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Contributed Paper

Selective Isolation of Cultivable Actinomycetes from Thai Coastal Marine Sediment

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ABSTRACT

This study was designed to improve isolation method for actinomycetes from coastal marine sediments. Five pretreatments, three enrichment media, and fifteen selective media were tested for isolation of actinomycetes from a coastal marine sediment sample from Thailand. The pretreatment methods were (1) Tenfold serial dilution, (2) Tenfold serial dilution with 10 minutes shaking at 125 rpm, (3) 1.5% phenol treatment, (4) Shaking sediment suspension for 10 minutes at 125 rpm, and (5) Shaking sediment suspension for 60 minutes at 125 rpm. Three enrichment media were used: (1) Marine Broth (MB), (2) Soil Extract Solution (SES), and (3) Marine Soil Extract Broth (MSB). All culture plates were incubated at 25°C for up to 30 days on 15 selective isolation media. Actinomycetes were isolated only by shaking sediment suspension for 60 minutes at 125 rpm (pretreatment 5). Marine broth was found to promote the isolation of actinomycetes with the highest ratio of total bacteria:actinomycetes (2:1). A total of 209 actinomycetes were isolated. In general, diluted media or low nutrient media were more effective for the isolation of actinomycetes. Molecular identification based on 16S rRNA gene sequences revealed that these isolates belonged to eight known actinomycete genera: *Curtobacterium, Dermacoccus, Micromonospora, Microbispora, Pseudonocardia, Rhodococcus, Streptomyces* and *Tsukamurella.*

Keywords: pretreatment, enrichment, selective isolation, actinomycete diversity, coastal marine sediment

1. INTRODUCTION

The oceans cover more than 70% of the earth's surface. These marine areas are the largest inhabitable space for living microorganisms including actinomycetes. Actinomycetes are a large group of bacteria that are well known for their high ability to produce secondary metabolites especially antibiotics. These Gram positive high GC bacteria are widely distributed in soils, but can be found in different marine environments such as surface sea water, lower or abyssal depth of coastal to offshore region, and other general oceanic area [1-3]. The increasing numbers of literatures on novel metabolites and the diversity of marine actinomycetes strongly support the view that the marine environment is a significant source for the search and discovery of both diversity and secondary metabolites [4-9].

The first actinomycete to be isolated from the oceanic sediment was not considered to be a marine microorganism. It was believed that they were spores of terrestrial actinomycetes that had simply washed out into the ocean and had remained dormant [10]. However, further investigations showed that many indigenous actinomycetes in marine environments exist [11-13]. The diversity of actinomycetes was studied from various parts of the marine ecosystem, for example, nearshore water and sediment, coastal sediment, deep sea, and mangrove environment [3,9,14]. Actinomycetes from marine sediments were reported to be associated mainly with the genera *Actinomyces*, *Actinopolyspora*, *Micromonospora*, *Micropolyspora*, *Nocardia*, *Rhodococcus*, *Salinispora*, *Serinicoccus*, *Streptomyces*, *Streptosporangium*, *Streptoverticillium*, and *Solwaraspora* [1, 15-16].

The knowledge about the diversity of marine actinomycetes in Thailand is inadequate, only limited data are available [17-20]. Most of marine ecosystems in Thailand are still understudied. The essential step in biodiversity study is an isolation procedure which is very important for the purpose of understanding "True Diversity" and its future beneficial applications of these diversities. Modern molecular studies revealed that most actinomycetes are vet to be isolated [1, 21]. Successful cultivation of actinomycetes from marine environments generally relies on pretreatment methods, enrichment and choice of selective isolation media [7-8]. This work described our attempt to find an effective method for the isolation of actinomycetes from Thai coastal marine sediment based on the combination of pretreatments, enrichments and selective media.

2. MATERIAL AND METHODS

2.1 Environmental Sample

Sediment sample was collected from Klong-Ta-Guan, a coastal area of Rayong province, Thailand (latitude: N12 39′ 44.6′, longitude: E101 10′ 59.6′) at the depth of 4.5 m. Sediment texture was loamy sand with percentage distribution of sand:silt:clay of 85.4:8.2:6.5 and pH of 8.2. The sample was stored in sterile plastic bags and kept at 4°C before transferring to the laboratory for isolation. The sediment sample was dried at room temperature for 1 week and processed using different pretreatment and enrichment methods.

2.2 Pretreatment Methods

This preliminary study on pretreatment methods was carried out with 8 selective media: Starch casein agar, Marine agar, Gause No.2 agar, 1/100 Starch casein agar, 1/100 Marine agar, 1/100 Gause No.2 agar, Humic acid vitamin (HV) agar and 1/100 HV agar. All media were used in triplicates and incubated at 25°C for 24-30 days .

2.2.1 *Method 1 (Tenfold serial dilution)*

One gram of the sediment sample was diluted in 9 ml of distilled water (10^{-1}) and serially diluted to 10^{-3}. Then, 100 µl of each diluted sample was spread on agar plates.

2.2.2 *Method 2 (Tenfold dilution and shaking 10 min)*

One gram of the sediment sample was tenfold diluted to 10^{-1} with 9 ml of distilled water. The suspension was shaken at 125 rpm for 10 min at room temperature and serially diluted up to 10^{-3} before spreading 100 µl on agar plates.

2.2.3 *Method 3 (1.5% phenol treatment)*

Sediment suspension was prepared by mixed 1 g of sediment with 3 ml of distilled water. The suspension was shaken at 125 rpm, at room temperature for 10 min. The sediment suspension was then treated with 1.5% phenol (v/v) [22-23], mixed for 1 min and 100 µl inoculated onto the surface of the agar plates.

2.2.4 ***Method 4 (Shaking suspension for 10 min)***

One gram of sediment sample was diluted with 3 ml of distilled water to prepare sediment suspension. The suspension was shaken at room temperature at 125 rpm for 10 min before 100 µl aliquot was spread on the agar plates.

2.2.5 ***Method 5 (Shaking suspension for 60 min)***

Sediment suspension was prepared as in 2.2.4, shaken at room temperature at 125 rpm for 60 min and 100 µl aliquot was spread on the surface of an agar medium.

