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ISOLATION AND SCREENING ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES FROM MARINE ORGANISMS SAMPLES OF THE AREA KHANH HOA, VIETNAM

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SUMMARY

The marine environmental conditions are particularly different from terrestrial ones; recent studies have showed that marine actinomycetes might produce many novel compounds with good biological activity. The objective of this study is to isolate and screen actinomycetes strains from the marine environment with activity against pathogenic microorganisms. Fifty strains of actinomycetes were isolated from 40 samples including: sediments, sponges, soft corals, echinoderms... collected from Van Phong Bay area of Khanh Hoa province, Vietnam. The strains fermented in A^+ medium and fermentation broths were extracted 5 times with ethyl acetate. The extracts were evaporated under reduced pressure to yield crude extracts. Quantitative assay was used to determine MIC (minimum inhibitory concentration) of extract against 7 test strains, including three Gram-negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076), three Gram-positive bacteria (*Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC 14579), and the yeast *Candida albicans* ATCC10231. The result of screening showed that: 18/50 strains have shown the antibacterial activities against at least 3 strains of tested microorganisms. In which, strains G650, G654, G657, G666, G675 inhibited three Gram-positive test microorganisms at MIC values from 8 to 64 µg/mL and *C. albicans* ATCC10231 with MIC values from 2 to 16 µg/mL. In addition, the two strains G657 and G666 had inhibitory effect on *P. aeruginosa* ATCC27853 và *S. enterica* ATCC13076 with MIC value 128 µg/mL. Morphological and phylogenetic investigations based on 16S rRNA gene sequences of the selected five strains showed that: strain G650 belonged to species *Streptomyces ardesiacus* and strain G666 high similarity to the genus *Streptomyces*; strain G654 showed the highest similarity to the *Salinispora arenicola* species; strain G657 highest similarity to the *Micromonospora aurantiaca*; whereas G675 belonged to the *Nocardiopsis flavescens* species.

Keywords: actinomycetes, antimicrobial activity, marine samples, MIC, 16S rRNA gene sequences

INTRODUCTION

Actinomycetes are a diverse group of Gram positive with high G+C content, free living, saprophytic, filamentous bacteria and are a major source for the production of antibiotics (Valli *et al.*, 2012). Since Waksman and Schatz discovered the broad-spectrum antibiotic Streptomycin, actinomycetes have received more attention to isolate more antibiotics. Actinomycetes have been exploited successfully for their biological potential secondary metabolites. They produce

diverse classes of antimicrobial metabolites, especially glycopeptides, beta-lactams, aminoglycosides, polyenes, polyketides, macrolides, actinomycins and tetracyclines.

Among the actinomycetes, *Streptomyces* accounted for more than 80% of the total antibiotic products, followed by *Actinopolyspora* and *Micromonospora* which are rare actinomycetes with less than one-tenth of *Streptomyces* population. It was estimated that about 42% of commercial metabolites are known to be produced by various actinomycetes, 16% by fungal strains and the remaining from bacterial sources. The discovery of antibiotics had a significant impact on the control of various infectious diseases and the development of the pharma industry (Alkubaisi *et al.,* 2019).

The ocean which covers about 70% of the Earth's surface is a particularly extreme living environment because of its poor nutrient content and high salinity. As marine microorganisms have been able to adapt to these extreme environmental conditions, they might have opened prospects of discovering new bioactive compounds including anti-tumor, antibacterial, antiviral, antifungal, anti-inflammatory, anti-cancer activity, and enzyme inhibitory (Prakash *et al.,* 2005). In which, marine actinomycetes are a potential source of novel compounds because marine environmental conditions are completely different from the terrestrial conditions. Many researchers have isolated new antibiotics from the marine environment. In recent years, there have been a number of publications on secondary compounds derived from marine actinomycetes and marine fungi of Vietnam (Trinh *et al.,* 2017; Quyen *et al.,* 2016; Tuan *et al.,* 2019)... This paper publishes the results on isolation and antimicrobial activity of the actinomycetes strains isolated from marine samples collected at Van Phong, Khanh Hoa of Vietnam.

