Testing the ability against *Bacillus cereus* of actinobacteria strains isolated from sponges in Kien Giang Sea, Vietnam

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***Abstract*—** *This study aimed to test the antibacterial activity of Bacillus cereus of actinobacterial isolates isolated from marine sponges in the Kien Giang Sea, Vietnam. That can select the strains with high resistance to identify them. There were 198 actinobacterial isolates tested. Based on the ability of antimicrobial activity to* B. cereus, *82/198 had the against B. cereus, in which there were six isolates with high (7.3%), 52 medium (25.6%), and 21 weak resistance (67.1%). Selection of six isolates with the best resistance to B. cereus (ND1.7a, ND2.7c, HD1-3e, HD1-6a, HD2.3b, and H6b) identified by PCR and 16S rRNA gene sequencing. The results identified five strains of* Streptomyces (*Streptomyces tateyamensis ND1.7a, Streptomyces althioticus HD1.3e, Streptomyces flaveolus HD1.6a, Streptomyces olivaceus HD2.3d,* and *Streptomyces albidoflavus H6b) and one strain of genus Microbacterium* (*Microbacterium tumbae ND2.7c).*

Keywords*— *Antimicrobial activity, Bacillus cereus, Kien Giang Sea, sponge, Streptomyces.

I. INTRODUCTION

According to the World Health Organization [1], more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. Microorganisms have the potential to cause diseases. The human body is very prone to viral, bacterial, and fungal infections. The discovery of antibiotics in the early twentieth century provided an increasingly important tool to combat bacterial diseases. However, due to the indiscriminate use of commercial antibacterial drugs treated for infectious diseases, resistance is becoming more common and severe [2]. Microbial natural products have been the source of most of the antibiotics in current use for the treatment of various infectious diseases. *Bacillus cereus*, family Bacillaceae, order Bacillales, class Bacilli, phylum Firmicutes, is a Gram-positive, rod-shaped, facultative, an anaerobic, motile, beta-hemolytic, spore-forming bacterium commonly found in soil and food. Some strains are harmful to humans and cause foodborne illness, while others can be beneficial as probiotics for animals [3].

Until recently, the majority of antimicrobial compounds were isolated from terrestrial microorganisms. The aquatic environment is now becoming increasingly appreciated as a rich and untapped reservoir of useful novel natural products. The marine environment alone is known to contain taxonomically diverse bacterial groups which exhibit unique physiological and structural characteristics that enable them to survive in extreme environmental conditions, with the potential production of novel secondary metabolites not observed in terrestrial microorganisms [4].

Marine bacteria are considered to play a central role as symbionts of most marine invertebrates and also represent one of the most novel biomedical resources remaining to be explored [5). Marine microorganisms have been an important study in recent years because of the production of novel metabolites which represent various biological properties such as antiviral, antitumor, or antimicrobial activities. These secondary metabolites serve as model systems in the discovery of new drugs [6]. The studies of the secondary substances produced by marine microorganisms have obtained many significant achievements in the world [7]. Among the secondary metabolites from marine microorganisms, many compounds are having interesting biological activities that should be useful for development for their pharmaceutical uses.

Therefore, in this study, the presence of potent antimicrobial metabolite-producing microorganisms with *Bacillus cereus* was reposted, a human pathogenic, especially microbes symbiosis in sponges at Kien Giang Sea, that is a resource not studied yet.

II. MATERIALS AND METHODS

2.1 Materials

The actinobacterial strains were isolated from sponge [8]. The *Bacillus cereus* (ATCC 11778) used for testing the agent of antibacterial isolates.

2.2 Screening assays for antibacterial activity

The liquid cultures were grown with shaking at 150 rpm for one day at 30°C. The broth was centrifuged at 5,000 rpm, 15 minutes. The supernatant was stored at 4°C. The bacterial test organism *(Bacillus cereus)* was plated in the LB medium. The antimicrobial extract was added to the wells, the plates were incubated at 4°C for 2h for the diffusion of antimicrobial extract and observed for the zones of inhibition at 28°C for 48h.