2.3 Enrichtment Methods

To increase the recovery rate of actinomycetes from the sediment sample, the enrichment method was also used. Four enrichment media: (1) Marine Broth (MB: 40 g marine broth which composition as follows: 1 g Yeast Extract, 5 g Bacteriological Peptone, 0.022 g Boric Acid, 19.40 g NaCl, 8.80 g $MgCl_2$, 3.24 g Na_2SO_4, 1.80 g $CaCl_2$, 0.55 g KCl, 0.16 g $NaHCO_3$, 0.1 g $FeC_6H_5O_7 \cdot 3H_2O$, 0.08 g KBr, 0.034 g $SrCl_2$, 0.008 g Na_2HPO_4, 0.004 g Na_2SiO_3, 0.0024 g FNa, and 0.0016 g NH_4NO_3 (Labscan Ltd., Thailand), 1 L distilled water), (2) Soil Extract Solution (SES: 1 kg garden soil, 1 L distilled water), (3) Marine Soil Extract Broth (MSB: 40 g marine broth (Labscan Ltd., Thailand), 1 L soil extract solution) (modified from [24]), and (4) Distilled water, were used in the preparation of the sediment suspension following the best pretreatment method (method 5). This enrichment procedure was carried out with 15 selective media as shown in Table 1 and incubated at 25°C for up to 30 days.

2.4 Selective Isolation of Actinomycetes

Fifteen media designed for the cultivation of actinomycetes were selected for the isolation of actinomycetes, namely, Marine Agar (MA), Starch Casein Agar (SCA), Gause No.2 Agar (GNO2A), Marine Soil Extract Agar (MSA), Starch Casein Soil Extract Agar (SCSA), Gause No.2 Soil Extract Agar (GNO2SA), Soil Extract Agar (SEA), 1:100MA, 1:100SCA, 1:100GNO2A, 1:100MSA, 1:100SCSA, 1:100GNO2SA 1:2 SEA (HSEA), and 1:100SEA (Table 1). All media were added with a final concentration of 10 µg/ml of anti-Gram negative bacteria nalidixic acid and 50 µg/ml of antifungal ninazol. The sediment suspension (100 µl) which was made to undergo the most effective pretreatment and enrichment from 2.2 and 2.3 (1 g sediment in 3 ml marine broth and shaken at 125 rpm for 60 min) was spread over the surface of 15 selective isolation media in triplicate and incubated at 25°C for 24-30 days. Colonies of actinomycetes and bacteria were counted, recorded, and expressed as colony forming unit per gram (CFU/g). Presumptive actinomycetes were purified on glucose yeast extract agar (GYEA: 10 g glucose, 10 g yeast extract, 15 g agar; [25]). The purified isolates were then stored in 20% glycerol at -20°C for long-term preservation.

2.5 Characterization of Actinomycete Isolates

Actinomycete isolates were subcultured onto glucose yeast extract agar (GYEA), incubated at 25°C for 1 week and then checked for purity based on their colony morphology and Gram stain results. All isolates were assigned to filamentous and non-filamentous actinomycetes based on their macroscopic characteristics (colony texture, substrate and aerial mycelium production, pigment production) and microscopic characteristics (Gram's stain, cell structure, spore production). These

Table 1. Composition of fifteen selective media used for isolation of actinomycetes.

Media	Composition	References
MA	40 g Marine broth (Labscan Ltd., Thailand), 15 g agar, 1 L distilled water	Pathom-aree *et al*. [9]
SCA	10 g soluble starch, 2 g KNO_3, 2 g K_2HPO_4, 2 g NaCl, 0.3 g casein, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, 15 g agar, 1 L distilled water	Pathom-aree *et al*. [9]
GNO2A	3 g tryptone, 5 g peptone, 10 g glucose, 5 g NaCl, 15 g agar, 1 L distilled water	Modified from Bredholt *et al*. [41]
MSA	40 g Marine broth (Labscan Ltd., Thailand), 15 g agar, 1 L soil extract solution	This study
SCSA	10 g soluble starch, 2 g KNO_3, 2 g K_2HPO_4, 2 g NaCl, 0.3 g casein, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 0.01 g $FeSO_4 \cdot 7H_2O$, 15 g agar, 1 L soil extract solution	This study
GNO2SA	3 g tryptone, 5 g peptone, 10 g glucose, 5 g NaCl, 15 g agar, 1 L soil extract solution	This study
SEA	1 L soil extract solution, 15 g agar	Starr *et al*. [24]
HSEA	500 ml soil extract solution, 15 g agar, 500 ml distilled water	Modified from Starr *et al*. [24]
1:100MA	100-fold dilution of Marine broth, 15 g agar, 1 L distilled water	Modified from Pathom-aree *et al*. [9]
1:100SCA	100-fold dilution of Starch casein broth, 15 g agar, 1 L distilled water	Modified from Pathom-aree *et al*. [9]
1:100GNO2A	100-fold dilution of Gause No. 2, 15 g agar, 1 L distilled water	Modified from Bredholt *et al*. [41]
1:100MSA	100-fold dilution of Marine broth, 15 g agar, 1 L soil extract solution	This study
1:100SCSA	100-fold dilution of Starch casein broth, 15 g agar, 1 L soil extract solution	This study
1:100GNO2SA	100-fold dilution of Gause No. 2, 15 g agar, 1 L soil extract solution	This study
1:100SEA	100-fold dilution of soil extract solution, 15 g agar, 1 L distilled water	This study

properties were examined according to the Bergey's Manual of Determinative Bacteriology [26]. Diaminopimelic acid isomers of filamentous actinomycetes were also determined based on the method of Hasegawa *et al*. [27] in order to assign them to either streptomycetes or non-streptomycetes.

2.6 DNA Extraction and PCR Amplification of 16S rRNA Gene

Actinomycete isolates were cultured on non-sporulating media [7] for 7-14 days and DNA was directly extracted from the

colony grown on the agar plates (Modified from [28]). The obtained DNA was amplified by PCR using universal bacterial primer 27F and 1492R [9]. The 16S rRNA gene sequences which obtain from 1st Base, Malaysia (Ward Medic Ltd. Partnership, Thailand), were compared with those in public databases using the BLAST option in EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) [29]. Related sequences were downloaded and used for phylogenetic analysis. The neighbor joining tree was generated using the MEGA version 4.0 software [14] and the robustness of the tree topology was evaluated by bootstrap analysis of 1,000 resamplings.

3. RESULTS AND DISCUSSION

3.1 Effect of Pretreatment

Pretreatment methods either physical or chemical pretreatments have been used to select specific actinomycetes from various habitats as it could inhibit and eliminate other non-actinomycetes [22]. In this study, actinomycetes were only recovered from method 5 in which sediment sample was shaken at 125 rpm for 60 min at room temperature in all selected media except for humic acid vitamin agar. No microorganisms were observed in method 3 (1.5% phenol treatment). The remaining methods yielded only bacteria.

