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Contributed Paper

Isolation and Diversity of Actinomycetes from Sediments of Different Depths Between 34 m and 3,235 m in South China Sea

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ABSTRACT

Diversity and isolation of actinomycetes from marine sediments collected from the South China Sea at depths ranging from 34 to 3,235 m were carried using dilution plate technique with heat and non-heat pretreatment on 9 isolation media, and plate stamping technique incubated on RH and M6 media at 28 °C and 10 °C. A total of 43 actinomycete strains were isolated from the different depth of marine sediments, among of which 32 were from the deep sea samples (1,645-3,235 m). Non-heat pretreatment was effective for the actinomycete isolation from deep sea sediment samples. RH and M6 were the more effective media regarding the number and diversity of isolates recovered. A higher percentage of actinomycete colonies (19.64%) were obtained by using plate stamping technique comparing to dilution plate method (5.29%). However, low-temperature incubation (10 °C) was also significantly effective on actinomycetes isolation from deep-sea samples with a higher percentage of the ratio actinomycetes to other total viable bacteria. Higher diversity of actinomycetes genera were found in deep-sea regions (depth 1,645 m-3,235 m) comparing to shallow regions marine sediments (depth <200 m). Partial 16S rRNA gene sequence data revealed that the isolates belong to the Class *Actinobacteria*, with genetic affiliations to five orders, six families, and six genera. *Micromonospora* (n= 23) and *Streptomyces* (n= 14) were the dominant genera, followed by *Dietzia* (n= 2), *Tsukamurella* (n=2), *Blastococcus* (n=1) and *Microbacterium* (n=1).

Keywords: marine actinomycetes, isolation, diversity, deep sea

1. INTRODUCTION

The vast oceans cover 70 % of the world's surface, with 95% greater than 1,000 m deep. Deep sea environments are divided into the bathyal zone (depths between 200 and 2,000 m), the abyssal (depths between 2,000 and 6,000 m) and the hadal zone (depths below 6,000 m) [1]. At about 200 m depth, the deep sea is characterized by high pressure, low temperature, lack of light and variable salinity and oxygen concentration.

Deep-sea organisms have developed unique biochemical metabolic and physiological capabilities, which not only ensure their survival in this habitat but also provide a potential for the production of novel metabolites absent in terrestrial microorganisms. The increasing numbers of literature on novel metabolites and diversity of marine actinomycetes strongly supported the view that the marine environments including deep sea are significant source for search and discovery of both diversity and novel secondary metabolites [2, 3].

Several factors influence the actinomycetes isolation from marine sediments, including heat pretreatment, composition of selective media and isolation methods. Heat pretreatment procedures have been effectively used for the selective isolation of members of several actinomycete taxa by decreasing the ratio of bacteria to actinomycetes on isolation plates, but these procedures may also reduce the number of actinomycetes [4]. Moreover, the isolation media were designed to favor the growth of actinomycetes and reduce the development of unwanted microorganisms [5]. For example, Vickers et al. [6] designed raffinose-histidine agar plates for the isolation of rare *streptomycetes* by reducing the frequently isolated species-group; plate stamping method was found to show good actinomycetes recovery from marine sediments [7]. Thus, the developments of efficient cultivation methods are important for better understanding of actinomycetes diversity of deep-sea habitats to obtain novel species or novel compounds.

Microbial diversity in deep-sea regions could be remarkably high with species richness. The expanding of microbial biodiversity reach to the 5,000 m depth to the abyssal zone than the peak amount of species at the depths of 3,000 m and beyond [8, 9]. Some previous reports on the isolation and diversity of actinomycetes from the deep-sea environment showed that the genus *Micromonospora* and *Streptomyces* were dominant genera in the deep-sea. For example, *Micromonospora* was found to be dominant in the Mediterranean (2,800 m and 4,400 m) and Trondheim Fjord (Norway) [10, 11]. In addition, Streptomycetes were reported to be dominant in deep-sea coral ecosystems of Cantabrian Sea (1,500-4,700 m) [12], and also presented in considerable number in the Mariana Trench (10,898 m) [13]. Novel actinomycete species isolated from deep sea environment between 2006 and 2016, have yielded an impressive array of novel species including 21 species under 13 genera. The highest number of deep sea actinomycete was found at depths of the abyssal zone and the deeper regions, with the maximum number from *Microbacterium*, followed by *Dermacoccus*, *Streptomyces* and *Verrucosispora*. Eight genera of actinomycetes were reported to produce secondary metabolites, among which *Streptomyces* was the richest producer [14].

The high number of novel actinomycete species isolated from the deeper regions of South China Sea showed a potentially abundant of novel genera/species. Their secondary metabolites are remarkably equal to that of Mariana Trench [2, 14]. However, few studies focused on the estimation of microbial diversity at different depths in the South China Sea. In this study, we report the diversity of culturable actinomycetes in the South China Sea marine sediment samples from different depths (34 m - 3,235 m). In addition, the dilution plate technique with heat/non-heat pretreatment, selective media and plate stamping technique are compared for the selective isolation of actinomycetes.

2. MATERIALS AND METHODS

2.1 Environmental Samples

Fourteen marine sediment samples were collected from different locations in the South China Sea, at depths ranging from 34 m to 3,235 m (Table 1). Sediment sample depths are classified as "Non-deep sea", "Bathyal (depths between 200-2,000 m)" and "Abyssal (depths between 2000-6000 m)". There are seven samples of "Non-deep sea" (depths ranging from 20 to 156 m), two samples of "Bathyal" (depths ranging from 1,471 m and 1,645 m), and five samples of "Abyssal" (depths ranging from 2,017 to 3,235 m). All samples were preserved at 4 °C until they were processed for isolation of actinomycetes.

Table 1. Source of sediment samples.

