Convenient, Simplified Preparation of Less Commonly Used Media

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A method is described for the preparation of some less commonly used media. This approach should encourage use of these media.

From time to time certain biochemical tests that are not a part of a diagnostic laboratory's routine battery of tests are needed for a more definitive identification of an unknown microbial isolate. Some of these tests are avoided because of the inconvenience and cost of preparing small batches of the necessary media for a limited usage. An example of this would be the addition of a test for starch hydrolysis (amylace) to the other tests used for the occasional identification of Pseudomonas stutzeri. Because we are interested in using some of these less commonly used tests to aid in our identifications of, particularly, nonfermentative gram-negative bacilli, we examined this situation for an approach that would conveniently provide the necessary media when needed. This paper describes our first suggestions for the preparation of some of these media. Descriptions and discussions of the conventional preparation and of the use of comparable media can be found in various laboratory manuals such as the Manual of Clinical Microbiology (2) or the American Public Health Association Diagnostic Procedures (1).

We selected, as a basal medium, a commercial medium that is readily available in most laboratories, Trypticase soy agar (TSA). Because calcium ion stimulates various proteolytic, lipolytic, and phospholipolytic enzymes, but mainly because it intensifies the positive zones with the Tweens and lecithin, we added calcium chloride to the TSA to a final concentration of 0.05%. This medium was tubed in 18- to 20-ml volumes in screw-cap tubes (150 by 20 or 150 by 25 mm), sterilized by autoclaving, and then refrigerated until needed.

The substrates were prepared as concentrated solutions and stored in bulk, usually in 50- or 100-ml amounts; however, preparation of smaller volumes consistent with anticipated needs would be appropriate. Small volumes were added to the melted and cooled (45 to 50°C) basal medium to prepare the final concentrations of the specific substrates for use. Table 1 shows the concentrations of the stock solutions and the volumes used to prepare 20 ml of each medium. Stock solutions of gelatin, Tween 80, amylepectin (or soluble starch), powdered milk, esculin, and ferric ammonium citrate were prepared in distilled water. Casein was dissolved in 0.1 N NaOH. These aqueous stock solutions were sterilized by autoclaving. The esculin stock solution was saturated at room temperature and had to be reheated to dissolve the esculin. Tween 85 and lecithin (Egg lecithin, Nutritional Biochemical Corp., Cleveland, Ohio) were prepared in 95% ethanol. These alcoholic solutions were used without further sterilization.

The media were usually used in petri dishes (100 by 15 mm). In these containers up to 12 isolates can be tested simultaneously by patching eight at the periphery and four in the central area. Since lethal reagents were used to test for gelatinase and amylase, the organisms to be tested for these reactions were sometimes applied to gelatin or to amylpectin agars in 100-mm dishes as single strokes across the surface (up to four organisms). This provides areas for testing, namely the opposite ends and the center of the strokes, at three intervals of incubation. Frazier reagent (HgCl₂, 12 g; distilled water, 80 ml; concentrated HCl, 20 ml) was used for testing for gelatin liquefaction and Gram iodine was used for demonstrating starch hydrolysis. These must be added to the appropriate media at the time the results are to be determined. Occasionally the media were used in smaller containers such as 35- and 60-mm plastic petri dishes. When these were used, the volume of medium was 3.5 to 4 ml for the 35-mm size and 7 to 8 ml for the 60-mm size. The number of organisms tested was reduced to one or two and up to five for the two sizes, respectively.

Positive reactions for Tween 80 esterase, Tween 85 lipase, and lecithinase were observed.
as zones of calcium soaps surrounding the patches of growth in and/or on the surface of the respective medium. Clear zones surrounding the growth were positive results for the digestion of casein and milk and of gelatin (with mercuric chloride reagent) and amylopectin (with iodine reagent). Hydrolysis of esculin, the initial step in the metabolism of this glycoside, was observed as a distinct brown to black discoloration beneath and surrounding the growth. Some strains of *Pseudomonas aeruginosa*, which form hydrogen sulfide, may produce darkened (ferrous sulfide) growth but no brown discoloration in the medium was observed.

All of these media, prepared as described above, were tested with known weak to strong positive and known negative controls from a broad spectrum of fermentative and nonfermentative gram-negative bacilli. Our use of gram-negative bacilli to evaluate this approach does not preclude the method for the characterization of gram-positive bacteria. The method is probably suitable for all microbial species able to grow on TSA. Results, after incubation periods appropriate to the specific control organisms but always within 72 h, were entirely satisfactory. In our experience clear zones are sharper with casein than with skim milk, but the latter, which is probably more readily available, is satisfactory for detecting casein hydrolysis. Only one, however, is needed. At the present time we are using Tween 80 (polyoxyethylene sorbitan mono-oleate) as a substrate for esterases (water-soluble esters). For those who wish to use Tween 20 (monolaurate), Tween 40 (monopalmitate), and/or Tween 60 (monostearate) for the same purpose, the same procedure may be used as for Tween 80; however, the stock solution of Tween 60 should be prepared in ethanol instead of in water. Tween 85 (polyoxyethylene sorbitan trioleate) is used as a substrate for lipases (water-soluble and immiscible esters). Egg lecithin is used for lecithinase activity in place of egg-yolk suspensions. The alcoholic solution of lecithin is more easily prepared than egg-yolk suspensions and both the subsurface precipitate and the iridescent surface film described for organisms on egg-yolk agar can be seen with the lecithin-TSA. Furthermore, the lecithin-TSA medium is free from the distracting proteolytic reactions (clearing) seen with egg-yolk agar. With many organisms, such as *Aeromonas hydrophila*, *Vibrio paraahaemolyticus*, *Serratia marcescens*, and *P. stutzeri*, amylase activity can be observed as a cloudy zone surrounding the growth; however, testing (detection) with iodine is recommended.

This approach for less frequently used tests can save time, money, and space, and perhaps, in some instances, increase the number of positive identifications. Time and money are saved because a single basal medium can be prepared in greater volume and can be available immediately when needed, thus eliminating the time- and money-consuming occasional preparation of small batches of different media. Loss due to aging of the media is avoided since only the basal medium needs to be prepared in amounts consistent with anticipated needs. Space is saved since all of the different media are not being stored separately for their occasional use. The concentrated stock solutions (100 ml) are occupying much less space than the equivalent 50 to 500 tubes of the various media. If these various tests have any value for the identification of species of bacteria, then the convenience of their availability should increase their use and thus increase the number of positive identifications.

**LITERATURE CITED**