Phenol treatment is one of the useful recognized isolation technique for rare actinomycetes. The pretreatment with 1.5% phenol was designed for selective isolation of *Microbispora*, *Micromonospora* and *Streptomyces violaceusniger* [22, 23]. It have been successfully used to isolate actinomycetes from mangrove sediments [14, 30] and marine sediments [31, 41]. However, in our study, chemical pretreatment with 1.5% phenol showed no growth of either actinomycetes or bacteria on all selective media used. Since phenol is commonly toxic to microbial cells and spores, it is possible that phenol reduced actinomycetes and bacterial population in our sample to a non-detectable level. Thus, phenol treatment may not be an appropriate pretreatment for coastal sediment sample.

Physical pretreatment by shaking in diluent followed by plating out on selective media is a simple way of separating microorganisms from soil particles [32]. The shaking time plays a crucial role on separation efficiency. If the shaking time is not sufficient, actinomycetes (cells and spores) could not be separated or only a small number of cells will be released from the sediment particles, which will result in none or few colonies growing on the agar plates. In our study, bacteria could be separated from the sediment particles into suspension (both for 1:10 fold and 1:4 fold dilutions) by shaking for 10 min. However, actinomycetes were isolated only after shaking at 125 rpm for 60 min. The ratio of actinomycetes and bacterial isolates obtained from pretreatment 5 was 1:5. This result suggested that 60 min shaking time was enough for the separation of actinomycetes from the sediment particles and recovered on isolation plates.

3.2 Effect of Enrichment Solution

Enrichment techniques have been developed to enhance the growth of desirable actinomycete from natural habitats [22]. Each enrichment medium showed different numbers of actinomycetes and bacterial isolates on the isolation media. Marine broth demonstrated the highest recovery efficiency with a number of actinomycetes and bacterial isolates in a ratio of 1:2, followed by marine soil extract solution (1:4) and sterile distilled water (1:4). Soil extract solution gave the poorest result with 1:7 ratio (Figure 1). This mimicking approach of natural marine environments was successful in several isolation and cultivation studies [6, 12-13,

33]. The use of marine broth was to mimic the *in situ* condition and also provide nutrients to promote growth of marine bacteria. Actinomycetes could grow in marine broth and it is also possible that damaged cells could be repaired. Marine broth could also induce spore germination which promote the growth of actinomycetes, thereby leading to an increased chance of actinomycetes recovery from the sample. We have carried out an experiment to prove that an enrichment with marine broth could promote the growth of actinomycetes in the sample. A spore suspension of *Streptomyces* sp. R1-1A/A106 ($5.87 \pm 2.52 \times 10^8$ cfu/ml) was inoculated into either 3 ml of distilled water or marine broth as described in method 5. The suspension was then shaken at 125 rpm for 60 min at room temperature and 100 µl aliquot of appropriate dilution was spread on the surface of a glucose yeast extract agar. The *Streptomyces* count from suspension enriched with marine broth was $1.15 \pm 0.70 \times 10^9$ cfu/ml compared to $1.17 \pm 0.47 \times 10^7$ cfu/ml obtained from distilled water. It was evident that at least 100 times increase in growth of actinomycetes could be achieved by simple enrichment of sample in marine broth. Therefore, marine broth was used as an enrichment medium in the subsequent experiment for the selective isolation of actinomycetes from coastal sediment.

3.3 Selective Isolation of Actinomycetes

Different groups of actinomycetes requires different kinds of media for their growth and differentiation. Suitable culture media promote successful isolation of actinomycetes from the environmental samples. In our study, the total actinomycetes count ranged between 40-440 cfu/g (Table 2) which is considerably low compared to previous studies [9, 39]. This is not surprising as actinomycetes usually make up only for a small fraction of bacterial communities [3, 9]. An approximate value of ratio between actinomycetes and bacteria was used to demonstrate the isolation efficiency of the proposed selective media (Figure 2). The highest ratio of actinomycetes and bacterial isolates (1:1) was found on the diluted media1:100 MA, 1:100MSA, 1:100 SCSA, 1:100GNO2SA, normal strength of SEA, and half strength of SEA. An increase in the recovery efficiency was observed based on the change in an approximate ratio of actinomycetes and bacterial isolates from 1:5 (sample undergone pretreatment) to 1:2 (sample undergone enrichment) to 1:1 (sample undergone pretreatment and

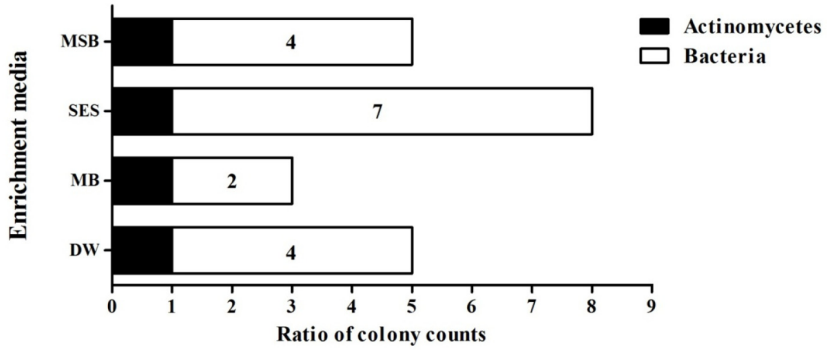

Figure 1. The ratio of actinomycetal and bacterial isolates using different enrichment broths, MB: marine broth, SES: soil extract solution, MSB: marine soil extract broth, and control DW: distilled water.

Table 2. Total actinomycete and bacterial counts on selective medium using marine broth as enrichment medium and shaking sediment suspension for 60 minutes at 125 rpm.

Selective Media	Total Counts ($\times 10^2$ CFU/g)	
	Actinomycetes	Bacteria
MA	3.87 ± 0.23 ^{de*}	14.13 ± 3.35 ^{c}
SCA	1.73 ± 1.22 ^{abc}	13.87 ± 2.57 ^{c}
GNO2A	2.00 ± 0.69 ^{abc}	4.80 ± 1.06 ^{ab}
MSA	4.40 ± 0.80 ^{e}	7.20 ± 1.20 ^{b}
SCSA	3.47 ± 0.23 ^{cde}	5.20 ± 1.06 ^{ab}
GNO2SA	2.80 ± 1.44 ^{cde}	5.20 ± 2.50 ^{ab}
SEA	2.67 ± 0.61 ^{cde}	2.53 ± 3.00 ^{a}
HSEA	2.53 ± 0.83 ^{bcd}	2.53 ± 3.03 ^{a}
1:100MA	2.53 ± 1.40 ^{bcd}	2.00 ± 0.80 ^{a}
1:100SCA	0.53 ± 0.23 ^{a}	4.00 ± 3.55 ^{ab}
1:100GNO2A	0.40 ± 0.40 ^{a}	1.33 ± 1.40 ^{a}
1:100MSA	3.87 ± 1.89 ^{de}	2.67 ± 2.90 ^{ab}
1:100SCSA	2.80 ± 0.70 ^{cde}	4.00 ± 1.74 ^{ab}
1:100GNO2SA	3.87 ± 0.61 ^{de}	4.53 ± 1.89 ^{ab}
1:100SEA	0.80 ± 1.06 ^{ab}	2.67 ± 2.95 ^{ab}

* Mean with the same letter are not significantly different for ANOVA test ($P < 0.05$).

enrichment). It is evident that the efficiency of our isolation procedure was improved by at least 50% with the combination of pretreatments, enrichment media and suitable selective media. The use of various isolation media would also increase the chance to obtain more actinomycetes [7, 34].

