Powdered Chitin Agar as a Selective Medium for Enumeration of Actinomycetes in Water and Soil

S. C. HSU and J. L. LOCKWOOD

Bureau of Laboratories, Michigan Department of Public Health, Lansing, Michigan 48914, and Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824

Received for publication 25 October 1974

Agar media made with 0.4% colloidal chitin plus mineral salts and adjusted to pH 8.0 was superior to four other commonly used media for the isolation and enumeration of actinomycetes from water samples. More actinomycetes developed on chitin agar, and the development of bacteria and fungi was suppressed.

Frozen and vacuum-dried chitin from aqueous colloidal suspensions was finely divided and gave results comparable to those obtained with media prepared from colloidal suspensions.

Several media have been proposed for the selective isolation of actinomycetes from soil (1-7, 9, 10, 12). However, in our hands only those containing chitin (7, 9, 10, 14) have proved to be highly selective. The selectivity of chitin agar is based on the apparently near universal ability of actinomycetes to hydrolyze this carbohydrate (8, 13), whereas relatively few bacteria and fungi among the soil population can utilize it. Most media used for enumeration of actinomycetes in water have not included chitin as the selective ingredient. Therefore, such media may be less selective than those containing chitin.

A barrier to the widespread use of chitin agar is the time-consuming preparation of colloidal suspensions of chitin, which are required for good development of colonies in agar media. As originally described (9, 10), chitin was dissolved in HCl and precipitated in cold water after passage with suction through glass wool on a Büchner funnel to remove undissolved impurities, the process being repeated several times. The precipitated chitin was washed free of acid and concentrated by sedimentation and decantation. Yields were about 35%. Later, solutions of more highly purified chitin in HCl were precipitated directly into cold water with yields of about 80% (11).

The purposes of this study were: (i) to attempt further simplification of the preparation of colloidal chitin; (ii) to test different preparations of colloidal chitin in agar for isolation of actinomycetes from water and soil; and (iii) to compare chitin agar with other selective media frequently used for this purpose.

MATERIALS AND METHODS

Preparation of bleached chitin. Sixty grams of unbleached chitin (ICN Nutritional Biochemicals Corp.) was stirred with 300 ml of commercial bleach (5.25% sodium hypochlorite) for 10 min. The mixture was added to 5 liters of tap water and filtered with suction onto a coarse filter paper. This procedure was repeated at least five times to remove the bleach. The chitin was then air-dried overnight; 95% of the chitin was recovered. The dried bleached chitin was ground at high speed in a Waring blender.

Preparation of colloidal chitin. Forty grams of unbleached chitin, bleached chitin, or partially purified chitin (Calbiochem Corp.), all ground dry in a Waring blender, was dissolved in 400 ml of concentrated HCl by stirring for 30 to 50 min. The chitin was precipitated as a colloidal suspension by adding it slowly to 2 liters of water at 5 to 10°C. The suspension was collected by filtration with suction on a coarse filter paper and then washed by suspending it in about 5 liters of tap water and refiltering. The washing was repeated at least three times or until the pH of the suspension was about 3.5. At least 85% of the chitin was recovered. Water content of the chitin was determined by drying a sample at 100°C. For use, sufficient water was added to resuspend the chitin, and the suspension was blended at high speed for about 10 min. Autoclaved filter cake or aqueous suspensions could be stored indefinitely at room temperature.

Preparation of powdered colloidal chitin. Two liters of colloidal chitin suspension prepared from bleached or partially purified chitin and containing 3 to 5% chitin was spread in a thin layer on a tray and frozen at -5°C. The frozen chitin was then vacuum-dried (F. J. Stokes Corp., model 338) for 3 or 4 days. When dry, the delicate, flake-like chitin particles readily disintegrated into powder.

Media. Chitin agar was usually prepared using 4 g of the dry preparation per liter, or a sufficient volume of colloidal chitin suspension to give 4 g of chitin. These were mixed with the following mineral salts:

1Journal article no. 7025 from the Michigan Agricultural Experiment Station.
K$_2$HPO$_4$ (0.7 g); KH$_2$PO$_4$ (0.3 g); MgSO$_4$·7H$_2$O (0.5 g); FeSO$_4$·7H$_2$O (0.01 g); ZnSO$_4$ (0.001 g); MnCl$_2$ (0.001 g); and 20 g of agar. After autoclaving, the melted agar medium was adjusted to pH 8.0 with 5 N sterile NaOH. To determine the optimum pH for chitin agar, colloidal bleached chitin with mineral salts was adjusted with NaOH or HCl to cover a range from pH 5.5 to 10.0.

**Soil and water samples.** Water samples were taken from Lake Lansing and from the Red Cedar River at Michigan State University, both in Ingham County, Mich. These were assayed after dilution to $10^{-2}$ in sterile buffer (0.5 M KH$_2$PO$_4$ adjusted with NaOH to pH 7.2) or were used undiluted. Conover loam from the Michigan State University Farm and a soil under grass near the shore of Lake Lansing were diluted to $10^{-4}$ in 100 ml of sterile buffer, after first shaking the original suspension (10$^{-1}$) for 15 min. One-milliliter aliquots of diluted soil and water suspensions were placed in petri dishes. Melted and cooled (40 to 42 C) agar was mixed with the suspensions, with swirling. Plates were incubated for 7 to 10 days at 25 to 28 C when counts of actinomyces, bacteria, and fungi were made, with the aid of a 1.5× lens on a dark-field colony counter. Identifications were based on gross colony morphology.

