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Improved Technique for Isolation and Identification of *Sphaerotilus*

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It is the opinion of a number of aquatic microbiologists that *Sphaerotilus* is a part of the natural flora of streams. This is true despite the fact that this organism has not been isolated consistently from waters in which the characteristic slime infestations are not apparent to the naked eye. In many instances, the available slime growth from an infestation must be shipped to a laboratory, necessitating a delay of 24 hr or more. These isolations are difficult and sometimes impossible owing to, in part, excessive concomitant growths. Because of the lack of success achieved by currently available methods (2-9; M. S. Cataldi, Ph.D. Thesis, Univ. of Buenos Aires, Argentina), an effort was made to develop a simple medium which would enhance the growth of *Sphaerotilus* while retarding the growth of the concomitant organisms.

Preliminary studies showed that a large variety of carbon sources added individually to biochemical oxygen demand (BOD) dilution water (1) have the ability to support growth of this organism when inoculated from pure cultures. Of the carbon compounds tried, sodium lactate provided the medium which permitted the best growth for a variety of strains. The following formulation was adapted for the first field trials: sodium lactate, 100 mg; NH_4Cl, 1.7 mg; KH_2PO_4, 8.5 mg; K_2HPO_4, 21.5 mg; $Na_2HPO_4 \cdot 7H_2O$, 34.4 mg; $MgSO_4 \cdot 7H_2O$, 22.5 mg; $CaCl_2$, 27.5 mg; $FeCl_3 \cdot 6H_2O$, 0.25 mg; distilled water, 1,000 ml (*p*H 7.1 to 7.2).

For the first field trial, 50 ml of the above medium was dispersed into French square bottles. Sterilization was accomplished by autoclaving at 10 lb/in^2 of steam for 15 min. The sampling sites for this trial consisted of two local rivers, two open county drains, and one roadside ditch, none of which had ever shown any visual evidence of *Sphaerotilus* infestations. In sampling, care was taken not to stir up the water or the stream sediment. At each sampling site, 25-ml portions were collected directly into duplicate bottles containing the culture medium.

The samples were incubated at room temperature (22 to 25 C) for a period of 5 days. Filamentous growth, identified microscopically as *Sphaerotilus*, appeared on the 2nd or 3rd day. Contamination was of a much lower magnitude, and in all cases the usual spore formers, always present with other methods, were totally absent. This would indicate that the procedure was at least partially selective for *Sphaerotilus*.

This selectivity can be attributed to the use of very low nutrient concentrations. In another series of experiments, using the same mineral base with 1,000 to 5,000 mg/liter of sodium lactate or other selected carbon sources, much more contamination grew and *Sphaerotilus* isolations decreased markedly. If 50 mg/liter, or more, of peptone was added, *Sphaerotilus* almost never was isolated from these waters, whereas *Bacillus* was prominent along with a myriad of other growth forms. Apparently, these other more rapidly growing organisms either use the essential ingredients necessary for *Sphaerotilus* or else they produce waste products which are inhibitory.

Isolation for pure cultures was accomplished by picking a filament from the BOD-lactate broth and streaking it on 0.05% meat extract agar. After incubation for 24 hr at 25 C, typical curling filaments of *Sphaerotilus* were easily picked with the aid of a dissecting microscope. These isolates were transferred to a Trypticase-glycerol broth, which has been found to be quite effective as a holding medium for stock cultures. The formula for this medium is as follows: Trypticase (BBL), 5 g; glycerol, 5 g; distilled water, 1,000 ml (*p*H 7.0 to 7.2).

In this medium, a heavy pellicle is formed in 2 to 3 days and the underlying broth remains clear. If turbidity develops in the broth, an immediate reisolation on agar is indicated. At times, the contaminating organisms may take up to 2 weeks to appear. Consequently, in this laboratory, it is routine to reisolate on the meat extract agar the first pellicle grown in the tryptose-glycerol broth before it is considered a pure culture.

Experience with this procedure has led to minor modifications. For example, in sampling raw sewage or the products of various stages of the plant process, it was found that samples of 1, 5, and 10 ml of the settled liquor would insure isolation in at least one portion of the series. If

greater volumes are used, the *Sphaerotilus* usually does not develop.

In a second series of five rivers and five county drains, a modification was made to permit larger samples. In addition to the previously described procedure, a 50-ml sample was collected in a bottle containing 50 ml of double-strength BOD lactate broth. Once again, at least one sample from every source was positive for *Sphaerotilus* despite the fact that there was no visual evidence of the organism.

The BOD lactate medium has also been found useful in a number of instances in shipping suspected materials from field studies to the laboratory in a viable state, even after several days. It is most important, however, that the specimen be quite small (no larger than a man's shirt button).

Identification of *Sphaerotilus* is still dependent on microscopic examination because there are no generally agreed upon cultural characteristics for these organisms. The most important characteristic for positive identification is the organic sheath which encloses the rod-shaped organisms in a tubule. Certain other organisms, mainly members of the genus *Bacillus*, produce chains of cells with a ghost cell or two in the chain which have frequently been mistaken for the *Sphaerotilus* sheath. Another problem is that *Sphaerotilus*, under certain environmental conditions, has been observed to grow in exceedingly long filaments with no apparent differentiation as to cells or sheath.

A wet-mount staining procedure has been developed to simplify the microscopic identification and to eliminate many of the false identifications. The microscope preparation is easily made by placing a small piece of the slime growth on a slide in a drop of water and pressing it flat under a cover slip. If an excess of water bulges around the edges of the cover slip, it must be picked up with blotting paper. A very small drop of aqueous 1% crystal violet stain is placed at the edge of the cover slip so that it will flow into the preparation by capillary action. After 30-sec contact, the preparation is pressed with blotting paper to remove excess dye. When viewed with the aid of a bright-phase, oil immersion objective, both the sheath and cells stand out vividly. However, if the preparation is to be viewed through a bright-field microscope, it is necessary to flush out the excess dye by placing a drop of water at one edge of the cover slip and picking up the dye with blotting paper at the opposite edge. Upon removal of the excess dye, both the cells and sheath are easily discernable.

In this technique, the cells shrink very slightly and it is frequently possible to see them actually sliding out of the sheath tubule. Large sections of sheath are always visible, and the confusion with ghost cells is cleared up. The long filaments break into chains of cells which are seen sliding along inside the sheath, and thus present many large sections of stained, empty sheath. Experience with this procedure has shown it to be particularly valuable in the examination of grossly contaminated material such as activated sluge flocs, since the characteristic *Sphaerotilus* can now be rapidly and easily identified even while in the midst of other filamentous forms.

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