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IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES STRAINS ISOLATED FROM SAMPLES COLLECTED IN THE COASTAL AREA OF HUE, DA NANG AND QUANG NAM PROVINCES, VIETNAM

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SUMMARY

Microorganisms are of particular interest because of their ability to synthesize high-value secondary compounds and provide us with novel and diverse chemical structures. The most common source of antibiotics is Actinomycetes which provide around two-third of naturally occurring antibiotics, including many of medical importance. In this study, 81 strains of actinomycetes were isolated from 145 samples including: sediments, sponges, soft corals, echinoderms and starfish collected from three sea areas of Vietnam: Hue, Da Nang and Quang Nam. The strains were 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 reference strains. From the results of screening, Seven strains of actinomycetes that have the highest biological activity (Code: G244, G246, G261, G266, G278, G280 and G290) were chosen to be identified by morphological and phylogenetic based on 16S rRNA gene sequences. The results showed that 6 strains G246, G261, G266, G278, G280 and G290 belonged to the genus *Streptomyces*; and the strain G244 belonged to the genus *Micromonospora*. In particular, strains G244, G278, G280 were resistant 5/7 strains of microorganisms test, with values MICs from 2 µg/mL to 256 µg/mL; and three strains G261, G266, G290 showed the inhibitory effect towards 4/7 strains of microorganisms test, with respective values MICs from 2 µg/mL to 256 µg/mL. Moreover, six of the seven selected strains were highly resistant to yeast *Candida albicans* ATCC10231 with MIC values from 2 µg/mL to 256 µg/mL. These results indicated that marine Actinomycetes in Vietnam are also a potential source to find bioactive substances.

Keywords: *16S rRNA gene sequences, Actinomycetes, Antimicrobial activity, Micromonospora, Streptomyces*

INTRODUCTION

Actinomycetes are diverse group of Gram - positive bacteria that usually grow by filament formation. They belong to the order Actinomycetales with high G+C (>55%) content in their DNA. In fact, the most common source of antibiotics is Actinomycetes which provide around two-third of naturally occurring antibiotics, including many of medical importance (Okami, Hotta 1988). Aquatic actinomycetes are of biological importance because of their efficiency in antibiotic production. They are considered highly valuable for producing various antibiotics and other therapeutically useful compounds with diverse biological activities. Many of the presently used antibiotics such as streptomycin, gentamicin, rifamycin and erythromycin are the products of actinomycetes. The genus *Streptomyces* is represented in nature by the largest number of species and varieties, producing the majority of known antibiotics among the family Actinomycetaceae. *Streptomyces* are well known sources of antibiotics and other important novel metabolites, including antifungal agents, antitumor agents, antihelminthic agents and herbicides (Lee *et al.*, 2003; Thakur *et al.,* 2007).

Though the recent search for novel antibiotics have established approach of target based discovery using bacterial genomics, combinatorial chemistry, these powerful tools have not yet yielded any antibiotics approved for clinical use, and the prospects for their success are not encouraging (Baltz, 2007). Another way, programs aimed at the discovery of antibiotics from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have application in human medicine and agriculture (Busti *et al*., 2006). Therefore, the traditional method of screening antibiotics from microorganisms is still very effective (Baltz, 2007).

It is obvious that actinomycetes serve as an abundant source of bioactive compounds. In the future, manifold novel compounds would be potentially discovered from them. Herein, we reported on the isolation, taxonomic characterization, extraction fermentation broths with ethyl acetate of these actinomycete strains isolated from samples collected in Hue, Da Nang and Quang Nam of Vietnaman also reported on their antimicrobial activity.

MATERIALS AND METHODS

Microorganism test

The microorganisms used for antibacterial test were from ATCC Collection: Three Gram negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeraginosa* ATCC27853, *Salmonella enteric* ATCC13076), and three Gram positive bacteria (*Enterococcus faecalis* ATCC29212, *Stapphylococus aureus* ATCC25923, *Bacillus cereus* ATCC 13245), one yeast strain *Candida albicans* ATCC10231.

Sample collection

The marine samples were collected using Ponar from three locations in Hue, Da Nang and Quang Nam at 4 - 24 m depth with different geographic coordinates (Table 1), the water at temperatures was 26-29°C. The samples were collected into 15 mL or 50 mL sterile Falcon tubes, preserved in ice-box and processed within 24 h.

Table 1. Detail of the samples collected from three different locations: Hue, Da Nang and Quang Nam.

