

METHODS FOR CHARACTERIZATION  
OF STREPTOMYCES SPECIES<sup>1</sup>

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ABSTRACT. The methods used by collaborators in the International Streptomyces Project (ISP) for emendation of descriptions of type and neotype strains of the genus Streptomyces (Actinomycetales) are presented.

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An international cooperative effort, now in progress, is directed toward collection of type cultures of the Streptomyces species for deposition with the Centraalbureau voor Schimmelcultures (CBS), Baarn. From this center the reference cultures will be supplied to other culture collections so that they are available throughout the world.

An essential adjunct to this activity is the redescription of each type culture in terms of currently acceptable criteria and methods. The urgent need for an authentic reference collection, accompanied by standardized characterizations for each species, has been pointed out by spokesmen for the several meetings and conferences which culminated in this project. (See, for example, Gottlieb, 1959, 1961; Küster, 1959; Krasil'nikov, 1961.) More than 40 investigators<sup>2</sup> representing 17 countries are participating in this research. Each culture is described independently by three of these cooperating specialists in different laboratories before it is deposited in the reference collection.

<sup>1</sup> This project is supported in part by a research grant from the National Science Foundation, U.S.A. The Subcommittee on Actinomycetes of the Committee on Taxonomy, A.S.M. and the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature are co-sponsoring advisors.

<sup>2</sup> Participants in the 1964-1965 studies are listed on p. 338.

This manual contains the criteria and methods adopted for the project. It reflects the results of two extensive co-operative studies directed toward selection of stable properties and reproducible procedures for characterization of streptomycetes. One study conducted under the direction of the Subcommittee on Actinomycetes of the Committee on Taxonomy, American Society for Microbiology was reported by the Chairman, Dr. D. Gottlieb (1961). A similar preliminary study on an international basis was reported for the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature by the Secretary, Dr. E. Küster (1961, 1964). The descriptive criteria are essentially the same as those included in the recommendations of this international subcommittee for descriptions of Actinomycetales appearing in patent applications (Gottlieb, 1963). The methods in mimeographed form have been used successfully for the description of type and neotype strains of more than 200 named species submitted to ISP collaborators during 1964 and 1965, and are now in use for a continuation of the project. Only minor editorial changes have been made except that the test for nitrate reduction (including medium 8, Bacto-nitrate broth) has been omitted. This characteristic proved unstable and has been dropped from the study.

It is hoped that the characterizations used in this manual will be included in future descriptions of Streptomyces species so that direct comparison can be made with descriptions for type cultures in the reference collections.

## MATERIALS AND GENERAL METHODS

### CULTURE MEDIA

Prepare Difco<sup>3, 4</sup> dehydrated culture media as instructed on labels of containers. If the dehydrated media are not used, use formulas and instructions in this manual as a guide to preparation of the media.

<sup>3</sup> All culture media described in this manual have been prepared especially for the I. S. P. by Difco Laboratories as preformulated dehydrated media. This important contribution by Difco Laboratories is gratefully acknowledged. When Difco dehydrated media are used, instructions on labels supersede instructions in the manual.

<sup>4</sup> Difco Laboratories, Detroit, Michigan, U.S.A. 48201

Sterilize culture media in the autoclave at 121°C. Sterilize loosely packed tubes or flasks containing less than 500 ml for 15 minutes; sterilize larger quantities for 20 minutes. (Do not autoclave carbon compounds to be used in the carbon utilization tests. Special instructions for sterilizing these compounds are given with medium 9.) Adjust pH of media with NaOH or HCl before addition of agar and before sterilization.

Trace salts solution (Use as directed in media 3, 4, 5 and 7 if prepared from formulas. Do not add to the corresponding Difco dehydrated media.)

FeSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0.1 g
MnCl <sub>2</sub> ·4H <sub>2</sub> O . . . . .	0.1 g
ZnSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0.1 g
Distilled water . . . . .	100.0 ml

Medium 1: Tryptone-yeast extract broth (Pridham and Gottlieb, 1948)

Bacto-Tryptone (Difco) . . . . .	5.0 g
Bacto-Yeast Extract (Difco) . . . . .	3.0 g
Distilled water . . . . .	1.0 liter

pH 7.0 to 7.2 before autoclaving

Dispense 5 ml of broth into test tubes with a diameter of 20 mm or more. Use these test tubes for initiating growth from lyophile pellet. One tube will be needed for each culture studied.

Dispense 50 ml of the broth into each 250 ml Erlenmeyer flask (or 25 ml into 125 ml flask). These flasks will be used for preparation of washed inoculum (p. 322). One flask will be needed for each culture studied.