Oceanic zone	Depth (m)	pH	Coordinates	
			longitude	latitude
Non-deep sea	34	7.83	114.38 E	22.10 N
	35	8.04	114.74 E	22.23 N
	62	7.87	114.78 E	21.91 N
	85	7.85	114.91 E	21.60 N
	96	7.27	110.00 E	18.00 N
	140	6.69	118.35 E	22.27 N
	156	6.23	110.50 E	18.00 N
Bathyal	1,471	5.93	111.00 E	18.00 N
	1,645	6.46	115.37 E	20.27 N
Abyssal	2,017	5.96	111.50 E	18.00 N
	2,093	5.70	113.00 E	18.00 N
	2,441	6.69	112.00 E	18.00 N
	3,004	6.46	118.00 E	20.00 N
	3,235	5.75	114.00 E	18.00 N

2.2 Selective Isolation and Enumeration of Actinomycetes from Marine Sediments

A total of fourteen sediment samples were subjected to three different methods for isolation and enumeration of actinomycetes. **(1) The dilution plate technique with heat/ non heat pretreatment**: 1 g of sediment was added to 9 ml of sterilized artificial sea water solution (1%; Sigma, Germany), and agitated on a shaker for 30 min at 150 rpm to disperse bacterial propagule, followed by heat-pretreatment at 50 °C in a water bath for 10 minutes [15] or left untreated (non- heat pretreatment). Serial dilutions of soil suspensions up to 10^{-3} were made and 0.1 ml suspensions from each dilution tube were spread on 9 selective isolation media, such as OA (oatmeal agar, ISP medium 3); oatmeal, 60 g; agar, 15 g; pH 7.2~7.4; GS1 (Gause modified medium 1); soluble starch, 20 g; K_2HPO_4, 0.5 g; KNO_3, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 20 g; pH7.4~7.6; SC (IM6); yeast extract, 4 g; malt extract, 30 g; glucose, 4 g; agar, 18 g; pH 7.0~7.4; HL2 (IM8); glucose, 10 g; peptone, 5 g; tryptone, 3 g; NaCl, 5 g; agar, 15 g; pH 7.0; RH (Raffinose-histidine medium); raffinose, 10 g; L-histidine, 1g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H2O$, 0.01 g; K_2HPO_4, 1g; agar, 20.0 g; pH 7.0~7.4; HV (Humic acid -vitamin

agar); humic acid, 1.0 g; $CaCO_3$, 0.02 g; Na_2HPO_4, 0.5 g; KC1, 1.7 g; $FeSO_4.7H_2O$, 0.01 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 18 g; pH7.2; SIM (Starch casein agar); starch, 10 g; casein, 3 g; agar, 18 g; pH 7.0~7.4 [16], M6; glycerine, 6 ml; arginine, 1 g; K_2HPO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; pH 7.2~7.4 [17] and 1:100 Marine agar; yeast extract, 0.01 g; peptone, 0.05 g boric acid, 0.02 mg; NaCl, 0.19 g; $MgCl_2$, 0.09 g; Na_2SO_4, 0.03 g; $CaCl_2$, 0.02 g; KCl, 5.50 mg; $NaHCO_3$, 1.6 mg; $FeC_6H_5O_7 \cdot 3H_2O$, 1 g KBr, 0.80 mg; $SrCl_2$, 0.34 mg; Na_2HPO_4, 0.08 mg; Na_2SiO_3, 0.04 mg; NH_4NO_3, 0.016 mg; agar, 18 g; pH 7.2~7.4 (adapted from [18]) in triplicate. Plates were incubated at 28 °C. **(2) Plate stamping technique**: This method was carried out as described by Jensen et al [7]; One gram of sediment was thawed, dried in the biosafety cabinet for 1 h, when clumping occurred, it was grounded lightly with an alcohol-sterilized mortar and pestle, and inoculated using sterilized sponge stamp on RH and M6 media. Plates were incubated at 28 °C and 10 °C up to 3 months. All selective isolation media were prepared with 1 L of sterilized artificial sea water solution (1%; Sigma, Germany) and amended with nalidixic acid (25 µg/ml), nystatin (50 µg/ml) and cycloheximide (50 µg/ml) to inhibit the growth of Gram-negative bacteria and fungi. Different colonies were chosen based on colony morphology and streaked on ISP medium 2 [19] to obtain pure isolates. The experimental data was analyzed by SPSS program version 16.0 (2007 SPSS Inc., Chicago, IL; free trial version). The data were subjected to analysis of variance (ANOVA) using Tukey HSD's test and T-test ($p<0.05$).

2.3 Phylogenetic Analysis Based on 16S rRNA Gene Sequencing

Extraction of genomic DNA from pure cultures and PCR amplification. For DNA extraction, total genomic DNA samples from all the actinomycetes isolates were extracted with Fast DNA®SPIN Kit followed manufacturer's protocol. The PCR reactions were performed in a final volume of 25 µl composed of DNA template (1 µl upper aqueous layer), I-5TM 2X High-Fidelity Master Mix (12.5 µl) and 20 µM of primer Eubac27F and primer Eubac1492R with the appropriate reaction buffer under the following conditions: initial denaturation at 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, and 72 °C for 90 s.

DNA Sequencing and phylogenetic analyses. The PCR products were purified using E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, USA) and were sent to TsingKe Biological Technology for gene sequencing. DNA sequences were deposited in GenBank under accession numbers MF769728-MF769770. The 16S rRNA gene sequences were compared to publicly available sequences of related type strains, and 16S rRNA gene sequence similarities were calculated using the EzTaxon-e server (http://www.ezbiocloud.net/., [20]). The alignment of 16S rRNA gene sequences and phylogenetic analysis were performed as described previously [21].

3. RESULTS AND DISCUSSION

3.1 Selective Isolation and Enumeration of Actinomycetes from Marine Sediments

Deep sea environments are a significant source for search and discovery of both diversity and novel secondary metabolites [2, 14] and few studies have attempted to estimate and compare actinomycetes diversity at different depths. Selective isolation procedures and actinomycetes diversity in different depths were intended in the present investigation. In this study, a total of 43 actinomycete isolates from 14 marine

South China Sea sediments at different depths (34 m -3,235 m) were considered in more details.

