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During the past generation, Bacteriologists and Plant Pathologists have developed a technique for isolating unicellular forms of life. The purposes of these isolations have been to study the life history, polymorphism, physiology, parasitism and finally the classification of organisms. A brief historical review of these methods of isolation, especially those of single spores, might aid investigators in their researches and stimulate them to construct better apparatus and employ a superior technique.

Early Methods

Single celled forms of Flagellata, Protozoa and Algae were the first to be isolated. They were isolated by drawing one of the individual cells with water into a pipette fitted with a rubber bulb. Records state that this was the method commonly employed previous to the 19th century. Many of these single celled organisms could be seen with the unaided eye but a magnifying glass was necessary to locate some. However, the microscpe was of little assistance since it was not in general use.

Development of Modern Methods

The development of single spore isolation of microscopic forms such as spores of bacteria and fungi may be divided into two phases or methods: the dilution or physiological and the mechanical.

The dilution method: Lister (1878) was the first to describe the dilution method as applied to his investigation of lactic acid fermentation. By this method, an accurately measured sample of the liquid is placed in a flask, from this one-tenth is removed to another flask containing sterile water equal to nine-tenths of the original sample. This latter form of dilution is continued to several attenuations by removing the solution from the last flask diluted. Counts of bacteria in a measured amount of liquid from any one of the dilution flasks can then be made by the plating-out method and the number of bacteria in the original culture estimated mathematically.

To de Bary (1872) belongs the honor of proclaiming the necessity of watching uninterruptedly, the development of a single spore from its germination to the formation of its hyphae and conidia (Van Tiegham 1873). In 1873, Van Tiegham published his classical work on the Mucors. In his researches, he cultured single conidia of Penicillium, Mucors, Achlya, Thamnidium, Circinella, Chaetocladium and Syncephalis from beerwort. By doing so, he proved that these are not different forms of one fungas but various fungi. As a result, Circinella, Chaetocladium and Syncephalis were named as new genera and set aside as new and separate plants. Thus the phylosophical idea of the polymorphism of these forms was exploded by scientific application of single spore isolations.

In 1881, Brefeld employed the dilution method whereby a spore suspension in sterile water was so diluted that one drop removed with a "spear needle" generally bore one spore. He employed hanging drop slides and used sterilized fresh horse dung decoction as a medium. Brefeld did his work so thoroughly and his results were so conclusive that they are still regarded as models for myceticulturists, plant pathologists and mycologists. For this reason, it seems as if very little progress was made in single spore isolation for the next quarter of a century.

In 1905, Lindner employed Brefeld's drop slide culture methods but used a writing pen to transfer drops of the beerwort culture to the drop slides. Rosenbaum (1912), culturing Thielavia, and Anderson (1913), culturing Endothia, isolated conidia by placing spore dilutions on solidified agar in Petri dishes. By inverting these dishes on the stage of the microscope, they located germinated and ungerminated conidia, marked their location on the Petri dish and transferred single conidia with a needle. Strasburg (1913) streaked a spore suspension on cover glasses and those that showed but one conidium when examined with a microscope were then placed on a medium so that the conidium could germinate.

In 1915, Keitt explained a cylinder loop and its use in transfering a single spore with its substrate of agar to a desired environment. He also emphasized and described a good method for clearing agar so as to remove undesirable particles which would obscure the view of conidia when the observer was locating them wtih a microscope. Langenon (1921) prepared a spore dilution, removed drops in a (needle) loop and examined them with a microscope. Those drops which showed only one conidium present were transferred to drop slide cultures.

In 1922, Kniep employed a 2 per cent malt extract and gelatin

(10 per cent?) as a culture medium for germinating smut spores. He observed that spores germinated better on gelatin than on agar so his dilutions were made on gelatin. Goldsmith (1928) employed in his smut spore studies a culture solution consisting of 2 per cent agar and 3 per cent malt. He germinated smut spores in fermenting tubes, collected the "ooze" and isolated sporidia by "bacteriological methods" (dilution).