Conventional culture media are generally rich in nutrients, for example, when they consist of more than 1% yeast extract, meat extract or peptone. Application of these media resulted in the failure in isolation of most microorganisms [35]. In this study, the total number of isolates recovered from each media was significantly different. The recovery efficiency of each selective media was compared in term of ratio of actinomycetes to bacteria on isolation plates as shown in Figure 2. It is evident that the diluted media with lower nutritional components were more effective for the isolation of actinomycetes than the non-diluted ones, which is consistent with the results from previous studies [36-37]. Soil extract was found to be a suitable substrate for isolation of many soil bacteria including actinomycetes as it contained several different growth-promoting substances [38]. Soil-extract agar medium was successfully used to isolate novel actinomycetes from forest soil [35]. These results suggested that nutritionally poor media or diluted media were more favorable for growth of actinomycetes. Our results were consistent with previous studies [4, 30, 33, 36-39]. It is known that actinomycetes can survive and grow to some extent on small amounts of nutrients [40]. Selective media for isolation of actinomycetes need not to be media on which their growth is abundant. It is preferable to be a medium that actinomycetes may have limited growth, but without other bacteria and fungi.

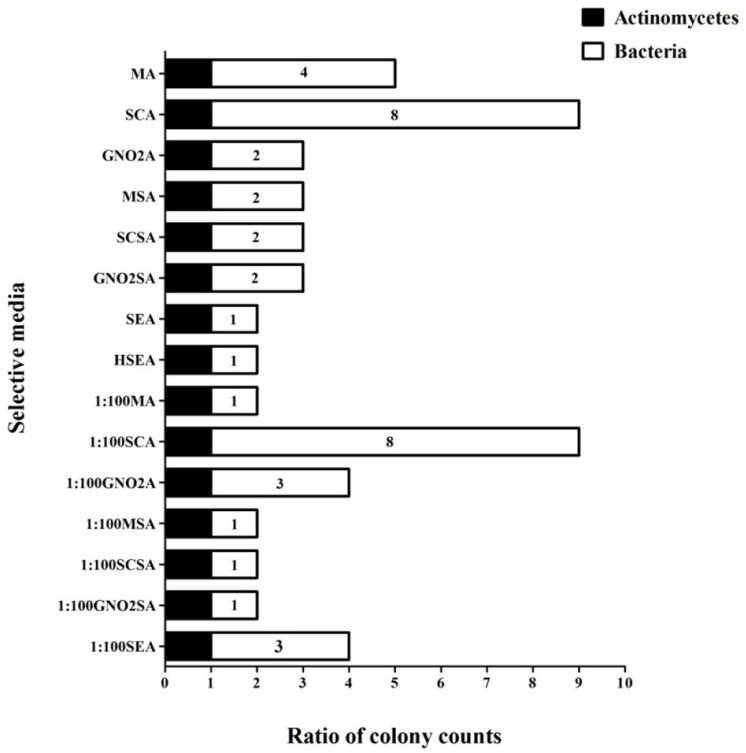

Figure 2. The ratio of total actinomycetal and bacterial counts from 15 selective media using pretreatment 5 and enrichment by marine broth; MA: marine agar, SCA: starch casein agar, GNO2A: Gause no.2 agar, MSA: marine soil extract agar, SCSA: starch casein soil extract agar, GNO2SA: Gause no.2 soil extract agar, SEA: soil extract agar, HSEA: haft soil extract agar and 1:100 mean diluted 100 fold of media.

3.4 Characterization of Actinomycete Isolates

A total of 209 actinomycete isolates were obtained from 15 selective media. Based on the morphological characteristics, they were assigned into 2 groups, namely, filamentous and non-filamentous actinomycetes. To validate genus level identification using light microscopy, these isolates were analyzed for the isomers of their cell wall diamiopimelic acid (DAP) using a rapid method of Hasegawa *et al*. [27]. Typical *Streptomyces* isolates contained *LL*-DAP in the cell wall. The majority of isolates (172) were filamentous actinomycetes, which composed of streptomycetes (38) and non-streptomycetes (134) as shown in Table 3. The presumptive streptomycetes were assigned to 8 colour groups (S1-S8) as shown in Table 4. The dominant streptomycetes were isolated with pale green spore mass colour. The remaining 134 isolates (64.12%) in the filamentous non-streptomycetes group which contained *meso*-DAP in their cell wall were assigned into five groups, A1-A5. This grouping was dominated by *Micromonospora* like isolates (A2) which showed typical orange colonies with black mucoid spores on agar plates (Table 5). The non-filamentous *meso*-DAP containing actinomycetes group with 37 isolates (17.70%) were assigned into four groups (B1-B4) based on their morphological characteristics (Table 5).

3.5 16S rRNA Gene Sequencing Analysis

Twenty six isolates, chosen as the representative from each assigned morphological group were sent for 16S rRNA gene sequencing analysis. The results of the partial 16S rRNA gene sequence analyses revealed considerable diversity among these isolates which were affiliated with 19 species in eight genera, namely *Curtobacterium Dermacoccus, Microbispora, Micromonospora, Pseudonocardia, Rhodococcus, Streptomyces,* and *Tsukamurella* (Table 6). Members of the filamentous like-streptomycetes group were confirmed to belong to the genus *Streptomyces*, in agreement with their phenotype assignment. Members of the filamentous non-streptomycetes group belonged to three genera, namely, *Microbispora*, *Micromonospora*, and *Pseudonocardia*. The non-filamentous group was represented by four genera, *Curtobacterium*, *Dermacoccus*, *Rhodococcus,* and *Tsukamurella* (Table 3). The results from 16S rRNA gene sequencing were in good agreement with morphological grouping, thus, provided the evidence for the usefulness of conventional characterization based on phenotypic properties (morphology and DAP analysis) of actinomycetes.