MATERIALS AND METHODS

Chemicals

Genomic DNA isolation kit was purchased from Promega (Madison, WI, USA). PCR master mix was purchased from Bioneer. All other chemicals (for media) were obtained from Himedia (India), Duc Giang (Vietnam) and Sigma-Aldrich (St. Louis, MO, USA).

Test microorganisms

Microorganisms used for antibacterial test were from ATCC including three Gram negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Salmonella enterica* ATCC13076), three Gram positive bacteria (*Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC 14579), one yeast strain *Candida albicans* ATCC10231.

Sample collection

Marine sediment samples were collected from three different locations at depth of 5 - 19 m (Table 1), seawater temperature at the sampling sites was 28 - 32^{o}C. The samples were put into 15 mL or 50 mL sterile Falcon tubes, preserved in an ice-box and processed within 24 h.

Isolation of actinomycetes

An amount of 0.5 g of sample was suspended in 4.5 mL of sterile distilled water, homogenized by vortexing for 1 min, and the suspension was treated at 60^{o}C for 6 min. Next, 0.5 mL of the heat-treated suspension was used for serial dilution in sterile distilled water to 10^{-3}. At the final dilution step, aliquots of 50 µL were spread on seven different solid media, including: **A1** (g/L): 10g soluble starch, 4 g yeast extract, 2 g peptone, 30 g instant ocean, 15 g agar), **M1** (g/L): 1g soluble starch, 0,4 g yeast extract, 0,2 g peptone, 30 g instant ocean, 15 g agar; **SWA** (g/L): 30 g instant ocean, 15 g agar; **SCA** (g/L): 30 g instant ocean, 2 mg $CaCO_3$, 10 mg $FeSO_4.7H_2O$, 50 mg $MgSO_4.7H_2O$, 300 mg casitone, 2 g K_2HPO_4, 2g KNO_3, 15 g agar; **ISP1** (g/L): 2g yeast extract, 5 g casitone, 30 g instant ocean, 15 g agar; **ISP2** (g/L): 5 g soluble starch, 2 g yeast extract, 10 g glucose, 10 g malt extract, 30 g instant ocean, 15 g agar; **NZSG**

(g/L): 20 g soluble starch, 5 g yeast extract, 10 g glucose 5 g NZ amine A, 30 g instant ocean, 15 g agar). Plates were incubated at 28°C for 7 - 30 days. Single colonies of actinomycetes were transferred onto new petri dishes of A1 medium for further purification steps.

Table 1. Detail of the samples collected from different locations at Van Phong Bay area of Khanh Hoa province.

SNo	geographic coordinates	No of samples	Water depth (m)	Collection time
1	12º40'45''-109º15'05''	1	8,5	22. 05. 2020
2	12º38'39''-109º22'38''	4	8,5	22. 05. 2020
3	12º38'43''-109º20'23''	5	12	22. 05. 2020
4	12º40'34''-109º15'42''	3	5	23. 05. 2020
5	12º41'36''-109º16'51''	5	5	23. 05. 2020
6	12º40'44''-109º20'33''	1	5	23. 05. 2020
7	12º37'25''-109º24'44''	6	7	24. 05. 2020
8	12º39'55''-109º13'39''	1	8,5	24. 05. 2020
9	12º37'34''-109º12'49''	9	8,5	24. 05. 2020
10	12º37'40''-109º13'46''	4	8,15	26. 05. 2020
11	12º38'00''-109º17'28''	1	19	26. 05. 2020

Preparation of crude extracts of culture broth

Actinomycete strains were cultivated at 30°C in sterile 1000 mL flasks containing 500 mL broth medium A1+(g/L): 10 g soluble starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 100mg $FeSO_4$, 40mg KBr, 30 g instant ocean, pH 7.0, shaked at 200 rpm. After 7 day cultivation, the culture broths were filtered by filter paper (thickness 0.35 - 0.5 mm, particle retention 3 µm) and then extracted with 300 mL ethyl acetate (5 times × 15 minutes). Extracts were then evaporated under reduced pressure (250 mbar, heating bath at 45ºC) to yield crude extracts.

