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Cultivation-Dependent Characterization of Endophytic Actinomycetes

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Abstract: The opportunity to find new endophytic microorganisms among myriads of plants in different settings and ecosystems is very much appealing. A research to isolate endophytic actinomycetes from ethnobotanical plants in Northern part of the Malay Peninsula has been carried out. We assessed the efficacy of different surface sterilization methods and determined the most suitable medium for isolation of endophytic actinomycetes. The root and stem samples of four plants, *Cinnamomum zeylanicum*, *Zingiber spectabile*, *Elettariopsis curtisii* and *Labisia pumila* were sterilized and cultured on five isolation media such as Starch Yeast Casein Agar (SYCA), Actinomycetes Isolation Agar (AIA), Humic Acid Vitamin Gellan Gum (HVG), Tap Water Yeast Extract agar (TWYE) and Coal Vitamin Agar (CVA) which were supplemented with cyclohexamide and nystatin. The macro and micro morphological of isolates was observed as well as the chemotaxonomic analysis of the isomer diaminopimelic acid. The surface sterilization method that incorporates an overnight incubation between the use of fungicide/sodium hypochlorite mix and sodium hypochlorite followed by 70% ethanol was effective to eliminate the epiphytic microorganisms. There were 66 isolates of *Streptomyces* sp. and 1 isolate with unknown genus successfully isolated with the highest recovery of 35.8% obtained from SYCA media followed by 32.8 and 23.9% from AIA and HVG media, respectively. The TWYE and CVA media showed the least isolation of actinomycetes with 6.0 and 1.5%, respectively. Of the total isolates obtained, 61.2% was isolated from the root and 38.8% were isolated from the stem part. Besides that, 56.7% of the endophytic actinomycetes were isolated from the external part of the surface-sterilized plants and 43.3% was isolated from the internal part of the plants. All the 66 isolates were phenotypically grouped into 11 groups based on the presence and color of aerial mycelium, pigment and the morphology of the spore chain. SYCA media is found to be the best among the media tested for isolation of endophytic actinomycetes. A good isolation media is important to increase the number of isolates of endophytic actinomycetes and helps in the discovery of potential bioactive metabolite.

Key words: Endophytes, actinomycetes, isolation media, chemotaxonomic

INTRODUCTION

Endophytic actinomycetes which reside in the tissues of living plants are relatively unstudied and potential sources of novel natural products for exploitation in medicine,

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agriculture and industry (Strobel and Daisy, 2003). The advent of drug resistance in most bacterial pathogens and the current increase in the number of fungal infections has caused a resurgence of interest in finding other reserves of biologically active compounds (NIH, 2001). One of the most promising endophytic micro-organisms to be isolated would be an actinomycete, or specifically a streptomycete, since these organisms often produce antibiotics (Ezra *et al*., 2004) such as alnumycin (Bieber *et al*., 1998), fistupyrone (Igarashi *et al*., 2000) and munumbicin (Castillo *et al*., 2002). There are more than 300,000 plant species on earth and only a few of these plants have ever been completely studied relative to their endophytic biology (Strobel and Daisy, 2003). Of the myriad of ecosystems on earth, Malaysia is an area of enormous plant biodiversity, with over 15000 plant species and specifically there are over 9000 on the Malay Peninsula alone (Mittermeier *et al*., 1999). Since the number of plant species is so great, thus increasing the effort to isolate new endophytic actinomycetes from plants. The isolation of actinomycetes from the mixed micro flora present in nature is complicated by their characteristic slow growth relative to that of other bacteria (Hirsch and Christensen, 1983). The reports by Janssen *et al*. (2002) and Sait *et al*. (2002) had found that members of previously unstudied groups of soil bacteria can be isolated by using different type of media. In addition, increasing the number of isolates studied has yielded further novel bacteria (Joseph *et al*., 2003). Therefore, the selection of isolation media is important to maximize the number of isolates thus increasing the possibility to discover new endophytic actinomycetes.

MATERIALS AND METHODS

Selection of Plants

Four plants namely *Cinnamomum zeylanicum* (BPP 02), *Zingiber spectabile* (BPP 06), *Elettariopsis curtisii* (BPP 07) and *Labisia pumila* (SM1057) have been selected. Those plants were collected from Bukit Panchor Country Park in Penang Island (5.14° North latitude and 100.54° East longitude) at the Northern part of the Malay Peninsula. Each plant is selected based on its ethno botanical properties that used as traditional medicine (Mat-Salleh and Latiff, 2002). The plants have been identified, tagged, stored in an airtight plastic bag and given a voucher specimen number accordingly.

