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Screening of actinomycetes from Ninh Thuan and Binh Thuan sea for antimicrobial producers

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ABSTRACT

The sea is a unique ecosystem for finding secondary metabolites from marine microorganisms. Many compounds produced by marine microorganisms with antibacterial activity have been applied in life. Furthermore, microorganisms are renewable raw materials and media for drug production, thus crucial for sustainable development in our life. In this study, we screened for antibacterial activity of microbial strains isolated from tern samples from Ninh Thuan and Binh Thuan seas. The antibacterial activity of the marine microbial crude extracts was performed by the Bioassay method in a 96-well tray. The minimum inhibitory concentration (MIC) test results showed that from 86 strains of microorganisms isolated from the waters of Ninh Thuan and Binh Thuan, 68 strains were able to inhibit the growth of 1 to 6 strains of the tested microorganisms, and 27 over 86 strains had antibacterial activity against at least three strains of microorganisms tested. In which strains G631, G756, G769, and G778 inhibited 4 to 6 tested microorganisms with MIC values equal to or lower than positive controls. Morphological characteristics and molecular biological analysis identified the four most active strains. The results showed that all four strains were actinomycetes.

Keywords: Actinomycetes, antimicrobial activity, bioassay, MIC, marine microbial, 16S rRNA.

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INTRODUCTION

A severe global health problem caused by pathogenic microorganisms is threatening human lives. Due to abusive uses of antibiotics and antimicrobial prescriptions, the effectiveness of drugs and vaccines has been sharply decreased. Antibiotic resistance leads to more extended hospital stays and increased mortality. Bioactive compounds obtained from marine actinomycetes are being explored for their potential for biotechnological, including anti-inflammatory, antivirus, antifungal, antiallergic, antibacterial, antitumor, and cytotoxic activities [1]. These compounds are produced in response to their environment, and many have shown value in biotechnological or pharmaceutical applications [1, 2]. Marine microbial isolated from extreme environments are considered a significant for searching and discovering new drugs. Because of living in the water environment with low nutrition, high salinity and high pressure, microbes can adapt and respond to their environment through natural selection and compete for defense and survival by changing their metabolism.

Marine microbial as source of structurally diverse antimicrobial products, some compounds had inhibitory activity against Gram-positive bacteria while others have higher effectiveness on Gram-negative bacteria. Some are also efficacious against both Gram-positive and Gram-negative bacteria and drug-resistant strains [2, 3]. *Actinomycetes* account for 10% of the total marine microorganisms and represent attractive source for isolation of novel microorganisms and production of potent bioactive secondary metabolites. Secondary metabolites from marine Actinomycetes have the ability to inhibit the growth or kill harmful bacteria and other microorganisms at low concentrations. Thus, these compounds can be essential in pharmaceutical research [3, 4].

MATERIALS AND METHODS

Sample collection and processing

The marine samples were collected by SCUBA at Ninh Thuan and Binh Thuan provinces in the Southeast of Vietnam. The sampling sizes and locations of some precious results were described in detail in the result part. The specimens were collected in cleaned, sanitized, and autoclaved bottles with 30% glycerol on an ice box during transported to the laboratory of Institute of Marine Biochemistry for further analysis. In the lab, the samples were stored at 4 °C until used (not longer than 3 weeks).

Microbial isolation

The culture media used for the isolation and cultivation of marine microorganisms were A1, M1, ISP1, ISP2, PDA, NZSG. The pH of the culture medium was adjusted to 7.0. Samples (0.5 g weight or smear pattern) was crushed and diluted ten times in sterile water, homogenized by vortexing for 5 minutes. Aliquots of 50 µL was spread on isolation media. The petri plates were incubated at 30 °C for 2 weeks. Isolated strains were observed according to spore mass, substrate mycelial and diffusible pigments, and growth. Colonies showed powder consistency and stick firmly to agar surface from the isolation plates were purified through several rounds of transfer to suitable culture media [3, 5].

