Malaysian Journal of Science **24**: 69 - 75 (2005)

Survey of Culturable Actinomycetes from Marine Macroorganisms and Mangrove Areas of Langkawi Islands

Geok Yuan Annie Tan*, **Christabel Loni, Ho Li Sim, Nik Zahirah binti Nik Abdul Aziz, Sarini binti Syafri** and **Vikineswary Sabaratnam**

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
* ygtan@um.edu.my

ABSTRACT Fifty-two strains of actinomycetes isolated from bryozoan and marine sponges collected from the waters of Langkawi Islands were studied. The cell wall hydrolysates of twenty-two strains were found to contain the *meso*-diaminopimelic acid indicating that they are non-streptomycete strains. Furthermore, ninety-six strains of actinomycetes were isolated from soil samples collected from various mangrove areas during the first and second Langkawi Expeditions and forty-three of these strains have the *meso*-diaminopimelic acid in the cell wall hydrolysates. The strains were isolated using various selective isolation media for actinomycetes and several different pretreatment techniques. All the strains were further characterized by colour grouping. This study showed that the mangrove soils and marine organisms from Langkawi Islands hold relatively high diversity of non-streptomycete isolates that could be potential producers of novel bioactive compounds.

ABSTRAK Lima puluh dua strain aktinomiset yang dipencilkan daripada bryozoan dan span marin yang dikutip daripada perairan Pulau Langkawi telah dikaji. Dinding sel daripada dua puluh dua pencilan aktinomiset didapati mengandungi asid *meso*-diaminopimelic dan ini menunjukkan pencilan tersebut bukan jenis streptomiset. Tambahan pula, sembilan puluh enam aktinomiset telah dipencilkan daripada sampel tanah yang dikutip daripada beberapa paya bakau semasa Ekspedisi Langkawi yang pertama serta kedua, dan empat puluh tiga daripada pencilan tersebut mengandungi asid *meso*-diaminopimelic dalam dinding selnya. Semua aktinomiset tersebut dipencilkan menggunakan beberapa media terpilih untuk aktinomiset dan beberapa teknik pra-perlakuan. Semua pencilan juga dibahagikan kepada kumpulan mengikut warna kultur. Kajian ini menunjukkan bahawa tanah paya bakau dan organisma marin dari Pulau Langkawi mempunyai diversiti aktinomiset bukan streptomiset yang cukup tinggi dan mempunyai potensi menghasilkan bahan bioaktf yang baru.

(actinomycetes, mangrove, sponges, Langkawi)

INTRODUCTION

Mangrove wetlands are one of the most significant components of Langkawi's coastal ecosystem. Mangroves play a crucial role in the regulation of the nutrient balance which involves interactive processes of plants, soil and microorganisms. Microorganisms, including actinomycetes, are responsible for the breakdown of organic matter and nutrient turnover [1].

Actinomycetes are a phylogenetically distinct group of gram-positive microorganisms with a unique capacity to produce novel bioactive compounds [2, 3]. Recent research [4-6] have revealed that Malaysian mangrove mud, rhizosphere, near shore sediments, soft coral and sponge samples contain diverse actinomycete populations. We report here the selective isolation and characterization of actinomycete strains from marine macroorganisms and mangrove mud soil collected during two different expeditions to Langkawi.

MATERIALS AND METHODS

The first and second Langkawi Expeditions were carried out from 10th to 19th April 2003 and from 4th to 10th April 2004, respectively.

Samples

A bryozoan and five marine sponge samples (Table 1) were collected from the waters off the Langkawi Island during the second Langkawi Expedition. The samples were kept on ice until they were brought back to the laboratory to be ground with a blender.

Table 1. Tentative identification of marine macroorganism samples collected during the second Langkawi Expedition

Code	Marine macroorganism	Scientific name
S1	Sponge (greenish black, porous, rock-like)	Unidentified
S2	Sponge (long branching, porous)	*Cribrochallina* sp.
S3	Sponge (mollusk boring sponge)	Unidentified
S4	Bryozoan	*Triphylozoon* sp.
S5	Sponge (greenish black)	Unidentified
S6	Sponge (brown, spiky, calcareous)	*Callyspongia* sp.

