ZIBELINE INTERNATIONAL PUBLISHING

ISSN: 2521-0912 (Print)
ISSN: 2521-0513 (Online)
CODEN: JCOLBF

Journal Clean WAS (JCleanWAS)

DOI: http://doi.org/10.26480/jcleanwas.02.2020.79.83

RESEARCH ARTICLE

RARE ACTINOMYCETES FROM KUANTAN MANGROVE FOREST SEDIMENT

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ARTICLE DETAILS

Article History:

Received 08 July 2020
Accepted 12 August 2020
Available online 26 August 2020

ABSTRACT

Actinomycetes are prolific secondary metabolites producer, and they are sought after for their unparalleled capability. Mangrove forests are among the underexplored locations in search of new resources of actinomycetes. Bioprospecting of actinomycetes in Kuantan mangrove forests has revealed highly diverse actinomycetes with high antimicrobial properties. Members of the rare genera successfully isolated include *Micromonospora* sp., *Pseudonocardia* sp., *Verrucosispora* sp., *Nocardiopsis* sp., *Actinophytocola* sp., *Dietzia* sp., *Gordonia* sp., *Micrococcus* sp., *Mycobacterium* sp., *Nocardia* sp., *Saccharopolyspora* sp. And *Rhodococcus* sp. These rare actinomycetes can also be added to the list of genera isolated from this ecological niche, providing solid evidence that considerable diversity of actinomycetes are distributed within Kuantan mangrove forest. PKS-I and NRPS genes are usually related to the ability to produce secondary metabolites in actinomycetes. Interestingly, several of these rare actinomycetes showed the presence of both PKS-I and NRPS genes or either gene, and this exemplifies the potential of these rare actinomycetes may possess. Further studies conducted on these rare actinomycetes may reveal their true potentials that can be exploited for natural product discovery.

KEYWORDS

rare actinomycetes, diversity, PKS-I and NRPS, mangrove forest.

1. INTRODUCTION

Actinomycetes can be categorized into two major groups: the dominant group and the rare actinomycetes group (Azman et al., 2015). In the natural habitat, *Streptomyces* and *Micromonospora* are among the dominant genus of actinomycetes with more than 600 and 30 species described respectively (Genilloud et al., 2011; Labeda et al., 2012; Hirsch and Valdes, 2010). On the other hand, genera including *Actinoplanes*, *Dactylsporangium*, *Kineosporia*, *Microbispora* and *Virgosporangium* that have lower isolation rates and are harder to cultivate due to their extremely slow growth are known as rare actinomycetes (Hayakawa, 2008; Khanna et al., 2011; Subramani and Aalbersberg, 2013; Tiwari and Gupta, 2013). Rare actinomycetes are defined as genera in which the isolation frequency by conventional methods is lower than the *Streptomyces* abundance. The conventional approach to the search for potentially valuable bioactive actinomycetes from natural habitats has been strictly restricted to the cultivation of small fraction of actinomycetes community, predominantly members of genus *Streptomyces* (Hames and Uzel, 2012).

The presence of other uncultured or rare actinomycetes taxa was solely known through their molecular fingerprints (Hayakawa, 2008; Subramani and Aalbersberg, 2013). Although genus *Streptomyces* is accounted for almost 70% of the commercial antibiotics, rare actinomycetes also contributed to bioactive compounds registry. To date, rare actinomycetes represented approximately 26% of the antimicrobial compounds with more than 50 rare actinomycete taxa have been reported as the producers of 2,500 antimicrobial compounds (Kurtboke, 2012; Subramani and Aalbersberg, 2013). Members of genus *Actinomadura*, *Actinoplanes*, *Saccharopolyspora* and *Streptoverticillium* are the most frequent producers among the rare actinomycete groups, and each produces hundreds of antibiotics (Berdy, 2005; Subramani and Aalbersberg, 2013). Studies suggested that considerable progress should be made to screen actinomycetes from poorly studied habitat and focusing on rare actinomycete groups that are hardly brought into culture, with an assumption that species novelty will lead to chemical novelty (Goodfellow and Fiedler, 2010; Subramani and Aalbersberg, 2013).

