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Selective isolation of *Actinomycetes* from mangrove sediment of Tanjung Lumpur, Kuantan, Malaysia.

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ABSTRACT

Aims: Mangroves of Tanjung Lumpur, Kuantan, Pahang is considered as a relatively underexplored resource of actinomycetes. Based on the above perspective, a study was conducted on mangrove sediments of Tanjung Lumpur, Kuantan to isolate potential actinomycetes using several pretreatments and various selective media.
Methodology and results: Sediments from five different sites at Tanjung Lumpur mangrove were collected and selectively pre-treated. The pretreated sediments were diluted and plated onto eight different selective media. A total of 172 potential actinomycetes were isolated from all the media. Antimicrobial activities of 61 selected strains were checked against 8 test microorganisms using cross streak method. Pretreatment of wet heat with seawater was the most effective method for the isolation of actinomycetes as it yielded a maximum of 105 actinomycete isolates and IM7 was the most suitable medium for actinomycete isolation with highest percentage of recovery (31 %). Forty three isolates (70.5 %) showed antimicrobial activities against one or more test microorganisms. Isolates IIUM B21 and IIUM B31 showed antimicrobial activity against all test microorganisms. Seven isolates showed antifungal activity as they inhibited only *C. albicans*. Ten isolates were randomly selected for identification based on partial sequences of 16S rRNA gene. Six isolates were found belong to the genus *Streptomyces*, two isolates belong to the genus *Micromonospora* and two isolates were identified as *Rhodococcus* spp.
Conclusion, significance and impact of study: These findings revealed the potential of mangrove sediment of Tanjung Lumpur as an important source of actinomycetes with biosynthetic capabilities which might be beneficial to pharmaceutical industries.

Keywords: Actinomycetes, Tanjung Lumpur, mangrove sediment, pretreatments, selective media

INTRODUCTION

The demand for new antibiotics continues to grow due to the rapid emergence of antibiotic resistant pathogens which cause life threatening infections and risk undermining the viability of healthcare systems in both developing and developed countries (Okeke *et al.*, 2005; Talbot *et al.*, 2006). Nature still remains the richest and the most versatile source for new antibiotics even though considerable progress is being made within the fields of engineered biosynthesis and chemical synthesis of antibacterial compounds (Baltz., 2006; Bredholt *et al.*, 2008). Actinomycetes are common soil inhabitants which play an important role in the degradation of organic matter, production of novel pharmaceuticals (Xu *et al.*, 2012; Adegboye and Babalola., 2013) and antitumor agents (Kekuda *et al.*, 2010; Ravikumar *et al.*, 2011; Schleissner *et al.*, 2011). Out of 23 000 bioactive compounds produced by microorganisms, 10 000 among these compounds were isolated from actinomycetes and almost 80% have been obtained from *Streptomyces* which is the most productive genus in the microbial world (Watve *et al.*, 2001; Berdy, 2005). However, extensive screening and isolation of actinomycetes from the terrestrial counterpart has led to exhaustive cultivars and rediscovery of known compounds. It is becoming increasingly difficult to discover commercially significant secondary metabolites from well-known actinomycetes as it leads to the wasteful rediscovery of known bioactive compounds, thereby, emphasizing the need to isolate undiscovered actinomycete taxa (Hong *et al.*, 2009). Therefore, researchers are now looking into underexplored habitats such as the oceans (Mincer *et al.*, 2002; Bian *et al.*, 2009), deserts (Hozzein *et al.*, 2004) and mangrove forests (Xu *et al.*, 2014; Prabhahar *et al.*, 2014) for new resources of actinomycetes. It is stipulated that new resources of actinomycetes will lead to the discovery of new bioactive compounds that are capable to produce chemically diverse compounds with a wide range of biological activities.

Majority of the isolates recovered on agar plates have been identified as genus *Streptomyces* which is the dominant actinomycetes in soil, when conventional isolation techniques were applied (Abou-elela and Ghanem, 2005). Hence, several factors must be

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considered for the purpose of screening novel bioactive molecules such as pretreatment, choice of screening source, selective media and culture condition (Vijayakumar *et al.*, 2007). As stated by Jensen *et al.* (2005), the pretreatment including enrichment, physical and selective media might be used to study the ecology of actinomycetes in natural habitat. Naikpatil and Rathod (2011) stated that employing pretreatments of soil by heating and drying stimulated the isolation of rare actinomycetes. Futhermore, the numbers of unwanted bacterial and fungal colonies were obtained when the mangrove sediments were cultured without pretreatment (Baskaran *et al*., 2011).

Due to their slow growth characteristic compared to the other soil bacteria, the isolation of actinomycetes in nature is complicated. Thus, selective isolation media were developed primarily based on nutritional selection, in which media were formulated with nutrients which are preferentially utilized by actinomycetes and selective inhibition, in which compounds such as antibiotics were added into media to selectively inhibit non-actinomycete bacteria (Hirsch and Christensen, 1983). As many of the actinomycetes have shown multiple resistances to antibiotics, several antibiotics such as cycloheximide and nystatin were used in selective medium to inhibit the competing bacteria including fast-growing actinomycetes.