Fermentation, production of crude extracts and antibacterial bioassay

The strains were cultivated in 1,000 mL flasks containing 500 mL A1 medium (soluble starch: 10 g/L; yeast extract: 4 g/L; peptone: 2 g/L; instant ocean: 30 g/L; pH 7.0) for 10 days at 30 °C and 150 rpm. The fermentation broths were filtered and extracted with ethyl acetate (5 times). This ethyl acetate extract was purified by column chromatography on silica gel, eluted with n-hexane/acetone gradient, and washed with CH_2Cl_2 /MeOH to give fractions. The extracts were evaporated under reduced pressure to yield crude extracts. Those extracts were used for antibacterial tests [6].

The antibacterial bioassay was carried out in flat-bottom 96-well microliter plates. For measurements of OD, transparent microliter

plates were used. Microorganisms used for the antibacterial test were three Gram-negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076), and three Gram-positive bacteria (*Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC 14579), one fungus strain *Candida albicans* ATCC10231. This dilution yielded a starting inoculum of approximately 2×10^5 CFU/mL.

The stock extracts were diluted in DMSO (Dimethyl sulfoxide) at a decreasing concentration range: 256 µg/mL, 128 µg/mL, 64 µg/mL, 32 µg/mL, 16 µg/mL, 8 µg/mL, 4 µg/mL, and 2 µg/mL with the number of experiments. Streptomycin (Sigma) and cycloheximide (Merck) were used as a positive control for bacteria and fungi, respectively.

Antimicrobial activities are often reported as MIC (Minimum Inhibition Concentration) values, which typically denote the lowest concentration of test compound, which inhibits growth (97–100%), reproducible and yielding values expressed in µg/mL. MIC denotes the lowest test compound concentration, totally inhibiting growth. MIC values were determined accurately based on turbidity measurements by BioTeck spectrophotometer and Raw Data Software. The percentage of inhibition compared to the control was determined from the calculation:

$$\left[\left(O_{sample}-OD_{blank}\right)/\left(OD_{control}-OD_{blank}\right)\right]\times100$$

Where the sample was the serially diluted crude extracts incubated for 24 hours, the stock solution of pure organic solvent was used as a control, and the blanks were media that were the same as media used for cultivating the microorganisms [7].

Taxonomic characterization of pure isolated strains

The description of a strain is based on applying of the taxonomic approach based on a combination of genotypic and phenotypic data.

Pure isolated Actinomycetes usually develop with different morphological features after being incubated for 2–3 weeks. Morphological characterization of pure isolated strains was observed according to spore-producing, diffusible pigments, melanin pigment formation, and hyphae and conidia in culture media [5]. 16S rRNA sequencing was used to identify the candidate strains commonly employed in the study of actinobacteria. 16S rRNA reference was considered as full-length if it covered the position of the primers 27F (5′AGAGTTTGA TCCTGGCTCAG 3′) and 1492R (5′ACGGCTACCTTGTTACGACTT 3′).

The conditions for thermal cycling were as follows: Denaturation of the target DNA at 94 °C for 5 minutes followed by 30 cycles at 94 °C for 45 seconds, primer annealing at 42 °C for 1 minute, and primer elongation at 72 °C for 40 seconds. At the end of the cycling, the reaction mixture was held at 72 °C for 10 minutes [8].

The 16S rRNA gene *sequencing* products were analyzed by capillary electrophoresis *on* DNA Analyzer (ABI PRISM 3100, Applied Bioscience). Gene sequences were analyzed by BioEdit version 7.2. and compared with bacterial 16S rRNA sequences in GenBank database by NCBI Blast online program. The analysis was conducted with MEGA 10 using the neighbor-joining method. Evolutionary analysis was performed using the Maximum Likelihood method, whereas the evolutionary history was inferred using the Maximum Likelihood method and JTT matrix-based model [9, 10].