Terrestrial rhizosphere samples from mangrove sites of Sungai Sireh and the Kisap Basin were collected during the first Langkawi Expedition. Mangrove mud samples were collected during the second Langkawi Expedition from mangrove areas at Sungai Kisap (6°23'N 99°51'E- hereafter referred to as site 1), Sungai Banjar Kilim (6°24'N 99°52'E- site 2) and Sungai Kilim (6°24'N 99°51'E- site 3). The pH of the soil samples were determined directly using pH probe HI99121 (Hanna Instruments). The soil samples were air-dried, ground and sieved prior to storage.

Pretreatment methods

Four different pretreatment methods were used on the marine samples. Pretreatment using moist heat at 70°C for 30 minutes was as described [7]. The saline pretreatment in which samples were vigorously shaken and settled for 10 minutes with or without heating at 50°C for 60 minutes was a slight modification of [8]. The heat shock pretreatment in which samples were vigorously shaken and heated at 55°C for 6 minutes were carried out as in [9]. Dilutions of 10^{-2} and 10^{-3} saline suspension were inoculated onto selective isolation media followed by incubation for 4 to 6 weeks at 28°C [6].

Terrestrial soil samples from the first expedition were pretreated using dry heat (60°C, 40 minutes) and moist heat method (45°C, 30 minutes) modified slightly from [6]; soil samples from the second expedition were pretreated by moist heat 1 (60°C, 20 minutes) and moist heat 2 (40°C, 20 minutes with YE-SDS [10]). The soil suspensions were diluted up to 10^{-3} prior to inoculation onto selective isolation media followed by incubation for 2 to 4 weeks at 28°C [6].

Selective isolation media

Five different media were used to selectively isolate various groups of actinomycetes from the marine samples. Starch-casein agar (SCA), SCA supplemented with novobiocin (25µg/ml) and Raffinose-Histidine agar (RHA) were used to isolate non-specific actinomycetes, *Micromonospora* species and rare streptomycetes, respectively [11]. Chitin agar (CA) with or without rifampicin (10µg/ml) was used to isolate rare, marine-derived actinomycetes [12]. Two selective media were used to isolate actinomycetes from the mangrove soil samples, SCA and Humic acid-Vitamin agar (HVA). All the media were supplemented with cycloheximide (50µg/ml) and nystatin (50µg/ml) [6].

Characterisation

Determination of isomers of diaminopimelic acid from whole-organism hydrolysates was as described by [13]. In addition, the strains were placed into colour groups based on the colour of aerial mycelia, substrate mycelia and diffusible pigments.

RESULTS AND DISCUSSION

Culturable actinomycete diversity in marine sponges

A total of 52 actinomycete strains were isolated from one bryozoan and five marine sponge samples using the various pretreatment methods and selective media. 42.3% of the strains

contained the meso-DAP while 50% contained the LL-DAP; the DAP isomer of the remaining strains could not be determined due to insufficient biomass. The detection of DAP isomers by TLC is one of the most useful methods for differentiating between streptomycete and non-streptomycete actinomycetes. These results showed that nearly half of the actinomycete strains isolated from marine macroorganisms did not belong to the ubiquitous *Streptomyces* genus.

Sponge sample S1 gave the highest number of actinomycete isolates, followed by bryozoan *Triphylozoon* sp. sample S4 and sponge sample S5 (which had the highest proportion of non-streptomycete) irrespective of the pretreatment and media used; sponge sample S6 has the least number of actinomycete isolates (Figure 1). This is an indication that bryozoans and greenish black sponges might be good sources of diverse and uncommon actinomycetes from marine environments.

The majority of the strains with *meso*-DAP were isolated using moist heat pretreatment and RHA (Figures 2 & 3). Saline and lengthy heat pretreatment yielded the least number of isolates. SCA gave rise to predominantly streptomycete isolates. However, when the antibiotic novobiocin was added to the medium, all the isolates recovered, though the least, were non-*Streptomyces* actinomycetes. Thus, it is recommended that moist heat pretreatment and the media RHA and SCA supplemented with novobiocin, are used to selectively isolate non-*Streptomyces* actinomycetes from marine macroorganisms.