3.1.1 The effect of dilution plate technique with heat and non-heat pretreatments

Few actinomycetes were isolated on selective media inoculated with heat pretreated suspensions. Actinomycete colonies were observed only from 12 of the 594 isolation plates (2.02%). In contrast, significantly higher counts were generally recorded by using non-heat pretreatment except two samples at a depth of 1,645 m and 85 m. From 756 isolation plates, 40 (5.29%) yielded actinomycete colonies. The actinomycete community formed between 0 and 40.95% of the total bacterial counts on 8 selective media. The highest number of actinomycetes with 40.95% of the total bacterial counts was recovered from the sample depth of 3,004 m (Table 2, Figure 1). None of actinomycete isolates were derived from heat pretreatment, while fifteen isolates were obtained from non-heat pretreatment procedures. Hence, heat pretreatment procedure was not the suitable procedure for these marine sediment samples, though it was proved effectively for selective isolation of several actinomycete taxa. While it could decrease the growth of other bacteria, it may reduce the number of actinomycetes as well [4]. Pathom-aree et al. isolated actinomycetes from Norwegian fjord sediments supported that the high actinomycetes counts were recorded on isolation plates seeded with non-heat pretreated suspensions [13].

Table 2. Actinomycete recovery using heat pretreatment and non-heat pretreatment.

Depth (m)	Ratio actinomycetes/total viable bacteria (%)[a]		*p* value [c]
	Heat pretreatment	Non-heat	
34	NT	10.00 ± 4.36	ND
35	0.87 ± 0.32	3.01 ± 0.96	0.02*
62	0	12.00 ± 2.64	0.001**
85	0.66 ± 0.29	0.46 ± 0.38	0.513
96	0	0	ND
140	NT	0	ND
156	0	0	ND
1,471	0	0	ND
1,645	3.08 ± 0.14	0.83 ± 0.18	0.000**
2,017	0	22.73 ± 6.51	ND
2,093	4.05 ± 0.90	9.35 ± 2.10	0.016*
2,441	0	0	ND
3,004	NT	40.95 ± 25.74	ND
3,235	3.66 ± 1.52	19.51 ± 1.62	0.000**

[a] Average ± standard error from three replicates

NT = Not tested (Heat pretreatment was not performed)

ND = Not determined

0 = No actinomycetes or bacteria grew

[b] No. of actinomycetes isolated from non-heat pretreatment procedures

[c] Comparison of the effects of heat and non-heat pretreatment within the same sample; * Statistically significant difference ($p<0.05$) and ** Statistically significant differenence ($p<0.01$) according to independent t-test

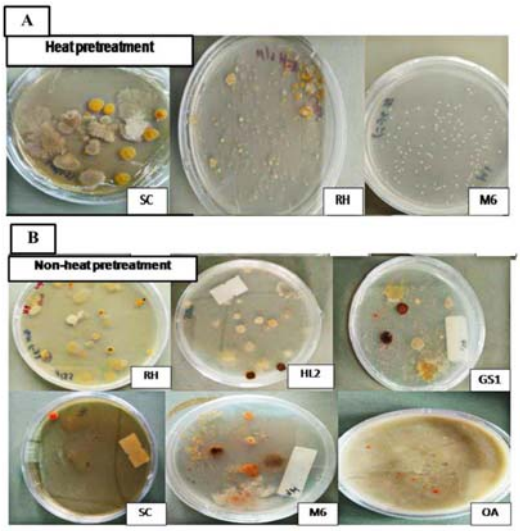

Figure 1. Isolation of actinomycetes from marine sediments on different media with heat (A) and non-heat (B) pretreatment.

3.1.2 Selective isolation media

Actinomycetes grew on 8 of the 9 selective media, with the exception of 1:100 marine agar. The ratios of total actinomycetes to other total viable bacteria on different media were 0.21, 0.09, 0.08, 0.07, 0.05, 0.05, 0.01 and 0.005 respectively, i.e.: RH > M6 > HL2 > OA > SC = GS1 > SIM > HV (Table 3). It is suggested that the 1:100 marine agar as low-nutrient medium was not suitable for actinomycetes isoaltion from these marine sediment samples because it showed no growth of either actinomycetes or other bacteria. Hence, using high nutrient media may be more effective than low nutrient media in this case.

Table 3. Total counts of actinomycetes and viable bacteria recorded for the marine sediment samples on selective media.

Medium	Total actinomycetes $\times 10^3$ (cfu/g)[a]	Total bacterial count $\times 10^3$ (cfu/g)[a]	Ratio actinomycetes/ total viablebacteria[a]
RH	9.80 ± 1.15	45.75 ± 4.02	0.21 ± 0.04
M6	0.55 ± 0.14	6.00 ± 1.32	0.09 ± 0.04
HL2	1.40 ± 0.91	17.80 ± 4.16	0.08 ± 0.05
OA	2.35 ± 0.95	31.30 ± 0.96	0.07 ± 0.03
SC	0.40 ± 0.10	7.60 ± 2.36	0.05 ± 0.02
GS1	0.65 ± 0.35	12.80 ± 7.23	0.05 ± 0.01
SIM	0.40 ± 0.17	26.80 ± 9.93	0.01 ± 0.01
HV	0.10 ± 0.10	20.00 ± 8.70	0.005 ± 0.0025

[a] Average ± standard error from three replicates.

In addition, RH and M6 were more effective media based on the number and diversity of actinomycete isolates recovered. RH medium contributed 29 actinomycete isolates which belong to 4 genera of *Micromonospora*, *Streptomyces*, *Dietzia* and *Blastococcus*. M6 medium supported 8 actinomycete isolates which belong to 4 genera of *Micromonospora*, *Dietzia*, *Tsukamurella* and *Microbacterium* (Figure 2). Therefore, these two media were used in plate stamping method for further isolation and the result was shown in Table 4.

The raffinose-histidine agar plate was used for the isolation of rare streptomycetes by Vickers et al. [6]. One study also showed that most strains (58%) of actinomycetes from sediment collected from the Mariana Trench (10, 898 m) can be isolated from raffinose-histidine agar plates [13].