Medium 2: Yeast extract—malt extract agar (Pridham et al., 1956-57)

Bacto-Yeast Extract (Difco) . . . . .	4.0 g
Bacto-Malt Extract (Difco) . . . . .	10.0 g
Bacto-Dextrose (Difco) . . . . .	4.0 g
Distilled water . . . . .	1.0 liter
Adjust to pH 7.3, then add --	
Bacto agar . . . . .	20.0 g
Liquefy agar by steaming at 100° C for 15-20 minutes.	

Dispense appropriate amount for a slanting into at least 6 tubes for each culture. Sterilize by autoclaving; cool tubes as slants. Use the agar slants for preparation of stock cultures (page 321).

Also sterilize medium 2 in flasks for pouring the sterilized medium into Petri dishes. Seven standard 90 mm dishes containing 25 ml per plate will be needed for each culture. (Pages 325 and 330).

Medium 3: Oatmeal agar (Küster, 1959a).

Oatmeal. . . . . 20.0 g

Agar. . . . . 18.0 g

Cook or steam 20 g oatmeal in 1000 ml distilled water for 20 minutes.

Filter through cheese cloth.

Add distilled water to restore volume of filtrate to 1000 ml.

Add trace salts solution (page 315). . . 1.0 ml

Adjust to pH 7.2 with NaOH.

Add 18 g agar; liquefy by steaming at 100°C for 15-20 minutes.

Sterilize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish will be needed for each culture. (Pages 325 and 330)

Swirl medium before pouring to assure even distribution of the oatmeal.

Medium 4: Inorganic salts-starch agar (Küster, 1959a.)

Solution I: Difco soluble starch 10.0 g. Make a paste of the starch with a small amount of cold distilled water and bring to a volume of 500 ml.

Solution II:

K <sub>2</sub> HPO <sub>4</sub> (anhydrous basis) . . . . .	1.0 g
MgSO <sub>4</sub> · 7H <sub>2</sub> O . . . . .	1.0 g
NaCl . . . . .	1.0 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> . . . . .	2.0 g
CaCO <sub>3</sub> . . . . .	2.0 g
Distilled water . . . . .	500 ml
Trace salts solution (p. 315) . . . . .	1.0 ml

pH should be between 7.0 and 7.4. Do not adjust if it is within this range.

Mix starch suspension and salts solution.

Add agar (Difco). . . . . 20.0 g

Liquefy agar by steaming at 100°C for 15-20 minutes.

Sterilize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish will be needed for each culture. (Pages 325 and 330)

Medium 5: Glycerol-asparagine agar (Pridham and Lyons, 1961)

L-asparagine (anhydrous basis) . . . . .	1.0 g
Glycerol . . . . .	10.0 g
K <sub>2</sub> HPO <sub>4</sub> (anhydrous basis) . . . . .	1.0 g
Distilled water . . . . .	1.0 liter
Trace salts solution (page 315). . . . .	1.0 ml

The pH of this solution is about 7.0-7.4. Do not adjust if it is within this range.

Agar . . . . . 20.0 g

Liquefy agar by steaming at 100°C for 15-20 minutes.

Sterilize in flasks for pouring into Petri dishes. Seven standard 90 mm dishes containing 25 ml per dish

will be needed for each culture. (Pages 325 and 330)

The final pH of the medium after sterilization with agar and solidification is about 7.4.

Medium 6: Peptone-yeast extract iron agar (Tresner and Danga, 1958)

Bacto-Peptone Iron Agar, dehydrated (Difco) 36.0 g  
 Bacto-Yeast Extract (Difco) . . . . . 1.0 g  
 Distilled water . . . . . 1.0 liter  
 pH should be 7.0-7.2 before autoclaving; adjust if necessary.  
 Liquefy agar by steaming at 100°C for 15-20 minutes.

Dispense appropriate amount for slanting into 2 tubes for each culture. Sterilize and solidify as slants. (Page 334). (Note that less than 1 liter of this medium is easily prepared by using a proportionately smaller amount of the dehydrated peptone iron agar and adding yeast extract in proportion of 0.1% of water used.)

Bacto-Peptone Iron Agar, dehydrated, contains the following ingredients when reconstituted as 36.58 grams per liter of water: Bacto-Peptone, 15 g; Proteose Peptone, Difco, 5 g; Ferric Ammonium Citrate, 0.5 g; Dipotassium Phosphate, 1 g; Sodium Thiosulfate, 0.08 g; Bacto-Agar, 15 g.