The 52 strains were sorted into 9 colour groups: brown, red, yellow, orange, cream, white, blue, green and grey series. The orange colour group contains the most number of isolates (11 strains) that were tentatively identified as members of the genus *Micromonospora* based on their morphology and presence of *meso*-DAP in their hydrolysates. The members of the brown, red, yellow, white and grey series with between 5 to 8 strains each were identified as *Streptomyces* spp. on the basis of their morphology and presence of *LL*-DAP.

Actinomycetes such as members of the genera *Dietzia*, *Gordonia*, *Micromonospora*, *Pseudonocardia*, *Streptomyces* and *Tsukamurella* have been isolated from deep-water marine invertebrates [14]. Our results provide further evidence that marine macroorganisms harbour great actinomycete diversity.

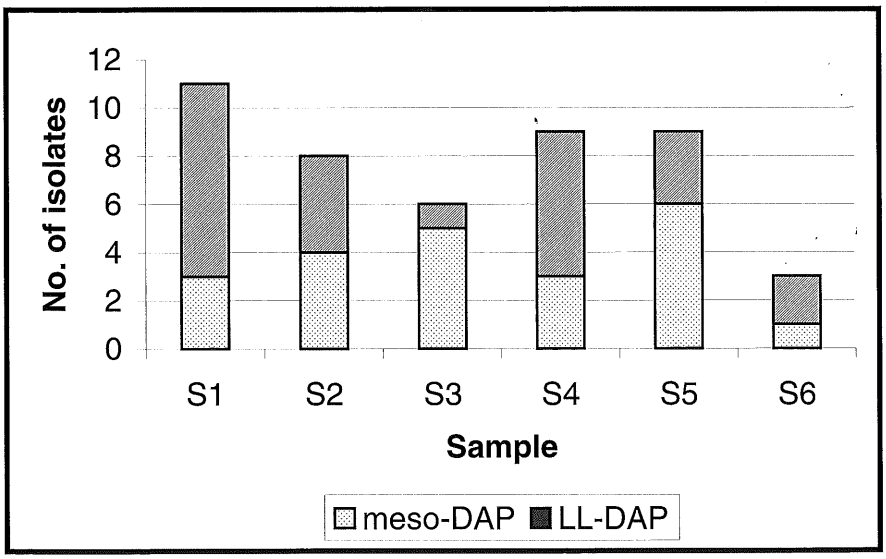

Figure 1. Numbers of actinomycete strains isolated from bryozoan and various sponge samples. S1-S6, please refer to Table 1.

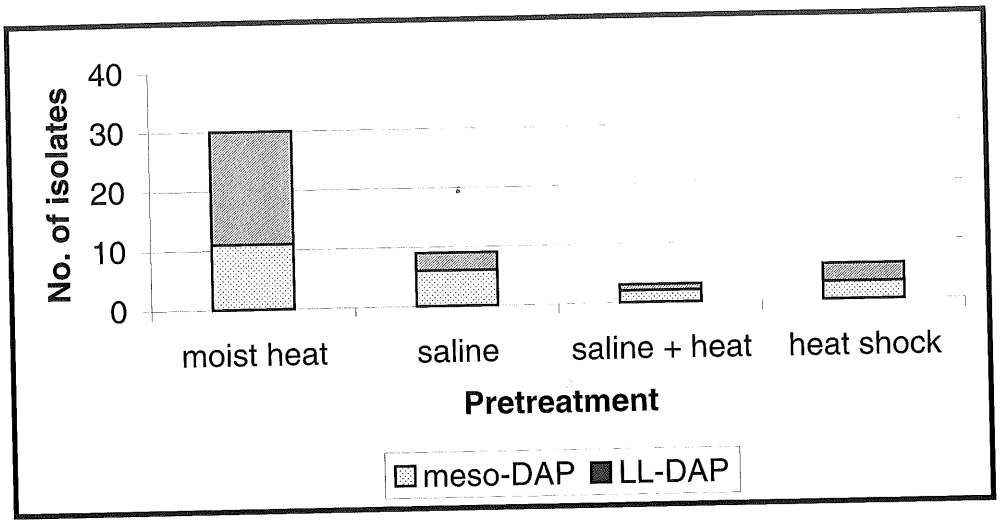

Figure 2. Numbers of actinomycete strains isolated from marine macroorganisms following various pretreatment methods.

