**MgCl₂**). As the salt concentration increased from 0 to 4.6%, the number of bacteria decreased. At 2.66% additive, a few *Streptomyces* colonies were on the rim, the rest being unable to compete, and a few white wisps were visible on the surface. At 3.32%, colonies in the center were still seen as white dots, and at 4% they were still small, with larger ones on the rim. At 4.66%, there was little bacterial growth, but the colony size was badly retarded in comparison with the same medium plus NaCl.

**Effect of trace metals on the colony count.** Trace metals were added because Waksman (9) mentioned their beneficial effect. These were in the form of zinc acetate, copper chloride, and ammonium molybdate. Even with such variables as agar concentration and salt concentration the trend is obvious in each of the four treatments. Only zinc ions enhanced *Streptomyces* colony yields (see Table 3).

It can be readily seen that salt (NaCl) addition is not a panacea for the prevention of bacterial overgrowth on all media. However, it has been found to be outstandingly beneficial when used with the starch casein medium (11). On an uncrowded plate, grey-white *Streptomyces* colonies with a maximum diameter of 5 mm develop and emit in all cases the strong, characteristically musty odors. Only *Streptomyces* spp. were encountered, with typical colonial growth patterns of concentric rings of spores developing as shown in Fig. 1. The complete formula is as follows: starch (Ajax Chemical Co.), 10 g; casein (fat and vitamin free; Hopkins & Williams Ltd.), 0.3 g; potassium nitrate, 2.0 g; sodium chloride, 2.0 g; sodium chloride (additional), 26.6 to 40.0 g; dipotassium hydrogen phosphate, 2.0 g; magnesium sulfate, 0.05 g; calcium carbonate, 0.02 g; ferrous sulfate, 0.01 g; agar (Oxoid no. 1), 18.0 g; zinc acetate, 1.0 mg; distilled water, 1,000 ml.

All ingredients, except starch, were combined, and the pH was adjusted to between 7.0 and 7.2. When the medium began to boil, starch was added as a paste in distilled water. After the medium was sterilized at 15 lb/in² for 15 min in an autoclave, the precipitate was resuspended before pouring. Four antibiotics were added to each petri dish (per milliliter of agar) before the molten medium was poured: nystatin (E. R. Squibb & Son), 50 μg; cycloheximide (Acti-Dione, Upjohn), 50 μg; aerosporin (Burroughs Wellcome), 5.0 μg; penicillin G (Glaxo Allenbury), 1 to 3.0 μg. For potable water 2.66% NaCl as additive was satisfactory, whereas up to 4.0% may be needed if the competing bacterial load is greater.

**DISCUSSION**

As mentioned above, tests need to be performed to find the most suitable concentration of NaCl to be used as additive. The counting error level is not relevant in this study as the number and clarity of *Streptomyces* colonies on medium (v) far surpasses those of any competitor. In cases when high counts were obtained, there may have been an error of about 8% due to crowding, but for lower counts no error occurred. The effect of an altered level of penicil-

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**Table 3. Effect of trace metals on the number of streptomyces colonies on starch casein agar modified with additional NaCl or MgCl₂**

<table>
<thead>
<tr>
<th>Metal (1 μg/ml)</th>
<th>Mean no. of colonies/plate per 100 ml at agar concn of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.8% (2.64% NaCl)</td>
</tr>
<tr>
<td>Blank</td>
<td>226</td>
</tr>
<tr>
<td>Zinc ions</td>
<td>330</td>
</tr>
<tr>
<td>Molybdenum and copper ions</td>
<td>155</td>
</tr>
<tr>
<td>Zinc, copper, and molybdenum ions</td>
<td>135</td>
</tr>
</tbody>
</table>

**Fig. 1. Streptomyces colonies growing on a membrane on starch casein agar plus NaCl.**

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lin G is irrelevant compared with the salt content effect.

ACKNOWLEDGMENTS

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LITERATURE CITED