The *Streptomyces* isolates are of interest as these groups of actinomycetes are a well known productive source of secondary metabolites. The 16S rRNA gene sequence analysis revealed that they were members of the uncommon *Streptomyces* subclades as shown in Figure 3 and 4. The similarity values of these isolates were between

Table 3. Groups of actinomycete isolates based on morphology, DAP isomers, and 16S rRNA gene sequence analysis.

Group		Taxonomic assignment	No. of isolates
Filamentous actinomycetes		(genus level)	
Filamentous streptomycetes	S1		3
	S2	*Streptomyces*	11
	S3	*Streptomyces*	15
	S4	*Streptomyces*	2
	S5	*Streptomyces*	1
	S6	*Streptomyces*	2
	S7	*Streptomyces*	2
	S8	*Streptomyces*	2
		Streptomyces	**38**
Filamentous non-streptomycetes	A1		1
	A2	*Pseudonocardia*	120
	A3	*Micromonospora*	3
	A4	*Microbispora*	3
	A5	*Microbispora*	7
		Microbispora	**134**
Non-filamentous actinomycetes	B1		1
	B2	*Curtobacterium*	8
	B3	*Dermacoccus*	3
	B4	*Tsukamurella*	25
		Rhodococcus	**37**

Table 4. Morphological characteristics of presumptive streptomycetes on various cultivation media.

Group	Morphological Characteristics														No. of isolates
	GYEA			ISP2			ISP3			ISP4					
	SM	Spore	Pig	SM	Spore	Pig	SM	Spore	Pig	SM	Spore	Pig	ISP6	Spore Morphology	
S1	Yellowish White	Gray	-	Yellowish White	Gray	-	Yellowish White	Gray	Yellowish White	Yellowish White	Gray	-	-	Spiral	3
S2	Moderate Greenish Yellow	White	-	Pale Greenish Yellow	White	-	Light Yellowish Green-Grayish Olive	Very Pale Green	Light Yellowish Green-Grayish Olive	Light Grayish Olive	Very Pale Green	-	-	Flexibilis	11
S3	Moderate Olive	Very Pale Green	-	Moderate Greenish Yellow	Very Pale Green	-	Light Greenish Yellow	Very Pale Green	Light Yellowish Green	Moderate Yellowish Green	Light Yellowish Green	-	-	Flexibilis	15
S4	Brilliant Orange Yellow	Greenish Gray	-	Strong Yellow	Gray	-	Vivid Yellow	Gray	-	Grayish Greenish Yellow-Dark Yellow	Gray	-	-	Spiral	2
S5	Dark Orange Yellow	Gray	-	Moderate Yellow-Grayish Greenish Yellow	Gray	-	Light Greenish Yellow-Moderate Olive	Gray	-	Moderate Yellow	Gray	-	-	Spiral	1
S6	Grayish Olive Green	Dark Gray	-	Grayish Yellowish Green	Gray	-	Dark Bluish Gray	Gray	-	Greenish Gray	Gray	-	-	Spiral	2
S7	Dark Purple	Very Pale Green	-	Dark Purple	Very Pale Green	-	Strong Purple	Very Pale Green	-	Dark Purple	Gray	-	-	Spiral	2
S8	Strong Yellowish Brown	Gray	-	Deep Yellowish Brown	Gray	Strong Yellowish Brown	Strong Yellow	Gray	Strong Yellowish Brown	Greenish Gray	Gray	-	-	Spiral	2

Note: "SM" = substrate mycelium and "Pig" = soluble pigment.

86.63-100% with the length of sequences used in the comparison ranged from 931 - 1471 bp. Nevertheless, it is likely that some of these isolates may represent a putative novel *Streptomyces* species which merit detailed taxonomic characterization, in particular, isolate R1-3/A401, which shared only 86.63% similarity with *Streptomyces griseorubens* NBRC 12780^T.

Members of the group A3-A5 were represented by isolates closely related to *Microbispora amethystogenes* JCM3021^T and *M. mesophila* JCM3151^T. Isolates R1-3/B403 and R1-2B/H805 were distantly related to *M. amethystogenes* JCM3021^T sharing only 76.81-77.97% 16S rRNA gene similarity from 1,106 nucleotides. It is also evident from the phylogenetic tree that these *Microbispora* isolates were well separated from their related type strains in their own subclade (Figure 3 and 4). These isolates merit a polyphasic taxonomic characterization to clarify their novelty. For non-filamentous actinomycetes, isolate R1-1A/A105 shared very low similarity (66.63%) with *Curtobacterium citreum* DSM20528^T and this isolate may represent a putative novel genus or family. Although the comparison of this isolate and their related type strains was based on partial 16S rRNA gene sequences (946 nt), it is possible that isolate R1-1A/A105 may represent a novel taxa considering its low similarity value.

Table 5. Morphological grouping of filamentous non-streptomycete and non-filamentous actinomycete isolates.

Grouping		Macroscopic Characterization	Microscopic Characterization			
			Spore mass colour	Cell	Spore	No. of isolate
Filamentous non-streptomycetes	A1	brownish orange colony, convex and filamentous	pinkish white	Filamentous	long chain spore	1
	A2	orange and moderately soft colony, convex and wrinkle	brown-black mucoid mass	Filamentous	only one spore on mycelium	120
	A3	yellowish white - soft pink colony, moderately soft, convex and wrinkle	white-pinkish white	Filamentous	two spores on mycelium	3
	A4	very dark reddish purple and moderately soft colony, convex and wrinkle	very dark reddish purple	Filamentous	two spores on mycelium	3
	A5	pale yellowish green - deep purple and hard colony, convex and smooth	white-gray	Filamentous	two spores on mycelium	7
Non-filamentous actinomycetes	B1	yellowish, shiny and soft colony, convex and smooth	-	Rod	-	1
	B2	yellow, shiny and soft colony, convex and smooth	-	Cocci	-	8
	B3	pale orange yellow, shiny and soft colony, convex and wrinkle	-	Rod	-	3
	B4	orange - deep pink and soft colony, convex and smooth	-	Cocci	-	25

4. CONCLUSION

Our results suggested that pretreatment by shaking the sediment suspension in marine broth as an enrichment medium for 60 min at 125 rpm at room temperature prior to isolation on agar plates is necessary. The use of such pretreatment in combination with diluted media or low nutrient media yielded the highest numbers of actinomycete isolates on the isolation plates. The success of the proposed method was based on the strategy of extracting actinomycetes from sediment particles and growth enhancement of the dormant spores and cells of actinomycetes through the combination of pretreatment, enrichment, and cultivation on suitable selective media. This proposed method will help expand our knowledge on the diversity of cultivable actinomycetes in marine environments and provide resources for further biotechnological exploitation.