Medium 7: Tyrosine agar (Shinobu, 1958)

Glycerol. . . . . 15.0 g  
 L-tyrosine (Difco) . . . . . 0.5 g  
 L-asparagine (Difco) . . . . . 1.0 g  
 K<sub>2</sub>HPO<sub>4</sub> (anhydrous basis). . . . . 0.5 g  
 MgSO<sub>4</sub>·7H<sub>2</sub>O . . . . . 0.5 g  
 NaCl . . . . . 0.5 g  
 FeSO<sub>4</sub>·7H<sub>2</sub>O . . . . . 0.01 g  
 Distilled water . . . . . 1.0 liter  
 Trace salts solution (page 315). . . . . 1.0 ml  
 Adjust to pH 7.2-7.4  
 Bacto-Agar . . . . . 20.0 g  
 Liquefy by steaming at 100°C for 15-20 minutes.

Dispense appropriate amount for slanting into 2 tubes for each culture; sterilize and solidify as slants. (Page 333).

Medium 8: Nitrate broth (Deleted because of unreliability of the nitrate-reduction test).

Medium 9: Carbon utilization medium (Modified from Pridham and Gottlieb, 1948)

A. Sterile carbon sources

Use chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials. Carbon sources for this test are:

No carbon source (negative control)	
D-glucose (positive control)	
L-arabinose	
Sucrose	D-fructose
D-xylose	Rhamnose
I-inositol	Raffinose
D-mannitol	Cellulose

Sterilize without heat by one of the following methods:

1. Filtration. Filter sterilize 10% solution through bacteriological filter. (i-Inositol and cellulose are not sufficiently soluble for sterilization by this method—use one of the methods described below.)
2. Ether sterilization. Weigh an appropriate amount of the dry carbon source and spread as a shallow layer in a pre-sterilized Erlenmeyer flask fitted with a loose cotton plug. Add sufficient acetone-free ethyl ether ( $C_2H_5)_2O$  to cover the carbohydrate. (OBSERVE PRECAUTIONS AGAINST FIRE! Allow ether to evaporate at room temperature under a ventilated fume hood overnight or longer. When all ether has evaporated add sterile distilled water aseptically to make a 10% w/v solution of the carbon source.
3. Ethylene oxide sterilization. (Judge and Pelczar, 1955). Make a 10% w/v solution of the carbon source. Cool the liquid in an ice bath to 3-5°C and add 1 volume per cent liquid (cold) ethylene oxide with a chilled pipette or syringe. Agitate the solution. Leave it in the cold ice bath under a ventilated fume hood for

1 hour. Transfer to a warm water bath (about 45°C) UNDER FUME HOOD to permit complete volatilization of the ethylene oxide. The vapors are toxic and explosive.

Carbon sources sterilized by one of these three methods will be added to the basal mineral salts agar to give a final concentration of 1%. For example, add 10 ml of 10% solution to 100 ml basal medium, or 100 ml of a 10% solution to 1000 ml basal medium.

B. Pridham and Gottlieb trace salts (only 1 ml of this solution is used per liter of final medium)

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . . . . .	0.64 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	0.11 g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ . . . . .	0.79 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	0.15 g
Distilled water . . . . .	100.0 ml

Store at 3-5°C until required for use. Bring to room temperature before using. Prepare fresh solution each month. Disregard any precipitate or scale (probably iron salts) that forms during storage. (Only 1 ml of this solution will be used in the medium.)

C. Basal mineral salts agar (use analytical reagent grade chemicals)

$(\text{NH}_4)_2\text{SO}_4$ . . . . .	2.64 g
$\text{KH}_2\text{PO}_4$ anhydrous . . . . .	2.38 g
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ . . . . .	5.65 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . . . . .	1.00 g
Pridham and Gottlieb trace salts (B)	1.00 ml
Distilled water . . . . .	1.00 liter
Dissolve ingredients and check pH. Adjust, if necessary, to 6.8-7.0 with 1 N NaOH or 1 N HCl.	
Add agar (Difco) . . . . .	15.0 g

D. Complete medium

Sterilize basal agar medium (C); cool it to 60°C and add sterile carbon source (A) aseptically to give a concentration of approximately 1%. Agitate the mixture and pour 25 ml of medium per dish into 9 cm Petri dishes. Each organism will require 2 Petri dishes with no carbon (as a negative control) plus duplicate plates for each carbon source tested. (Page 335)