Unexplored and underexplored environments such as extreme environments, marine ecosystems and mangrove forest are considered a promising source of rare actinomycete that may lead to the discovery of structurally new and interesting compounds. Recently, underexplored mangrove ecosystems have been looked up as potential sources of high diversity actinomycetes (Thatoi et al., 2013). A number of reports from different geographical areas have described the occurrence of actinomycetes in various mangrove ecosystems. From 2007 to 2013, the isolation of 14 new rare actinomycetes species have been described from the mangrove ecosystem, and the number keeps on growing. Among the new species belong to 7 families which include *Acidimicrobiaceae, Demequinaceae, Micrococcineae, Micromonosporaceae,*

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	Website: www.jcleanwas.com	**DOI:** 10.26480/jcleanwas.02.2020.79.83

Cite the Article: Zaima Azira Zainal Abidin, Nurfathiah Abdul Malek, Nur Huda Mohd Zin, Ahmed Jalal Khan Chowdhury (2020). Rare Actinomycetes From Kuantan Mangrove Forest Sediment. *Journal Clean WAS, 4(2): 79-83*.

Promicromonosporaceae, Streptosporangiaceae and Thermonosporaceae (Subramani and Aalbersberg, 2013). Moreover, two novel genera - Ilumatobacter and Lysinimicrobioum were reported from mangrove sediments (Matsumoto et al., 2009; Hamada et al., 2012). Isolation of various novel taxonomic groups displays that mangrove sediments are an abundant source for the isolation of less exploited mangrove actinomycetes. Kuantan mangrove forest is foreseen as a virtually underexplored habitat for rare and novel actinomycete taxa. Hence, this study was conducted to assess the diversity of rare actinomycetes present in Kuantan mangrove forest with biosynthetic potentials.

2. Materials and Methods

2.1 Rare Actinomycetes

Rare actinomycetes in this study were previously isolated from mangrove sediment collected from 7 locations along the Kuantan estuary as depicted in Table 1 (Zainal, et al., 2016). Region1 (K1, K2 and K3) is located at the dense mangrove forest which is further from residential areas, while Region 2 (K4, K5, K6 and K7) is situated at the intertidal region under the influenced of terrestrial run-off.

Table 1: Location of the sampling sites at Kuantan mangrove forest

Sampling location code	GPS coordinate	
	Latitude	Longitude
K1	N 03° 47.210'	E 103° 18.410'
K2	N 03° 46.965'	E 103° 18.976'
K3	N 03° 47.355'	E 103° 19.527'
K4	N 03° 48.074'	E 103° 19.682'
K5	N 03° 48.068'	E 103° 19.671'
K6	N 03° 48.145'	E 103° 19.804'
K7	N 03° 48.237'	E 103° 19.933'

2.2 Detection of PKS-I and NRPS Genes

Two sets of degenerate primers as recommended by Ayuso-Sacido and Genilloud (2005) were used in a multiplex PCR amplification of both NRPS and PKS-I genes in actinomycete isolates. Adenylation domains of NRPS gene were PCR amplified from the genomic DNA using the primers A3F and A7R, while the PCR primers KIF and M6R were used to target the ketoacyl synthase and malonyl-malonyl-CoA transferase domains of PKS-I gene. The PCR reaction mixture consisted of 200 ng genomic DNA, 0.4 µM of each primer, 25 µl of 2X MyTaq™ Mix (Bioline, UK), 5 µl DMSO and sterile ultrapure water was added to a final volume of 50 µl. PCR amplifications were carried using the Eppendorf Mastercycler Gradient (Germany) under the following conditions: initial denaturation at 95°C for 5 minutes followed by 35 cycles of 95°C for 30 seconds, 58°C for 2 minutes, and 72°C for 2 minutes and final extension step at 72°C for 10 minute. Amplified products were examined by electrophoresis in 1.0 % (w/v) agarose gel.