Screening for antimicrobial activity of extracts from actinomycetes

Crude extracts were diluted in DMSO at 1% concentration (10 mg/mL DMSO) and used in screening experiments for antagonistic properties against the test microorganisms. Thus, the test microbes were grown in 96 well plates containing LB broth –supplemented with the crude extracts at different concentrations. Streptomycin was used as a positive control for bacteria and cycloheximide for the yeast *C. albicans* ATCC10231. Quantitative assay was performed by dilution series on 96 well plates for the determination of MIC values of extracts against the test bacteria. The UV absorption of each sample was measured at 610 nm and compared with the UV absorption of the media as a negative control. A MIC value was determined in wells containing the extract at the lowest concentration completely inhibited the growth of the test microorganisms after 24 hours of incubation and was correctly calculated based on the turbidity measurement on spectrophotometer Biotek (Hadacek *et al.*, 2000).

Identification of actinomycetes

Genomic DNA of five potential isolates was extracted by Wizard® Genomic DNA Purification Kit was purchased from Promega (USA). Sequences of 16S rRNA was used for taxonomical identification of the actinomycetes strains. Gene amplifications were performed in a 25.0 µL mixture containing 10 µL of sdH2O, 12.5 µL of PCR Master mix, 1.0 µL of 0.05 mM for both primers 9 F (5'-GAGTTTGATCCTGGCTCAG3') and 1541R (5'-AAGGAGGTGATCCAACC3') and 0.5 µL of genomic DNA. Thermocycling was performed on MJ Thermal cycler (Bio - Rad), with a preheating

step at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 62ºC for 30 sec and extension at 72ºC for 45 sec before a final extension of 72ºC for 8 min. The PCR product size was about 1500 bp. PCR products were purified by DNA purification kit (Invitrogen) and sequenced by DNA Analyzer (ABI PRISM 3100, Applied Bioscience). Gene sequences were handled by BioEdit v.2.7.5. and compared with fungal 16S rRNA sequences available in GenBank database by using NBCI Blast program. The alignment was manually verified and adjusted prior to the reconstruction of a phylogenetic neighbor-joining tree by using the MEGA program version 7.2.

RESULTS AND DISCUSSION

Isolation and screening for antimicrobial activity of marine actinomycetes

From 40 marine samples randomly collected, including: sediments (9 samples), sponges (9 samples), soft corals (6 samples), molluscas (8 samples), seaweeds (5 samples), sea cucumber (2 samples) and crinoids (1 sample) collected from Van Phong Bay area of Khanh Hoa province, Vietnam. We carried out dilution and serial plating on different media to isolate actinomycetes. After 1 to 4 weeks of incubation, 50 actinomycete strains were isolated. About 15 actinomycetes were isolated from sediment samples, 14 from sponges, 9 from molluscas, 8 from seaweed samples, 4 from soft corals and 3 from sea cucumber. The number of actinomycetes isolated from each type of sample depends on the number of each type of sample collected and the sample collection area. No general rule was found for the distribution of actinomycetes strains for each type of sample.

Among the seven different media used in this study for the isolation of actinomycetes, M1 and A1 were shown to be effective in isolating marine actinomycetes when compared with other media ISP1, ISP2, SWA, SCA and NZSG. Approximately 19 actinomycetes were isolated using M1, 14 with A1, 7 isolated using ISP1, 5 using SWA, 2 isolates with ISP2 and NZSG medium (Figure 1A). The isolates well grown on A1 agar and produce aerial mycelium and substrate filaments in the medium, with diverse colony morphology and color such as: grey, orange, yellow, white and black. In which, orange and gray colonies predominate (Figure 1B). Similar study by Gunasekaran et al. (2013) isolated 52 actinomycetes from 28 marine samples. In which, strains with gray and orange colony morphology are the majority. In total of 41 isolates, colony color varied from yellow (48%), brown (10%), gray (13%), greenish brown (6%) and 3% others (Sapkota *et al.*, 2020).