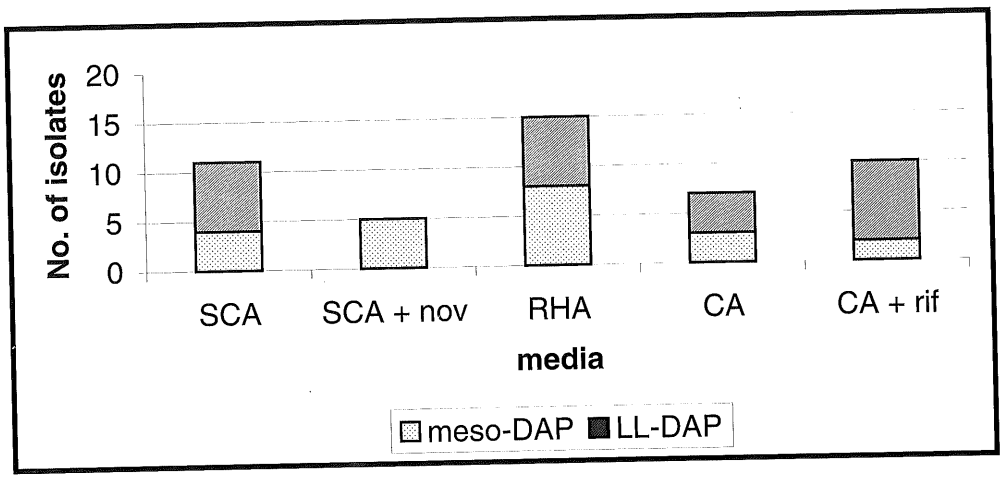

Figure 3. Numbers of actinomycete strains isolated from marine macroorganisms using various Selective isolation media. SCA, Starch-casein agar; nov, novobiocin; RHA, Raffinose-Histidine agar; CA, Chitin agar; rif, rifampicin.

Culturable actinomycete diversity from mangrove soil samples

A total of 97 strains of actinomycetes were isolated from the terrestrial soil samples and characterized by morphology and chemically. The pH of the soil samples were between 5 and 6.5. 44.3 % of the strains contained the *meso*-DAP while the remaining contained the *LL*-DAP. Interestingly, similar to the results obtained from macroorganism samples, almost half of the isolates belong to 'rare' actinomycetes.

The moist heat with YE-SDS pretreatment of soil samples collected from the first expedition gave rise to 4.5 times more isolates compared to that obtained by using dry heat pretreatment (Figure 4). Similarly, for the soil samples collected from the second expedition, the moist heat 2 method containing YE-SDS in the soil suspension yielded 3 times more isolates than the moist heat 1 method in which the soil suspension was treated to a higher temperature of 60°C (Figure 5). These findings are generally in good agreement with those from other studies [6, 10 and 15]. Both the moist heat pretreatment also gave rise to proportionally more *meso*-DAP actinomycetes, though pretreatment with moist heat 1 on soil samples from sites 2 and 3 yielded zero isolates and only *meso*-DAP isolates, respectively (Figure 5).

Dark, mud samples from site 1 which had predominantly mangroves of the *Rhizophora mucronata* Lamk and *Ceriops* spp., gave rise to the most number of isolates, followed by site 3 (predominantly mangroves of the *Rhizophora*

apiculata Blume and *Ceriops* spp.) with 10 isolates. Only 6 strains were isolated from mud samples from site 2 which had mainly mangroves of the *Bruguiera* spp.

The medium SCA gave rise to 6.3 times more isolate than HVA inoculated with suspensions of soil samples from the first expedition. Similarly, SCA yielded more isolates than HVA, albeit lower proportion, when using the soil samples from the second expedition (Figure 6). Thus, SCA is perhaps the medium of choice for the isolation of large populations of actinomycetes from mangrove soil samples.