MECHANICAL METHODS OF SPORE ISOLATION: One versed in the technique commonly employed in the dilution method and in culturing by the drop slide or Van Tiegham cell knows that there are numerous chances for the entrance of foreign fungi and other organisms. Furthermore, since these chances exist, the investigator's results would be uncertain and often inconsistent. Scientists could not be satisfied until these chances were eliminated and the results consistent when the experiment was duplicated. To meet these demands, scientists felt that they must actually see the spore before, during and after it was transferred so that they could be sure that one and only the one was present in the culture. Then and only then could the investigator be satisfied that he possessed a pure monosporous culture as a starting point. So this demand lead to a partial substitution of the mechanical method for the dilution or physiological method. However, the two are somewhat combined by some investigators.

In 1914, when it seemed as if the possibilities of the spore dilution method for isolating single spores had been exhausted, Barber invented the micromanipulator. This machine enabled its operator to control one glass pipette by adjusting delicate controls. This machine aided him in carrying out his claim that an individual spore of a bacterium "gave better cultures than vegetative individuals." Chamber's modification of Barber's micromanipulator was described by Kahn in 1922. These bacteriologists were interested in isolating spores of bacteria and not especially concerned with spores of fungi.

The micromanipulator was a further development of the pipette and needle methods of spore isolation. In 1909, Lindner transferred yeast cells on the point of a needle for his pure cultures. However, Küster (1907) mentioned the needle method (p. 58) together with the operation of Byerinck's capillary tube (pipette).

Edgerton (1914) was a pioneer of isolating single spores of fungi with a micromanipulator. He described the laboratory construction of a micromanipulator and its operation by means of capillary tubes. In 1923, Roberts published his modification of

Edgerton's apparatus showing how the condenser of a microscope could be employed for assisting in the manipulation of the glass tube with a bulb. Furthermore, he described how to draw a spore from the spore dilution by placing ether on the bulb of the tube and, after microscopic examination, expel the spore on the agar by heating the bulb. A good illustration of the apparatus is shown. Bauch (1923) and Kniep employed an excellent single spore technique in isolating sporidia of the smut fungi under investigation. Their single spore isolations and culture work has fairly revolutionized our former ideas of smut fungi. Bauch described an attachment for the objective of the microscope by which single spores could be isolated.

In 1924, Funcke painted cover glasses with a two per cent gelatin and a drop of glycerin solution. The spores (Hypholoma, and Collybia) were either collected or dusted on the prepared cover glass. With a glass tube drawn to a needle-point, he collected individual spores with a micromanipulator, then transferred each to malt extract gelatin cooled to 40° C. Hanna (1924) while working with Coprinus employed a method similar to Funcke's only he transferred the spores to drop slide cultures. These drop slide cultures were composed of ring cells several of which were cemented to the lower surface within a Petri dish which was lined with damp filter paper. Brown (1924) allowed the spores to germinate and then cut-out hyphal tips from the colonies and transferred them to suitable agar. Dunn (1924) made a loop from a capillary tube, removed loop-fulls from his spore dilutions and those that contained but one spore, when examined by the microscope, were transferred to agar.

In 1926, Dickinson described the construction and operation of a micromanipulator which he employed. A portion of the side wall in a large Van Tiegham cell of paraffin was removed so that the arm of the micromanipulator could be so regulated as to insert a glass needle for isolating. The cover glass of the Van Tiegham cell was coated with agar and spores spread on it. The glass needle of the micromanipulator was so controlled as to float a spore from the drop of liquid to an isolated position on the agar cooled to 40° C. An individual spore together with the melted agar was drawn in a capillary tube with a diameter slightly larger than the spore. The glass tube was then carefully examined microscopically and if it contained only one spore, it was sterilized externally with alcohol and broken in suitable agar.

There are comparatively few operative micromanipulators in

our American colleges of to-day. For this reason, scientific work by the faculty and graduate students in spore isolation has been handicapped. Furthermore, too little attention has been given to monosporous cultures in our phytopathological laboratories. Every now and then we are startled by the results of investigators who have investigated some homothallic or heterothallic form. Much remains to be done in the future for there are many homothallic and heterothallic forms, especially of basidiomycetes and ascomycetes, to be investigated and interesting problems to be solved.