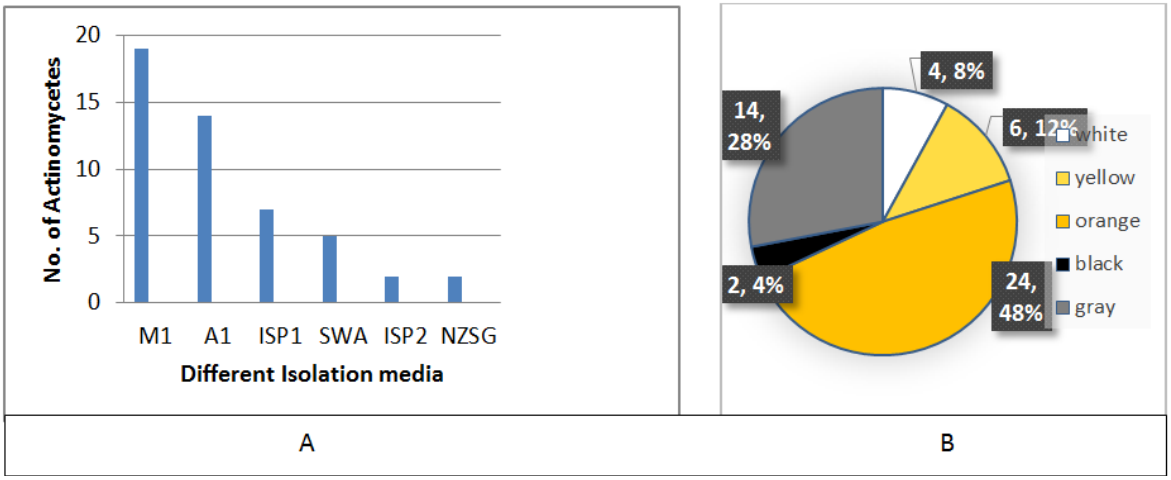

Figure 1. Preference of media for isolation of marine actinomycetes (A) and aerial mycelial color of the isolates grown on A1 medium (B).

The result of screening showed that out of 50 isolates, 47 isolates inhibited 1 to 5 strains of the test microorganisms; 18/50 strains have shown antibacterial activities against at least 3 strains of the test microorganisms. In which, strains G650, G654, G657, G666, and G675 inhibited 4 to 5 test microorganisms with MIC values equal to or lower than the positive control. In particular, all 5 strains inhibited all three Gram-positive test microorganisms at MIC values from 8 to 64 µg/mL and *C. albicans* ATCC10231 with MIC values from 2 to 16 µg/mL. In addition, the two strains G657 and G666 had an inhibitory effect on *P. aeruginosa* ATCC27853 và *S. enterica* ATCC13076 with a MIC value of 128 µg/mL (Table 2).

Table 2. Antimicrobial activity of crude ethyl acetate extracts from 18 actinomycete strains.

STT	Tên chủng	Gram +			Gram -			Nấm men
		***E. faecalis* ATCC29212**	***S. aureus* ATCC25923**	***B. cereus* ATCC14579**	***E. coli* ATCC25922**	***P. aeruginosa* ATCC27853**	***S. enterica* ATCC13076**	***C. albicans* ATCC10231**
Đơn vị		**MIC(µg/mL)**	**MIC(µg/mL)**	**MIC(µg/mL)**	**MIC(µg/mL)**	**MIC(µg/mL)**	**MIC(µg/mL)**	**MIC(µg/mL)**
1	G647	128	-	256	-	-	-	128
2	G648	128	128	-		-		64
3	**G650**	**8**	**32**	**32**		**-**		**8**
4	G653	128	256	-				256
5	**G654**	**32**	**64**	**32**				**2**
6	**G657**	**8**	**32**	**64**				**16**
7	G659	64	-	32				256
8	**G666**	**32**	**8**	**8**				**16**
9	G667	128	256	256				32
10	G668	128	64	64	-	-	-	128
11	G669	32	256	256				16
12	G671	32	256	-				32
13	G672	32	-	256				8
14	G673	256	32	-				16
15	**G675**	**32**	**32**	**64**				**16**
16	G686	32	-	256				128
17	G688	32	128	-		-		64
18	G693	128	256	-				256
Streptomycin		*256*	*256*	*128*	*32*	*256*	*128*	-
Cyclohexamide								*32*