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Table 6. Sequence analysis of selected actinomycete isolates from coastal marine sediment based on 16S rRNA gene.

Groups	Isolates code	Accession No.	Length (bp)	Nearest type strain (accession number)	Sequence identity (%)
S1	R1-3/A401	AB915622	1,019	*Streptomyces griseorubens* NBRC 12780(T) (AB184139)	86.63
S1	R1-3/H405	AB915627	1,474	*Streptomyces olivaceus* NBRC 12805(T) (AB249920)	99.79
S2	R1-1A/A106	AB922838	1,173	*Streptomyces exfoliatus* NBRC 13475(T) (AB184868)	100.00
S3	R1-2B/H204	AB915618	1,159	*Streptomyces koyangensis* VK-A60(T) (AY079156)	98.26
S3	R1-2A/O104	AB915616	931	*Streptomyces somaliensis* NBRC 12916(T) (AB184243)	99.24
S4	R1-2B/B106	AB915613	1,458	*Streptomyces enissocaesilis* NRRL B-16365(T) (DQ026641)	98.95
S5	R1-2A/D104	AB922840	1,458	*Streptomyces rochei* NBRC 12908(T) (AB184237)	99.65
S6	R1-2B/A502	AB915617	1471	*Streptomyces pactum* NBRC 13433(T) (AB184398)	99.93
S7	R1-2A/B102	AB922839	1,447	*Streptomyces puniceus* NBRC 12811(T)(AB184163)	99.65
S8	R1-3/K306	AB915631	1,119	*Streptomyces erythrogriseus* LMG 19406(T) (AJ781328)	99.82
A1	R1-2B/O805	AB915621	1,148	*Pseudonocardia carboxydivorans* Y8(T)(EF114314)	99.73
A2	R1-2A/D103	AB915614	1,453	*Micromonospora chalcea* DSM 43026(T) (X92594)	99.79
A2	R1-3/N201	AB915632	1,400	*Micromonospora aurantiaca* ATCC 27029(T) (CP002162)	99.93
A3	R1-2B/H805	AB915619	1,107	*Microbispora amethystogenes* JCM 3021(T) (U48988)	77.97
A4	R1-3/B403	AB915623	1,106	*Microbispora amethystogenes* JCM 3021(T) (U48988)	76.81
A4	R1-3/J202	AB915629	1,109	*Microbispora amethystogenes* JCM 3021(T) (U48988)	99.44
A5	R1-1A/A116	AB915610	938	*Microbispora amethystogenes* JCM 3021(T) (U48988)	92.06
A5	R1-3/H406	AB915628	1,108	*Microbispora mesophila* JCM 3151(T) (AF002266)	99.54
A5	R1-3/J401	AB915630	938	*Microbispora mesophila* JCM 3151(T) (AF002266)	95.83
A5	R1-2B/M603	AB915620	1,465	*Microbispora mesophila* JCM 3151(T) (AF002266)	99.09
B1	R1-1A/A105	AB915609	946	*Curtobacterium citreum* DSM 20528 (T) (X77436)	66.63
B2	R1-2A/N707	AB915615	1,146	*Dermacoccus profundi* MT2.2(T) (AY894329)	99.79
B3	R1-1A/B119	AB915611	1,424	*Tsukamurella paurometabola* DSM 20162(T) (CP001966)	99.36
B4	R1-3/D102	AB915624	1,452	*Rhodococcus corynebacterioides DSM 20151(T)* (AF430066)	99.37
B4	R1-3/G103	AB915625	1,449	*Rhodococcus rhodochrous* DSM 43241(T) (X79288)	99.86

REFERENCES

[1] Prieto-Davó A., Fenical W., Jensen P.R., Comparative actinomycete diversity in marine sediments, *Aquat. Microb. Ecol.*, 2008; **52**: 1-11.

[2] Bull A.T. and Stach J.E.M., Marine actinobacteria: New opportunities for natural product search and discovery, *Trends Microbiol.*, 2007; **15(11)**: 491-499.

[3] Bull A.T., Stach J.E., Ward A.C. and Goodfellow M., Marine actinobacteria: Perspectives, challenges, future directions, *Antonie van Leeuwenhoek*, 2005; **87**: 65-79.

[4] Zhang G., Cao T., Ying J., Yang Y. and Ma L., Diversity and novelty of actinobacteria in Arctic marine sediments, *Antonie van Leeuwenhoek,* 2014; **105**: 743-754.

[5] Manivasagan P., Venkatesan J., Sivakumar K. and Kim S.K., Marine actinobacterial metabolites: Current status and future perspectives, *Microbiol. Res.,* 2013; **168**: 311-332.

[6] Özcan K., Aksoy S.C., Kalkan O., Uzel A., Hames-Kocabas E.E. and Bedir E., Diversity and antibiotic-producing potential of cultivable marine-derived actinomycetes from coastal sediments of Turkey, *J. Soils Sediments*, 2013; **13**: 1493-1501.

[7] Maldonado L.A., Fragoso-Yáñez D., Pérez-García A., Rosellón-Druker J. and Quintana E.T., Actinobacterial diversity from marine sediments collected in Mexico, *Antonie van Leeuwenhoek*, 2009; **95**: 111-120.

[8] Ward A.C. and Bora N., Diversity and biogeography of marine actinobacteria, *Curr. Opin. Microbiol.,* 2006; **9**: 279-286.

[9] Pathom-aree W., Stach J.E., Ward A.C., Horikoshi K., Bull A.T. and Goodfellow M., Diversity of actinomycetes isolated from Challenger Deep sediment (10, 898 m) from the Mariana Trench, *Extremophiles,* 2006; **10**: 181-189.

