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A NOVEL BAITING TECHNIQUE FOR DIVERSITY ASSESSMENT OF SOIL ACTINOBACTERIA IN A LABORATORY MICROCOSM

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Abstract
A novel slide technique which employed Arginine Vitamin Agar was used for isolation of diverse actinobacteria from tropical soil habitat in a laboratory microcosm. About ~60% of the total actinobacteria were recovered comprising mainly Streptomyces, Streptosporangium and Actinomadura as the common genera. The technique demonstrates the potential for 'ex situ' visualization and rapid diversity assessment of soil actinobacteria, especially under demanding tropical conditions.

Keywords: *Actinobacteria, Arginine Vitamin Agar, Microcosm, Tropical diversity, Isolation*

INTRODUCTION

Actinobacteria are producers of biologically useful substances, such as antibiotics, vitamins and enzymes, (Basilio et al 2003; Takahashi and Omura 2003; Berdy 2005) thereby necessitating development of novel methods to access new representatives of this group (Gavrish et al 2008). Laborious and time consuming techniques such as dilution plating are commonly being used for isolating soil actinobacteria (Waksman and Martin 1939). Novel technologies like high throughput screening, whole genome sequencing, make targeted isolation possible but being expensive these are beyond the reach of large culture collections in Third World Countries. Difficulties encountered using older techniques for specific isolation, have led to development of baiting techniques which uses addition of natural substrates to soil as baits to study the population and succession of microbes in different habitats (Warcup 1965; Tribe 1957; Warnes and Randles 1980). This note describes a technique with slides specifically coated with an actinobacterial isolation medium combining direct visualization with isolation techniques. Instead of glass slides, previous modifications had employed, nylon mesh (Warcup 1965), pieces of glass, or cover slips (Warcup 1965; Linford 1942; Takahashi and Omura 2003), or strips of cellophane attached to cover slips (Tribe 1957, 1961) and hair (Griffin 1960) which were buried in soil to study the succession of microbes by direct examination. These techniques however do not permit isolation of morphological dissimilar colonies on plate. Gavrish et al (2008) developed semi-permeable membrane trap for specifically capturing and cultivating actinobacteria *in situ,* but it employs relatively expensive polycarbonate membrane filters and a diffusion chamber. Lewis et al (2010) on the other hand, introduced isolation chips (ichips) which are more advanced than diffusion chambers for invitro isolation of actinobacteria but in the same year, Nichols et al (2010) showed the limitations of the technique in isolating the same. Thus, emphasizing the need for an effective equipment for specific actinobacterial isolation (Hameº-Kocabaº and Uzel 2012). We describe a simple, inexpensive, 'ex situ' slide based technique with defined coating of AVA medium to capture actinobacterial diversity under 'Laboratory Soil Microcosm' (LSM) setup. AVA is fairly clear medium, yields a large number of actively growing actinobacterial colonies (Nonomura and Ohara 1969), supports good sporulation and permits microscopic detection of the individual colonies

including spores/cells diversity with relative ease and had emerged in our routine isolation work as a suitable candidate for slide coating medium (Velho-Pereira and Kamat 2011).

For testing the technique, humus rich soils from the lateritic plateau Taleigao, Goa University campus, Goa, India were collected at a depth of 10-20 cm in clean polythene bags from 5 x 5 m quadrat below the canopy of two indigenous keystone plant species namely *Ficus benghalensis* L., a sacred tree of 400 years age as per local knowledge and *Bombax ceiba* L. Samples were transported to the laboratory and stored at 22°C before use. The LSM was prepared in triplicates in brand new Garden PVC pots (length x width x depth; 13.5 x 13.5 x 10.5 cm, 1.2 kg capacity) which were filled with the moistened soil samples. Equidistant (1cm) vertical slits of upto 5-8 cm depth were made radially with a surface sterilized knife blade to create seven identical 'Slide Insertion Slots' (SIS) to enable periodic removal of colonized slides for observations. AVA was prepared and used as 'Slide Coating Medium' (SCM) (Velho-Pereira and Kamat 2011).