2.3 The agar well diffusion method

The active isolates were cultured by the method given in the previous step. The supernatants were used for testing extracellular antimicrobial activity by the agar well diffusion method. By using a sterile cork borer, wells were punctured in the appropriate agar medium previously seeded with one of the test organisms. One hundred microliters of the culture supernatants were added to each well. The plates were then incubated at 4^oC for at least 2 h to allow the diffusion of crude extracts followed by incubation for 24 h at 37^oC for bacteria and 48 h at 28^oC for yeast. The diameters of inhibition zones were monitored and measured [9]. Positive control was penicillin.

Screening of isolated microorganisms for inhibitory activity the isolates were screened for antibacterial metabolite production using the agar well diffusion method. The inoculate was prepared by growing the various test organisms on separate agar plates and colonies from the plate were transferred with inoculating loop into 3 mL of normal saline in a test tube. The density of these suspensions was adjusted to 0.5 McFarland standards.

Using a sterile cork borer wells (8 mm in diameter) were made in the agar and filled with 0.2 ml of 72 h culture of the isolated microorganism. Two replicates of the experiment were done, and the plates were incubated at 37°C for 18 h. The diameters of the zone of growth-inhibition produced were measured and the mean values calculated (Table 1).

2.4 Genomic DNA extraction

Bacterial cells from these cultures were collected by centrifugation, and genomic DNA was extracted [10].

2.5 16S rDNA gene amplification and sequencing

The PCR was performed in a final volume of 25 µl which was composed of about 50ng template DNA, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 ρM of Actinomycetes specific primers S-C-Act-0235-a-S-20 (5'-CGCGGCCTATCAGCTTGTTG-3') and S-C-Act-0878-a-A-19 (5'-CCGTACTCCCCAGGCGGGG-3') [11] and 1U of Taq polymerase with the appropriate reaction buffer under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 50s, annealing at 52°C for 50s, and 72°C for 90s. The amplified products were separated by gel electrophoresis in 1.2% agarose gels which were stained with Safeview dye.

2.6 Sequence analysis

The 16S rRNA gene sequences compared with those from the type strains available in NCBI (http://www.ncbi.nlm.nih.gov/) using the Basic Local Alignment Search Tool (BLAST) [12].

For phylogenetic analysis, multiple sequence alignment performed using CLUSTALX, version 1.81. The Phylogenetic tree constructed using Mega 7.0. The consistency of the trees was verified by bootstrapping (1000 replicates) for the UPGMA method.

2.7 Statistical analysis

The experimental results analyzed the ANOVA with the isolates and levels of diameters of inhibition zones. All analyses conducted using the statgraphics program. The data were considered significantly different at $P<0.01$. Duncan's test at $P = 0.01$ using to differentiate.

III. RESULTS AND DISCUSSION

3.1 Screening assays for antibacterial activity

There were 82/198 actinobacterial isolates with antimicrobial activity against *Bacillus cereus* (41.4%) (Table 1). Among 82 isolates, there were 6/82 (strong resistance), 55/82 isolates (medium resistance), and 21/82 (resistance); 116 isolates were

without resistance. Six isolates had the ability resistance to *Bacillus cereus* through a diameter of halo [sterile ring] (Table 1, Figure 1).