A study conducted Maldonado *et al.* (2009) used seventeen different media such as glucose-yeast extract agar, IM7, ISP3, Mueller-Hinton agar and ISP2 that were known to support growth and isolation of members of the *Actinobacteria* from marine sediment of Gulf of California and the Gulf of Mexico. Consequently, they managed to isolate several actinobacterial taxa, notably to the genera *Actinomadura*, *Streptomyces*, *Micromonospora*, *Gordonia*, *Salinispora*, *Rhodococcus* and *Saccharomonospora*. Therefore, the results of these reports gave clear picture about the significance of pretreatment methods and selective isolation media for the isolation of actinomycetes.

Mangrove is a high moisture, high salinity and hypoxia tolerant environment which is a highly complex ecosystem comprising of unique woody plant communities, marine animals and diverse microorganisms (Liu *et al*., 2007; Wu and Jiang, 2012). Studies conducted on mangrove sediments showed the presence of high populations of *Micromonospora* and novel actinomycetes as illustrated by the isolation of *Asanoa iriomotensis* and *Nonomuraea maheshkhaliensis* (Hong *et al*., 2009). According to Hong *et al*. (2009), mangroves are highly productive ecosystems as more than 2000 actinomycetes were isolated from mangroves and their secondary metabolites showed anti-infection, anti-tumour and protein tyrosine phosphatase 1B (PTP1B) inhibitory activity. However, surprisingly little is known about the microbial communities living therein and less attention has been given into the diversity of actinomycetes present in mangrove sediment particularly in Malaysia. Mangroves in Tanjung Lumpur which were located near to urban area, polluted with industrial and domestic waste (Kamaruzzaman *et al.*, 2011). Hence, actinomycetes present in this mangrove are adapted to extreme environment and might lead to the discovery of new bioactive compounds. This study was conducted to assess the potential of mangrove sediment of Tanjung Lumpur as resource of actinomycetes. Different types of pretreatments and media were used in this study to attain maximum recovery of actinomycetes preferably the rare species to determine the best pretreatment and medium for isolation of actinomycetes. It is expected that this study could expose a new discovery of potential actinomycetes which is valuable for pharmaceutical industry and the fore front to improve human healthcare systems as well.

MATERIALS AND METHODS

Collection of samples

A total of five sediment core samples at a depth of 0-30 cm were collected at the mangrove of Tanjung Lumpur within a 100 m² area in March 2012. The five locations of the sampling sites were marked using Global Positioning System (GPS*)* which were started at site IIUM-TLL1 (N 03° 48’ 22.9” E 103° 20’ 07.3”) until IIUM-TLL5 S5 (N 03° 48’ 22.3” E 103° 20’ 08.0”) respectively. Sediment samples were transported to the laboratory in sterile polyethylene bags for further analysis.

Isolation of actinomycetes from mangrove sediment samples

Sediment samples were air-dried for 1 week prior to isolation. Then, the samples were sieved to remove large mineral and organic matter particles. Selective pretreatments of sediment samples were conducted following the methods described by Hong *et al.* (2009); (i) dry heat at 120 °C, 60 min (Pisano *et al*., 1986); (ii) wet heat in sterilized sea water (55 °C, 15 min) (Takahashi *et al*., 1996); and (iii) addition of phenol (1.5 %, 30 min at 30 °C) (Pisano *et al.*, 1986). The pretreated sediment samples were diluted 1:10 v/v with saline followed by serial dilutions (10^{-2} to 10^{-5}) and plating on media. Plating of each dilution was carried out in triplicates. Eight selective isolation media including yeast-extract-malt-extract agar (ISP2) (Shirling and Gottlieb, 1966), oatmeal agar (ISP3) (Shirling and Gottlieb, 1966), inorganic-salt-starch agar (ISP4) (Shirling and Gottlieb, 1966), starch-yeast-extract agar (SYE) (Emerson, 1958), marine agar (MA) (Difco, New Jersey), actinomycetes isolation agar (AIA) (Difco, New Jersey), Gause-modified medium (IM2) (Ivantiskaya, *et al*., 1978) and starch-casein agar (IM7) (Kuster and Williams, 1964) were used for the selective isolation of potential actinomycetes. All media were supplemented with 50 mg/L cycloheximide to inhibit fungal growth followed by incubation at 30 °C for two weeks. Based on the colony morphology, the actinomycetes cultures were selected and purified on the selective media.