## METHODS FOR INITIATING GROWTH AND PREPARING STOCK CULTURES FROM LYOPHILE PELLET

1. Make a file scratch on ampoule at a location near upper part of looped cord (see Fig. 1).
2. Immerse the unbroken ampoule into 70% ethyl alcohol.
3. Enclose the alcohol-moistened ampoule with a piece of sterile cotton or cotton and gauze; then snap or break it at the file scratch (Fig. 2).
4. Use sterile forceps or a sterile stiff wire hook to transfer the looped string and pellet to the labeled tube containing 5 ml of sterile tryptone—yeast extract broth (Fig. 3). If a pellet fails to come out attached to the string, the cells or spores on the string will usually be adequate to start a good culture.
5. Shake the tube by hand until the pellet dissolves.
6. Incubate the tubes in a slanted position or on a mechanical shaker to give good aeration. Use of a shaker is the preferred method. Incubate at 25-28°C for 24-28 hours (or until there is evidence of spore germination or growth).
7. Look for possible contaminants with the microscope. Also streak one loopful of the broth culture onto the agar surface of a Petri dish containing medium 2 (yeast extract—malt extract agar). This plate can be examined after a few days to confirm absence of contaminants.
8. Inoculate 6 or more test tube slants of medium 2 (yeast extract—malt extract agar) and of medium 3 (oatmeal agar) with 0.1-0.2 ml of the 24-48 hour growth. Streak material over entire surface of agar slant.
9. Incubate the slants at 25-28°C for 14 days to get mature stock cultures for use in preparation of inoculum (see section which follows). Then store stock slants in refrigerator (6-10°C) until ready for use. Generally stock cultures for preparation of inoculum for characterization tests should be used within one month.

### PREPARATION OF INOCULUM

Use stock culture slants (prepared as described in the preceding section) for preparation of (A) general inoculum for all inoculations except carbon utilization test, and (B) a special washed inoculum for determining carbon utilization patterns.

#### A. Preparation of general inoculum

1. Prepare a supply of stoppered test tubes containing 3-5 ml of sterile distilled water.
2. Use a wire loop and standard aseptic technique to transfer spores, or mycelial growth, from a stock culture slant to one of the tubes of sterile distilled water.
  - a. If sporulation on the stock slant is good, transfer sufficient spore material to make a very turbid suspension in the distilled water. Normally most of the spore surface from a stock slant will be required. If necessary, use more than one slant to get a turbid suspension.
  - b. If spores are not formed, use the wire loop to transfer mycelial material to the tube of sterile distilled water. Triturate the mycelium in the distilled water with a sterile glass rod or the tip of a sterile pipette. Produce a very turbid suspension of mycelial fragments. Do not use a mycelial suspension if a good spore suspension (a) can be obtained.
3. This distilled water suspension of spores or mycelial fragments may be used immediately as general inoculum or may be held at room temperature 3-4 hours. Prepare fresh inoculum suspensions for tests performed on different days.

#### B. Preparation of washed inoculum

1. Prepare 5 ml of turbid suspension of spores or mycelium in sterile water as described for general inoculum.
2. Transfer 4-5 ml of this suspension to 50 ml of medium 1 (tryptone—yeast extract broth) in a 250 ml Erlenmeyer flask (or 25 ml in a 125 ml flask).

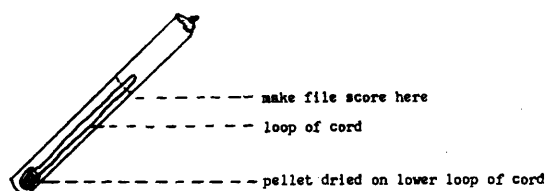


Figure 1: Sealed evacuated ampoule containing lyophilized pellet and cord

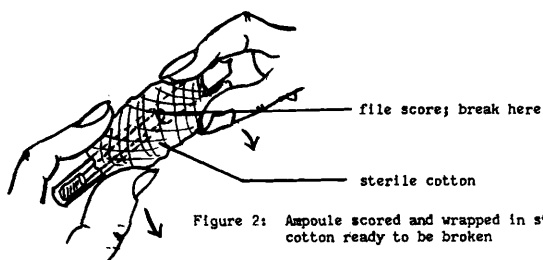


Figure 2: Ampoule scored and wrapped in sterile cotton ready to be broken

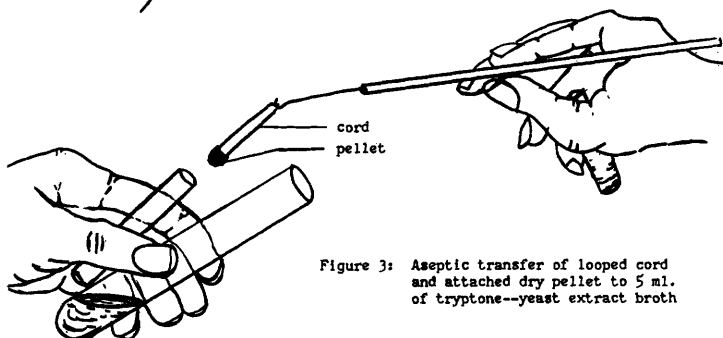


Figure 3: Aseptic transfer of looped cord and attached dry pellet to 5 ml. of tryptone--yeast extract broth

3. Incubate the flask for 48 hours at 25-28°C. If possible the flask should be aerated on a mechanical shaker during the incubation.
4. Use vigorous agitation with sterile glass beads, a mechanical blender such as the Waring Blendor, or other appropriate means to break up the 48-hour growth.
5. Transfer 5-10 ml of this fragmented broth culture into sterile centrifuge tubes equipped with sterile caps.
6. Centrifuge the suspension.
7. Decant the supernatant broth. Add sterile distilled water (or sterile 0.85% NaCl) to restore the original volume in the centrifuge tube. Mix and resuspend the washed sediment with a sterile rod or pipette.
8. Repeat steps 6 and 7. (Compare amount of sediment from different cultures. When amount of sediment is much less than normal amount, use proportionately less water for the final resuspension.)
9. Use the resuspended inoculum at once or within 3 hours to inoculate carbon utilization tests.