Two treatments were used-in first the slides coated with AVA homogeneously mixed with antibacterials and antifungals and the second without. Standard plain microscope glass slides (HiMedia, Mumbai, India) 75 x 25 mm and 1.0 mm thick were used as inert coating surface. To permit routine direct stereomicroscopic observations in a marked pre-defined area, a quadrat of the size of a standard cover slip, app. 324 mm^2 (Corning Glass, USA, Type n No.1, 18 x 18 mm, thickness 0.16 mm) was chosen at the centre of the reverse side of the area to be coated and was marked carefully. The slides were then sterilized by autoclaving at 121°C for 20 min. All the operations were performed on a laminar air flow bench. The slides were transferred in an 11 cm diameter sterile petri plate (Himedia, Mumbai, India) for coating of the medium to the marked central area. Two sets of slides in triplicates one with the coating medium incorporated with antibacterial and antifungals and other without were prepared. Sterile homogenous molten AVA medium cooled to 40°C was carefully poured in a single thin stream from a 500 ml Erlenmeyer flask on each slide to get a uniform coating. The coated slides were then transferred back to the petri plate for solidifying and excess medium crossing the edges of the marked area was removed. The solidified coated slides were designated as per a preset scheme and used within 15-30 min. Preselected and labeled pair of coated test slides (one with and another without antibiotics) was then gently inserted in SIS in the LSM, taking care to bring the medium in the contact with the surrounding soil without disturbing coated surface. The LSM was then covered with aluminum foil and incubated under ambient conditions on a clean laboratory platform. A pair of slides from each of the three LSM was sequentially extricated from respective SIS in LSM on 4, 7, 12, 15, 22, 26 and 28 days. After removing loose extraneous soil particles, these were observed under Olympus BX41TF microscope (Tokyo, Japan) with digital photomicrographic attachment. Actinobacterial (AB) colonization was detected by presence of typical submerged, aerial mycelium and spore bearing chains. 'Non Actinobacterial' (NAB) colonization was detected by presence of typical growth or the colour, texture and overall appearance of non filamentous microcolonies. Fungal (F) colonization was detected by presence of typical filamentous microcolonies. The extent of microbial colonization was scored by noting the relative area under AB, NAB and F in each of the 200+ random optical fields and expressed as average percentage colonization by AB, NAB and F (results not shown). For sampling, AB colony forming units (CFUs) from colonized surface, jets of 2 ml of sterile distilled water were directed (for each pair of extricated slides) using a sterile accupipet model T1000 (Tarsons Products Pvt. Ltd., Kolkata, India). Washings were collected in a sterile glass beaker and an aliquot (0.1 ml) was then plated on AVA medium with antibiotics (as described previously) and incubated at 28-30°C for upto 30 days. Plates were observed daily under Olympus SZ2-ILST stereomicroscope (Tokyo, Japan) and morphologically dissimilar colonies were identified by recording growth, aerial spores-mass colour, texture, elevation; substrate mycelium colour, margin, pigment production and presence or absence of exudates (Cross and Goodfellow 1973). Isolates representing dissimilar morphotypes were then identified, selected and purified on AVA plates. Pure colonies were transferred to AVA slants. Isolated pure strains were designated and maintained at the ambient temperature of 26-34°C.