TABLE 1
ANTIMICROBIAL ACTIVITY OF 82 ACTINOBACTERIAL ISOLATES TO *BACILLUS CEREUS*

No	Bacterial isolates	Inhibition zone	Antibacterial Level*	No	Bacterial isolates	Inhibition zone	Antibacterial Level*
01	ND1.1a	15.0 f	++	42	HD1.6a	21.0 a	+++
02	ND1.1b	16.0 e	++	43	HD2.1a	14.0 h	++
03	ND1.3b	14.0 h	++	44	HD2.2b	7.0 q	++
04	ND1.4a	5.3 rs	+	45	HD2.3a	13.0 i	++
05	ND1.4c	5.7 rs	+	46	HD2.3b	20.0 b	+++
06	ND1.5a	17.0 d	++	47	HD2.3c	18.0 c	++
07	ND1.5b	9.0 m	++	48	HD2.3e	6.0 r	++
08	ND1.5c	15.0 f	++	49	HD2.4a	17.0 d	++
09	ND1.5d	13.0 i	++	50	HD2.5a	15.0 f	++
10	ND1.6b	10.0 l	++	51	HD2.5b	5.0 st	+
11	ND1.7a	21.0 a	+++	52	HD2.5c	8.0 op	++
12	ND1.7b	13.0 i	++	53	HD2.5d	15.0 f	++
13	ND1.7c	4.0 u	+	54	HD2.6b	4.0 u	+
14	ND2.6a	15.0 f	++	55	HD2.7c	7.0 q	++
15	ND2.6c	17.0 d	++	56	HD2.7d	16.0 e	++
16	ND2.7b	4.0 u	+	57	HD2.8a	6.0 r	++
17	ND2.7c	21.0 a	+++	58	HD2.8p	14.0 h	++
18	ND2.8a	14.0 h	++	59	HD2.9a	7.0 q	++
19	ND2.8c	3.0 v	+	60	HD2.9c	8.0 op	++
20	RL1c	4.0 u	+	61	H6a	18.0 c	++
21	RL2b	14.0 h	++	62	H6b	20.0 b	+++
22	RL3a	3.0 v	+	63	H10a	10.0 l	++
23	RL3d	5.3 rs	+	64	N1a	10.0 l	++
24	RN1a	5.7 rs	+	65	N2a	7.0 q	++
25	RN1c	10.0 l	++	66	N6a	14.3 gh	++
26	RN1d	4.0 u	+	67	N7a	5.3 rs	+
27	RN1f	5.0 st	+	68	N7b	9.3 lm	++
28	RN3a	5.7 rs	+	69	N8b	11.0 l	++
29	RN3c	4.0 u	+	70	N8c	9.0 m	++
30	RN4c	6.0 r	++	71	N8d	14.0 h	++
31	RN5a	14.0 h	++	72	N8e	8.0 op	++
32	RN5c	3.0 v	+	73	N9a	8.7 mo	++
33	RN6a	7.0 q	++	74	N9c	10.0 l	++
34	RN6b	4.3 tu	+	75	N9d	11.0 k	++
35	HD1.2a	4.0 u	+	76	N9e	3.0 v	+
36	HD1.2c	9.0 m	++	77	N9f	6.0 r	++
37	HD1.3d	15.0 f	++	78	N9g	7.3 pq	++
38	HD1.3e	20.0 b	+++	79	N9h	14.0 h	++
39	HD1.4b	6.0 r	++	80	N10b	7.0 q	++
40	HD1.4d	4.0 u	+	81	N10d	12.0 j	++
41	HD1.5c	17.0 d	++	82	N11b	6.0 r	++
	CV (%) = 4.22%			Positive control		8.0 op	

In Means within a column followed by the same letter/s are not significantly different at p < 0.01
Inhibition zone: diameter [D = d_1 - d_2] (mm)

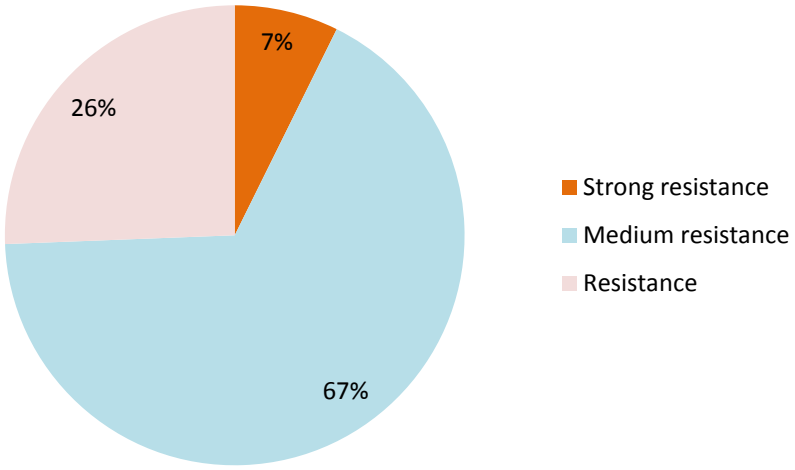

FIGURE 1: Ratio of number of actinobacterial isolates against *Bacillus cereus*

Six best isolates as ND1.7a, ND2.7c, HD1-3e, HD1-6a, HD2.3b and H6b with diameter of sterile ring (20-21 mm) were chosen to identify by PCR technique and sequencing.

