Selective Medium for the Isolation of *Prototheca*

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A medium was devised which permitted the selective isolation of *Prototheca* spp. from nature.

*Prototheca*, which has been mentioned as a common microorganism in nature (1) and a rare cause of infection in man and animals (2), has not commonly been reported in microbial flora surveys. Consequently, little is known of its ecology. The failure to isolate *Prototheca* spp. may be explained by the fact that they are readily overgrown by bacteria and fungi when culture is attempted from contaminated sources, as well as the fact that they superficially resemble yeast. Finally, although *Prototheca* spp. have simple nutritional requirements, many commonly used culture media are unsatisfactory because they contain unsuitable nutrients or inhibitors.

Two requirements considered important in devising a medium suitable for isolation of *Prototheca* spp. were the establishment of the minimum essential nutrients required for growth, and the selective inhibition of other microorganisms capable of growing in this medium. Thirty-five strains of *Prototheca* representing all authentic known species were obtained, primarily from other investigators. None of these strains were isolated utilizing the selective factors found effective in this research. Optimum ingredient concentrations from *Prototheca* spp. strains were determined in broth by densitometric methods (Table 1). Optimum concentrations of bacterial and fungal inhibitors were determined by visual observation of growth from sewage on agar medium. Most bacteria, algae, and fungi were inhibited by the combination of potassium hydrogen phthalate and 5-fluorocytosine (5-FC).

The phthalate and NaOH were dissolved first with stirring, and then the remaining ingredients were added, dissolved with heat and stirring, autoclaved for 15 min (121 °C, 15 lb of pressure), and cooled to 50 °C. The 5-FC was added and the resulting medium was poured into petri dishes.

The 5-FC obtained as Ancobon (Roche Laboratories, Nutley, N.J.), had to be purified of insoluble matter and lactose before use. Two 500-mg Ancobon capsules were dissolved in 30 ml of distilled water at 45 °C with vigorous stirring for 15 min, filtered under pressure while hot through a membrane filter (3 μm pore size, 47 mm) (Millipore Co., Bedford, Mass.), and followed with 5 ml of boiling distilled water. The filtrate was allowed to crystallize overnight at 5 °C. The 5-FC crystals were recovered by vacuum filtration with Millipore filters (0.45 μm pore size, 47 mm), washed with 3 ml of cold water, and then air-dried at 40 °C (approximate yield 75%). The amount to be used was redissolved in distilled water (45 °C), filter sterilized (0.45 μm pore size), and added to medium before pouring. The optimum selective concentration of 5-FC was 250 μg/ml; however, less was also effective, and none of the 35 *Prototheca* strains tested were inhibited by up to 500 μg of 5-FC per ml.

Hexachlorocyclohexane (0.01 g/liter before autoclaving) was used as an optional ingredient, valuable for control of arthropod contaminants, as was Fumagillin Bicyclohexylamine (Abbott Laboratory, W. Chicago, Ill.) (0.005 g/liter dissolved in ethanol and added before autoclaving) for cyst-forming amoebae.

A few 5-FC-resistant yeasts were observed, but these comprised less than 2% of the

**Table 1. Chemical constituents of *Prototheca* isolation medium (PIM)**

| Constituent                  | Grams/liter |
|-----------------------------|-------------|
| Distilled water             | 10.0        |
| Potassium hydrogen phthalate | 0.9         |
| Sodium hydroxide            | 0.1         |
| Magnesium sulfate           | 0.2         |
| Potassium hydrogen phosphate| 0.3         |
| Ammonium chloride           | 10.0        |
| Glucose                     | 0.001       |
| Thiamine hydrochloride      | 20.0        |
| Agar                        | 0.25        |

5-fluorocytosine (5-FC), pH 5.2

a Essential nutrient.
Prototheca colonies present on Prototheca isolation medium (PIM) from sewage, for example. Bacteria were not a problem, but should additional antibacterials be required, chloramphenicol is recommended. The occasional overgrowth by filamentous fungi may be circumvented by early (24 to 48 h) counting or subculture of microcolonies, with the aid of a stereomicroscope (Fig. 1).

It was found that, with PIM, *Prototheca* spp. could be isolated directly from sources as densely contaminated as sewage and soil or from sources needing concentration (centrifugation or filtration), such as stream water. Liquid specimens were pipetted or streaked directly onto petri dishes, and good results were obtained when solid specimens were homogenized, resuspended in water, and plated. Incubation at 30°C for 72 h was adequate for most *Prototheca* spp. However, there were strains which preferred 25°C, and there were slow-growing strains requiring up to 7 days for visible colonies. Using PIM, hundreds of *Prototheca* spp. isolates, representing several species, have been cultured from numerous sources.

An incidental finding of the investigation was that five strains of *Chlorella protothecoides* would grow well on PIM, but seven strains of other *Chlorella* spp. would not. This provides additional support for a purported (3) relationship of *C. protothecoides* and *Prototheca*. Subsequently, *C. protothecoides* was isolated from nature on PIM.

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