Surface Sterilization Procedures

Both representative root and stem plant samples were subjected to four different surface sterilization procedures. For procedure I (Zin *et al*., 2007), samples were immersed in ethanol 70% for 5 min. In procedure II (Coombs and Franco, 2003), samples were immersed in ethanol 99% for 1 min, followed by NaOCl 3.15% for 6 min and 3-5 sec in Ethanol 99%. For procedure III (Webster *et al*., 2003), samples were immersed in cycloheximide (50 μg mL) for 60 min followed by NaOCl 3.15% for 15 min and ethanol 70% for 3-5 sec. In procedure IV (Webster *et al*., 2003), samples were immersed in a solution of cycloheximide (50 μg mL) and NaOCl 3.15% (1:1) for 4 h and stored in a refrigerator (4°C) overnight. In all procedures, the samples were washed in sterile distilled water and shaken for 30 sec at the final step of each procedure. Then, 10 μL of washing water was cultured on the nutrient agar and the numbers of Colony Forming Unit (CFU) was counted after overnight incubation at 37°C. This was to assessed the procedure effectiveness to eliminate the surface contaminating microorganisms.

Isolation Procedure

The inner tissues and outer tissues of the root and stem of four plants samples were carefully excise into 0.5-1.0 cm tissue blocks. Each representative tissue of each plant was

placed on the agar surface of Humic Acid Vitamin Gellan gum (HVG), Starch Yeast Casein Agar (SYCA), Actinomycetes Isolation Agar (AIA), Tap Water Yeast Extract Agar (TWYE) and Coal Vitamin Agar (CVA) that were supplemented with cycloheximide and nystatin, respectively Then it was incubated for 3 weeks at 28°C. Colonies with the properties of actinomycetes were subcultured on Nutrient Agar (NA) and stored in 15% glycerol solution at -80°C until used.

Macromorphology and Micromorphology Observation

The macromorphology of the actinomycetes colonies was examined by naked eyes and light microscopy (40X). Then, the colonies were subcultured on Actinomycetes Isolation Agar (AIA) to obtain pure culture and to observe the presence and color of aerial mycelium, substrate mycelium and the pigment. The mircomorphology of filamentous structure of actinomycetes was observed using Gram staining method. Characteristics of the spore-bearing hyphae and spore chains could be determined by using direct microscopic examination (40X) of the culture surface on AIA plate. The magnification of 400X was used to establish the presence or absence of spore chains and to observe the nature of sporophores (Kumar *et al*., 2005).

Determination of Diaminopimelic Acid (DAP) Stereoisomers

Analysis for diaminopemilic acid was carried out by using the procedure described by Staneck and Robert (1974) with modification. Pure culture of actinomycetes from AIA media was transferred into 100 mL of nutrient broth, incubated at 28°C for 2 weeks. Cell suspension obtained was treated by formalin for 24 h, then centrifuged for 25 min at 2000 rpm. The pellet was washed with distilled water and the process was repeated again by washing with ethanol 95% and air dried. Then, the pellet was mixed with 1 mL of 6N HCl and kept at 121°C in an oven for 15 min. The hydrolysate was filtered through Whatman no. 1 filter paper and was evaporated to dryness at 100°C. The residue was dissolved in 0.3 mL of distilled water and 2 μL of sample was applied at the base line of the Thin Layer Chromatography (TLC) paper. Ascending TLC was performed with the solvent system methanol-distilled water-6 N HCl-pyridine (80:26:4: 10, v/v) for approximately 2 h 30 min. A spot of 10 μL 0.01 M meso-diaminopimelic acid was added on a TLC paper to run alongside the sample as a reference standard. After the chromatogram was air-dried, spots visualized by spraying with 0.2% ninhydrin in acetone and heating at 50-60°C for 10 min. The DAP spots were seen as gray-green fading to yellow, with the L isomer moving ahead of the meso isomer.

RESULTS

Efficacy of Different Surface Sterilization Methods

Procedure I produced the highest bacterial contamination (97.6%) (Table 1). It showed that sterilization method with ethanol 70% alone was not effective to eliminate microorganisms on the plants surface. On the other hand, procedures II and III showed a very low contamination of bacteria and fungus with 0.82 and 2.47% contamination, respectively. For procedure III in which the samples have been immersed in cycloheximide showed low percentage of contamination due to it inhibiting protein biosynthesis in eukaryotic organisms. Procedure IV which used two steps of sterilization process was 100% effective as surface sterilization method.