## PROCEDURES FOR CHARACTERIZATION OF CULTURES

### I. MORPHOLOGICAL CHARACTERIZATIONS

Accurate morphological characterization of the Actinomycetes producing catenulate spores is obviously dependent upon use of a culture medium giving good sporulation. Four media which gave good performance in this respect in previous cooperative studies are listed below as "standard" media. If these four media all fail to give good development of the sporulating aerial mycelium, then an additional medium promoting good spore formation should be used.<sup>5</sup> If it is necessary to use an additional medium, also include a record of observations of the growth on the standard media. Spore chain and sporophore morphology should be determined by observation of a fully matured culture with good

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<sup>5</sup> Cooperators in the ISP will please communicate the formula of any additional medium used to Dr. Shirling, who will then forward the formula to the other two cooperating investigators studying the culture.

spore formation. Do not use a culture in which degeneration through autolysis, hygroscopic properties, or extreme dehydration may have altered the morphology.

A. Culture media for morphological studies

1. The "standard" culture media for morphological studies for all cultures will be medium 2 (yeast extract—malt extract agar); medium 3 (oatmeal agar); medium 4 (inorganic salts—starch agar); and medium 5 (glycerol—asparagine agar).
2. Pour 7 plates of each medium using 25 ml of medium in each 9 cm diameter Petri dish.

Media should be cooled to about 50°C and dispensed aseptically into sterile Petri dishes. Poured media should be held for a minimum of 24 hours at 25-28°C to promote moderate drying and to check sterility before inoculation.

B. Inoculation of plates for morphological studies

1. Use general inoculum prepared according to directions on page 322.
2. Place about 0.05 ml of the inoculum onto the agar surface near one edge of the Petri dish (1 drop from a 1 ml serological pipette). This drop will serve as a "pool" of inoculum.
3. Use a flame-sterilized wire loop to make 5 equally-spaced streaks across the plate, dipping the loop into the pool of inoculum prior to each streak (see Fig. 4).
4. Make crosshatch streaks as shown in Fig. 5.
5. Inoculate a given culture onto at least 7 Petri dishes for each medium.
6. Incubate plates in the dark at 25-28°C. For each culture observe two plates of each medium after 7 days, two at the end of 14 days, and two at the end of 21 days. One extra plate is inoculated in case of accident.

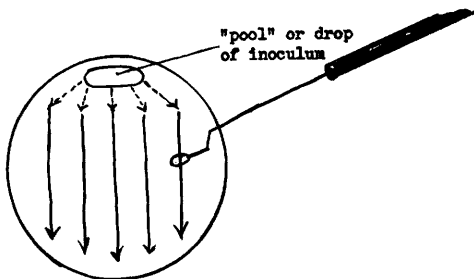


Figure 4: Five initial streaks; loop dipped into inoculum for each streak

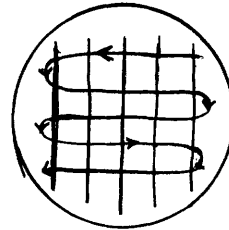


Figure 5: Cross streaks made without picking up additional inoculum

C. Determination of micromorphological characteristics of the spore-bearing hyphae

1. Determine the characteristics of the spore-bearing hyphae and spore chains by direct microscopic examination of the culture surface on opened dishes of the crosshatched cultures. Use a magnification adequate to establish the presence (or absence) of chains of spores. This will usually be 100x - 700x. A minimum of 10 microscope fields at 100x should be examined.
2. Determine the number of spores at the end of mature hyphae. State whether spores occur (a) singly, (b) in pairs, (c) in chains of 3-10, or (d) in chains of more than 10. If there is variation state the range of variation and then choose the most representative spore number (a, b, c, or d) for the culture on a given medium. Make the observation on each medium which shows good sporulation.
3. The form of the spore chain and spore-bearing hyphae should be described only with mature cultures on which many well-defined spore chains can be seen. Describe sporulating cultures in terms of the morphological groups of Pridham *et al.* (1958), modified according to Baldacci (see Figs. 6-13). In some cases, particularly with cultures producing open loops and

primitive spirals, more than one morphological type may be observed. On the basis of extensive observation of the culture surface, record all mature sporophore-spore chain types seen with an estimate of the percent of spore-bearing aerial hyphae falling into each category.