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Screening for antimicrobial activity

The potency of mangrove actinomycetes to produce antimicrobial substances was investigated in 61 isolates which were chosen randomly based on their morphologies. Antimicrobial activity of the selected strains were checked using cross streak method as described by Oskay (2009), against three Gram positive bacteria; *Bacillus subtilis* (IMR O 145/11C), *Streptococcus pyogenes* (ATCC 19615), *Staphylococcus aureus* (IMR S 1414/08A), four Gram negative bacteria; *Escherichia coli* (ATCC 25922), *Serratia marcescens* (IMR S 1406/08A), *Salmonella typhimurium* (IMR S 974/105B), *Klebsiella pneumoniae* (IMR K 46/09A) and one fungus; *Candida albicans* (IMR C 523/11A). Nutrient agar (NA) plates were prepared and all selected isolates were streaked as a single line at the centre of the petri plate. These plates were incubated at 30 °C for 7 days. After observing a good ribbon like growth of the actinomycetes, the plates were then inoculated with the test bacteria by a single streak at 90° angles to the actinomycete strains. This step was done in triplicates for each test microorganisms followed by incubation at 37 °C (bacteria) for 24 h and 30 °C (fungus) for 48 h. The decrease in length between the edge of the test bacterial growth and the actinomycete colony formed were measured and recorded in millimetre (mm). Control plates were also maintained without inoculating actinomycetes to assess the normal growth of the test pathogens.

Molecular identification

Ten selected isolates (IIUM A08, IIUM A11, IIUM A44, IIUM B15, IIUM B21, IIUM B24, IIUM B25, IIUM B31, IIUM E01 and IIUM E05) were identified using PCR amplification of 16S rRNA gene. The genomic DNA used for the PCR was prepared from the single colonies grown in the marine broth for 7 days. The total genomic DNA for putative actinomycetes was extracted using the GF-1 Nucleic Acid Extraction Kit (Vivantis) according to the manufacturer's protocol. The 16S rRNA gene fragment was amplified using the following primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Wilson *et al.*, 1990). The reaction mixture was prepared in a total volume of 50 µL containing 10% of extracted DNA, 15 µM of each primer, 10 mM of dNTPs, 10× PCR buffer, 25 mM of $MgCl_2$ and 5 U *Taq* DNA polymerase (PROMEGA, USA). The PCR temperature profiles were 94 °C for 3 min, 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and finally an extension step at 72 °C for 5 min. Amplification products were analyzed by electrophoresis in 1 % (w/v) agarose gel stained with ethidium bromide. PCR products obtained were then purified using QIAquick PCR Purification Kit (QIAGEN, German) according to the protocol provided by the manufacturer. The purified PCR products were then sent for sequencing (1st BASE DNA Sequencing Service). Nucleotide sequences obtained were analysed and edited by using BioEdit Sequence Alignment Editor. Sequentially, BLAST analysis on partial 16S rRNA sequences of the isolates was carried out via GenBank BLASTn (http://www.ncbi.nlm.nih.gov) search tool.

RESULTS AND DISCUSSION

A total of 172 potential actinomycetes were isolated from 5 different sampling sites in Tanjung Lumpur. Several potential actinomycetes were illustrated in Figure 1. The presence of relatively large populations of actinomycetes in the sediment samples of Tanjung Lumpur mangrove indicates that the source is an eminently suitable ecosystem.

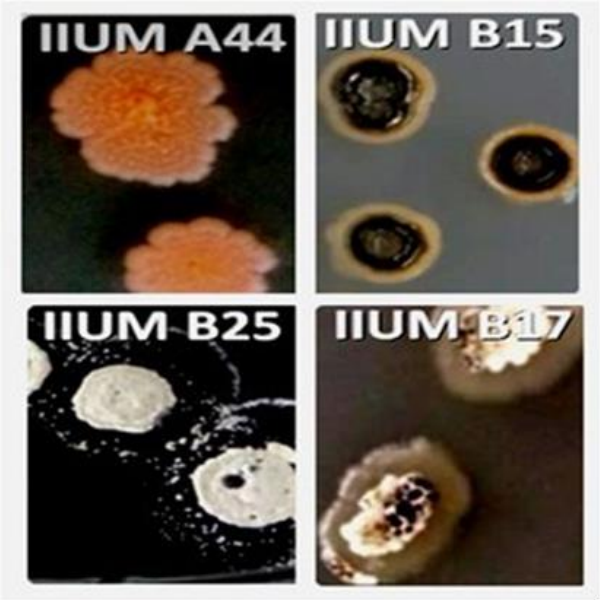

Figure 1: Among the potential actinomycetes isolated.

Effect of pretreatment of sediment

Pretreatments generally stimulate the isolation of rare actinomycetes by inhibiting or eliminating unwanted microorganisms. The number of colonies recovered was in the order of other bacteria, *Streptomyces*, fungi and non-streptomycete actinomycetes when the mangrove soils were cultured without pretreatment (Seong *et al.*, 2001). When the soil was air-dried, other bacterial numbers decreased, while the growth of the slow growing actinomycete colonies was enhanced.

In this study, pretreatment method using wet heat in sterilized sea water at 55 °C for 15 min was the most effective method for the isolation of actinomycetes as indicated by high number of isolates (105 isolates) whereas dry heat treatment yielded only 67 isolates. However, pretreament with phenol produced no actinomycetes growth, instead led to high recovery of fungi. The results were as shown in Figure 2. Previous studies have also reported, that pretreatment of wet-heating for 55 °C for 15 min and IM7 medium were the most effective for the isolation of actinomycetes (Kalyani

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et al., 2012; Gebreyohannes *et al.*, 2013; Jagan *et al*., 2013). When pretreatments were carried out, the number of bacterial and fungal contamination was reduced and allowed selective isolation of actinomycetes which included *Streptomyces* spp*., Nocardia, Micromonospora* and *Rhodococcus* (Jagan *et al*., 2013).

