An Effective Use of Petri Dishes for Microcultures

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SUMMARY: A microtechnique has been developed for the growth of many cultures in one Petri dish. The agar substrate is in the form of small plaques pipetted so that each plaque is distinct and separate from its neighbour in the Petri dish. The method is used for the assay of antifungal materials and has been adapted to the isolation and biochemical characterization of micro-organisms. Work is in progress to adapt the procedure to yeast and bacterial counts.

During work on the isolation and characterization of antibiotic substances, it was found essential to develop methods whereby the time of the investigator and the necessary materials were used more efficiently. Microtechniques appeared to hold the most promise. Microtechniques developed before and on the turn of the century have, for the most part, been neglected by later bacteriologists.

Koch (1881) was the first to recommend the use of slide cultures of semi-solid media for the microscopic observation of growth. The ‘Little Plate Method’ (Frost, 1921) is essentially a modification of Koch’s technique. An accurate volume of milk (0.05 ml) is mixed with a drop of agar on an accurately defined area on a sterile microscope slide. After incubation in a moist chamber for 4–16 hr., the area is dried and stained, and the colonies are counted under the microscope. In Burri’s slant method (1928) an accurately shaped loop of a known diameter is dipped in milk and then run across the surface of an agar slant in a zig-zag manner so that the milk is spread evenly. After incubation, the colonies on the surface are counted.

Beijerinck (1889) introduced the auxanogram for the biochemical characterization of yeasts. The agar medium, heavily seeded with yeast, is poured into Petri dishes; when it is set, small amounts of the powdered test sugars are placed on the agar sufficiently far apart to prevent mixing when the powders have dissolved. Growth results in the sections where the utilizable sugar has diffused into the agar.

Lindner (1905) suggested a microtechnique for broth cultures. The substrate is inoculated with the test organism and drops of the resulting suspension are placed on a cover-glass by means of a sterile pen; the cover-glass is inverted on a hollow ground slide so that the hanging drops do not come in contact with the slide. Appropriate dilutions are made so that when observed microscopically, the growth developing from a single cell is seen. Henneberg (1934) adapted the procedure for his ‘Federstrichkulture’ technique. A broth medium is inoculated with the test organism and the suspension is streaked on a sterile cover-glass with a sterile pen point. Between streaks, the point is rinsed with uninoculated broth so that each successive streak is more dilute. The cover-glass is inverted on a hanging-drop slide and is sealed with a vaseline ring. After incubation, observation is made under the microscope.
Bronfenbrenner & Schlesinger (1918) described a micromethod for the identification of bacteria by fermentation tests; the substrate containing the agar, indicator and the sugar were deposited in droplets from a sterile capillary pipette in a Petri dish. These droplets, identified by markings on the outside of the Petri dish, were inoculated with the culture, and a piece of filter-paper soaked in 2 % NaOH was placed in the inverted Petri dish to absorb the CO₂ produced. This technique for the identification of coliform bacteria is very similar to the one developed independently in this laboratory, but it is the purpose of this paper to give the method a wider range of application.

The universally accepted cylinder plate method for antibiotic assay described by Heatley (1944) is essentially a modification of the auxanogram of Beijerinck. Small cylinders on an agar medium preseeded with the test organism are filled with the test solution. This diffuses through the agar and the resultant zones of inhibition are measured. The procedure to be described was an attempt to improve this method.

METHODS

Preparation of Petri dishes. Agar containing the nutrient materials and reagents or indicators to be used, is pipetted aseptically and falls as droplets on to the flat bottom of a Petri dish. The average volume of these droplets is 0.05 ml. and usually 2 droplets (0.1 ml.) are placed on the same site to cover an area of 1.0–1.2 cm. in diameter. Measured volumes of the agar may be pipetted when greater accuracy is desired. The agar solidifies almost immediately into circular plaques which are raised when the temperature of the agar droplets is close to the solidification point and flat when the temperature is higher. When comparisons are not quantitative, the agar may be poured directly from the test-tube containing the medium. The shape of the plaques may be varied at the time the droplets fall; an oval or narrow strip is obtained by placing 2 or 3 drops in a row.

The agar concentration in the original substrate is adjusted so that the solution will not be too fluid when the reagent under test is added. Generally a 2 % agar medium is used when the reagent is to be added up to 20 % concentration. When the reagent concentration is above this percentage, the agar in the original substrate is raised to 3 or 4 %.

With this technique, 8–30 plaques can be poured in each plate. As the agar solidifies almost immediately, the Petri dish may be inverted and agar plaques poured on the cover, so that the number of plaques is doubled.

The procedure may be adapted to the cylinder plate method of assay: the quantity of agar in each plaque is 0.2 ml. and the surface area correspondingly greater. A cylinder is firmly sealed in the centre of each plaque before the agar sets and the test solution is pipetted into the cylinder instead of being mixed with the substrate. The agar concentration used is 1.5 %.