DIRECTIONS FOR MAKING A SINGLE SPORE ISOLATION

The preceding brief descriptions of variations in the physiological and mechanical methods would be confusing to one wishing to perform a single spore isolation. A brief outline of the procedure follows but it must be assumed that the experimenter will observe the usual necessary aseptic precautions practiced by bacteriologists.

1. Employ germinable spores.
   Be sure the spores are after-ripened and will germinate; know the optimun temperature, moisture, periodicity of germination, proper substrate; how to sterilize the surfaces if necessary; washing methods; specific gravity — sink or float; the sample is free from other conida or pure.
2. Prepare spore dilutions.
   a. Four test-tubes; each containing about 10 cc. distilled water, plugged, sterilized, cooled.
   b. Place enough of the spore sample in test-tube A so that when well agitated and a loopfull is removed, five or more spores are present.
   c. Place five or more loopfulls from test tube A into another test tube of the sterile water, B. Agitate and examine loopfulls to determine the number of spores in each loop.
   d. Continue the dilutions until one loopful generally contains a single spore.
3. Prepare agar plates and slants of the culture medium and the isolating medium. Note; if the culture medium is clear, it may be used for both processes; otherwise, prepare a clear agar for an isolating medium;
   a. The proper agar, culture solution or substratum for the fungus should be known. Prepare Petri dishes and test tubes of the culture medium.
   b. Also, prepare 8 Petri dishes containing isolating or cleared agar on which the spore dilution is to be poured. Isolating medium or agar may be prepared by one of the following methods:
      1. By employing Keitt's method of clearing with the white of an egg.
      2. By allowing the agar (potato, oat, etc.) to settle and use the clear portion which melts first, in a water bath.
      3. By allowing the agar to harden in an open jar and with a spoon

remove the clear portion at the surface and discard the sediment.

4. By careful preparation of the agar; filtering the ingredients.
5. By using 2 per cent agar agar in water (plane agar).

c. Two or more drops of lactic acid may be added to each 20 cc. of melted agar in the Petri dishes to check the growth of bacteria.

4. Distribute the spores on agar or mix them with the agar.

a. Pour just enough of the spore dilution from test tube A on the isolating agar in each of two Petri dishes to moisten the surface. Surplus water (dilution) should be poured off after flaming the edge of the Petri dish. Likewise, prepare two Petri dishes from each test

b. If one prefers, test tubes of the culturing agar may be melted, cooled in water at 40° C. and various amounts of the spore dilutions added until several loopfulls must be removed before a spore is located. However, if too much of the spore dilution is added, the agar will not harden unless to it has been added an extra amount of agar agar in its preparation (3-5 per cent). While this agar agar spore dilution is still in a liquid state, it is poured in a Petri dish and allowed to cool. Then the spores may be isolated as in the methods described below.

5. Transfer ungerminated spores.

Invert a Petri dish, which contains isolating agar with spore dilution on its surface, on the stage of the microscope so that the bottom is uppermost; focus downward until the surface of the agar is reached then move the dish around until spores are located. If individual spores are located in a field where no others are nearby, mark their location with India ink. This is done by placing the inked pen in the field of the microscope and drawing an inkline, on the glass dish, extending from the spore to the edge of the microscopic field. Remove the Petri dish and draw a circle around the spore at the end of the line. Reexamine and be sure that no other spore is within the inked circle. Likewise mark the desired number of spores to be isolated. If the spores are too numerous for isolating individuals, use one of the other isolating agar plates which may contain spores distributed suitable for isolation. With a needle, small spatula, or cylinder loop as described by Keitt, remove the agar over a marked circle with the spore on its surface and transfer it to a desirable substrate and environment. Spores in the hardened agar may be isolated in a similar manner.

6. Transfer germinated spores, colonies or portions of colonies.

Many investigators prefer to transfer germinated spores for the following reasons:

a. Viable transfers are guaranteed.
b. Time is saved.
c. Contaminations can be located if present and avoided in the transfers.
d. Germinated spores are easier to locate and mark.
e. Higher percentage of the transfers is successful.