Locations	geographic coordinates	No of samples	Water depth (m)	Collection time
Hue (Mui Tho Lo in Hai Van)	16^{0}13'3''-108^{0}7'57''	21	5 – 24	26. 05. 2016
Hue (Bai Chuoi, Son Cha in Hai Van)	16^{0}13'1''-108^{0}8'37''	8	6	27. 05. 2016
Hue (BanhTranh, Son Cha in Hai Van)	16^{0}12'58''-108^{0}7'59''	3	5	27. 05. 2016
Quang Nam (Hon Tai in Cu Lao Cham)	15^{0}54'13"-108 ^{0}31'54"	16	4	19. 09. 2016
Quang Nam (Hon La in Cu Lao Cham)	15^{0}58'19"-108 ^{0}27'7"	11	3	01. 10. 2016
Quang Nam (Hon Mo in Cu Lao Cham)	15^{0}55'50"-108^{0}28'30''	19	3 – 7	01. 10. 2016
Quang Nam (Hon Dai in Cu Lao Cham)	15^{0}56'24"-108 ^{0}28'56''	24	4 – 90	02. 10. 2016
Đa Nang (Son Tra)	16^{0}11'37 – 108^{0}11'43	5	16 – 20	24. 08. 2016
Đa Nang (Northeast of the Son Tra)	16^{0}09'11 – 108^{0}13'50	23	10	24. 08. 2016
Đa Nang (Northeast of the Son Tra)	16^{0}34'50 – 108^{0}11'4	15	7	25. 08. 2016

Isolation of actinomycetes

First, 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 using a wet-heat technique (60°C for 6 min). Next, 0.5 mL of this suspension was transferred to another 4.5 mL sterile distilled water and this step was repeated to set up a ten fold dilution series to 10^{-3}. At the final dilution step, aliquots of 50 µL were spread on six different media including A1 (soluble starch: 10 g/L; yeast extract: 4 g/L peptone: 2 g/L; instant ocean: 30

g/L; agar: 15 g/L); M1 (soluble starch: 5 g/L; yeast extract: 2 g/L; peptone: 1 g/L; instant ocean: 30 g/L; agar: 15 g/L), SWA (instant ocean: 30 g/L; agar: 15 g/L); A+(soluble starch: 10 g/L; yeast extract: 4 g/L; peptone: 2 g/L; instant ocean: 30 g/L; $CaCO_3$: 1 g/L; agar: 15 g/L), SCA (soluble starch: 10 g/L; K_2HPO_4: 2 g/L; KNO_3: 2 g/L; casitone: 300 mg/L; $MgSO_4{\cdot}7H_2O$: 50 mg/L; $FeSO_4{\cdot}7H_2O$: 10 mg/L; instant ocean: 30 g/lL $CaCO_3$: 2 mg/L; agar: 15 g/L), NZSG (soluble starch: 20 g/L; yeast extract: 5 g/L glucose: 10 g/L; NZ amine A: 5 g/L; instant ocean: 30 g/L; agar: 15 g/L); ISP1 (soluble starch: 5 g/L; yeast extract: 2 g/L; casitone: 5 g/lL instant ocean: 30 g/L; agar: 15 g/L), ISP2 (soluble starch: 5 g/L; yeast extract: 2 g/L; malt extract: 10 g/L; glucose: 10 g/L; instant ocean: 30 g/L; agar: 15 g/L). These media were supplemented with 50 µg/mL polymycin B and cycloheximide to inhibit Gram - negative bacterial and fungal contamination. After 21 days of aerobic incubation at 28°C, the colonies of actinomycete strains were transferred onto A1 agar medium (Williams *et al*., 1965, 1971).

Extraction crude and screening the antimicrobial activity of the extracts

The actinomycetes strains were cultivated at 28°C in sterile 1000 mL flasks containing 500 mL media A^+ with glucose 1%, pH 7.0, at 200 rpm. After 7 days of cultivation, the fermentation broths were filtered and then extracted 5 times with ethyl acetate. The extracts were evaporated under reduced pressure to yield crude extracts (Cédric *et al.,* 2013).

Crude extracts were tested against the Gram-positive bacteria (*B. cereus* ATCC13245, *E. faecalis* ATCC29212, *S. aureus* ATCC25923), the Gram-negative bacteria (*P. aeruginosa* ATCC27853, *E. coli* ATCC25922, *S. enterica* ATCC13076) and the fungi *C. albicans* ATCC10231. The positive control was streptomycin for bacteria, and nystatin for fungi *C. albicans* ATCC10231. Quantitative assay was done by dilution method for determination of MIC (Minimum Inhibition Concentration) values of extracts against the test bacteria. MIC means the lowest concentration of extract at which the test microorganism did not show any visible. The density of cells was read at 610 nm and adjusted to an optical density (OD) of 0.04 for Gram-positive bacteria, and 0.05 for Gram-negative bacteria and *C. albicans*. Aliquots of 50 µL of bacterial or fungal suspension were incubated with each crude extract for 24 h at 30ºC. The UV absorption of each sample was read at 610 nm and compared against the UV absorption of the media as control. MIC value was determined in wells with the lowest concentration of reagents that completely inhibits the growth of microorganisms after 24 h of incubation and was correctly identified based on data of cell turbidity measured by spectrophotometer Biotek and GraphPadPrism DaTa software (Hadacek *et al.,* 2000).