2.3 PCR amplification of 16S rRNA gene

Universal primers of 27F (5'–AGAGTTTGATCCTGGCTGGTCCAG–3') and 1492R (5'–GGTTACCTTGTTACGACTT–3') designed to target the conserved region of 16S rRNA gene were used to amplify an approximately 1.5 kb long DNA fragment (Wilson et al., 1990). Amplification was performed in a final volume of 50 µl which consist 200 ng of template genomic DNA, 0.4 µM of each primer, 25 µl of 2 ×MyTaq™ Mix (Bioline, UK) and sterile ultrapure water. The 16S rRNA gene fragment amplification reaction was carried out in an automated thermal cycler (Eppendorf Mastercycler Gradient, Germany) using the following temperature profile: initial denaturation at 94°C, followed by 30 cycles of 94°C for 30 seconds, 55°C for 60 seconds, and 72°C for 4 minutes, and final extension step at 72°C for 1 minute. The PCR products were visualized with 1.0 % (w/v) agarose gel electrophoresis. The resultant 16S rRNA sequences were manually verified using Sequence Scanner software version 2.0 (Applied Biosystems) to remove low-quality bases. The resultant forward and reverse sequences were assembled using the Bioedit Sequence Alignment Editor program. The sequences were matched with the GenBank database using BLASTn (htttp://www.ncbi.mlm.nih.gov) search tool. Partial 16S rRNA gene sequences were deposited to GenBank for accession numbers.

3. Results and Discussion

Bioprospecting programme of actinomycetes in Kuantan mangrove forests successfully produced high recovery of actinomycetes, with genus *Streptomyces* dominating the overall actinomycetes composition from both highly bioactive regions. Concurrently, several rare actinomycetes were also recovered through this bioprospecting programme which was distributed among 7 families and 12 genera: Dietziaceae (*Dietzia*), Micrococcaceae (*Micrococcus*), Micromonosporaceae (*Micromonospora*, *Verrucosispora*), Mycobacteriaceae (*Mycobacterium*), Nocardiaceae (*Gordonia*, *Nocardia*, *Rhodococcus*), Nocardiopsaceae (*Nocardiopsis*) and Pseudonocardiaceae (*Actinophytocola, Pseudonocardia, Saccharopolyspora*) (Table 2) (Figure 1). When analyzing the diversity of rare actinomycetes from specific sites, it was observed that members of 4 genera which include *Micromonospora*, *Gordonia*, *Pseudonocardia,* and *Verrucosispora* were isolated from both Region 1 and Region 2. A total of three genera were found exclusively in Region 1 (*Micrococcus*, *Nocardia* and *Saccharopolyspora*) and five exclusively in Region 2 (*Actinophytocola*, *Dietzia*, *Mycobacterium*, *Nocardiopsis* and *Rhodococcus*).