## SIMPLE

Straight - Rectus (R)  
Flexible - Flexibilis (F)  
Open loops - Retinaculum-  
Apertum (RA)  
Spirals - Spira (S)

## VERTICILLATE

Monoverticillus (MV)  
Monoverticillus-  
Spira (MV-S)  
Biverticillus (BIV)  
Biverticillus-Spira  
(BIV-S)

Undetermined (U)

4. Photograph typical morphology of the sporulating hyphae, or make simple drawings.
5. Retinaculum-Apertum: Record the approximate diameter of characteristic open loops and primitive spirals of RA cultures.
6. Verticillate: (a) Record average diameter of the main hyphae from which verticils originate. (b) Record whether or not verticils are evenly spaced along the main hyphae.

D. Special morphological observations

It is also important to look for the following characteristics (usually identified with other actinomycete groups):

1. Presence of globular sporangia as in Actinoplanaceae
2. Presence of flagellated spores, as in Actinoplanes
3. Formation of conidia-like spores on the substrate hyphae
4. Tendency of the substrate mycelium to fragment
5. Occurrence of sclerotia.

## SIMPLE



Figure 6. Rectus (R)  
or straight



Figure 7. Flexibilis (F)  
or flexuous



Figure 8.

Retinaculum-Apertum (RA)  
Open loops, hooks, or extended  
spirals of wide diameter



Figure 9. Spira (S)  
Simple spirals (not on verticils)  
Spirals may be short and compact  
or long, extended, or open

## VERTICILLATE



Figure 10. Monoverticillus (MV)  
Primary verticils or whorls  
distributed on a long axis or  
branch; no spirals

True verticillates have main  
axis of wider diameter than  
branching hyphae, and have  
uniform spacing between  
verticils.



Figure 11.  
Monoverticillus-Spira (MW-S)  
Primary verticils or whorls  
distributed on a long axis;  
elements of verticils spiralled

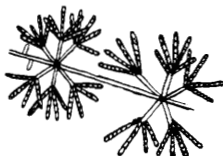


Figure 12. Biverticillus (BIV)  
Compound verticils or whorls on  
a long axis; no spirals



Figure 13.  
Biverticillus-Spira (BIV-S)  
Compound verticils; elements of  
secondary verticils spiralled

Figures 6-13. Reproduced from Pridham *et al.* (1958) with  
permission of authors and publisher.



### E. Spore morphology and surface

Laboratories having access to an electron microscope should include electron micrographs of the spore surface as one of the descriptive characterizations for each type culture. Electron micrographs should also show spore chains. Suggested method:

1. Use the same crosshatch Petri dish cultures prepared for observation under the light microscope.
2. Electron microscope specimen grids coated with Formvar or collodion are gently pressed to the aerial surface of a culture with mature spores.
3. Spore chains which adhere to the coated surface of the grids are observed and photographed in the electron microscope without fixing or shadowing at a magnification of 8,000x - 10,000x.
4. Preserve the picture record to include with the report. In addition to the photographic record, spore silhouettes should be characterized as smooth, spiny, hairy, warty (see Fig. 14).



Figure 14.

## II. COLOR DETERMINATIONS

Color determinations should be made for (1) the mass color of mature, sporulating aerial surface growth, (2) for the color of the substrate mycelium as viewed from the reverse side, and (3) for diffusible soluble pigments other than melanins. The Tresner-Backus color series, adopted by participants in the 1962 Montreal Workshop (Küster, 1964; Tresner and Backus, 1963) is specified for spore mass color determinations. In this system, seven color series

are delineated by color wheels made with tabs from the 3rd or 4th edition of the Color Harmony Manual (Jacobson et al. 1948; Eckerstrom and Foss, 1958).<sup>6</sup> The Tresner-Backus color guide is not designed for determination of substrate mycelium colors or colors of soluble pigments. Although a color guide is not required for these colors, the substrate mycelium color may be identified in terms of a special guide<sup>7</sup> prepared for the I.S.P. by H. Prauser (1964) or in terms of the Bondartsev (1954) color scale.

A. Culture media for color determinations

Use the following media: Medium 2 (yeast extract—malt extract agar); medium 3 (oatmeal agar); medium 4 (inorganic salts—starch agar); and medium 5 (glycerol—asparagine agar). The crosshatch plates that were prepared for morphological studies may be used for this purpose.

B. Observations

1. Observe plates at 7, 14 and 21 days as prescribed for morphological studies. Color determinations should be recorded only for mature cultures.
2. Use north-window daylight on a bright day. If this is not possible, use a balanced and reproducible system of artificial light simulating north sky daylight.