RESULTS

Sample collection and processing

Marine samples were collected with SCUBA diving at a depth of 12 m under sea level, and the water temperature ranged from 26 - 30°C in different geographic coordinates in Ninh Thuan and Binh Thuan provinces in Vietnam.

Microbial isolation

The isolated strains were observed for diffusible pigments, spore mass, substrate mycelial, and growth. Colonies showed powder consistency and stuck firmly to agar surface from the isolation plates were purified through several rounds of transfer to suitable culture media. A total of 86 strains were isolated from the marine samples. The numbers of samples and isolates in each sample are presented in Table 1.

Table 1. Number of isolated strains from Ninh Thuan and Binh Thuan Sea

Samples	Number of isolates
Seaweed	31
Molluscs	7
Sediment	7
Echinoderm	4
Soft coral	17
Sponge	20
Total	86

Antibacterial bioassay

The antibacterial activities of the obtained raw extracts were evaluated at some concentrations using the bioassay method against test microorganisms. Experiments were performed in triplicate and *repeated three times* with similar results. The screening results showed that out of 86 isolates, 68/86 isolates inhibited 1 to 6 strains of tested microorganisms, and 27/86 strains showed antibacterial activities against at least 3 strains of tested microorganisms. In which strains G631, G756, G769, and G778 inhibited 4 to 6 tested microorganisms with MIC values equal to or lower than the positive control (Table 2). Concentration used 1.0 mg/1.0 mL of DMSO, size of well 6 mm (diameter). The results are presented in Table 2.

Among all the marine microbial crude extracts tested, G631, G756, G769, and G778 showed good to excellent activity with values MICs from 2 µg/mL to 256 µg/mL. From Table 2, it was observed that: The most significant antibacterial activity against 4/6 and 5/6 strains of reference microorganisms was obtained from aqueous extract of strains G631 and G756.

Specially, all of the four strains had resistant to *S. aureus.* ATCC25923 with low MIC values. The evidence *suggests* that: Secondary compounds from the culture of these strains may be potential targets for anti-*S. aureus* drug discovery. *S. aureus* can cause a range of illnesses, from minor skin infections. It is still one of the five most common causes of hospital-acquired infections and is often the cause of wound infections following surgery. Up to 50,000 deaths yearly in the United States are linked to *S. aureus* infections [11]. Researchers observed that the test fungi were inhibited by all of four strains, G631, G756, G769, and G778 extracts, in very low MIC values for antifungal activity.

Out of the 86 isolates, the 4 strains G631, G756, G769, and G778 were selected for further characterization and analysis since they showed good control efficacy against tested organisms.

Table 2. Minimum inhibitory concentration of the most significant crude extracts of selected marine microbial

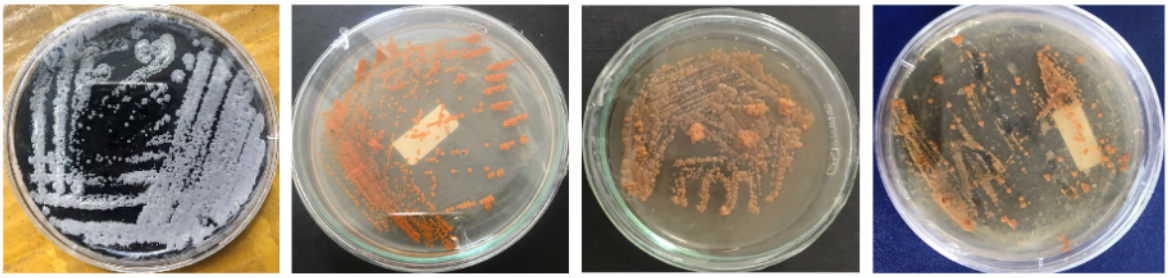
Samples	MIC (µg/mL)						
	Gram (+)			Gram (-)			Fungi
	E. faecalis	*S. aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. enterica*	*C. albicans*
G631 extract	64	2	16	-	-	256	2
G756 extract	4	4	8	256	-	128	2
G769 extract	16	16	64	-	-	-	16
G778 extract	8	16	32	256	-	128	4
Streptomycin	*256*	*256*	*128*	*32*	*256*	*128*	-
Cycloheximide	-	-	-	-	-	-	*32*

Notes: Positive control: Streptomycin, Cycloheximide; (-): inactive.