In the study of Sapkota et al. (2020), among 41 pure isolates, 19 isolates showed antibacterial activity against the test organisms. Of which, 13 isolates showed inhibition against Gram-positive bacteria (*S. aureus*) and 7 strains showed inhibition against Gram-negative bacteria (*E. coli*). In another study, 8 out of 20 strains showed activity against Gram-positive test organisms and only 4 strains showed activity against Gram-negative organisms (Wahab *et al.,* 2015). Similarly, out of 106 isolates, only 36 isolates showed activity against test organisms among which only 2 were active against only Gram-negative organisms (Pandey *et al.,* 2004). According to Shirling and Gottlieb, the reason for the different sensitivity between Gram-positive and Gram-negative bacteria could be explained with respect to the morphological differences between these microorganisms. Gram-negatives have an outer polysaccharide

membrane making the cell wall impermeable to lipophilic solutes; however, the Gram-positives have only an outer peptidoglycan layer which is not an effective permeability barrier (Shirling *et al.*, 1966).

Identification of the actinomycetes

From the results of activity screening, 5 strains (G650, G675, G666, G654 và G657) showed the highest antimicrobial activity. They were chosen to be identified by phylogenetic based on 16S rRNA gene sequences analyses. Colonies of five strains well grown on the A1 medium had diameters from 2 – 5 mm after 7 days at 30°C. Mature colonies were grey colored mycelial (G650, G675) light brown (G666) and yellow (G654 và G657) (Figure 2).

The 16S rRNA genes were amplified by PCR by using specific primers 9 F and 1541R, giving products about 1500 bp (Figure 3). Comparative analyses of 16S rRNA gene sequences of these five isolates on GenBank database showed that: strain G650 exhibited the highest similarity (99.30%) to the species *Streptomyces ardesiacus* NBRC 15402 (NR112454) and strain G666 (99.22%) to the species *Streptomyces palmae* CMU-AB204 (NR152026); strain G654 showed the highest similarity (99.65%) to the species *Salinispora arenicola* ATCC BAA-917 (NR042725); strain G657 highest similarity (99.78%) to the species *Micromonospora aurantiaca* ATCC 27029 (NR074415); whereas G675 highest similarity (100%) to the species *Nocardiopsis flavescens* BA6 (NR108853) (Figure 4). The sequences of 16S rRNA gene of G650, G654, G666, G657 and G675 isolates were registered on GenBank database with the accession numbers ON619538, ON619537, OM899717, ON619539 and ON619540, respectively.