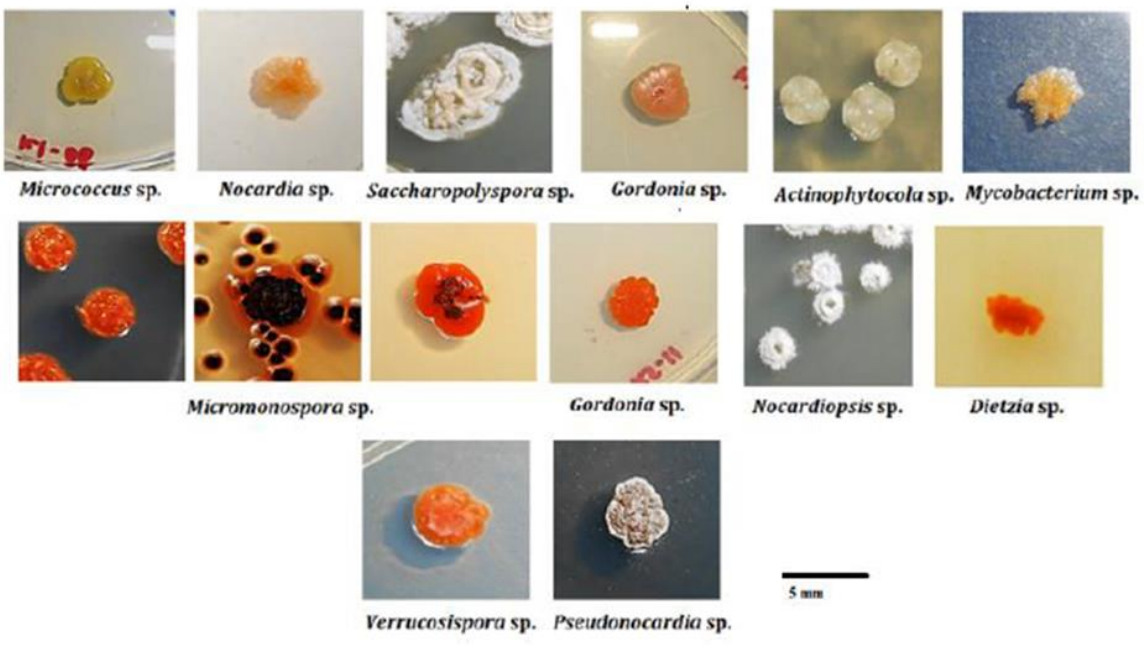

Figure 1: Colony morphology of rare mangrove actinomycetes

In terms of number, genus *Micromonospora* dominated the total number of rare actinomycetes with 11 isolates. It is therefore not a surprise to obtain a high number of *Micromonospor*a isolates as this genus was also commonly isolated actinomycete from any habitats, alongside with *Streptomyces* isolates. Pairwise comparison of partial 16S rRNA gene sequences of both isolates K2–04 and K7–12 showed 99% similarity to *Verrucosispora gifhornensis* strain 9–2. Studies have shown that *Verrucosispora* can be found in various habitats, including mangrove ecosystem (Xie *et al.* 2012). *Verrucosispora* has drawn much attention in recent years as it has proved to produce cyclopeptide antimicrobial compound and antitumor compounds including gifhornenolons and proximicins (Dai et al., 2010; Schneider et al., 2008; Shirai et al., 2010). Genera *Gordonia* and *Pseudonocardia* were also distributed in both regions of Kuantan mangrove forest. Isolate K3–01 and K5-11 are closely related to *Gordonia terrae* ATCC 25594 (99% similarity), while isolates K7-03 and K7-05 were similar to *G. desulfuricans* 213E (98%) and *G. lacunae* BS2 (99%).

Table 2: Identification and presence of NRPS and PKS-I gene in mangrove rare actinomycetes of Kuantan mangrove forests