Table 1: Percentage of bacteria and fungi contamination from the surface of plants tissue when using different surface sterilization procedures

	Surface sterilization procedures			
Contamination (%)	I	II	III	IV
Bacteria contamination	97.6	0.21	1.85	0
Fungi contamination	0	0.61	0.62	0
Total	97.6	0.82	2.47	0

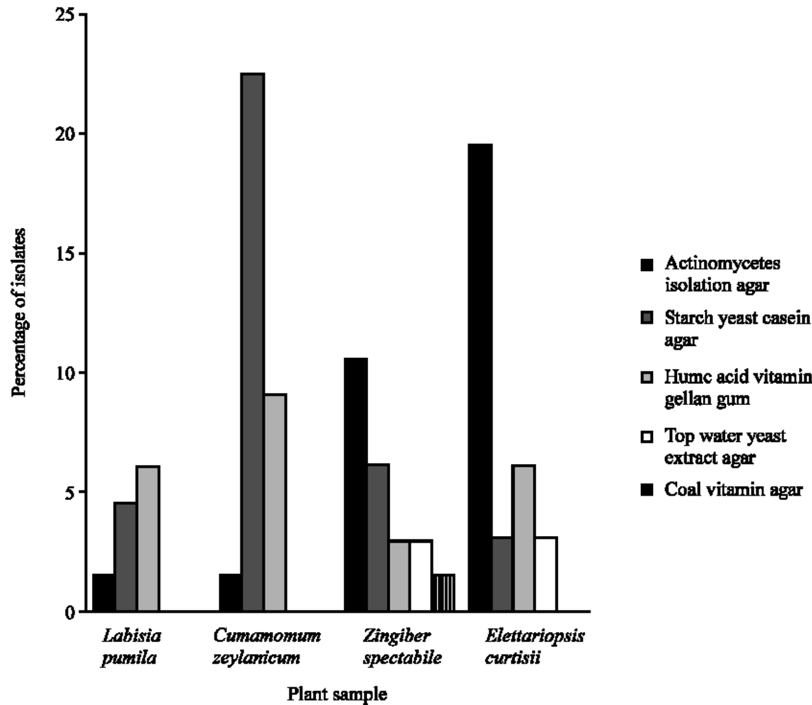

Fig. 1: Percentage of actinomycetes isolated from different plant species

Isolates of Endophytic Actinomycetes

There were 67 actinomycetes successfully isolated from the samples (Fig. 1). *Cinnamomum zeylanicum* yielded the highest number of isolates with 22 isolates (32.8%), followed by *Elettariopsis curtisii* with 21 isolates (31.34%). *Zingiber spectabile* and *Labisia pumila* showed the least isolation of actinomycetes with 16 isolates (23.88%) and 8 isolates (11.94%), respectively. From the isolates obtained, 61.2% was isolated from the root and 38.8% from the stem portion, respectively (Fig. 2). In this study, the external part of the four plants yielded 56.7% of isolates while the internal part was 43.3%, respectively. The highest number of actinomycetes isolates (35.8%) was obtained from SYCA media followed by AIA and HVG media with 32.8 and 23.9%, respectively (Fig. 3). The TWYE and CVA media showed the least isolation of actinomycetes with 6 and 1.5%, respectively.

Out of 67 isolates, 58 of them produced aerial mycelium with grey, white, pinkish or yellow-greenish colour on AIA media. Six isolates with sparse aerial mycelium and another three without aerial mycelium. Almost all of the isolates in this study produced dark brown or yellow-brown pigment, only one of the isolates producing red pigment. Observation under light microscope (40X) revealed the edge of the colony was opaque with many filaments

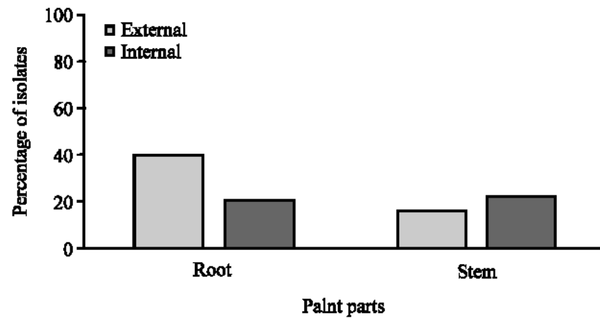

Fig. 2: Percentage of Actinomycetes isolated from different parts and depth of four plants tissues