Heat treatments by moist-heating and dry-heating have long been employed to select for various actinomycete groups. The relatively mild heating regimens (50 °C, 10 min) are extremely useful standard procedures for routine isolations and have been proven to eliminate bacterial competitors from actinomycete isolation plates (Labeda and Shearer, 1990). It was also proven in previous study that the recovery of actinomycetes, specifically, rare actinomycetes such as *Micromonospora*, *Actinoplanes*, and *Actinomadura*, increased up to 50% of the total microorganisms and inhibited the fungal and bacterial colonies by heating the soil suspension at 70 °C for 15 min (Naikpatil and Rathod, 2011). Some of the selective pretreatments, for example dry heat and exposure to 1.5% of phenol were indeed designed for the selective isolation of *Micromonospora.*

A study conducted by Bredholt *et al.* (2008) reported that it was possible to obtain isolation plates containing actinomycetes with morphologies typical for the genera *Micromonospora* using phenol pretreatment. However, very few *Micromonospora* isolates from deep water samples survived these treatments. This could suggest that the potential actinomycetes in this mangrove sediment could not survive through phenol pretreatment due to the signs of adaptation to their marine ecosystem. Even though previous studies have found that phenol treatment successfully recovered actinomycetes (Seong *et al*., 2001; Bredholt *et al*., 2008), this study however showed otherwise. No actinomycetes were recovered using this pretreatment, instead high number of fungi were obtained. This suggested that the selective pretreatments designed to isolate actinomycetes were not optimal for different type of samples (soil, sediments or water) and more research is required in order to establish methods allowing specific enrichment of mangrove actinomycetes.

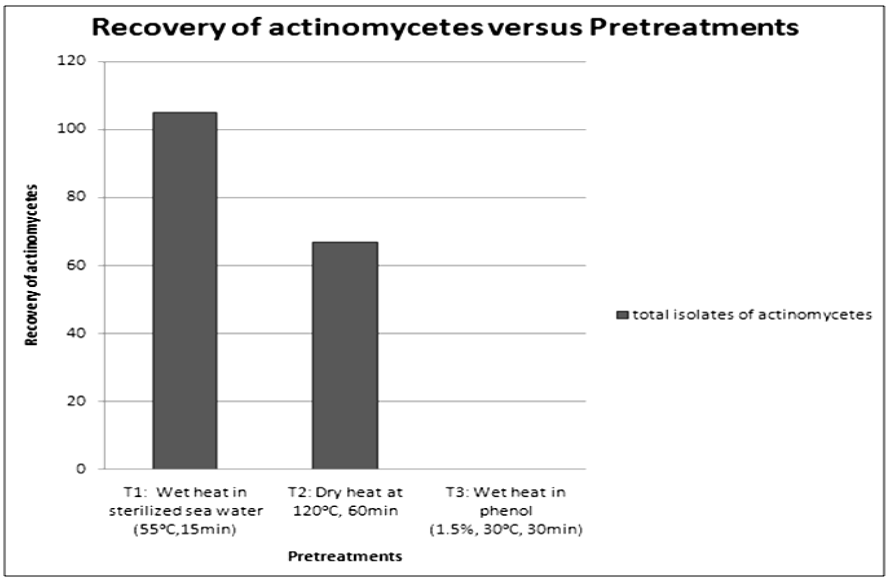

Figure 2: The recovery of actinomycetes based on pretreatments.

Selective isolation media for potential actinomycetes

In recent years, the most interesting and significant advances have been made in the area of selective media (Seong *et al*., 2001; Sivakumar *et al*., 2005; Kalyani *et al*., 2012). An isolation medium carries a variety of bacteria colonies growing in intense competition and a modification to the medium can influence the growth of several species, which in turn can encourage or discourage the growth of others species.

In this study, eight different selective media were applied to assess the optimal conditions for the isolation of rare actinomycetes from sediment. As shown in Figure 3, IM7 was the most suitable medium as it showed the highest percentage for the recovery of actinomycetes (31 %). Naikpatil and Rathod (2011) stated that IM7 improved the growth of actinomycetes including *Streptomyces*. Moreover, this medium also seems to be specific and suitable for actinomycetes, because it contains starch that most actinomycetes use as a carbohydrate source and casein as nitrogen source (Sweetline *et al.*, 2012). This finding was also in consistent with a recent study by Prabhar *et al.* (2014) which indicated that IM7 promotes good growth of actinomycetes compared to other media employed in this study.