Isolate	GenBank accession number	Closest relative	Sequence identity (%)	NRPS	PKS-I
K1–02	KT803945	*Micromonospora tulbaghiae* TVU1(T)	99	+	+
K1–06	KU289068	*Micrococcus yunnanensis* YIM 65004	99	-	-
K1–12	KU289070	*Micromonospora echinospora* ATCC 15837	99	-	-
K1–14	KU289071	*Saccharopolyspora dendrathemae* KLBMP 1305	98	-	-
K1–16	KU289073	*Pseudonocardia hydrocarbonoxydans* IMSNU22140	99	-	-
K2–02	KU289074	*Nocardia higoensis* DSM44732	99	-	-
K2–04	KU289075	*Verrucosispora gifhornensis* HR1-2	99	-	+
K2–05	KU289076	*Micromonospora rosaria* DSM 803	99	-	-
K3–01	KU289077	*Gordonia terrae* 3612	99	+	-
K3–10	KU308265	*Micromonospora sediminicola* SH2-13	99	-	-
K3–12	KT803939	*Pseudonocardia antitumoralis* SCSIO 01299	100	-	-
K3–13	KU289079	*Micromonospora carbonacea* DSM43168	99	+	+
K3–16	KU289080	*Micromonospora krabiensis* MA-2	99	-	-
K4–07	KR902624	*Mycobacterium peregrinum* ATCC14467	98	-	-
K4–08	KR902625	*Actinophytocola oryzae* GMKU367	99	+	+
K4–09	KT803941	*Nocardiopsis alba* DSM 43377	100	-	-
K4–16	KR902628	*Pseudonocardia antitumoralis* SCSIO01299	99	-	-
K4–19	KR902629	*Micromonospora carbonacea* DSM43168	99	-	-
K5–11	KR902632	*Gordonia terrae* DSM43249	99	-	-
K5–13	KR902633	*Micromonospora carbonacea* DSM43168	99	-	-
K5–14	KR902634	*Rhodococcus rhodochrous* DSM43241	99	+	+
K5–19	KR902635	*Micromonospora aurantiaca* ATCC27029	100	+	+
K6–02	KU289081	*Micromonospora aurantiaca* DSM 43813	99	-	-
K6–17	KU289084	*Dietzia timorensis* IDO-A0528	99	-	-
K7–03	KU289086	*Gordonia desulfuricans* 213E	98	+	+
K7–04	KT803952	*Micromonospora carbonacea* DSM43168	99	-	-
K7–05	KU289087	*Gordonia lacunae* BS2	99	+	-
K7–12	KU289088	*Verrucosispora gifhornensis* 9-2	99	-	+
K7–13	KT803943	*Pseudonocardia antitumoralis* SCSIO 01299	100	+	+

"+" indicates the presence of biosynthetic genes, while "–" indicates the absence of biosynthetic genes.

It was also observed that 3 *Pseudonocardia* isolates (K3–12, K4–16 and K7–13) were highly similar to *Pseudonocardia antitumoralis* SCSIO 01299 and isolate K1-16 was closely related to *P. hydrocarbonoxydans*. This finding provides further evidence about the presence of these rare genera in mangrove ecosystem as reported by previous studies. Isolation of *Gordonia* and *Pseudonocardia* strains from mangrove ecosystem has been reported from sediment of Hainan Province mangrove and Nizampatnam mangrove, respectively (Hong et al., 2009; Mangamuri et al., 2012). Isolate K2–02 was closely related to *Nocardia higoensis* DSM 44732 with a similarity value of 99%. Members of genus *Nocardia* is a ubiquitous group of microorganism that was mostly isolated from terrestrial soil (Wilson, 2012). They have been isolated from various mangrove habitats (Arifuzzaman et al., 2010; Hong et al., 2009). Isolate K1–14 displayed 98% similarity to *Saccharopolyspora dendrathemae* KLBMP 1305.

Members of genus *Saccharopolyspora* are of special interest as they are known producers of medically important antibiotics, including erythromycin produced by *Saccharopolyspora erythrae* (Oliynyk et al., 2007). Members of this genus have been isolated from terrestrial soil and marine ecosystem including sponges suggesting that they are widely distributed in nature (Yuan et al., 2008; Pimentel-Elardo et al., 2008). Hence, it is not surprising to recover genus *Saccharopolyspora* from mangrove habitat as reported by whom isolated *Saccharopolyspora* sp. RL78 from mangrove sediment in Ishigaki Island, Japan (Izumikawa et al., 2012). In this present study, both *Nocardia* and *Saccharopolyspora* were exclusively found in the dense mangrove region of Kuantan mangrove forest (K1, K2 and K3) alongside the common *Micrococcus* isolate.