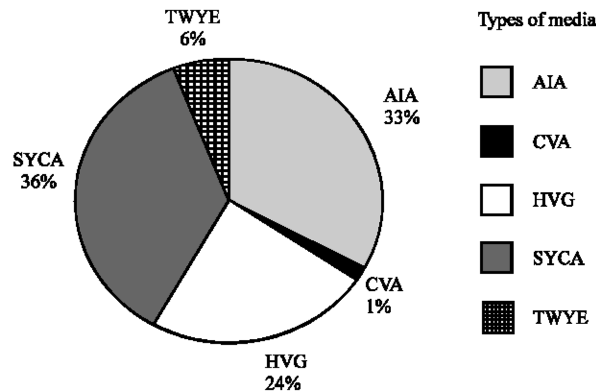

Fig. 3: Percentage of Actinomycetes isolated on different types of media

protruding out of f the colony. After staining with Gram stain, all the isolates were classified as filamentous Gram-positive bacteria. The structure of filaments varied among the bacteria with different forms of branching, length and thickness. The shapes of spore chains observed on the isolates were flexous, rectusflexibilis, retinaculumapertum and spiral. According to Shirling and Gottlieb (1966), streptomyces have spore chains classified as rectusflexibilis (RF), retinaculumapertum (RA), Spira (S) and Verticillate (V). All the 66 isolates were phenotypically grouped into 11 groups based on the presence and color of aerial mycelium, pigment and morphology of the spore chain.

Analysis of Diaminopimelic Acid (DAP) Stereoisomers

All of the 66 isolates contained LL-diaminopimelic acid as a major constituent. The presence of LL-DAP in peptidoglycan cell wall ascertained that all the isolates were streptomyces (Becker *et al*., 1965). Only one isolate was not identified in this study according to this method and at present, this isolate is undergone further study using molecular characterization.

DISCUSSION

Efficacy of Different Surface Sterilization Methods

Procedure I showed that sterilization method with ethanol 70% alone was not effective to eliminate microorganisms on the plants surface. As reported by McDonnell and Russell

(1999), ethanols 70% do not possess sporadically activities and was not able to eliminate spore-forming bacteria. procedures II and III showed a very low contamination of bacteria and fungus because sodium hypochlorite (NaOCl) was effective as disinfectant agent against many bacteria. Hypoclorite (OCl^-) was considered as a strong oxidant and can denature and aggregating essential proteins of bacteria (Winter *et al*., 2008). For procedure III in which the samples have been immersed in cycloheximide showed low percentage of contamination due to it inhibiting protein biosynthesis in eukaryotic organisms. However, some fungus and bacteria still survived due to resistant towards cycloheximide (Coppin-Raynal, 1977). Procedure IV that used two steps of sterilization process with the second step that involved storage at 40°C for overnight ensured all bacteria and fungus were killed.

Isolates of Endophytic Actinomycetes

This result indicates that more actinomycetes colonized on the external part rather than the internal part of the plant. Endophytic bacteria usually originated from epiphytic bacteria colonising soil, any wound or opening on the plant surface give the opportunities of the epiphytic bacteria to enter the plant tissue and become endophytic bacteria, in line with finding reported by Hallmann *et al.* (1997).

The SYCA, AIA and HVG media composition contained all sources of carbon, amino acids and minerals needed for the cultivation of actinomycetes. The SYCA and HVG were also supplemented with vitamins. The TWYE media contained tap water and yeast extract as their nutrient source while CVA contained coal and vitamin as their nutrient source. Yeast extract was the source of carbohydrates, amino acids, peptides and minerals for SYCA media. In this study, SYCA is found to be the most suitable media for isolation of endophytic actinomycetes. The results differ from previous study (Zin *et al*., 2007) because the usage of different media for isolation of endophytic actinomycetes. Previous study only used Starch Casein Agar (SCA) for isolation.

Analysis of Diaminopimelic Acid (DAP) Stereoisomers

The chemical composition of cell walls has been accepted by Lechevalier and Lechevalier (1970) as a criterion for the classification of aerobic actinomycetes. Becker *et al.* (1964) introduced a system of paper chromatography able to separate the taxonomically important stereoisomers of diaminopimelic acid (DAP). The test showed DAP spots with olive-green fading to yellow, with the L isomer moving ahead of the meso isomer while amino acid spots appeared purple or red and migrated ahead of the DAP spot.

In conclusion, a good isolation media is important to increase the number of isolates of endophytic actinomycetes and helps in the discovery of potential bioactive metabolite. These results demonstrated that some of endophytic actinomycetes belonging to both known and new groups can be isolated, suggesting that our methods extend the range of culturability among endophytic bacteria from the ethnobotanical topical plants. These results should now allow physiological and genetic characterization of representatives of these endophytic bacterial groups and help elucidate their roles in the plants. We are currently carrying out more detailed phenotypic and phylogenetic characterizations of our novel isolates to begin to characterize them, as well as to assess their antimicrobial activity.

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