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Appropriate selective media containing macromolecules like casein, chitin, humic acid are widely used for promoting growth of rare actinomycetes present in the soil samples and simultaneously suppressing or hindering the contaminant bacterial or fungal colonies (Qiu *et al.*, 2008; Cuesta *et al*., 2012). Usage of these selective media greatly reduces the number of unwanted bacteria present on isolation plates, because other bacteria, in contrast to actinomycetes, grow better on media with low carbon-to-nitrogen ratios. Enhancement of actinomycetes growth by addition of calcium carbonate and chitin to the growth medium has also been known (Khanna *et al*., 2011). These inorganic or organic substances can be utilized by actinomycetes as sources of nitrogen and carbon. However in this study, the composition of substances in ISP4 might not suitable for isolation of actinomycetes from mangrove sediments as it recovered only 8 isolates.

All 172 isolates were then categorized based on the production of spore and diffusible pigment (Table 1). Based on the results, there were 132 actinomycetes that produces spore and 40 non spore-forming actinomycetes. All of the selective media used in this study produced more spore-forming isolates than non spore-forming isolates and such results were expected due to their abundance in soil (Wang *et al*., 2001; Ceylan *et al*., 2008).

All selective media produced both spore-forming and nonspore-forming isolates except IM2 which only produced spore-forming isolates. According to Zhang and Zhang (2011), generally strains isolated from IM2 were mainly belonging to *Streptomyces*, while other rare actinomycetes were difficult to isolate. Moreover, many novel and potential *Streptomyces* which have important capabilities for natural product discovery had been isolated using IM2 (Zhu *et al*., 2007; Liang *et al.*, 2009; Lin *et al*., 2011). Out of 16 isolates originated from ISP2 agar, 9 (56.3%) isolates belonged to spore-forming actinomycetes and 7 (43.7%) isolates were non spore-forming actinomycetes. The use of ISP2 promoted growth of actinomycetes in a significant way and resulted in wide range of colonies (Algafari, 2014).

Actinomycetes are known to produce many types of antibiotics and pigments. Production of pigments by actinomycetes has been utilized as an important cultural characteristic in describing the organisms (Perumal *et al*., 2009). Dark brown substance released into the medium is generally referred to as melanin or melanoid pigment. Other coloured diffusible pigments are sometimes related to antimicrobial compounds (Shabaan, 2011). Among the total recovery of actinomycetes isolated from the mangrove sediment samples, 29 isolates produced diffusible pigments which were light brown, dark brown, red, light green, black and yellow in color. IM7 yielded the highest number of actinomycetes producing diffusible pigment with 11 isolates that produced brown and red pigments. Most of the isolates were capable of producing brown pigment which were derived from MA (6 isolates), ISP2 (4 isolates), AIA (2 isolates) and SYE (1 isolate).

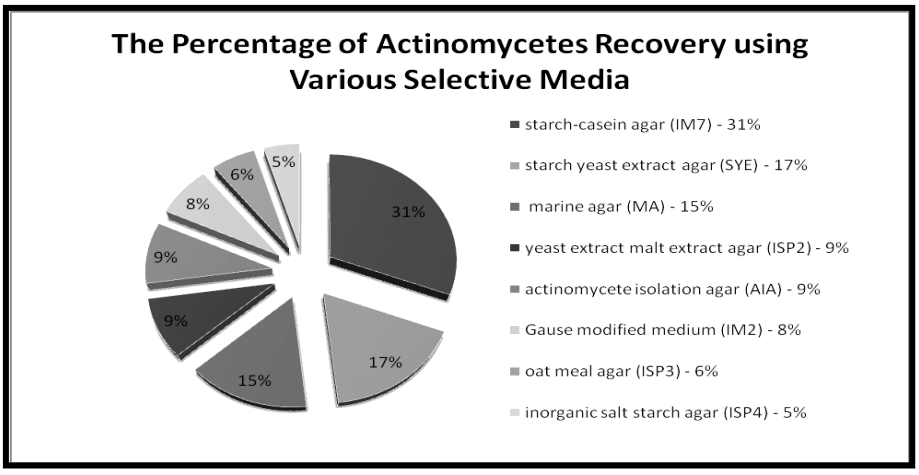

Figure 3: The recovery of potential actinomycete isolation using different selective media.

Antimicrobial activity of actinomycetes isolates

Among the 61 isolates tested, 43 isolates (70.5%) showed antimicrobial activities against more than one genus of test microorganisms (Tables 2 and 3). Isolates IIUM B21 and IIUM B31 showed antimicrobial activity against all the test microorganisms. They demonstrated good activity against *B. subtilis* (IIUM B21: >32.0±2.6 mm, IIUM B31: 26.3±1.5 mm), *S. pyogenes* (IIUM B21: >31.7±2.9 mm, IIUM B31: 23.0±6.1 mm) and *C. albicans* (IIUM B21: 29.3±1.2 mm, IIUM B31: 26.7±2.9 mm). Eight Isolates (IIUM E01, IIUM B33, IIUM B40, IIUM E67, IIUM B02, IIUM B03, IIUM B23, IIUM B25) were found showing antagonistic activity against both Gram positive and Gram negative test bacteria while 21 isolates showed antagonistic activity against both Gram positive test bacteria and *C. albicans*. In addition, among the 61 isolates tested, 4 isolates (IIUM A23, IIUM A24, IIUM A67,

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IIUM E11) only displayed inhibitory activity against Gram positive test bacteria.