The procedure in isolating germinated spores is similar to that described for ungerminated spores. The spore dilutions on, and sometimes in, the isolating agar are incubated and when the spores have germinated sufficiently, a block of agar with the germinated spore is transferred to culture tubes.

7. Loops and streaks.
   a. Place eight or more loopfulls of the different spore dilutions on eight marked sectors of the agar in each of several Petri dishes. Each of these areas or sectors is examined and those containing one spore are marked and when the spore has germinated, the colony is transferred to culture tubes. Students who have used this method prefer it to any of those already described.
   b. Loops of a concentrated or diluted spore dilution may be streaked on the surface of agar, examined with a microscope and single spores isolated by one of the methods mentioned above.

8. Before operating a micromanipulator, the investigator should read the references, following this article, relating to its structure and operation. Space does not permit detailed description or the instruments and their operation.

SUMMARY

1. Macroscopic, unicellular animals and plants were first isolated in a pipette fitted with a rubber bulb.

2. Microscopic forms of bacteria and fungi have been isolated by two types of technique: dilution methods and mechanical methods.

3. The idea of the dilution method for isolating fungus spores was borrowed from bacteriologists but modified to meet the needs of myceticulturists.

4. Lister was first to describe the details of the dilution method for the isolation of single spores. De Bary was first to show need for single spore isolation in his observations of fungi.

5. Van Tiegham in 1873, exploded the philisophical idea of polymorphism of the mucors' and showed yeast, Mucor, Thamnidium, Penicillium, Achyla, Circinella, Chaetocladium and Syncephalis are not different forms of one Mucor.

6. Brefeld's isolations and culture work has remained a model for fifty years. It has been stated that "he was first to show that polymorphism of Mucors did not exist" (1872).

7. Modification of Brefeld's isolation and culture methods have been employed by Rosenbaum, Anderson, Keitt, Kniep and others. Each modification has added to the efficiency of the method.

8. Directions for making single spore isolations by the dilution method and its modifications are outlined. Suggestions for the operation of the micromanipulator are also given.

9. The first micromanipulator in America was constructed by Barber: Chambers modified it and his apparatus was described by Kahn.

10. Edgerton (1912) was the first investigator to construct his own micromanipulator for isolating spores of fungi and describe its operation in his research work.

11. Roberts modified Edgerton's apparatus and employed Byerinck's idea of capillary tubes instead of glass needles.

12. Dickinson also described a micromanipulator which can be constructed by an investigator. It combined the Van Tiegham cell and glass needle methods.

13. Monosporous cultures of fungi, more especially of the basidiomycetes and ascomycetes, have not received sufficient attention in our mycological and

plant pathological laboratories. There is still opportunity for further inventions of apparatus which will successfully isolate single spores and remain reasonable in price.