Members of 4 rare genera which include genus *Dietzia, Mycobacterium, Nocardiopsis* and *Actinophytocola* were successfully discovered exclusively from the mangrove region near to anthropogenic activities (K4, K5, K6 and K7). Pairwise comparison of the 16S rRNA gene sequences of isolate K4–07 and K6–17 displayed similarity to *Mycobacterium peregrinum* ATCC 14467 and *Dietzia timorensis* ID05-A0528 at the similarity of 98% and 99%, respectively. A group researchers isolated *Mycobacterium* sp. from mangrove in Hong Kong, while isolated *Dietzia* sp. from mangrove sediment of Guanabara Bay, Brazil (Guo et al., 2011; Brito et al., 2006). Respective studies demonstrated that members of these two genera were known for their capability to degrade hydrocarbons. Isolate K4–09 showed (100 % similarity) to *Nocardiopsis alba* DSM 43377. *Nocardiopsis* strains have been isolated from various natural habitats, including mangrove ecosystem (Azman et al., 2015; Huang et al., 2015; Tiwari and Gupta, 2012).

Members of genus *Nocardiopsis* were known to produce antimicrobial metabolite as reported by Engelhardt *et al.* (2010), whom recovered a thiopeptide compound designated as TP-1661 from *Nocardiopsis* strain isolated from marine sediment (Engelhardt et al., 2010). Findings in this study raise the enquiry of why some genera are found on both mangrove regions while others just on one region as lack of recovery cannot be assumed as the absence of the organism in a particular site. Even though no clear clarification can be proposed for this observation, one possible explanation is that some of these genera might present at ubiquitous distribution compared to the others (Maldonado et al., 2005). The difference in the composition of actinomycetes between the regions might be the result of the interaction of several factors which include the ecosystem characteristics of the sampling sites.

The physical, chemical and biological characteristics of the sampling site could influence the life forms that developed on their respective regions. The K1, K2 and K3 sampling sites that are located within the dense mangrove forest might have different salinity, tidal influence, nutrient and organic matter content to those located near the anthropogenic activities. The differences could provoke the functional changes of sediment-living bacteria within the respective sampling sites in order to adapt to the specific ecological niche, which might be complemented with the development of speciation processes (Solano et al., 2009). Assessment of the biosynthetic capabilities of these rare actinomycetes was conducted through the amplification of nonribosomal peptide synthetase (NRPS) and polyketide synthetase type I (PKS-I) genes. Genome analyses of actinomycetes revealed that various biosynthetic gene clusters for secondary metabolites are encoded in their genomes.

Moreover, nearly half to three-quarters of the clusters are associated with NRPS and PKS pathways (Nett et al., 2009). Thus, detecting the presence of these genes in actinomycetes will aid in identifying potential actinomycetes that can be utilized for natural product discovery. Of the 29 rare actinomycetes screened for the presence of PKS-I and NRPS, only seven isolates (*Micromonospor*a sp. K1-02, *Micromonospora* sp. K3-13, *Micromonospora* sp. K5-19, *Actinophytocola* sp. K4-08, *Rhodococcus* sp. K5-14, *Gordonia* sp. K7-03, Pseudonocardia sp. K7-13) showed positive results for both genes, two isolates (*Gordonia* sp. K3-01, *Gordonia* K7-05) for PKS-I gene only, two isolates (*Verrucosispor*a sp. K2-04, *Verrucosispor*a sp. K7-12) for NRPS gene only and the remaining showed no presence of either gene (Table 2). The absence of an amplification product of these genes did not necessarily mean that these actinomycetes lacked biosynthetic capabilities. Perhaps, degenerate primers used in this were

unsuitable for that particular actinomycete isolates or probably the secondary metabolites are regulated by systems other than NRPS and PKS-I genes, such as type II and type III PKS gene and aminoglycoside-resistance gene (Komaki et al., 2018; Koudri et al., 2014). Nevertheless, detection of these genes helps to focus on potential rare actinomycetes that can be further applied in the production of bioactive compounds.

4. Conclusion

Findings from this study demonstrate that an unexpected diversity of rare actinomycetes colonizing the sediments of Kuantan mangrove forest. Promising rare actinomycetes may pave the way in search of novel bioactive compounds. Moreover, this study also suggested that these isolates may represent a valuable source of biologically active compounds.

Acknowledgement

The authors would like to acknowledge the Ministry of Higher Education, Malaysia for funding under the grant FRGS/1/2017/STG03/UIAM/03/1.

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