Most of the selected isolates showed more antimicrobial activities against Gram positive bacteria (35 isolates) than Gram negative bacteria (10 isolates). This is because of the cell wall of Gram negative bacteria possess an outer membrane of lipopolysaccharide and phospholipids which form a lipid bilayer that serves as barrier against antibacterial drugs (Struelens, 2003; Mohseni *et al*., 2013). However, some antibacterial compounds could still be effective against Gram negative bacteria. In this study, isolates IIUM B03 and IIUM E01 were found to have good activity against *E. coli* (IIUM B03: 23.3±3.1 mm, IIUM E01: 26.0±4.4 mm), IIUM B33 against *S. marcescens* (27.0±2.6 mm), IIUM E67 against *S. thypimurium* (20.7±0.6 mm) and IIUM B25 against *K. pneumoniae* (27.7±1.2 mm). Of 61 isolates tested, 38 isolates produced anti-*Candida albicans* metabolites. Out of 38 isolates, 8 isolates only had the potential of producing anticandidial metabolite including IIUM A29 (24.3±4.9 mm), IIUM A48 (7.7±2.1 mm), IIUM A60 (22.3±9.1 mm), IIUM B05 (21.0 ±9.6 mm), IIUM E06 (21.7±1.5 mm), IIUM E18 (26.7±2.5 mm), IIUM E28 (15.3±0.6 mm) and IIUM E32 (15.3±0.6 mm), as they were found to inhibit only the growth of *C. albicans*.

Analysis of the 16S rRNA gene sequence of the selected isolates

The partial 16S rRNA sequence of 10 selected isolates were aligned and compared with all the 16S rRNA gene sequence available in the GenBank database by using the multi sequence advanced BLAST comparison tool that is available in the website of National Centre for Biotechnology Information (NCBI). Partial 16S rRNA sequences of the isolates were deposited to GenBank database under the following accession numbers: IIUM A08 (KP085604), IIUM B21 (KP085609), IIUM B25 (KP085611), IIUM B31 (KP085612), IIUM E01 (KP085614), IIUM E05 (KP085615), IIUM B15 (KP085608), IIUM B24 (KP085610), IIUM A11 (KP085605), IIUM A44 (KP085607). Comparison of partial sequence of 16S rRNA gene revealed 6 isolates (IIUM A08, IIUM B21, IIUM B25, IIUM B31, IIUM E01, IIUM E05) belong to genus *Streptomyces,* 2 isolates (IIUM B15 and IIUM B24) belong to genus *Micromonospora* and another 2 isolates (IIUM A11 and IIUM A44) belong to genus *Rhodococcus.* The morphological characterizations and identification of these isolates were illustrated in Table 4.

Among the Streptomyces isolates, IIUM B21 and IIUM B31 possessed high antimicrobial activity against *B. subtilis*, *S. pyogenes* and *C. Albicans.* Partial sequence of 16S rRNA gene of isolate IIUM B21 matched *Streptomyces variabilis* strain 7525 (GenBank accession number: JN 180216) with 99 % identity, and IIUM B31 matched *Streptomyces erythrogriseus* strain CTF13 (EU 301830) with 99% identity in GenBank database respectively. Isolates IIUM B15 and IIUM B24 were found to be closely related to *Micromonospora* spp. while isolates IIUM A11 and IIUM A44 were closely related to *Rhodococcus* spp.

In this study, *Micromonospora* strains were isolated using dry heat method and both isolates IIUM B15 and IIUM B24 were recovered from IM7 medium which indicate that IM7 is the medium of choice for isolation of *Micromonospora* spp. However, only IIUM B24 showed antimicrobial activities against *B. subtilis* (15.3±1.5 mm), *S. pyogenes* (12.0±1.7 mm), *S. aureus* (4.0±2.0 mm) and *C. albicans* (8.7±1.2 mm), while no inhibition zone was detected in IIUM B15. Comparison of partial sequence of 16S rRNA gene revealed a close relationship between strains IIUM B15 and *Micromonospora marina* JSM1-1 (NR 112537, 98 %). *Micromonospora marina* JSM1-1 is a novel species isolated from sea sand, which has similar morphology with IIUM B15 strain (Tanasupawat *et al.*, 2010). Isolates IIUM A11 and IIUM A44 showed their similarities to members of the genus *Rhodococcus.* In particular, IIUM A11 was 99 % identical to *Rhodococcus ruber* H4a (JX 979148) while IIUM A44 was 99% identical to *Rhodococcus rhodochrous* SR-6 (JQ 040005). However, no antimicrobial activity was detected in both isolates. The genus *Rhodococcus* is usually associated with biotransformation of xenobiotic (Finnerty, 1992) and have significant values in biodegradation of diverse pollutants (Gilan *et al*., 2004; Loubna and Abdel, 2013).

Table 1: The recovery of actinomycete isolates on each medium for each pretreatment.