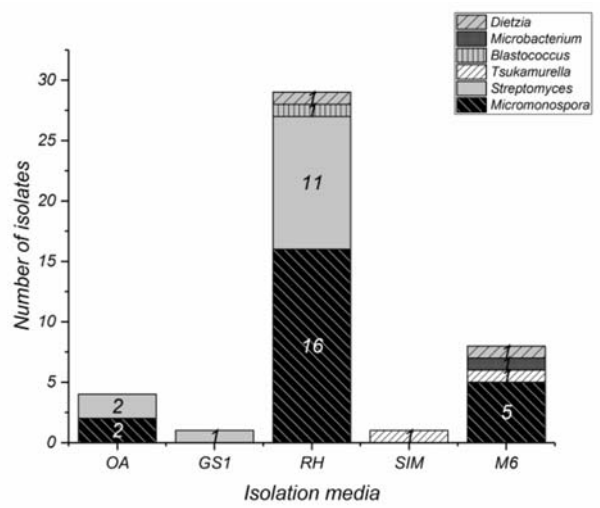

Figure 2. Number of actinomycetes of various genera isolated using different selective isolation media.

Table 4. Actinomycete recovery on RH and M6 media using plate stamping method (incubated at 28 °C and 10 °C).

Depth (m)	No. of actinomycetes isolated			Ratio actinomycetes/ total viable bacteria (%)[a]			*p*-value [b]		
	28 °C		10 °C	28 °C		10 °C	Different levels		
	RH	M6		RH	M6	M6	A	B	C
34	1	2	0	4.00±2.00	3.00±2.00	11.00±3.60	0.891	0.041*	0.023*
35	0	0	0	0	0	11.00±6.00	1.0	0.019*	0.019*
62	7	0	0	19.00±11.53	0.60±0.36	0	0.034*	0.030*	0.993
85	1	0	0	26.00±4.00	0	0	0.000**	0.000**	1.0
96	0	0	0	0	0	0	ND	ND	ND
140	0	0	0	7.70±3.46	12.00±4.36	0	0.301	0.05*	0.009**
156	0	0	0	0	0	0	ND	ND	ND
1,471	0	0	0	0	0	12.00±9.85	1.0	0.042*	0.042*
1,645	0	1	0	0	5.00±2.64	7.10±1.30	0.027*	0.005**	0.352
2,017	0	3	0	0	0.60±0.10	0	0.000**	1.0	0.000**
2,093	0	1	1	0	16.00±4.00	25.00±4.58	0.003**	0.000**	0.046*
2,441	0	0	0	0	0	0	ND	ND	ND
3,004	0	8	0	0	12.00±4.00	0	0.002**	1.0	0.002**
3,235	0	1	2	0	5.00±3.60	33.00±8.89	0.173	0.004**	0.038*
Total	25		3						

[a] Average ± standard error from three replicates
A = Difference between RH (28°C) and M6 (28°C)
B = Difference between RH (28°C) and M6 (10°C)
C = Difference between M6 (28°C) and M6 (10°C)
0 = No actinomycetes or bacteria grew
ND = Not determined
[b] Comparison of the effects of incubation temperature within the same sample, * Statistically significant difference ($p<0.05$) and ** Statistically significant difference ($p<0.01$) according to Tukey HSD's test

3.1.3 The plate stamping technique (incubated at 28 °C and 10 °C)

Dilution plate technique with heat/non heat pretreatment was used first with 28 °C incubation as the general temperature for actinomycetes growth. Jensen et al. [7] found that plate stamping method showed good actinomycetes recovery from marine sediments. Additionally, in the ocean, temperature decrease with increasing depth which correlated with changes in microbial diversity [2]. Jensen et al. [7] have successfully cultivated *Salinispora* which was in the obligate requirement for salt at low temperature 10 °C, the optimal growth temperature of deep sea actinomycetes was generally ranging from 25 to 30 °C for successful cultivation [14]. Hence, the selection of incubation temperature at 28 °C and 10 °C were used for plate stamping method. Moreover, RH and M6 media were used for stamping method because RH and M6 were shown the most effective media from the number and diversity of isolates recovered when using dilution plate technique.

A total of 14 marine sediment samples were processed using plate stamping technique incubated on RH and M6 media at 28 °C and 10 °C (Figure 3). Then, the 10 °C incubated-samples were inoculated onto RH and M6 media adding up to a total of 56 isolation plates of which 6 (10.71%) yielded actinomycete colonies. The actinomycete community formed 0-33**%** of the total bacterial counts on the M6 medium, but no colony observed on RH medium. So we only showed the result of M6 medium in Table 4. Moreover, more colonies were observed from the deep sea samples (4 of 6), with the highest number of actinomycetes recovered from the deepest sample at depth 3,235 m (Table 4). Three isolates from deep sea samples at depth 2,093 m and 3,235 m were obtained by using 10 °C for incubation. In contrast, 11 (19.64%) of 56 isolation plates incubation at 28 °C yielded actinomycete colonies, which were from the samples at depth 34 m, 62 m, 85 m, 140 m, 1,645 m, 2,017 m, 2,093 m, 3,004 and 3,235 m. The highest number of actinomycetes was recovered from the sample of 85 m depth, and the actinomycete community formed 0-26% of the total bacterial counts on RH medium from only non-deep sea samples, while 0-16% of the total bacterial counts on M6 medium from different depth range samples. The highest diversity of actinomycetes yielding 25 isolates comprised of 5 different genera (*Micromonospora, Streptomyces, Dietzia, Tsukamurella* and *Microbacterium*) (Figure 4).

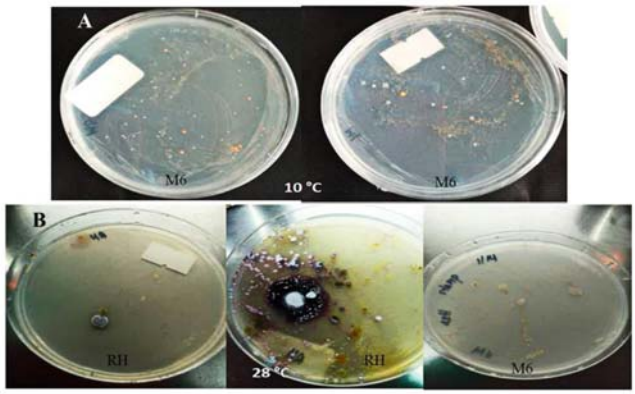

Figure 3. Isolation of actinomycetes by using plate stamping technique on M6 and RH media inoculated at 10 °C (A) and 28 °C (B).