All experiments were done at least twice, and each experiment usually had six replications.

**RESULTS**

**Isolation of actinomyces from water and soil.** Colloidal chitin preparations supported the development of more actinomyces colonies than media containing noncolloidal chitin (Table 1). There were no differences among the colloidal chitin preparations, including powdered colloidal chitin. However, the zones of chitin utilization surrounding actinomyces colonies were clearer when bleached or partially purified chitin was used than when unbleached chitin was used.

Chitin agar prepared with mineral salts gave higher numbers of actinomyces from water samples than that without mineral salts, but there were no differences in numbers of actinomyces from soil whether or not chitin agar contained mineral salts. More than 85% of the actinomyces growing on the colloidal chitin agars developed clear zones surrounding the colonies which enhanced their visibility (Fig. 1). Agar prepared without chitin supported growth of far fewer actinomyces than agar containing colloidal chitin. Although we have not attempted to identify all isolates from either water or soil, most were members of the genera Streptomyces, Micromonospora, and Nocardia.

**Optimum pH of chitin agar.** Figure 2 shows the mean number of actinomyces from water and soil in two different experiments. Few actinomyces developed on chitin agar below pH 6.5. Above pH 6.5, colony numbers from both soil and water increased to a maximum at approximately pH 8.0 to 8.5. The numbers isolated from water decreased as the pH was increased above 8.0 to 8.5, whereas those from soil did not decrease until the pH exceeded 9.5.

**Optimum concentration of chitin.** Numbers of actinomyces detected were greatest when the medium contained 0.2 to 0.8% chitin (Fig. 3). Above 1 to 2%, numbers decreased. At 0.1% chitin, clear zones surrounding the actinomyces colonies were indistinct. At 0.2 and 0.3%, they showed increasing clarity, and at 0.4% or more chitin zones of maximum clarity were formed.

**Comparison of selective media for isolation of actinomyces from water and soil.** Chitin agar was compared with four other media commonly used for enumeration of actinomyces from soil and water. Colloidal chitin-mineral salts agar gave the largest numbers and the most easily recognizable actinomyces colonies from two samples each of water and soil (Table 2). It also suppressed the growth of bacteria and fungi more than any of the other media. By contrast, egg albumin agar (15), arginine-glycerol-salts agar (5), starch-casein agar (6), and actinomyces isolation agar (4) consistently allowed the growth of more bacteria than actinomyces, and of numerous fungi.

**DISCUSSION**

Two improvements in the preparation of colloidal chitin are reported. (i) The use of

| Table 1. Comparison of media made with different preparations of chitin for enumeration of actinomyces from water and soil |
|--------------------------------------------------|
| Chitin prepn | Mineral salts* | Water (×10^6/ml) | Soil (×10^6/g) |
|--------------|---------------|----------------|---------------|
| Colloidal, partially purified                    | +             | 27.6           | 14.3          |
| Colloidal, bleached                              | -             | 4.6            | 11.8          |
| Colloidal, unbleached                            | +             | 28.2           | 13.2          |
| Colloidal, unbleached, powdered                  | +             | 25.6           | 14.8          |
| Bleached, ground                                | +             | 7.6            | 15.6          |
| Unbleached, ground                              | -             | 24.4           | 12.2          |
| No chitin                                       | -             | 3.2            | 10.3          |

* With (+); without (-).

* Mean of two experiments. Least significant range (P = 0.05) using Tukey’s “w” procedure: water, 4.6 × 10^6; soil, 4.5 × 10^6.
Actinomycetes isolated from a water sample in chitin-mineral salts agar. Note zones cleared of chitin surrounding the colonies, nearly all of which are actinomycetes.

filtration, rather than sedimentation and decanting, to remove HCl from the precipitated chitin saved much time and resulted in more highly concentrated preparations. (ii) Pulverized colloidal chitin was sufficiently finely particulate to give results comparable to those obtained with colloidal chitin from aqueous suspensions. This, for the first time, permits use of a dried product for preparation of chitin agar and has obvious convenience and storage advantages. Agar made with ground (noncolloidal) chitin was relatively coarse and proved
unsatisfactory for enumeration of actinomycetes.

Chitin agar with mineral salts was more effective than that without mineral salts for isolating actinomycetes from water. As previously reported (10), mineral salts gave no advantage for isolating actinomycetes from soils. This suggests that the aquatic actinomycetes may differ physiologically from the terrestrial isolates. It is also possible that substances carried over from soil in the dilution series had a stimulatory effect of actinomycete growth, but this seems unlikely in view of the high dilution used.