C. Determination of aerial mass color

1. Observations should be limited to mature cultures with a heavy spore mass surface.

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<sup>6</sup> A complete set of Tresner-Backus color guides has been provided to each participant in the I.S.P. Printed patterns for assembling the color wheels, together with reprints of the Tresner-Backus paper can be obtained without cost from Prof. E. B. Shirling, I.S.P., Ohio Wesleyan University, Delaware, Ohio 43015, U.S.A. Color Harmony Manual chips used in the guide may be purchased from Color Standards Department, Container Corporation of America, 38 South Dearborn St., Chicago, Illinois 60603, U.S.A.

<sup>7</sup> Prepared for I.S.P. participants by H. Prauser (1964) from color tabs of Baumanns Farbtonkarte Atlas I.

2. Determine the Tresner-Backus color series (Red, Yellow, Green, Blue, Violet, Gray or White) by comparing mature spore mass color on all appropriate media with the Tresner-Backus color wheels. Use only the mat (dull) surface of the color tabs. Most cultures will fall within the range delineated by one wheel. There are a few instances in which color tabs assigned to two different wheels are similar in color. If a culture is intermediate between these two tabs from different wheels, it may be difficult to choose one series as better than the other for describing the culture. In these rare cases classify the culture in both series.
3. Identify by number-letter code (printed on the tab), the one color tab which most nearly characterizes the spore-mass color. Also record the ISCC-NBS (U.S. Dept. of Commerce, 1955) name listed in the color wheel folder for the tab chosen to characterize the spore-mass color. If the color is intermediate between two tabs, designate both.

D. Color of substrate mycelium (reverse color)

The color of the substrate mycelium is determined by observing the reverse (under) side of mass growth on the various media. A method for removing most of the agar medium is described below. A simple guide for use in cutting off the agar is provided.

1. Place the cutting guide onto layers of filter paper or paper towel saturated with wet disinfectant solution (see Fig. 16). (Also provide a beaker of disinfectant for discarding work tools.)
2. Use a cork borer (or similar sharpened cylinder) with a diameter slightly less than the hole in the template to cut an agar plug from typical mature mycelial growth on a Petri dish culture (see Fig. 15). Determine color for (a) mature substrate mycelium and (b) the relatively young edges of the spreading growth.
3. Push the plug from the cylinder with a glass rod so that it turns over and drops inverted onto the cutting guide (Fig. 16). Push the inverted plug into the hole in the cutting guide.

4. Slide a razor blade across the guide slicing off all agar medium which extends above the level of the surface (Fig. 17).
5. It is now possible to observe the reverse side of the culture plug remaining in the cutting guide. Very little agar will remain to interfere with color determination.
6. Assign the culture to one of the following color groups: (Szabó and Marton, 1964)
  - yellow-brown
  - yellow-brown + red (or orange)
  - yellow-brown + blue or violet
  - yellow-brown + green
7. In addition, if either Prauser or the Bondartsev color code or the complete Color Harmony Manual is available, identify the color with the nearest matching panel on one of these color guides.
8. Determine the response of color of substrate mycelium to pH change by adding a drop or more of 0.05 N NaOH and 0.05 N HCl to the specimen prepared as described in steps 1-4 above. Make observations immediately and after 10 to 15 minutes.

E. Soluble colors other than melanoid pigmentation

If soluble colors other than brown or black are produced on any medium (or if brown is distinctly modified with red, yellow, green, blue, or violet), 1) record the medium; 2) record the color as the simple unmodified color name (red, orange, yellow, green, blue, violet—use no other color terminology); and 3) in every case of soluble color other than melanoid pigment, determine response of color to pH change by addition of a drop or more of 0.05 N NaOH and 0.05 N HCl to the colored agar. Record whether or not soluble color is affected by pH change. Make observations immediately and after 10 to 15 minutes.

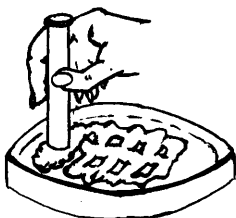


Figure 15: Cut agar plug from a broad mass of mature growth.

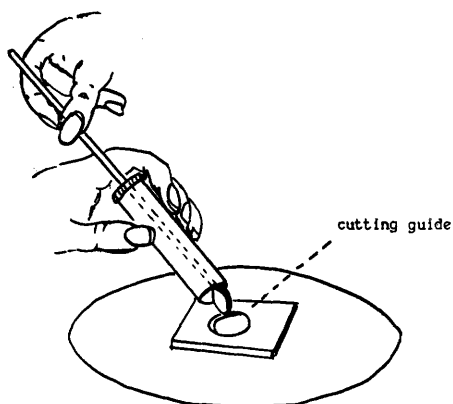


Figure 16: Push plug so that it drops inverted into the cutting guide.