For recording micromorphological details, air dried smears were stained in 1% (w/v) crystal violet and the spore bearing hyphae were identified with entire spore chain along with other microstructures (Locci 1989). The technique demonstrated the usefulness of AVA as a coating medium for selective growth of actinobacteria. Selection of a marked quadrat allowed focused, prolonged, reproducible observations and quick visual estimates of colonization under 40 X objective. Placement of coated slides inside defined SIS within LSM was also an improvement over previous techniques. Rossi-Cholodny technique was limited by the inability of microbes to attach to the slide and the difficulty in further isolation, as the mycelia growing over the slides were usually sterile (Garrett 1951; Alexander and Jackson 1954) but in the present technique use of AVA with antibiotics helped to reduce competing microbes. Around 60-70 % colonization by actinobacteria was seen in all the slides extricated from the soil of *F. benghalensis* L., with soil from *B. ceiba* L. exhibiting the same percentage of colonization, indicating that the technique is not specific to rhizosphere soils and actinobacteria could be isolated from varied soil samples. This could be highly beneficial for rapid isolation of such forms. The actinobacterial diversity predominated on the 4^{th} and 12^{th} day of incubation with decrease in colonization after 26^{th} day and sudden increase on 28^{th} day. The decrease in colonization could be attributed to medium depletion caused by presumptive grazing of soil arthropods which could be avoided by preheating the soils prior to setting up the experiment. Distribution of actinobacterial colonies at different time intervals could be due to the appearance of late colonizers. Majority of actinobacterial colonies formed well developed microscopically distinguishable vegetative mycelia. Fructifications such as the formation of branching, stable substrate hyphae and aerial hyphae with long spore chains could be categorized as *rectiflexibiles, rectinaculiaperti* or *spirales* on basis of their characteristic morphology (Locci 1989).

Spiral spore chains with spores of Genus *Streptomyces* approx 1-1.5 ìm in length predominated the slides (Fig. 1). Around 30 isolates representing 24 dissimilar morphotypes and 39 isolates representing 15 dissimilar morphotypes were recovered from respective LSM of *F. benghalensis* and *B. ceiba*. Majority of these belonged

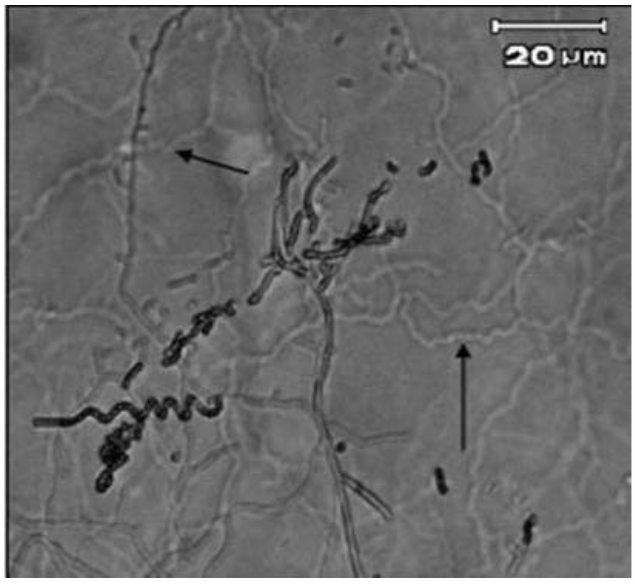

Fig. 1. Spiral spore chains with spores of *Streptomyces* sp. Approx 1-1.5 µm in length as seen under Phase Contrast Microscopy. Arrows indicates submerged mycelium.

to genus *Streptomyces*. Other actinobacteria isolated in small numbers included *Streptosporangium* and *Actinomadura*. The technique also permitted real time still image and video documentation of the growing actinobacterial colonies (www.youtube.com/watch?v=bJ8pDnXamPw). According to Durbin, the buried slide technique could also be used to obtain selective isolation of a particular organism rather than to study general microbial colonization by using many natural substrates as baits (Warcup 1965). Incorporating specific enzyme substrates in the baiting medium so as to isolate potential enzymatic actinobacterial producers has also been carried out using this technique (results not incorporated).Thus this novel baiting technique facilitated specific capturing of actinobacterial diversity and quick insight into community structure from mixed populations of microorganisms from different soil samples. In tropical countries it may be found useful for high frequency exploration and isolation of actinobacteria.

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