Taxonomic characterization of pure isolated strains

The four mentioned strains grew well in the A1 medium, and the diameter of colonies reached from 0.5–1.5 mm after 14 incubation days at 30 °C. Mature colonies were grey-colored mycelial (G631), deep orange (G756), yellow to orange (G769), and pink to orange (G778).

The results of Gram stain showed that: all of the 4 selected strains belonged to Gram-positive bacteria with thick cell walls. These isolates were subjected to identification by 16S rRNA gene sequences. The 16S rRNA genes were amplified by PCR, using specific primers primer 27F and 1492R. 16S rRNA studies help determine the phylogenetic relationship and make possible the recognition up to species level [9].



A B C D

Figure 1. Colony morphological characteristics of the strains G631 (A), G756 (B), G769 (C) and G778 (D) grown on A1 medium for 2 weeks at 30 °C

Table 3. Description of the isolated strains in this study

Strains	Origin of isolated/Coordinates	Media of isolated	Description
G631	Soft coral 11°28'70.52" 108°86'24.02"	ISP1 (Casitone: 5 g/L; Yeast extract: 3 g/L; Instant ocean: 30 g/L; Agar: 16 g/L)	Colony color: White. The vegetative mycelia are brown to grey and the aerial mycelia are blue to grey. Dry colonies on agar. Coccus-shaped. Colony diameter: 2 mm. Diffusible pigment: Black.
G756	Seaweed 10°54'13.61" 108°91'62.97"	SWA (Instant ocean: 30 g/L; Agar: 16 g/L)	Colony color: Deep orange Dry, rough colonies on agar. Coccus-shaped. Colony diameter: 0.5 mm.
G769	Seaweed 11°26'65.64" 108°86'27.07"	ISP1 (Casitone: 5 g/L; Yeast extract: 3 g/l; Instant ocean: 30 g/L; Agar: 16 g/L)	Colony color: Yellow to orange Dry, rough colonies on agar. Coccus-shaped. Colony diameter: 1.5 mm.
G778	Sponge 10°54'13.61" 108°91'62.97"	M1 (Soluble starch: 1 g/L; Peptone: 0.2 g/L; Yeast extract: 0.4 g/L; Instant ocean: 30 g/L; Agar: 16 g/L)	Colony color: Pink to orange. Dry, rough colonies on agar. Coccus-shaped. Colony diameter: 1.5 mm.

Agarose gel *electrophoresis* of PCR products of DNA of the four strains showed about 1,500 bp nucleotide fragment, corresponding theoretical size 16S rDNA gene of specific primers.

Sequence alignment was done for all retrieved sequences using BioEdit software version 7.2. Based on sequencing analysis of 16S rRNA genes, the 16S rRNA gene sequence of four potential strains was compared with the

available sequences in GenBank (NCBI, USA), indicating that: G631 was closely related to the genus *Actinoalloteichus*. This strain showed 16S rRNA gene sequence similarity values of 99.71 % with respect to *Actinoalloteichus cyanogriseus* IFO 14455, NR 024650.1. The phylogenetic analysis showed that the strain G756 had the highest similarity with Salinispora *arenicola* ATCC BAA-917 strain CNH-643, NR_042725.1 (99.65 %).

The isolated G769 was closely related to the genus *Micromonospora*. The phylogenetic analysis showed that the strain G769 had the highest similarity with *Micromonospora tulbaghiae* strain TVU1, NR 116241.1 (99.93%). An analysis of the 16S rDNA sequence of strain G778 showed a similarity level ranging between 98.85% and 97.77% within *Blastococcus* species, with *Blastococcus aggregatus* strain DSM 4725 NR_114864.1 the most closely related.