The 97 strains were sorted into eight colour groups: brown, red, yellow, orange, cream, white, green and grey series based on the colour of aerial and substrate mycelia. The white and brown series contained 35 and 23 strains, respectively, while the red and orange series of *Micromonospora* spp. had 9 strains each. These results again showed that mangrove soils in Langkawi are an excellent source for the isolation of diverse actinomycetes.

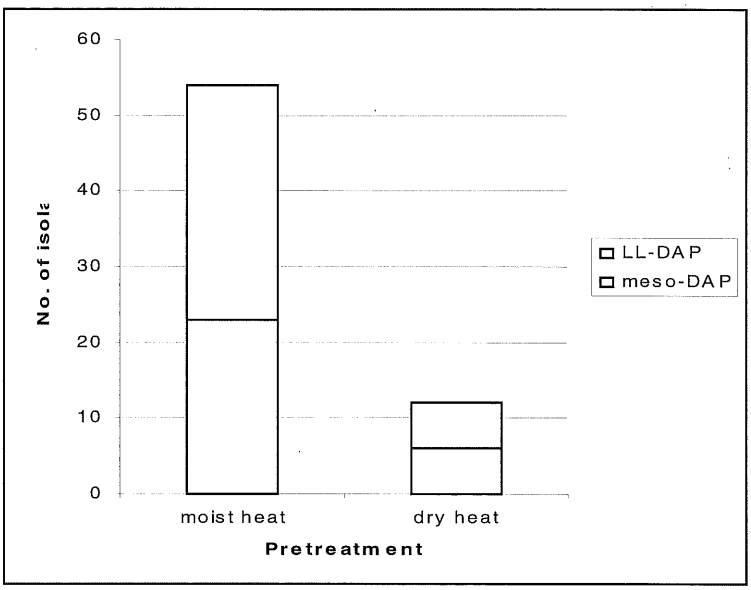

Figure 4. Numbers of actinomycete strains isolated following various pretreatment methods on the soil suspension from the first expedition.

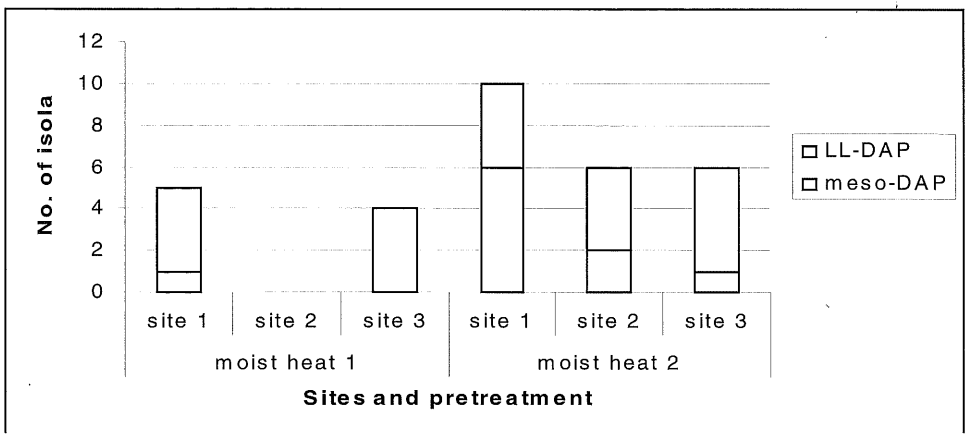

Figure 5. Numbers of actinomycete strains obtained from the three sites according to pretreatments.