3.2 Identify actinobacterial isolates

The result from Table 2 showed that 5/6 strains belonged to *Streptomyces*, and one strain was *Microbacterium*.

TABLE 2
PHYLOGENETIC AFFILIATION OF 6 ACTINOBACTERIAL ISOLATES ON THE BASIS OF 16S RDNA GENE SEQUENCES BY USING BLAST PROGRAMME IN THE GENBANK DATABASE BASED ON SEQUENCE SIMILARITY.

No	Actinobacterial isolates	Closest species relative	Similarity (%)
	Actinomycetaceae		
1	ND1.7a	*Streptomyces tateyamensis* strain 18I (MG009024.1)	100
		Streptomyces chumphonensis strain HQA999 (MH041238.1)	100
2	HD1.3e	*Streptomyces althioticus* P54-7 (LC551871.1)	100
		Streptomyces griseoincarnatus P49-18 (LC551868.1)	100
3	HD1.6a	*Streptomyces flaveolus* strain ADIP1 (KF732809.2)	100
		Streptomyces ambofaciens strain M (MK929483.1)	100
4	HD2.3b	*Streptomyces olivaceus* strain LEP7 (MW767828.1)	100
		Streptomyces coelicoflavus strain ROA061 (MW757213.1)	100
5	H6b	Streptomyces albidoflavus strain HQA017 (KT758349.1)	100
		Streptomyces saprophyticus strain DE2 (MW797316.1)	100
	Microbacteriaceae		
6	ND2.7c	*Microbacterium tumbae* strain C3 (MG958700)	100
		Microbacterium kyungheense strain MK (MF373498)	100

The results from Table 2 showed that 5/6 belonged to *Streptomyces*, and 1/6 was *Microbacterium.* The phylogenetic tree of 6 strains showed that 2 clusters: cluster A: 5 strains were genus *Streptomyces* and cluster B: *Microbacterium tumbae* ND2.7c.

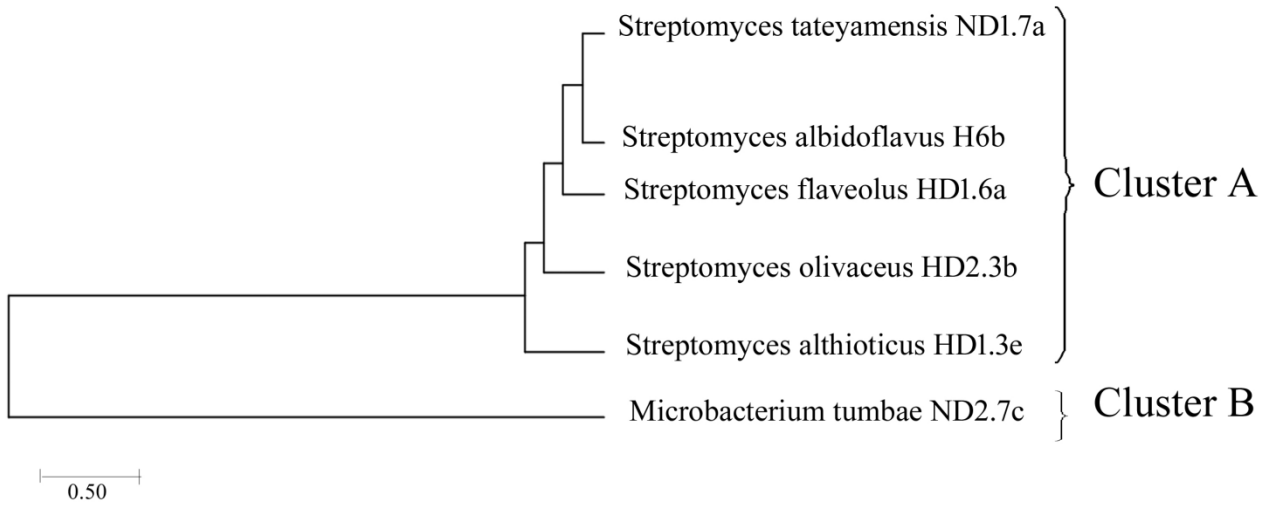

FIGURE 2: The Neighbor-joining phylogenetic tree of partial 16S rRNA gene sequences of actinobacteria isolated from sponges of the Kien Giang and closely related type strains. Numbers in the figure refers to percentage bootstrap values which were calculated for 1000 replicates. Bar, 0.02 was per nucleotide position