The evolutionary history was inferred using the Neighbor-Joining method [30]. The optimal tree is shown on Fig. 3. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Nei-Gojobori method and are in the units of the number of synonymous substitutions per synonymous site. This analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 479 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [12].

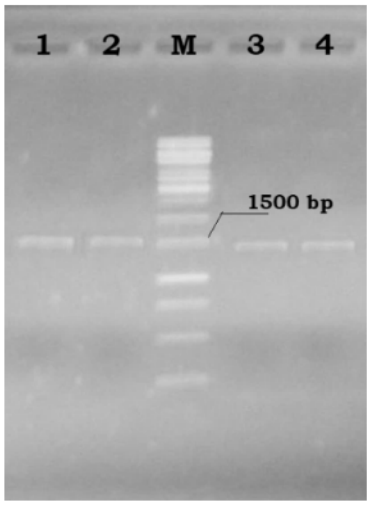


Figure 2. 1% (w/v) agarose gel showing the PCR products amplified from 16S rRNA gene of strain G631 (Lane 1), strain G756 (Lane 2), strain G769 (Lane 3), and strain G778 (Lane 4). Lane M, 1 kb plus molecular marker (Goldbio)

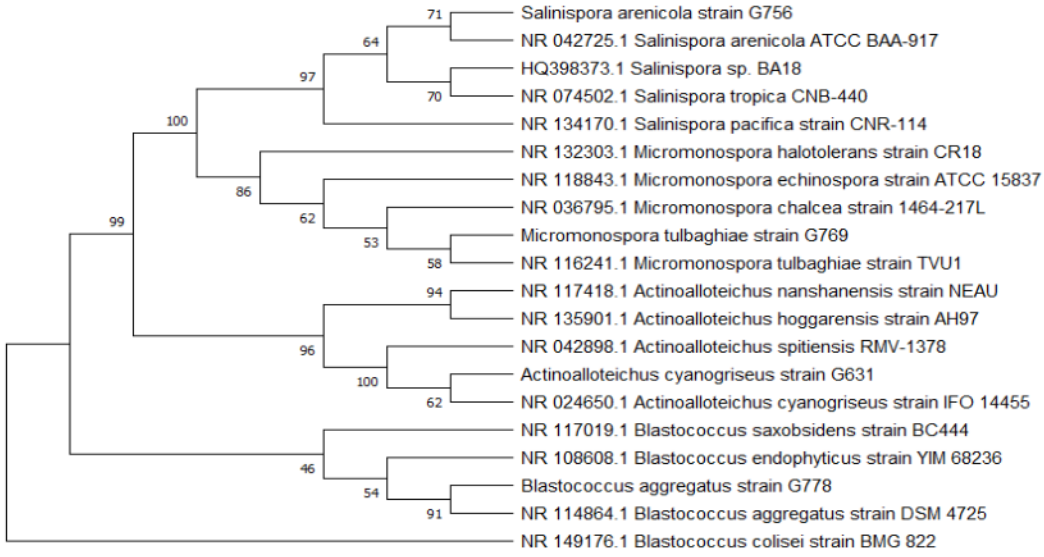


Figure 3. Phylogenetic analysis of 16S rRNA sequences of the actinomycetes isolate G631, G756, G769 and G778 with the sequences from NCBI. The analysis was conducted with MEGA X using Neighbor-Joining method

DISCUSSIONS

The *concentration* of *salt* in seawater is about 35 parts per thousand. Marine environmental components are very different from terrestrial ones. Thus, marine is a unique and extreme ecosystem for hunting marine microbial resources and secondary metabolites. In recent years, searching for new drugs has increased to control the spread of antibiotic resistance among the *microorganisms* that cause *infectious* diseases. *Studying marine microbial has been significant because they generate a wide* variety of *bioactive* secondary metabolites. Investigating marine microbial has been significantly increased due to their developed variety of promised bioactive metabolites.