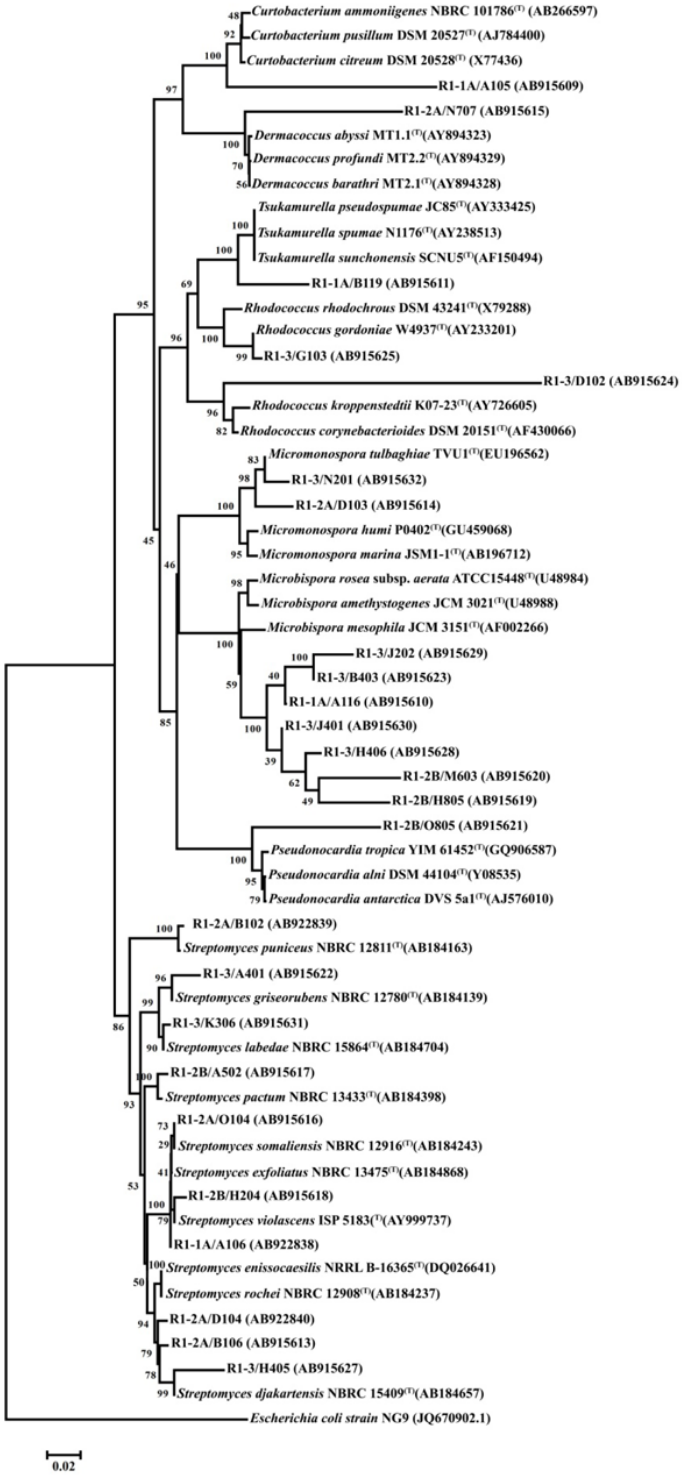

Figure 3. The neighbor-joining tree based on the partial 16S rRNA gene sequences of selected actinomycetes**.** The numbers at the node indicate the level of bootstrap support (%) based on the neighbor-joining analysis of 1,000 resampled data sets. The scale bar indicates 0.02 nucleotide substitutions per nucleotide position.

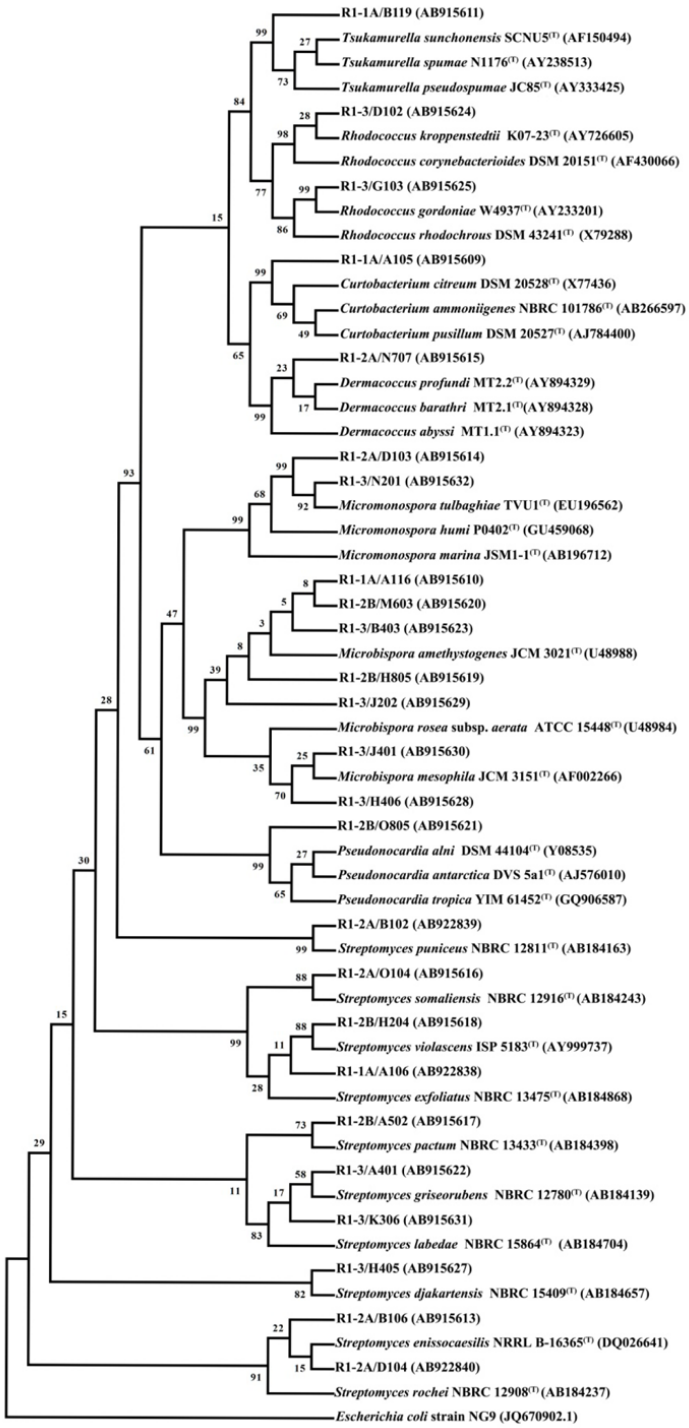

Figure 4. The maximum parsimony tree based on the partial 16S rRNA gene sequences of selected actinomycetes**.** The numbers at the node indicate the level of bootstrap support (%) based on the maximum parsimony of 1,000 resampled data sets.

[10] Goodfellow M. and Haynes J.A., Actinomycetes in Marine Sediments; in Ortiz-Ortiz L., Bojalil L.F. and Yakoleff V., eds., *Biological, Biochemical, and Biomedical Aspects of Actinomycetes*, Academic Press, London, 1984: 453-472.

[11] Stach J.E., Maldonado L.A., Ward A.C., Goodfellow M. and Bull A.T., New primers for the class *Actinobacteria*: Application to marine and terrestrial environments, *Environ. Microbiol.*, 2003; **5**: 828-841.