Inoculation. The test organism may be inoculated in any of the following manners: (1) An active cell suspension is added to the agar before it is poured. (2) Each plaque is inoculated from an inoculating needle which has been dipped in a cell suspension of the test organism. (3) A sterilized cotton-wool
Microcultures in Petri dishes

A ball, about 2 mm. in diameter, is dipped in a suspension of the test organism and then smeared evenly on the plaques so that the whole surface of each is covered. Sterile technique is used throughout.

This last procedure was found especially useful when the microcylinder plate technique was used. After the cylinder had been set in the agar and filled, the surrounding agar was smeared with the cell suspension.

**Incubation.** Inoculated Petri dishes are incubated at the optimal temperature of the test organism. For temperatures up to 37° the plaques retain sufficient moisture for the duration of the assay (up to 2 weeks at room temperature); above 37°, the plaques dry out before clear results can be obtained. For temperatures above 37° a beaker about 20 mm. in diameter and 8 mm. high, containing a small piece of cotton-wool, is partially sterilized in boiling water and then removed with flamed forceps. The excess water is poured off and after being flamed momentarily, the beaker is placed in the inoculated Petri dish on a drop of agar, which is melted by the heat of the beaker. When the agar sets it prevents movement of the beaker in the dish. It is not necessary to ensure absolute sterility of the beaker because it does not come in contact with any of the test plaques.

Cultures may be grown under anaerobic conditions with this technique, but evacuation of the jar will cause the plaques to dry out. The procedure can, nevertheless, be applied if the jar is not evacuated and the internal atmosphere is changed without a reduction in gas pressure. Partial reduction of O₂ tension is obtained if the inoculation is made in the liquid agar before the plaques are poured, or a drop of agar or a sterile cover-glass may be used to cover the inoculated plaque.

The inhibitory or stimulatory action of the test solutions may be determined by noting the incubation time necessary for visible growth to appear and by comparing the colony size with that of a control. Where indicators are added, the change in the indicator is clearly seen. In the cylinder cup application, the zone of inhibition is measured.

**Application to the assay of antifungal substances**

Wort agar at pH 7.0 is mixed with the material under test (pH 5–7) in varying proportions so that its final concentration varies from 1 to 50 %. The agar concentration is adjusted as previously described, and is further increased by 25 % when the final pH value is 5.5 as the agar is partly hydrolysed at this pH.

When the plaques are set they are labelled by symbols on the outside of the Petri dish. Identification may be made with a glass-marking pencil or Indian ink, but it was found more convenient to prepare written adhesive labels before the plates were poured since these could be glued to the plates without inverting them.

The plaques are now inoculated from a spore suspension of *Penicillium roqueforti* with an inoculating needle. Incubation is at 25°. Preliminary results may be noted after 48 hr. incubation but after 72 or 96 hr. results are much clearer. Pl. 1 shows the appearance of inoculated and uninoculated plaques after incubation.
The volume of substrate and test solution is 1–2 ml. Plaques are poured in duplicate or triplicate and the agar remaining in a small test-tube is inoculated with an inoculating needle. The test tube culture serves as a check of the results obtained with the plaques. The microcylinder cup technique is occasionally used as an additional check.

When the antagonism between two organisms is being determined, the plaques are poured in an elongated shape (two or three drops of agar in a row). The two organisms are inoculated at opposite ends of the plaque and the resulting antagonism may be determined after incubation.

**DISCUSSION**

Several drawbacks are evident if the microtechniques described by earlier authors are critically examined. The slide-cells used by Koch (1881), Frost (1921), Lindner (1905) and Henneberg (1934) require great care and accuracy, and it is doubtful whether complete sterility is maintained. Special moist chamber incubators are often required and the procedure as a whole is not adaptable to routine work with many samples. In the Burri (1928) procedure, only one inoculation can be made per tube and only aerobic organisms develop.

In the auxanogram of Beijerinck (1889) and in the cylinder plate method for antibiotic assay, diffusion through the agar may reach such proportions that the results in one segment are completely obscured by an adjoining segment. In addition, spreading organisms may cover the whole surface of the agar and completely obscure results. Finally, relatively large volumes of the substrate are used.

The microtechnique advocated in this paper has the following advantages:

(1) Time, space on tables and in incubators, dishes and substrate material are saved. (2) All the manipulations can be made by one worker except in the microcylinder cup assay, for which assistance is needed to insert the cylinder before the agar has solidified. (3) The results are concentrated in a small area so that the examination of the growth is more distinct. (4) The agar solidifies immediately and inoculation can be made at once. (5) The danger of condensation water dropping on to the agar is virtually eliminated. (6) Each plaque is distinct from its neighbour so that the results on one are not influenced by any other. (7) The danger of air contamination is lessened (less nutrient area).

The technique of smearing the plaque with cotton-wool assures an even inoculation without the danger of breaking the surface of the agar. A sterile glass rod or sterile brush are not as satisfactory because the former cools only slowly after flaming and the latter can only be used once.

When this paper was in preparation, Dingle & Solomons (1951) published a micromethod for measuring the biochemical activities of micro-organisms. The organism is grown in small cylinders of agar. The culture is then extruded with a glass rod and the test agar medium is added to enclose completely the agar plugs. The biochemical activity can then be determined with suitable developing agents in the agar. The manipulations are more complicated than