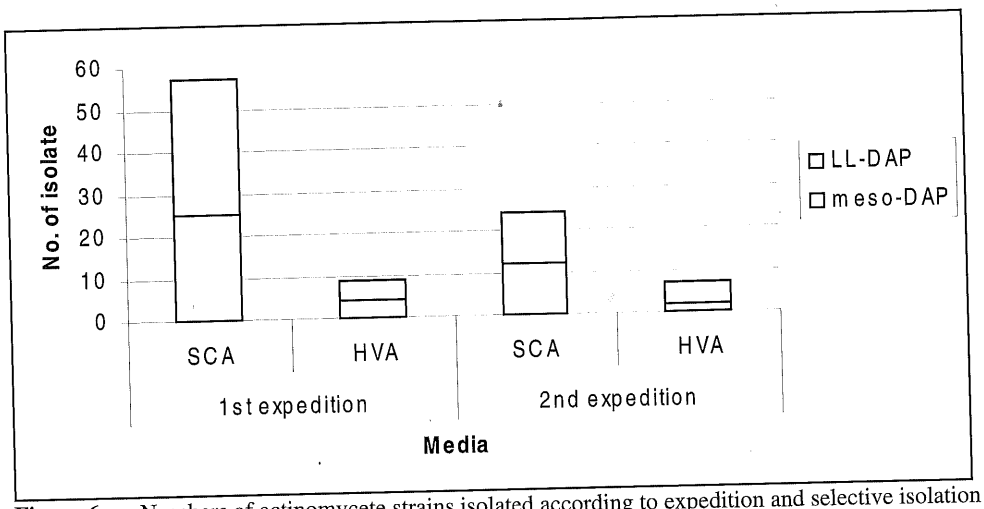

Figure 6. Numbers of actinomycete strains isolated according to expedition and selective isolation media. SCA, Starch-casein agar; HVA, Humic-acid Vitamin agar.

CONCLUSION

Further studies will be carried out to fully characterize the isolates phylogenetically and to screen for their ability to produce bioactive compounds. The discovery and characterization of novel actinomycetes from marine macroorganisms and mangrove samples in this study can lead to the detection of new commercially significant therapeutics and provide useful information for understanding mangrove microbial ecosystems.

Acknowledgements This work is supported by the vote F0367/2004A and the IRPA grant 01-02-03-1002.

REFERENCES

1. Alongi D.M. (1994). The role of bacteria in nutrient recycling in tropical mangrove and other coastal benthic ecosystems. *Hydrobiologia* **285**: 19–32.
2. Lazzarini A., Cavaletti L., Toppo G., and Marinelli F. (2000). Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie van Leeuwenhoek* **78**: 399-405.
3. Demain A.L. (2000). Microbial biotechnology. *Trends in Biotechnology* **18**: 26-31.
4. Vikineswary S., Nadaraj P., Wong W.H., and Balabaskaran S. (1997). Actinomycetes from a tropical mangrove ecosystem- antifungal activity of selected strains. *Asia Pacific Journal of Molecular Biology and Biotechnology* **5**: 81-86.
5. Ismet A., Paramaswari S., and Vikineswary S. (2002). Diversity of *Micromonospora* in Malaysian mangrove rhizosphere soil. *Malaysian Journal of Science* **21**: 51-59.
6. Vikineswary S., Ismet A., Thong K.L., and Paramaswari S. (2003). Rare actinomycetes in mangrove soils and leaf litter. *Jurnal Biosains* **14**: 105-109.
7. Tan C.J. (2001). *Personal communication*.
8. Jensen P.R. (1991). Distribution of actinomycetes in near-shore tropical marine sediments. *Applied and Environmental Microbiology* **57**: 1103-1108.
9. Mincer T.J. (2002). Widespread and persistent populations of a major new marine actinomycetes taxon in ocean sediments. *Applied and Environmental Microbiology* **68**: 5005-5011.
10. Hayakawa M. and Nonomura H. (1989). A new method for the intensive isolation of actinomycetes from soil. *Actinomycetologica* **3**: 95-104
11. Kuster E. and Williams S.T. (1964). Selective media for the isolation of Streptomycetes. *Nature* **202**: 928-929.
12. Hsu S.C. and Lockwood J.L. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied Microbiology* **29**: 422-426.
13. Staneck J.L. and Roberts G.D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer

chromatography. *Applied Microbiology* 28: 226-231.

14. Sfanos K., Harmody D., Dang P., Ledger A., Pomponi S., McCarthy P. and Lopez J. (2005). A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates. *Systematic and Applied Microbiology* **28**: 242-264.
15. Nakagaito Y. and Hasegawa T. (1991). Actinomycetes strains isolated from fallen leaves in mangrove areas. *IFO Research Communications* **15**: 52-56.