Selective Media	Diffusible pigment	Total recovery actinomycetes			Spore production	
		Pretreatment 1 (T1)	Pretreatment 2 (T2)	Pretreatment 3 (T3)	No. of Spore-forming isolates	No. of non spore-forming isolates
IM7	11	23	30	-	40	13
SYE	1	25	5	-	21	9
MA	6	25	1	-	24	2
ISP2	4	-	16	-	9	7
AIA	2	11	5	-	11	5
IM2	2	7	6	-	13	-
ISP3	1	7	3	-	7	3
ISP4	2	7	1	-	7	1

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Table 2: Selected actinomycetes in Pretreatment 1 (T1) that showed antimicrobial activities using cross streak method.

Media	Isolates	Inhibition zone (mm)							
		B. subtilis	*S. pyogenes*	*S. aureus*	*C. albicans*	*E. coli*	*S. marcescens*	*S. thypimurium*	*K. pneumoniae*
SYE	IIUM A23	6.0±1.0	5.3±0.6	-	-	-	-	-	-
	IIUM A24	27.5±2.5	20.0±6.0	-	-	-	-	-	-
MA	IIUM A29	-	-	-	24.3±4.9	-	-	-	-
	IIUM A33	24.7±0.6	-	-	24.7±0.6	-	-	-	-
	IIUM A34	28.0±2.6	15.7±3.1	4.0±3.6	28.7±7.8	-	-	-	-
	IIUM A48	-	-	-	7.7±2.1	-	-	-	-
	IIUM A55	20.0±5.0	-	-	12.7±11.0	-	-	-	-
IM2	IIUM A60	-	-	-	22.3±9.1	-	-	-	-
ISP3	IIUM A67	15.0±6.2	-	-	-	-	-	-	-
IM7	IIUM E01	29.7±3.2	26.3±1.2	-	>31.3±1.2	26.0±4.4	-	-	-
	IIUM E03	>33.3±1.5	-	-	>30.3±0.6	-	-	-	-
	IIUM E05	26.3±3.1	>30.3±0.6	17.7±2.1	26.3±3.1	-	-	-	-
	IIUM E06	-	-	-	21.7±1.5	-	-	-	-
	IIUM E11	23.0±3.6	-	-	-	-	-	-	-
	IIUM E14	>30.3±2.1	-	-	20.3±0.6	-	-	-	-
	IIUM E18	-	-	-	26.7±2.5	-	-	-	-
	IIUM E23	28.3±3.1	19.7±8.1	-	26.3±1.5	-	-	-	-
ISP4	IIUM E28	-	-	-	15.3±0.6	-	-	-	-
AIA	IIUM E54	>31.7±3.5	26.0±3.6	-	>30±1.0	-	-	-	-
	IIUM E55	>35.3±1.5	-	-	17.7±2.5	-	-	-	-
	IIUM E57	27.7±2.1	18.3±2.9	-	16.7±6.1	-	-	-	-
	IIUM E60	>36.7±1.5	13.3±1.5	14.3±3.8	22.7±2.5	-	-	-	-
	IIUM E64	26.7±1.5	15.7±1.5	-	28.3±4.0	-	-	-	-

*- , No activity; ≥16mm, Good activity; ≤ 11-15mm, Moderate activity; ≤ 10mm, Weak activity.

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Table 3: Selected actinomycetes that showed antimicrobial activities in Pre-treatment 2 (T2) using cross streak method.

Media	Isolates	Inhibition zone (mm)							
		B. subtilis	*S. pyogenes*	*S. aureus*	*C. albicans*	*E. coli*	*S. marcescens*	*S. thypimurium*	*K. pneumoniae*
SYE	IIUM B31	26.3±1.5	23.0±6.1	13.7±12.3	26.7±2.9	13.7±1.2	7.3±1.5	4.0±1.0	4.7±0.6
	IIUM B33	27.0±2.6	10.0±2.0	16.0±3.5	28.0±4.4	-	27.0±2.6	-	-
	IIUM B34	27.7±2.1	13.7±1.2	17.3±1.5	27.7±2.5	-	-	-	-
	IIUM B40	27.7±2.5	18.0±0.0	23.0±4.0	24.7±2.5	12.0±10.4	10.7±12.4	-	-
MA	IIUM B38	-	-	-	-	-	-	-	-
IM2	IIUM E32	-	-	-	15.3±0.6	-	-	-	-
	IIUM E34	>33.3±1.5	7.0±2.0	-	25.7±5.1	-	-	-	-
	IIUM E67	4.0±1.0	14.7±0.6	17.7±0.6	-	14.7±0.6	-	20.7±0.6	3.7±0.6
	IIUM E68	>37.3±1.5	19.7±1.5	-	25.0±3.0	-	-	-	-
ISP3	IIUM B35	7.3 ±8.7	17.0 ±15.4	17.0 ±14.7	5.3 ±9.2	-	-	-	-
IM7	IIUM B02	27.3±2.5	15.3±2.1	16.3±1.5	>30.3±2.1	-	4.3±0.6	2.0±0.0	3.7±1.5
	IIUM B03	29.3±4.5	11.7±1.2	23.3±1.5	27.7±2.5	23.3±3.1	14.7±0.6	-	-
	IIUM B05	-	-	-	21.0±9.6	-	-	-	-
	IIUM B08	>32.7±3.1	12.3±2.1	21.0±1.0	27.7±2.5	-	-	-	-
	IIUM B21	>32.0±2.6	>31.7±2.9	26.0±2.0	29.3±1.2	15.3±2.1	11.0±1.0	1.7±0.6	2.0±0.0
	IIUM B23	17.0±1.7	28.7±3.2	-	>31.7±2.9	13.0±3.5	13.0±1.7	-	-
	IIUM B24	15.3±1.5	12.0±1.7	4.0±2.0	8.7±1.2	-	-	-	-
	IIUM B25	29.0±2.6	25.3±0.6	-	16.3±1.5	-	15.7±1.5	-	27.7±1.2
	IIUM B27	>34.0±3.5	7.7±0.6	-	22.3±1.2	-	-	-	-
ISP4	IIUM B39	>32.0±2.0	7.0±1.7	19.7±17.0	>31.0±1.7				
AIA	IIUM E51	>33.0±3.6	24.7±1.5	17.0±2.0	>30.3±0.6	-	-	-	-