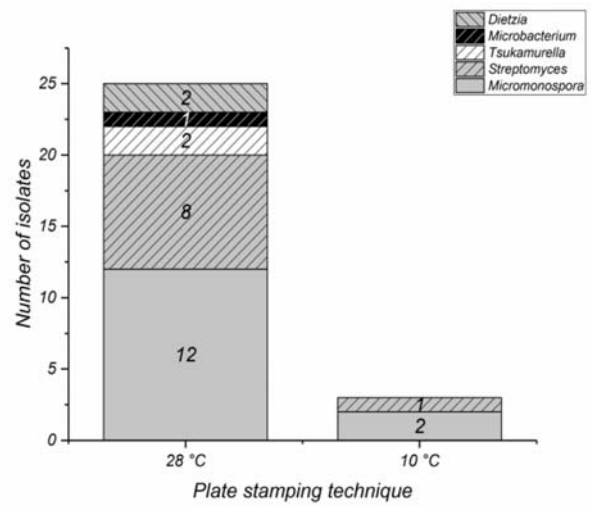

Figure 4. Number of actinomycetes of various genera isolated using plate stamping technique (incubated at 28 °C and 10 °C).

Hence, plate stamping technique was able to provide higher number and diversity of actinomycetes. The results were similar to previous reports that showed good marine actinomycetes recovery from tropical Pacific Ocean and subseafloor at the Nankai and Okinawa Troughs [7, 22]. Thus, these isolates are likely to be indigenous of tested sediment samples, attached directly from marine sediment particles and more effective for isolation without sediment washing (Base on dilution method) [22]. Some marine sediment samples including the samples depth of 140 and 1,471 failed for actinomycetes isolation by using dilution plate technique, but actinomycete colonies were observed while using plate stamping method. Hence, the isolation method and selective media could affect actinomycete recovery at different depth of marine sediments.

Although our results showed that the highest diversity and number of actinomycete isolates were obtained from isolates incubated at 28 °C comparing to 10 °C, more actinomycetes colonies from deeper sediments were obtained at 10 °C. The plate stamping method at 28 °C incubation on RH medium may be more effective for shallow marine sediment, but the M6 medium could be used for all depth marine sediments especially for deep-sea sediments together with incubation at low- temperature. Moreover, table 4 revealed that low temperature incubation (10 °C) was significantly effective for actinomycetes isolation from deep-sea samples producing a higher percentage of the ratio actinomycetes to the total viable bacteria, except two deep-sea samples at a depth of 2,017 m and 3,004 m. However, only three isolates were identified at 10 °C, a few colonies grew on the isolation plates but cannot be cultured later. Therefore, it is important to improve and optimize the conditions after colonies-picking from isolation plates.

3.2 Taxonomic Diversity of Actinomycete Isolates from Marine Sediments

Partial 16S rRNA gene sequences were obtained for all of the marine isolates from different depth zones. Figure 5 shows amplification bands of 16S rRNA genes, as

demonstrated by the presence of the PCR products at 1,500 bp. BLAST analyses of partial 16S rRNA gene sequence data revealed that all isolates shared 99.11-100.0% sequence similarities with the length of sequences used in the comparison ranged from 785-1,329 bp (Table 5). Among these, a total of 5 orders, 6 families, and 6 genera were detected (Table 6). When focusing on the relationship of number of isolates with the depth, all of the marine isolates from marine sediments (<200 m depth) belonged to 3 different genera and, the most abundant genera were *Micromonospora* (5 isolates) and *Streptomyces* (5 isolates), followed by *Dietzia* (1 isolate). All of the isolates from deep sea marine sediments (1,645-3,235 m depth) belonged to 6 different genera. *Micromonospora* (18 isolates) was the dominant genus followed by *Streptomyces* (9 isolates) and *Tsukamurella* (2 isolates), one isolate for each belonged to the genera *Microbacterium*, *Dietzia* and *Blastococcus*. In addition, the high number of actinomycetse isolates (31) were obtained from the samples in the Abyssal zone (Table 6). The previous report on the diversity of actinomycetes from deep-sea correlating to our results showed that the genus *Micromonospora* also dominates in the deep Mediterranean sea (2,800 m and 4,400 m) and Trondheim Fjord (Norway) [10, 11]. *Streptomyces* species were presented in considerable number in the deepest part of the ocean; Mariana Trench (10,898 m) [13] and also dominated in deep-sea coral ecosystems of Cantabrian Sea (1,500-4,700 m) [12]. Moreover, the results of some studies which considering depth similarly with our results; for example, Bredholt et al. [10] found that the deep water sediments at depth 450 m contained a higher relative amount of *Micromonospora* compared to the shallow water samples and *streptomycete* numbers decreased with increasing depth [23]. However, this finding was in contrast with Vizcaino [12] who found *Streptomyces* to be the dominant genus from all depths tested in the deep sea (1500-4700 m).

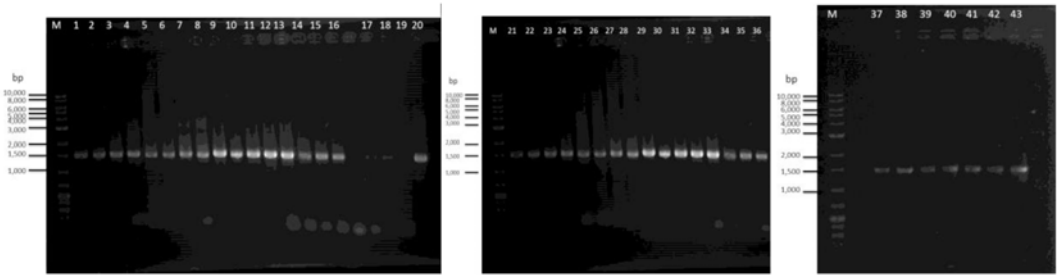

Figure 5. Gel electrophoresis of PCR amplified products of the actinomycete isolates Lane M = 1kb ladder plus, Lane 1-5, 18-21, 37-38, = isolates from the marine sediments (<200 m), Lane 6 = isolates from the deep-sea marine sediments in the Bathyal zone, Lane 7-17, 22-36, 39-43 = isolates from the deep-sea marine sediments in the Abyssal zone.