[12] Mincer T.J., Fenical W. and Jensen P.R., Culture-dependent and culture-independent diversity within the obligate marine actinomycete genus *Salinispora*, *Appl. Environ. Microbiol.*, 2005; **71(11)**: 7019-7028.

[13] Mincer T.J., Jensen P.R., Kauffman C.A. and Fenical W., Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments, *Appl. Environ. Microbiol.*, 2002; **68(10)**: 5005-5011.

[14] 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., Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China, *Mar. Drugs*, 2009; **7(1)**: 24-44.

[15] Jensen P.R., Mincer T.J., Williams P.G. and Fenical W., Marine actinomycete diversity and natural product discovery, *Antonie van Leeuwenhoek*, 2005; **87**: 43-48.

[16] Maldonado L.A., Stach J.E., Pathom-aree W., Ward A.C., Bull A.T. and Goodfellow M., Diversity of cultivable actinobacteria in geographically widespread marine sediments, *Antonie van Leeuwenhoek*, 2005; **87**: 11-18.

[17] Jongrungruangchok S., Tanasupawat S. and Kudo T., *Micromonospora krabiensis* sp. nov., isolated from marine soil in Thailand, *J. Gen. Appl. Microbiol.*, 2008; **54**: 127-133.

[18] Srivibool R. and Sukchotiratana M., Bioperspective of actinomycetes isolates from coastal soils: A new source of antimicrobial producers, *Songklanakarin J. Sci. Technol.*, 2006; **28(3)**: 493-499.

[19] Srivibool R., Jaidee K., Sukchotiratana M., Tokuyama S. and Pathom-aree W., Taxonomic characterization of *Streptomyces* strain CH54-4 isolated from mangrove sediment, *Ann. Microbiol.*, 2010; **60**: 299-305.

[20] Tanasupawat S., Jongrungruangchok S. and Kudo T., *Micromonospora marina* sp. nov., isolated from sea sand, *Int. J. Syst. Evol. Microbiol.*, 2010; **60**: 648-652.

[21] Duncan K., Haltli B., Gill K.A. and Kerr R.G., Bioprospecting from marine sediments of New Brunswick, Canada: Exploring the relationship between total bacterial diversity and actinobacteria diversity, *Mar. Drugs*, 2014; **12**: 899-925.

[22] Hayakawa M., Studies on the isolation and distribution of rare actinomycetes in soil, *Actinomycetologica*, 2008; **22(1)**: 12-19.

[23] Hayakawa M., Yoshida Y. and Iimura Y., Selective isolation of bioactive soil actinomycetes belonging to the *Streptomyces violaceusniger* phenotypic cluster, *J. Appl. Microbiol.*, 2004; **96**: 973-981.

[24] Starr M.P., Stolp H., Truper H.G., Balows A. and Schlegel H.G., *The Prokaryotes: A Handbook on Habitats, Isolation and Identification of Bacteria*, Springer-Verlag, New York, 1981.

[25] Gordon R.E. and Mihm J.M., Identification of *Nocardia caviae* (Erikson) nov. comb., *Ann. New York Acad. Sci.*, 1962; **98**: 628-636.

[26] Holt J.G., Krieg N.R., Sneath P.H.A., Staley J.T. and Williams S.T., *Bergey's Manual of Determinative Bacteriology*, 9th Edn., Williams&Wilkins, USA, 1994.

[27] Hasegawa T., Takizawa M. and Tanida A., A rapid analysis for chemical grouping of aerobic actinomycetes, *J. Gen. Appl. Microbiol.*, 1983; **29**: 319-322.

[28] Singka D., Kumdhitiahutsawakul L., Rekkriangkrai P. and Pathom-aree W., A simple method for DNA extraction from activated sludge, *Chiang Mai J. Sci.*, 2012; **39(1)**: 111-118.

[29] Kim O.S., Cho Y.J., Lee K., Yoon S.H., Kim M., Na H., Park S.C., Jeon Y.S., Lee J.H., Yi H., Won S. and Chun J., Introducing EzTaxon-e: A prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species, *Int. J. Syst. Evol. Microbiol.*, 2012; **62**: 716-721.

[30] Qiu D., Ruan J. and Huang Y., Selective isolation and rapid identification of members of the genus *Micromonospora*, *Appl. Environ. Microbiol.*, 2008; **74(17)**: 5593-5597.

[31] Pisano M.A., Sommer M.J. and Lopez M.M., Application of pretreatments for the isolation of bioactive actinomycetes from marine sediments, *Appl. Microbiol. Biotechnol.*, 1986; **25**: 285-288.

[32] Macnaughton S.J. and O'Donnell A.G., Tuberculostearic acid as a means of estimating the recovery (using dispersion and differential centrifugation) of actinomycetes from soil, *J. Microbiol. Methods*, 1994; **20**: 69-77.

[33] Hameş-Kocabaş E.E. and Uzel A., Isolation strategies of marine-derived actinomycetes from sponge and sediment samples, *J. Microbiol. Methods*, 2012; **88**: 342-347.

[34] Park S..H., Kwon K..K., Lee D.S. and Lee H.K., Morphological diversity of marine microorganisms on different isolation media, *J. Microbiol.*, 2002; **40(2)**: 161-165.

[35] Hamaki T., Suzuki M., Fudou R., Jojima Y., Kajiura T., Tabuchi A., Sen K. and Shibai H., Isolation of novel bacteria and actinomycetes using soil-extract agar medium, *J. Biosci. Bioeng.*, 2005; **99(5)**: 485-492.

[36] Gontang E.A., Fenical W. and Jensen P.R., Phylogenetic diversity of gram-positive bacteria cultured from marine sediments, *Appl. Environ. Microbiol.*, 2007; **73(10)**: 3272-3282.

[37] Connon S.A. and Giovannoni S.J., High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates, *Appl. Environ. Microbiol.*, 2002; **68(8)**: 3878-3885.

[38] Taylor C.B., Nature of the factor in soil-extract responsible for bacterial growth-stimulation, *Nature*, 1951; **168**: 115-116.

[39] Janssen P.H., Yates P.S., Grinton B.E., Grinton B.E., Taylor P.M. and Sait M., Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*, *Appl. Environ. Microbiol.*, 2002; **68(5)**: 2391-2396.

[40] 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.

[41] Bredholt H., Fjærvik E., Johnsen G. and Zotchev S.B., Actinomycetes from sediments in the Trondheim fjord, Norway: Diversity and biological activity, *Mar. Drugs*, 2008; **6(1)**: 12-24.