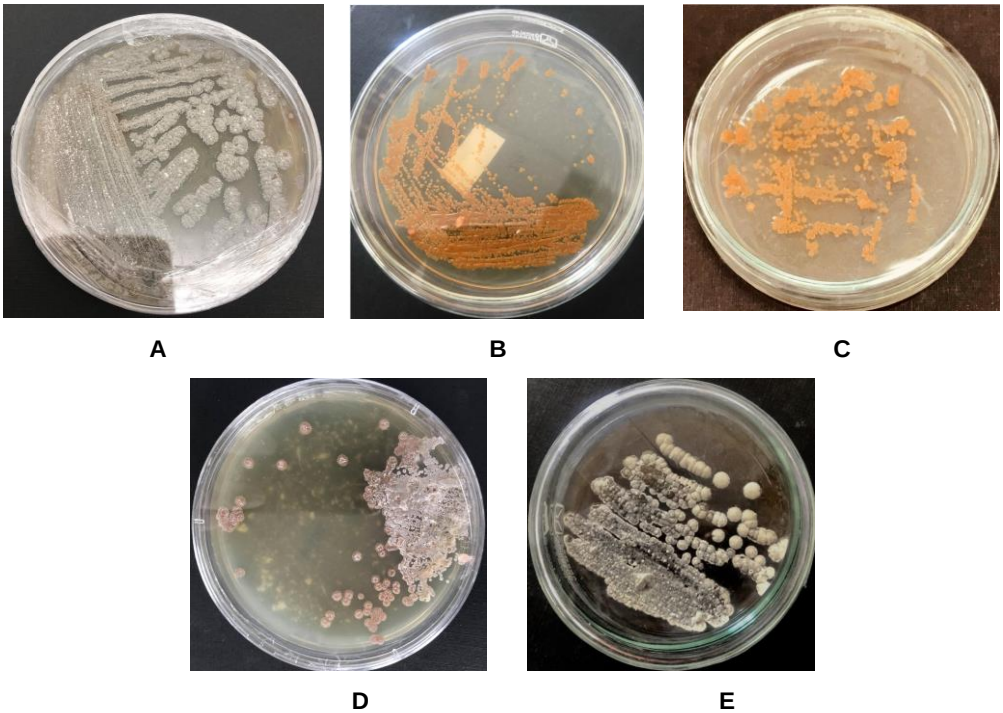

Figure 2. Colony morphological characteristics of the strains G650 (A), G654 (B), G657 (C) G666 (D), and G675 (E) grown on A1 medium for 1 week at 30°C.

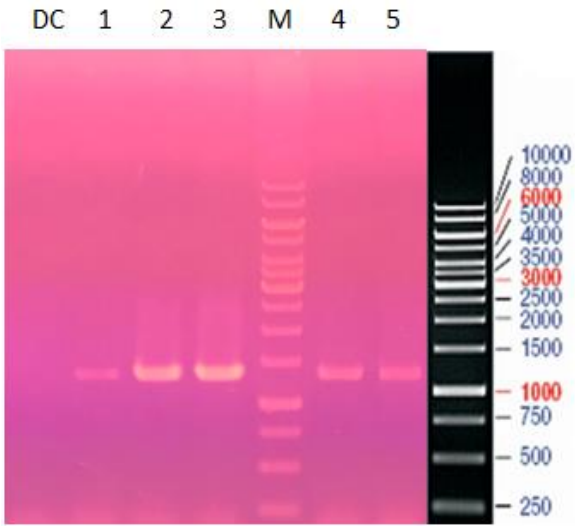

Figure 3. Electrophoresis image of PCR products16S rRNA gene of isolates. M: The 1 Kb DNA ladder of Fisher Scientific. DC: PCR product control without DNA template. 1-3: PCR products of G650, G654 and G657 isolates. 4-5: PCR products of G666 and G675 isolates

About 70% of the world's antibiotics are known to come from actinomycetes, mainly from the genera *Streptomyces* and *Micromonospora*. Previously, researchers focused more on discovering terrestrial actinomycetes species. Today, new antibiotics have been found from marine actinomycetes. Although the exploitation of marine actinomycetes as a source for the discovery of new secondary metabolites is in the early stages, many new metabolites have been isolated in the past few years. For example, Diazepinomicin is a unique dibenzodiazepinone produced by a strain of *Micromonospora*. It has antibacterial, anti-inflammatory and anti-tumor activity. Salinosporamide A is a new β-lactone-γ-lactam isolated from the fermentation medium of the new obligate marine actinomycetes *Salinispora tropica* (Sunaryanto *et al.*, 2010). Studies in marine-derived natural products chemistry all over the world have been significantly developed and achieved many positive results. However, research on secondary compounds from marine microbes in Vietnam has just been started and there are only few publications, in particular about marine actinomycetes. Vietnamese scientists, currently, have been focused on study about marine microbes to explore the great potential of this natural resource (Danh *et al.*, 2018; Luyen *et al.*, 2012; Tuan *et al.*, 2018).