Table 5. Partial 16S rRNA gene sequence similarity analysis of actinomycete isolates from South China Sea marine sediment samples.

Depth (m)	Isolate name	Acession No.	Related species	Length (bp)	Similarity (%)
34 m	MK-20-1	MF769728	*Micromonospora aurantiaca* ATCC 27029^T	872	99.89
	MK-20-2	MF769729	*Micromonospora aurantiaca* ATCC 27029^T	924	99.24
	MK-20-3	MF769730	*Micromonospora echinospora* ATCC 15837 ^{T}	894	99.11
62 m	MK-18-1	MF769731	*Micromonospora echinospora* ATCC 15837^T	867	99.54
	MK-18-2	MF769732	*Micromonospora echinospora* ATCC 15837^T	856	99.65
	MK-18-3	MF769757	*Streptomyces coelescens* DSM 40421^T	913	99.67
	MK-18-4	MF769758	*Streptomyces tricolor* NBRC 15461 ^{T}	1,296	99.23
	MK-18-5	MF769759	*Streptomyces coelescens* DSM 40421^T	900	99.56
	MK-18-6	MF769760	*Streptomyces coelescens* DSM 40421^T	868	100.00
	MK-18-7	MF769761	*Streptomyces coelescens* DSM 40421^T	849	99.76
85 m	MK-17-1	MF769751	*Dietzia maris* DSM 43672 ^{T}	845	99.64
1,645 m	MK-14-1	MF769752	*Dietzia maris* DSM 43672^T	934	99.25
2,017 m	MK-46-1	MF769762	*Streptomyces coelescens* DSM 40421^T	885	99.89
	MK-46-2	MF769763	*Streptomyces luridiscabiei* NRRL B-24455 ^{T}	928	100.00
	MK-46-3	MF769753	*Tsukamurella carboxydivorans* JCM 15482^T	885	99.89
3,004 m	MK-8-1	MF769733	*Micromonospora aurantiaca* ATCC 27029 ^{T}	908	99.89
	MK-8-2	MF769734	*Micromonospora saelicesensis* Lupac 09 ^{T}	905	99.78
	MK-8-3	MF769735	Micromonospora aurantiaca ATCC 27029^T	868	99.54
	MK-8-4	MF769736	*Micromonospora aurantiaca* ATCC 27029 ^{T}	894	99.55
	MK-8-5	MF769764	*Streptomyces luridiscabiei* NRRL B-24455 ^{T}	885	99.21
	MK-8-6	MF769765	*Streptomyces luridiscabiei* NRRL B-24455^T	922	99.46
	MK-8-7	MF769737	*Micromonospora noduli* GUI43^T	910	99.56
	MK-8-8	MF769766	*Streptomyces luridiscabiei* NRRL B-24455 ^{T}	887	99.77
	MK-8-9	MF769738	*Micromonospora zamorensis* CR38 ^{T}	892	99.89
	MK-8-10	MF769739	*Micromonospora zamorensis* CR38 ^{T}	899	99.78
	MK-8-11	MF769754	*Blastococcus aggregates* ATCC 25902^T	785	99.62
	MK-8-12	MF769740	*Micromonospora lupini* lupac 14N ^{T}	857	99.30
	MK-8-13	MF769767	*Streptomyces tricolor* NBRC 15461 ^{T}	882	99.66
	MK-8-14	MF769741	*Micromonospora zamorensis* CR38^T	862	99.77
	MK-8-15	MF769742	*Micromonospora zamorensis* CR38^T	856	100.00
	MK-8-16	MF769743	*Micromonospora chokoriensis* 2-19/6^T	856	99.42
	MK-8-17	MF769744	*Micromonospora maritima* D10-9-5^T	890	99.78
	MK-8-18	MF769745	*Micromonospora maritima* D10-9-5^T	859	99.65
	MK-8-19	MF769746	*Micromonospora maritima* D10-9-5^T	905	99.67
	MK-8-20	MF769755	*Microbacterium resistens* DMMZ 1710 ^{T}	923	99.13
	MK-8-21	MF769768	*Streptomyces pratensis* ch24^T	874	100.00
2,093 m	MK-43-1	MF769756	*Tsukamurella carboxydivorans* JCM 15482^T	856	99.18
	MK-43-2	MF769769	*Streptomyces tricolor* NBRC 15461^T	902	99.67
3,235 m	MK-41-2	MF769770	*Streptomyces coelescens* DSM 40421^T	884	99.43
	MK-41-3	MF769747	*Micromonospora saelicesensis* Lupac 09 ^{T}	840	99.64
	MK-41-4	MF769748	*Micromonospora aurantiaca* ATCC 27029^T	924	100.00
	MK-41-5	MF769749	*Micromonospora chalcea* DSM 43026^T	1,329	99.62
	MK-41-6	MF769750	*Micromonospora saelicesensis* Lupac 09^T	893	99.33

Table 6. Taxonomic diversity of actinomycete isolates from different samples.