Extreme marine environments, characterized by high or low temperatures, high-pressure, pH < 4 or > 9, and high salt concentrations, would result in that these microorganisms produce an incredible amount *of potential natural products* applicable to human life [3]. Some isolates from marine habitats in Mexico were observed against the food-borne poisoning strains S*taphylococcus aureus* and *Vibrio parahaemolyticus* [13]. By fermentation of the marine strains *Streptomyces* sp. with *Bacillus* sp. collected from the shore of a muddy wetland, Dentigerumycin E, a novel *compound, was* discovered; this compound showed antiproliferative and antimetastatic activities against human carcinoma [14].

Micromonospora was discovered almost 100 years ago, this genus are best known for synthesize of antibiotics and has demonstrated very potent antibacterial effects against pathogenic bacteria, including *Mycobacterium tuberculosis* [4, 15]. A strain *Micromonospora* sp. was isolated from marine sediments collected in the Canary Islands. The acetone extract from a culture of this strain showed antagonistic activity against methicillin-resistant *Staphylococcus aureus* (MRSA), *Mycobacterium tuberculosis* H37Ra, and *Mycobacterium bovis* [16]. Marine *Micromonospora* isolates were found to produce bioactive compounds; these compounds served as cytotoxic, antibacterial, antiparasitic, chemopreventive, or antioxidant activities [17].

Blastococcus is a Gram-positive, coccoid, and aerobic genus of bacteria; this genus belongs to the family of *Geodermatophilaceae* [18]. The antibacterial activities of the genus *Blastococcus* have not been widely reported. However, this genus has been found and described in several papers. For example, Blastococcus genera were reported and described from the Atacama Desert in Chile [19]. *Blastococcus deserti* sp. nov., isolated from a desert sample [20]. *Blastococcus* spp. has been isolated from marine sediment and stone interiors; those strains are resistant to some heavy metals and metalloids [21].

Salinispora spp. were frequently reported from marine samples. *Salinispora* strains produce novel natural products with biological activity. Seventy-one isolates as members of the genus *Salinispora* were isolated from marine sediment samples. These strains displayed activity against *Staphylococcus epidermidis, Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacer baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. [22, 23].

Actinoalloteichus strains have been isolated from different habitats: soil, Saharan soil, rhizosphere of a fig tree, soil in the cold desert of the Indian Himalayas, and marine samples [24]. Several secondary metabolites have been isolated from *Actinoalloteichus* spp., including the cytotoxic macrolactam BE-14106 from soil-derived *A. cyanogriseus* [25], cytotoxic cyclopentenones from *A. nanshanensis* sp. nov. NEAU 119 [26], antifungal neomaclafungins from marine *Actinoalloteichus* sp. NPS702 [27], and cytotoxic bipyridine and cyanogramide alkaloids from marine-derived *A. cyanogriseus* WH1-2216-6 [28, 29]. A new deep-sea-derived *Actinoalloteichus cyanogriseus* 12A22 was isolated from the sediment collected in the South China Sea. That strain showed potential cytotoxic and antimicrobial activities. The actinomycete 12A22 exhibited significant inhibitory activities against fungi (*Fusarium oxysporum* f. sp. *cucumerinum*, *Setosphaeria turcica*, and

Botrytis cinerea) and Gram-positive bacterium *Bacillus subtilis* [30].

CONCLUSIONS

A total of 86 isolates were isolated from the marine samples from Ninh Thuan and Binh Thuan Sea. The four strains, G631, G756, G769, and G778, had significantly antagonistic activities against various tested microorganisms with MICs values from 2 µg/mL to 256 µg/mL. Based on sequencing and phylogenetic analysis of the 16S rRNA gene, the Strain G631 was identified as *Actinoalloteichus cyanogriseus*, strain G756 belonged to *Salinispora arenicola*, and strain G769 was identified as a member of *Micromonospora tulbaghiae*, and strain G778 closely related to the genus *Blastococcus*. The results of this study indicated that isolated strains could be a promising marine microbial to the exploited potential of bioactive metabolites.

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