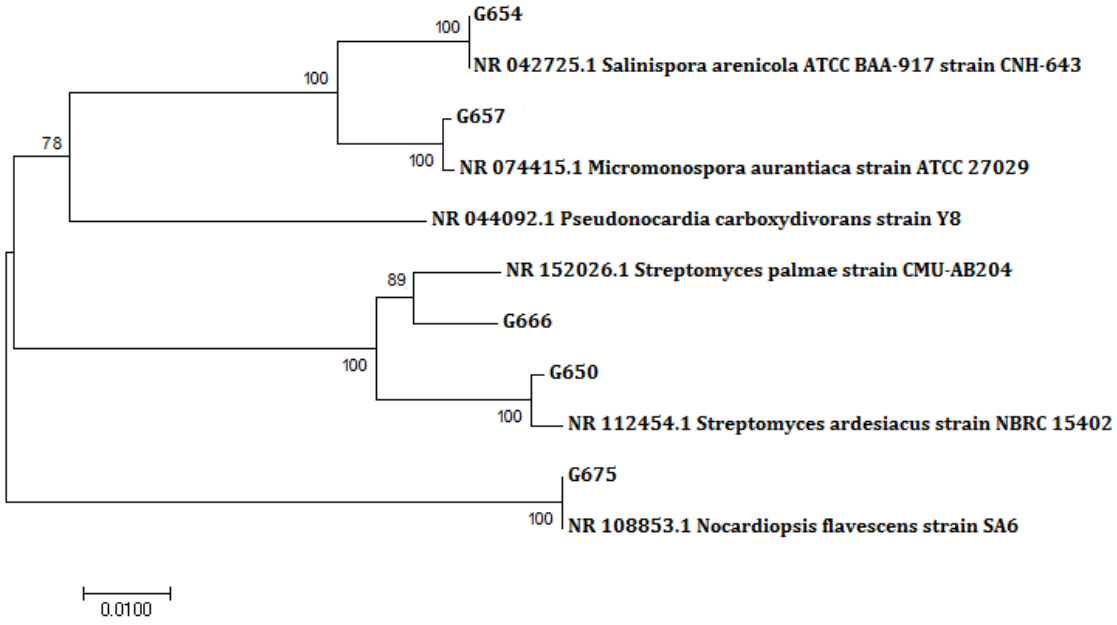

Figure 4. Neighbor-joining tree based on almost-complete 16S rRNA gene sequences showing relationships between the strains in groups and representative members of the genera *Streptomyces, Salinispora, Nocardiopsis* and *Micromonospora. Pseudokineococcus carboxydivorans* was used as an outgroup.

The genus *Salinispora* is one of the first salt obligatory marine species worldwide and is typically found in tropical and subtropical marine environments including sediments and marine organisms. This genus produces a wide range of bioactive compounds such as lomaiviticins, cyclomarins, rifamycins, salinaphthoquinones, and salinosporamides (Kim et al., 2020). The first description of the genus *Nocardiopsis* was in 1976 by J. Mayer. *Nocardiopsis* belongs to the order *Actinomycetales*, family *Nocardiopsaceae*, and morphologically, it is similar to members of the genera *Actinomadura* and *Nocardia*. Reviewing the literature on the genus *Nocardiopsis*, it could produce a wide variety of chemical classes of compounds with diverse biological activities, which are responsible for a wide spectrum of pharmacological and biological effects, mainly as antibacterial, antifungal, anticancer antitumor, cytotoxic, immunomodulatory and protein kinase inhibitory (Ibrahim *et al.*, 2018).

The results obtained and published studies showed that marine actinomycetes could be a potential source for producing antibiotics based on inhibiting microbial pathogens. However, there is a need for research in determining the chemical structure of bioactive compounds from these potential actinomycetes.

CONCLUSION

From 40 marine organisms samples randomly collected from Van Phong Bay area of Khanh Hoa province, Vietnam, 50 actinomycetes strains were isolated. The results of screening for antimicrobial activity showed that most of the isolates were active against 1 to 5 strains of test microorganisms. In which, 18/50 strains have shown antibacterial activities against at least 3 strains of test microorganisms. Specifically, strains G650, G654, G657, G666, G675 inhibited all three Gram-positive test microorganisms and *C. albicans* ATCC10231 with MIC values ranging from 2 to 64 µg/mL, depending on the test microbes. In addition, two strains G657 and G666 had inhibitory effects on *P. aeruginosa* ATCC27853 and *S. enterica* ATCC13076 with a MIC value of 128 µg/mL. The five strains were identified as members of the genus *Streptomyces* (strains G650 and G666), genus *Salinispora* (strain G654), genus *Micromonospora* (G657), and genus *Nocardiopsis* (G675) based on morphological and 16S rRNA gene sequence analyzes.

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