Orders	Families	Genera	Marine sediments (<200 m)	Deep-sea marine sediments (1,645-3,235 m)	
				Bathyal zone	Abyssal zone
Corynebacteriales	*Dietziaceae*	*Dietzia*	1	1	-
	Tsukamurellaceae	*Tsukamurella*	-	-	2
Frankiales	*Geodermatophilaceae*	*Blastococcus*	-	-	1
Micrococcales	*Microbacteriaceae*	*Microbacterium*	-	-	1
Micromonosporales	*Micromonosporaceae*	*Micromonospora*	5	-	18
Streptomycetales	*Streptomycetaceae*	*Streptomyces*	5	-	9
Total			11	1	31

Higher diversity of actinomycetes genera and bioactivity (data not shown) was found in deep sea zones (depth 1,675-3,235 m) comparing to shallow marine sediments (depth <200 m). It is interesting from the results that Abyssal zone at depth 3,000 m shows the highest of genera richness that may as a significant source for search and discovery of both diversity and secondary metabolites. Moreover, it is supported in previous studies that the microbial biodiversity reach to the peak amount of species at the depths of 3,000 m and beyond [8, 9]. In addition, abyssal hills possess the most abundant of biomass on earth [24]. Size of sediment particles, oxygen concentration and organic carbon flux are main factors associated with the microbial community. Goo et al. [22] explained that deeper sediments consisted of fine particles which would be easier to homogenize by grinding. Although oxygen concentration drops along with the depth, the oxygen concentration is slightly higher at the below oxygen minimum layer. Food materials sinking from the surface ocean have a substantial impact on the biodiversity in Abyssal. Sites of high abundance seem to occur where the flux of organic carbon is high [9].

The phylogenetic relationships of the isolates are shown in Figure 6. It is apparent that the isolates can be assigned to genera *Micromonospora*, *Streptomyces*, *Tsukamurella*, *Microbacterium*, *Dietzia* and *Blastococcus*. The genus *Micromonospora* was the most abundant group of isolates. Although, pairwise similarity showed relatively high similarities (99-100%) to the type strains, the phylogenetic analysis indicated that some isolates formed a distinct single cluster in the phylogenetic tree or formed a separate cluster from the type strain.

For example, isolates MK-8-16 and MK-8-7 affiliating with *Micromonospora chokoriensis* 2-19/6^T and *Micromonospora noduli* GUI43^T as next relative with >99% sequence similarity but formed a separate cluster from these two type strains, respectively; and isolate MK-8-7 in this cluster displayed antimicrobial activity against *Candida albicans* (data not shown). The isolate MK-8-12 in single cluster related to *Micromonospora lupini* lupac 14N^T with 99.30% sequence similarity and displayed antimicrobial activity against *Staphylococcus aureus* (data not shown). Moreover, the cluster of isolates MK-20-3, MK-18-2 and MK-18-1 affiliating to *Micromonospora echinospora* ATCC 15837^T with

>99% sequence similarity but formed a separate cluster from this type strain. Isolated actinomycete species were divided into different clusters indicates the degree of dissimilarity in sequences and the diversity among the organisms. As Kasai et al. [25] demonstrated that *Micromonospora* highly related by 16S rRNA gene similarity, up to 99.3%, shared low DNA-DNA relatedness. It is presumed that these isolates may possibly belong to novel species and provided further studies in identification to confirm them.

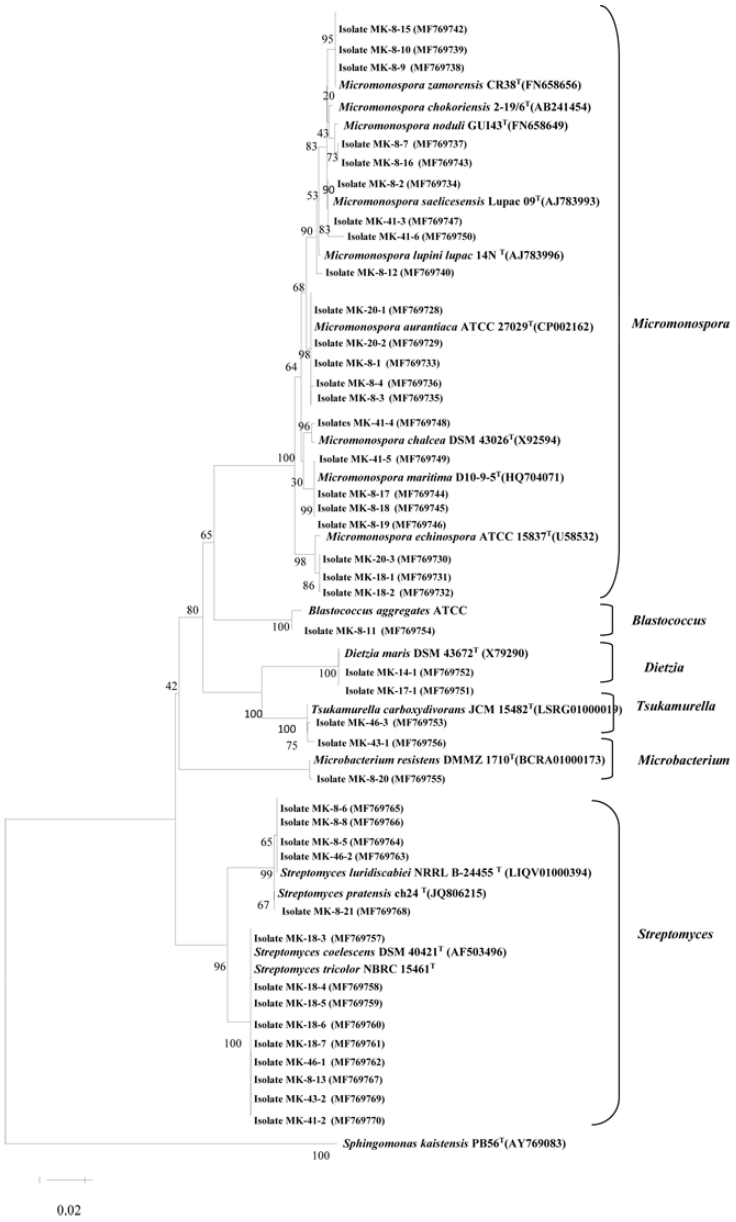

Figure 6. Neighbour-joining phylogenetic tree of partial 16S rRNA gene sequences from the marine isolates derived from the South China Sea and closely related type strains. Numbers are percentage bootstrap values which were calculated for 1000 replicates. Bar, 0.02 was per nucleotide position. *Sphingomonas kaistensis* PB56^T(AY769083) was used as the out-group.