REFERENCES

1. Anderson, P. J. and Anderson, H. W. The chestnut blight fungus and a related saprophyte.
Penn. Chestnut Blight Comm. Bull. 4: 11.1913. Also, Phytopath. *2*: 5: 204-205. 1912.
2. Andrews, F. M. Devices for sowing and growing spores.
Pl. Physiology *2*: 216-219. 1927.
3. Barber, D. The micromanipulator.
Philippine Jour. of Science. *9*: 307. 1914.
4. Bauch, R. Ustilago longissima und ihre varietät Microspora.
Zeit. f. Bot. *15*: 5: 254-256. 1923.
5. Brefeld, O. Botanische Untersuchungen über Schimmelpilze.
1: 25. 1872.
4: 14. 1881.
6. Brown, W. A. Two mycological methods. I. A simple method of freeing fungal cultures from Bacteria. II. A method of isolating strains of fungi by cutting out a hyphal tip.
Ann. Bot. *38*: 401-409. 1924.
7. Chambers, R. A micromanipulator.
Journ. Bacter. *8*: 1-5. 1923.
Journ. Inf. Dis. *21*: 334. 1922.
8. Cook, M. T. A method in micro-technique.
Jour. Dept. Agr. Port Rico. *4*: 11. 1924.
9. De Bary, A. Recherches sur le development les ustilaginées.
Ann. d. sci. nat. bot. *20*: 5. 1863.
10. Dickinson, S. A simple method of isolating and handling individual fungal spores and bacteria.
Ann. Bot. *40*: 273. 1926. Also, Proc. Roy. Soc. Medicine *19*: 1-4. 1926.
11. Dunn, M. S. The microloop. A rapid method for isolating single spores.
Phytopath. *14*: 338. 1924.
12. Duggar, B. M. Fungus diseases of plants.
p. 10-12. 1909.
13. Edgerton, C. W. The bean anthracnose.
La. Agr. Exp. Sta. Bull. 119: 18-27. 1910. (About spores)
14. Edgerton, C. W. A method of picking up single spores.
Phytopath. *4*: 115. 1914.
15. Funcke, G. L. Ueber die Isolierung von Basidiosporen mit dem Mikromanipulator nach Janse und Péterfi.
Zeit. f. Bot. *16*: 619-623. 1924. No. 10. (Aleurodiscus, Collybia, Hypholoma.)
16. Goldschmidt, V. Vererbungsversuche mit den biologischen Arten des Antherenbrandes (Ustilago violacea Pers.).
Zeit. f. Bot. *21*: 16. 1928-29.
17. Hanna, W. F. The dry needle method of making monosporous cultures of Hymenomycetes and other fungi.

Ann. Bot. *38*: 791. 1924.

18. HANNA, W. F. A simple apparatus for isolating single spores.
Phytopath. *18*: 1017. 1928. No. 12. Illustrated.
19. HANSEN, H. N. A simple method of obtaining single spore cultures.
Science *64*: 384. 1926. O, 15.
Phytopath. *16*: 10: 763. 1926.
20. KAHN, M. C. Chambers' micromanipulator for the isolation of a single bacterium.
Jour. of Inf. Dis. *31*: 4: 344-348. 1922.
21. KEITT, G. W. Technique for isolating fungi.
Phytopath. *5*: 5: 266-269. 1915.
22. KLEBS, E. Beiträge zur Kenntniss der Micrococcen.
Arch. f. Exp. Path. u. Pharmakol. *1*: 31-64. 1873.
23. KNIEP, H. Über Geschlechtesbestimmung und Reduktionsteilung.
Verh. d. Phys. Medic. Gesell. z. Würzburg *47*: 3. 1922.
24. KUSTER, E. Kultur der Microorganismen.
Leipzig und Berlin. 1907. p. 54-62.
25. LANGENON, M. Précis de Microscopie.
Masson & Cie. Paris, 1921. p. 845.
26. LINDNER, W. Mikroskopische Betriebskontrolle in dem Gärungsgeuerben.
Berlin, 1909. p. 230.
27. LISTER, J. On the lactic fermentation and its bearings on pathology.
Trans. Pathol. Soc. London. *29*: 425-467. 1878.
28. POVAH, A. M. A critical study of certain spores of Mucor.
Bull. Torr. Bot. Club *44*: 242-245. 1917.
29. PALLADIN, V. I. Plant physiology (Authorized English edition).
1918. p. 56.
30. ROBERTS, J. W. A method of isolating single spores.
Phytopath. *13*: 12: 558. 1923.
31. ROSENBAUM, J. Infection experiments with Thielavia basicola on ginseng.
Phytopath. *2*: 5: 194. 1912.
32. STAKMAN, E. C. Species concept.
Amer. Jour. Bot. *10*: 243. My. 1923.
33. STRASBURG-KOERNECKE Botanisches Praktikum.
Fünft tuflage 1913. p. 506.
34. TEMPLE, C. F. Methods of determining the time of discharge of apple scab spores.
Rept. of Maryland Sta. Hort. Soc. *29*: 203-214. 1927.
35. VAN TIEGHAM AND LE MONNIER. Recherches sur les Mucorinées.
Ann. d. soc. natur. (5 ser.) *17*: 273. 1873.

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