Chitin agar showed selectivity superior to that of several other media for isolating actinomycetes from water and soil, by favoring these organisms and suppressing development.

![Graph](image1)

**Fig. 2.** Effect of pH of chitin-mineral salts agar on numbers of actinomycetes isolated from water and soil.

![Graph](image2)

**Fig. 3.** Effect of concentration of chitin in chitin-mineral salts agar on numbers of actinomycetes isolated from water and soil.

**Table 2.** Comparison of five selective media for enumeration of actinomycetes from water and soil

| Agar medium                  | Water source       | Soil source       |
|------------------------------|--------------------|-------------------|
|                              | Red Cedar River   | Lake Lansing      | Mich. State Univ. | Lake Lansing |
|                              | (x10⁴/ml)         | (x1/ml)           | (x10⁴/g)         | (x10⁴/g)     |
|                             | A      B  F   | A      B  F   | A      B  F   | A      B  F   |
| Chitin-mineral salts         | 94 17  9   | 284 15  3   | 158 23  3   | 24 2  2     |
| Egg albumin                  | 33 58  16  | 82 44  11  | 56 85  48  | 8 38  14    |
| Arginine-glycerol-salts      | 19 82  49  | 42 88  16  | 42 187 28  | 6 79  16    |
| Starch-casein                | 38 54  21  | 86 60  9   | 68 93  32  | 10 14  8     |
| Actinomycete isolation       | 40 48  31  | 99 42  6   | 82 79  25  | 16 9  12    |
| LSRᵇ                         | 8 15  7   | 16 6  4   | 11 12  6   | 3 10  4     |

*a Means of two experiments. Abbreviations: A, actinomycetes; B, bacteria; and F, fungi.

ᵇLeast significant range (P = 0.05) by using Tukey's "w" procedure.
of most bacteria and fungi. Our results differ from those of El-Nakeeb and Lechevalier (5) who found their arginine-glycerol-salt medium to be more selective than chitin agar for isolating actinomycetes from soil. They also found that mineral salts agar without chitin gave as many actinomycetes as chitin agar, though they acknowledged that growth was better with chitin in the medium. These discrepancies may lie in some unknown difference in the preparations of chitin agar used, or in the fact that El-Nakeeb and Lechevalier sampled only the supernatant of soil suspensions allowed to stand for 30 min, whereas we sampled from the entire suspension without allowing sedimentation to occur.

Nearly all actinomycete colonies on chitin agar were surrounded by a zone cleared of chitin which facilitated macroscopic recognition of the actinomycetes. Thus, this characteristic was a good indicator for these organisms in the medium and may be utilized in the routine analysis of water for contamination by actinomycetes.

LITERATURE CITED

1. Corke, C. T., and F. E. Chase. 1956. The selective enumeration of actinomycetes in the presence of large numbers of fungi. Can. J. Microbiol. 2:12-16.
2. Crook, P., C. C. Carpenter, and P. F. Klens. 1950. The use of sodium propionate in isolating actinomycetes from soil. Science 112:856.
3. Dulaney, E. L., A. H. Larsen, and E. O. Stapley. 1955. A note on the isolation of microorganisms from natural sources. Mycologia 47:420-422.
4. Difco Laboratories. 1962. Bacto-actinomycete isolation agar. Code 0957. Difco Supplementary Literature. Difco Laboratories, Detroit, Mich.
5. El-Nakeeb, M. A., and H. A. Lechevalier. 1963. Selective isolation of aerobic actinomycetes. Appl. Microbiol. 11:75-77.
6. Küster, E., and S. T. Williams. 1964. Selection of medium for isolation of streptomycetes. Nature (London) 202:928-929.
7. Kuznetsov, V. D., and I. V. Yangulova. 1970. Utilization of medium containing chitin for isolation and quantitative enumeration of actinomycetes from soil. Mikrobiologiya 39:902-906.
8. Jeuniaux, C. 1955. Production of exochitinase by Streptomyces. C. R. Soc. Biol. 149:1307-1308.
9. Lingappa, Y., and J. L. Lockwood. 1961. A chitin medium for isolation, growth and maintenance of actinomycetes. Nature (London) 190:158-159.
10. Lingappa, Y., and J. L. Lockwood. 1962. Chitin media for selective isolation and culture of actinomycetes. Phytopathology 52:317-323.
11. Lloyd, A. B., R. L. Noveroske, and J. L. Lockwood. 1965. Lysis of fungal mycelium by Streptomyces spp. and their chitinase systems. Phytopathology 55:871-875.
12. Phillips, G. B., and E. Hanel, Jr. 1950. Control of mold contaminants on solid media by use of actidione. J. Bacteriol. 60:104-105.
13. Reynolds, D. M. 1954. Exocellular chitinase from Streptomyces sp. J. Gen. Microbiol. 11:150-159.
14. Sykes, G., and F. A. Skinner. 1973. Actinomycetales: characteristics and practical importance. Academic Press Inc., New York.
15. Waksman, S. A. 1950. The actinomycetes, their nature, occurrence, activities and importance. Chronica Botanica Co., Waltham, Mass.