4. CONCLUSIONS

This report was carried out to investigate the diversity of cultural actinomycetes in South China Sea sediments at different depths between 34 m and 3,235 m. The results have revealed that higher diversity of actinomycetes genera was found in deep-sea zones (depth 1,675-3,235 m) with 6 different genera comparing to shallow marine sediments (depth < 200 m) with 3 different genera. It indicated that microbial diversity in deep-sea zones can be remarkably high with genera richness. The development of efficient cultivation methods is continually important to guide the understanding of actinomycetes diversity and get more opportunity to obtain novel species. It is worth to notice that pretreatment without heat, plate stamping method, RH selective medium and low temperature (10 °C) incubation were more productive for actinomycetes isolation from deep-sea samples.

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REFERENCES

[1] Harino H., Arai T., Ohji M. and Miyazaki N., Organotin Contamination in Deep Sea Environment; in Arai T., Harino H., Ohji M. and Langston W.J., eds., *Ecotoxicology of Antifouling Biocides*, Springer, New York, 2009: 95-97.

[2] Skropeta D. and Wei L., *Nat. Prod. Rep.*, 2014; **31(8)**: 999-1025. DOI 10.1039/c3np70118b.

[3] Xu D.B., Ye W.W., Han Y., Deng Z.X. and Hong K., *Mar. Drugs*, 2014; **12**: 2590-2613. DOI 10.3390/md12052590.

[4] Williams S.T., Shameemullah M., Watson E.T. and Mayfield C.I., *Soil Biol. Biochem.*, 1972; **4**: 215-225. DOI 10.1016/0038-0717(72)90014-4.

[5] Goodfellow M. and O'Donnell A.G., Search and Discovery of Industrially-Significant Actinomycetes; in Baumberg S., Hunter I.S. and Rhodes P.M., eds., *Microbial Products: New Approach*, Cambridge University Press, Cambridge, United Kingdom, 1989: 343-383.

[6] Vickers J.C., Williams S.T. and Ross G.W., A Taxonomic Approach to Selective Isolation of Streptomycetes from Soil; in Ortiz-Ortiz L., Bojalil L.F. and Yakoleff V., eds., *Biological, Biochemical and Biomedical Aspects of Actinomycetes*, Academic Press, New York, 1984: 553-561.

[7] Jensen P.R., Mincer T.J., Williams P.G. and Fenical W., *Antonie van Leeuwenhoek*, 2005; **87**: 43-48. DOI 10.1007/s10482-004-6540-1.

[8] Knox G.A., Benthic Community; in Knox G.A. ed., *Biology of the Southern Ocean* 2nd Edn., CRC Press, 2006: 300.

[9] Thistle D., The Deep-sea Floor: An Overview; in Tyler P.A., ed., *Ecosystems of the Deep Ocean*, Elsevier Science, Amsterdam, 2003: 5-39.

[10] Bredholt H., Fjaervik E., Jonhsen G. and Zotchev S.B., *Mar. Drugs*, 2008; **6**: 12-14. DOI 10.3390/md6010012.

[11] Gartner A., Wiese J. and Imhoff J.F., *AIMS Microbiology*, 2016; **2(2)**: 205-221. DOI 10.3934/microbiol.2016.2.205.

[12] Vizcaino A.S., Gonzalez V., Brana A.F., Palacios J.J., Otero L., Fernandez J., Molina A., Kulik A., Vazquez F., Acuna J.L., Garcia L.A. and Blanco G., *Microb. Ecol.*, 2016; **73(2)**: 338-352. DOI 10.1007/s00248-016-0845-2.

[13] Pathom-aree W., Stach J.E., Ward A.C., Horikoshi K., Bull A.T. and Goodfellow M., *Extremophiles*, 2006; **10**: 181-189. DOI 10.1007/s00792-005-0482-z.

[14] Kamjam M., Sivalingam P., Deng Z.X. and Hong K., *Front. Microbiol.*, 2017; **8**: 760. DOI 10.3389/fmicb.2017.00760.

[15] Labeda D.P. and Shearer M.C., Isolation of Actinomycetes for Biotechnological Applications; in Labeda D.P., ed., *Isolation of Biotechnological Organisms From Nature*, McGraw-Hill, New York, 1990: 1-19.

[16] Hong K., Gao A.H., Xie Q.Y., Gao H., Zhuang L., Lin H.P., Yu H.P., Li J., Yao X.S., Goodfellow M. and Ruan J.S., *Mar. Drugs*, 2009; **7**: 24-44. DOI 10.3390/md7010024.

[17] Chang X.B., Liu R.C., Liu W.Z. and Zhang X.H., *Hubei Agric. Sci.*, 2014; **53(13)**: 3014-3018. DOI 10.3969/j.issn.0439-8114.2014.13.011.

[18] Ruttanasutja R. and Pathom-aree W., *Chiang Mai J. Sci.*, 2015; **42(1)**: 88-103.

[19] Shirling E.B. and Gottlieb D., *Int. J. Syst. Bact.*, 1966; **16(3)**: 313-340. DOI 10.1099/00207713-16-3-313.

[20] Yoon S.H., Ha S.M., Kwon S., Lim J., Kim Y., Seo H. and Chun J., *Int. J. Syst. Evol. Microbiol.*, 2017. DOI 10.1099/ijsem.0.001755.

[21] Zhang L., Li L., Deng Z.X. and Hong K., *Int. J. Syst. Evol. Microbiol.*, 2015; **65**: 4880-4885. DOI 10.1099/ijsem.0.000667.

[22] Goo K.S., Tsuda M. and Ulanova D., *Antonie van Leeuwenhoek*, 2014; **105**: 207-219. DOI 10.1007/s10482-013-0067-2.

[23] Weyland H., *Zentral. Bakteriol. Suppl.*, 1981; **11**: 185-193.

[24] Durden J.M., Bett B.J., Jones D.O.B., Huvenne V.A.I. and Ruhl H.A., *Prog. Oceanogr.*, 2015; **137**: 209-218. DOI 10.1016/j.pocean.2015.06.006.

[25] Kasai H., Tamura T. and Harayama S., *Int. J. Syst. Evol. Microbiol.*, 2000; **50(1)**: 127-134. DOI 10.1099/00207713-50-1-127.