*- , No activity; ≥16mm, Good activity; ≤ 11-15mm, Moderate activity; ≤ 10mm, Weak activity.

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Table 4: Morphological characterization of the identified isolates.

Isolates	Media	Aerial mycelium	Substrate mycelium	Diffusible pigment	Closest BLAST match	Identity (%)
IIUM A08 (KP085604)	SYE	Red white	Creamy yellow	-	*Streptomyces fradiae* G0S1	99
IIUM B21 (KP085609)	IM7	Brownish white	Brown	Brown	*Streptomyces variabilis* 7525	99
IIUM B25 (KP085611)	IM7	Grey	Dark maroon	Red	*Streptomyces carpaticus* BTSS-501	98
IIUM B31 (KP085612)	SYE	Whitish grey	Creamy grey	-	*Streptomyces erythrogriseus* CTF13	99
IIUM E01 (KP085614)	IM7	White	Cream	-	*Streptomyces parvulus* KUAP106	99
IIUM E05 (KP085615)	IM7	White	White	-	*Streptomyces owasiensis* NBRC 13832	97
IIUM B15 (KP085608)	IM7	Oranged black	Oranged black	-	*Micromonospora marina* JSM1-1	98
IIUM B24 (KP085610)	IM7	Oranged black	Orange	-	*Micromonospora aurantiaca* Z9-4	98
IIUM A11 (KP085605)	SYE	Colony colour: pink		-	*Rhodococcus ruber* H4a	99
IIUM A44 (KP085607)	MA	Colony colour: pink		-	*Rhodococcus rhodochrous* SR-6	99

* -, Not detected.

Tanjung Lumpur mangrove as a potential actinomycetes resource

The mangrove ecosystem is becoming a hot spot for natural product studies and bioactivity discovery as diverse mangrove actinomycetes have been explored and discovered as promising and productive sources. Xu *et al.* (2014) reported that until now there were 73 novel compounds and 49 known compounds isolated from mangrove actinomycetes including alkaloids (Xie *et al.*, 2008), benzene derivatives (Chen *et al.*, 2011), cyclopentenone derivatives (Lin *et al.*, 2005), dilactones (Yan *et al.*, 2011), macrolides (Ding *et al*., 2011), 2-pyranones and sesquiterpenes (Guan *et al*., 2005).

Potential actinomycetes found in Tanjung Lumpur mangrove sediment had attracted a great deal of attention as several actinomycetes showed distinct and unusual morphologies and characteristics with high antimicrobial activities. Although few studies have been conducted on actinomycetes from mangrove soils in Malaysia (Vikineswary *et al*., 2003; Suhaidi *et al*., 2012), this study undoubtedly adds more information on suitable pretreatments and selective media to be utilized for high recovery of actinomycetes. Moreover, this study also corroborates the potential of Tanjung Lumpur mangrove actinomycetes hold as source of antimicrobial agents. Thus, mangrove sediment of Tanjung Lumpur seems to be unique habitat for the isolation of novel actinomycetes with the potential to yield useful for pharmaceutical products.

CONCLUSION

In conclusion, this study was designed to prepare the ground for such studies by isolating a diverse range of actinomycetes and investigating the antimicrobial potential of actinomycetes isolated from mangrove sediments in Tanjung Lumpur, Kuantan. A substantial diversity of actinomycetes was isolated in this research, and several isolates have high potential for further investigation pertaining to natural product discovery. The findings indicate that mangrove habitat is valuable source of discovery for diverse genera of actinomycetes with promising potential to produce antimicrobial metabolites that useful for future treatments of multidrug resistant human pathogens. Therefore, this research also provided comprehensive survey of culturable actinomycetes present in mangrove forests especially in Malaysia.

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