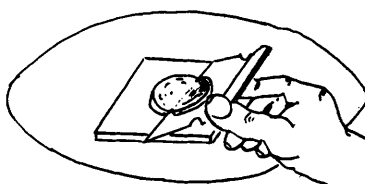


Figure 17: Use razor blade to cut off and discard all agar which extends above the surface of the cutting guide. Observe the exposed reverse side of the mycelium which remains in the cutting guide.

### III. PHYSIOLOGICAL CHARACTERISTICS

#### A. MELANIN PRODUCTION

##### 1. Culture media and inoculation for melanin production

Determine production of melanoid pigments on agar slants of medium 6 (peptone iron agar, Difco, supplemented with 0.1% yeast extract), and medium 7 (Shinobu's modification of Masumoto's tyrosine agar). Also observe other organic media, especially medium 1 (tryptone—yeast extract broth, used for obtaining growth from lyophile pellet).

Use agar slant cultures prepared as described under MATERIALS AND GENERAL METHODS as inoculum for media 6 and 7. Cultures used as inoculum source should be less than 3 weeks old (except for cultures unusually slow in producing mature aerial growth). Use a heavy inoculum of spores and aerial mycelium picked up on a standard wire loop. Streak this inoculum up the surface of the agar slant. Inoculate each experimental culture onto 2 slants of medium 6, and 2 slants of medium 7.

##### 2. Observations and interpretations

- a. Observe melanoid pigments on medium 6 and medium 7 after 2 days and after 4 days. Compare inoculated tubes with uninoculated controls. Cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color shall be recorded as positive (+). Absence of brown to black colors, or total absence of diffusible pigment, shall be recorded as negative (-) for melanoid pigment production.
- b. Other organic media containing peptones or tyrosine, including medium 1 (tryptone—yeast extract broth) used for initial growth from the lyophile pellet, should be observed for production of melanoid pigments. The presence of pigments in these media should be included in the record.

## B. CARBON UTILIZATION

### 1. Basal medium and carbon sources

Detailed instructions for medium 9 (Pridham and Gottlieb carbon utilization medium) including carbon source sterilization are given on pages 318-319.

After autoclaving the basal agar medium, cool it to 60°C and add sterile carbon source aseptically to give a concentration of approximately 1%. Agitate the mixture and pour 25 ml of medium per dish into 9 cm Petri dishes. Prepare duplicate plates of each carbon compound for each culture to be tested. Store medium in refrigerator.

Carbon sources and controls required for the test are repeated below:

No carbon source (negative control)	
D-glucose (positive control)	
L-arabinose	
Sucrose	D-fructose
D-xylose	Rhamnose
I-inositol	Raffinose
D-mannitol	Cellulose

### 2. Procedures for carbon utilization tests

- a. Prepare a washed inoculum as described on pages 323-324.
- b. Dry the uninoculated plates by leaving them at room temperature for 4 hours after they are freshly poured or after removing them from refrigerator storage.
- c. Place approximately 0.05 ml of washed inoculum (1 drop from a sterile 1 ml serological pipette or dropping pipette) onto one edge of the agar surface. Streak the drop straight across the dish (see Fig. 18). Repeat with a second drop. Inoculate duplicate plates. Use only one culture per plate to avoid false positives due to cross feeding.
- d. Observe plates at 10-16 days. Always compare growth on a given carbon source with the two controls; growth on basal medium alone, and growth on basal medium plus glucose.

e. Record results as follows:

Strongly positive utilization (++), when growth on tested carbon in basal medium is equal to or greater than growth on basal medium plus glucose.

Positive utilization (+), when growth on tested carbon is significantly better than on basal medium without carbon, but somewhat less than on glucose.

Utilization doubtful ( $\pm$ ), when growth on tested carbon is only slightly better than on the basal medium without carbon and significantly less than with glucose.

Utilization negative (-), when growth is similar to or less than growth on basal medium without carbon. (Always record utilization as negative if growth is not better than no-carbon control.)

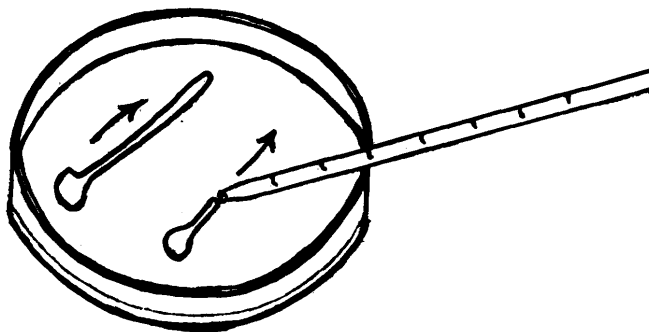


Figure 18.

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