The UPGMA phylogenetic tree (Figure 2) of these isolates described in the two clusters, Cluster A had five strains of Actinomycetaceae genus, in which *Streptomyces althioticus* HD1.3e strain had a high relationship *Streptomyces tateyamensis* ND1.7a, *Streptomyces flaveolus strain* HD1.6a, *Streptomyces olivaceus* HD2.3b, and *Streptomyces albidoflavus* H6b, but cluster B only had one *Microbacterium tumbae* ND2.7c strain of Microbacteriaceae genus.

World Health Organization (WHO) global surveillance report pointed to an increase in morbidity and mortality of infectious diseases due to AMR, which could result in a worldwide economic loss of up to 100 trillion US dollars (USD) in 2050 due to a 2%–3% reduction in the gross domestic product [1]. It is estimated that AMR now annually contributes to 700,000 deaths worldwide, with a potential increase to 10 million in 2050.

Actinobacteria are prolific antibiotic producers, which produce about 45% of the antibiotics currently in use. They produce diverse natural products that account for approximately 10,000 compounds [13-15]. The production of antibiotics by microbes is considered to be one of the most powerful biocontrol attributes of beneficial microbiomes against phytopathogens and has become increasingly better understood over the past three decades [16]. A variety of antibiotics have been identified, including compounds such as amphisin, 2,4-diacetylphloroglucinol, phenazine, pyoluteorin, pyrrolnitrin, tensin, and cyclic lipopeptides produced by *Arthobacter* and *Streptomyces* [17].

Poosarla et al., [18] have identified actinomycetes from marine sediments of the Andaman Islands with strong inhibitory activity against bacteria *Streptococcus, Staphylococcus aureus, Bacillus subtilis, Escherichia coli*, and *Proteus vulgaris*, and fungi *Aspergillus niger, Candida albicans, Penicillium, Mucor,* and *Rhizopus.*

According to Berdy [19] over 5.5% of the antibiotics detected between 1945 and 1978 originated from the genus *Streptomyces*, representing a total of more than 5,000 compounds. Other bioactive compounds were obtained from endophytic Actinobacteria, as anguciclines with antimicrobial activity against *Bacillus cereus* and *Listeria monocytogeneses* [20-21] analysis of antibacterial compound derived from marine actinobacteria isolated from the sediments of salterns of Ongole, Andhra Pradesh, India selected the antibacterial activity of isolate SJP4 showed inhibitory activity against all the test pathogens viz., *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacillus cereus* and the structure of the compounds extracted from SJP4 was identified as 8-diaza-2,9-dibenzoyl-5,6-diphenyl-2,8-decadienedioicacid diethyl ester and (1,2,4)triazol-1-ylethanone through GCMS analysis and The potential actinobacteria isolate was identified as *Nocardiopsis dassonvillei* SJPB4 strain (Accession no. MG434671) using 16s rRNA sequencing.

It is observed that *Streptomyces* has been greatly exploited for the production of antibiotics, fungicides, bactericides, herbicides, and insecticides. The members of actinobacteria can be applied to the biofortification of minerals for different cereal crops, and additionally, most dominant actinobacteria can be used as probiotics—as functional foods—for human health.

IV. CONCLUSION

In conclusion, the culturable diversity of sponge-associated actinobacteria from the Kien Giang Sea was established. Streptomyces isolates were found as the predominant strains showing antibacterial activity. Besides, Microbacterium tumbae performed as a rare actinomycete which displayed antifungal activity. It is indicated that marine sponges are a potent source of endophytic actinomycetes with wide biological activity against pathogenic fungi as well as Gram-positive bacteria, Bacillus cereus. This makes it a promising application of such newly functional sponge-associated actinobacteria as a novel source of bioactives.

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