Identification of actinomycetes

The actinomycete strains were grown for 14 days at 28ºC on starch casein agar (SCA) and examined using scanning electron microscopy (model JSM-5410 LV; JEOL). Samples for scanning electron microscopy (SEM) were prepared as described by Itoh (1989).

Sequences of the 16S rRNA gene were used for identification of choosen strains. PCR amplifications were performed in a 25.0 µL mixture containing: 16.3 µL of sdH_2O, 2.5 µL of 10X PCR buffer, 1.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP's, 0.2 µL of Taq polymerase, 1.0 µl for both 0.05 mM of 9 F (5'-GAGTTTGATCCTGGCTCAG3') and 0.05 mM of 1541R (5'-AAGGAGGTGATCCAACC3') primers (Rajesh *et al.,* 2013) and 2.0 µL of genomic DNA. The reaction tube was then put into MJ Thermal Cycler, which had been programmed to preheat at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s and elongation at 72°C for 45 s before a final extension of 72°C for 10 min. The estimated PCR product size was about 1500 bp. PCR products were purified by DNA purification kit (Invitrogen) then sequenced by DNA Analyzer (ABI PRISM 3100, Applied Bioscience). Gene sequences were handled by BioEdit v.2.7.5. and compared with bacterial 16S rRNA sequences in GeneBank database by NBCI Blast program. The alignment was manually verified and adjusted prior to the construction of a phylogenetic tree. The phylogenetic tree was constructed by using the neighbor-joining the MEGA program version 4.1 (Saitou *et al.,* 1987).

RESULTS AND DISCUSSION

Isolation and screening the antimicrobial activity of of actinomycetes

From 145 marine samples collected in Hue, Da Nang and Quang Nam, 81 actinomycete strains were isolated.These strains then were cultured and extracted to screen biological activity. From the

results of screening, seven strains of actinomycetes that have the highest biological activity (Code: G244, G246, G261, G266, G278, G280 and G290) were chosen (Table 2).

Table 2. Antimicrobial activity of crude ethyl acetate extracts from 7 strains.

S.No.	Isolates	Gram-positive			Gram-negative			Yeast
		E.faecalis ATCC29212	*S.aureus* ATCC25923	*B.cereus* ATCC13245	*E.coli* ATCC25922	*P.aeruginosa* ATCC27853	*S.enterica* ATCC13076	*C.albicans* ATCC10231
Unit		MIC(µg/mL)	MIC(µg/mL)	MIC(µg/mL)	MIC(µg/mL)	MIC(µg/mL)	MIC(µg/mL)	MIC(µg/mL)
1	G244	64	-	256	128	-	32	32
2	G246	64	-	128	-	-	-	32
3	G261	64	-	256	-	16	-	16
4	G266	-	16	256	-	32	16	-
5	G278	256	32	-	16	-	16	2
6	G280	256	-	256	16	32	-	2
7	G290	-	-	32	-	16	8	16
Steptomycin		256	256	128	32	256	128	-
Nistatin		-	-	-	-	-	-	8

The result reveals that most of the isolates were active against both Gram positive and Gram negative bacteria. Strains G244, G278, G280 were resistant 5/7 strains of microorganisms test, with values MICs from 2 µg/mL to 256 µg/mL; and three strains G261, G266, G290 showed the inhibitory effect towards 4/7 strains of microorganisms test, with respective values MICs from 2 µg/mL to 256 µg/mL. In addition, six of the seven strains selected were highly resistant to *C. albicans* ATCC10231 with MIC values from 2 µg/mL to 256 µg/mL. Comparison of antimicrobial activity among screening strains in Hue, Quang Nam and Da Nang with isolated strains in the North - East Coast of Vietnam showed that: 7 strains of actinomycetes selected above have potent activity against both Gram-positive and Gram-negative bacteria. Of the 15 strains screened in the North-East Coast of Vietnam, only four strains of G057, G115, G119, and G120 were resistant to *P. auruginosa* ATCC27853 with a MIC value of 64 - 32µg/mL (Le Thi Hong Minh *et al.,* 2016). This result shows that the biological activity of the strains depends very much on geographic location during sample collection.

Identification of actinomycetes by morphological characteristic

The spore morphology is considered as one of the important characteristics in the identification of Streptomyces and it greatly varies among the species. It has been found that the majority of the marine isolates produced aerial coiled mycelia and the spores arranged in chains as already reported by Mukherjee and Sen, 2004 (Fig. 1B, 1C, 1D). *Micromonospora* species produced well-developed and branched substrate hyphae on yeast extract-malt extract medium, but no aerial hyphae. Spores were borne singly on the substrate hyphae having an approximate diameter of 0.5 - 1 µm. The spores were nodular and smooth on the surface and non-motile (Fig.1A).

The colors of the substrate mycelium were yellowish white to vivid orange and turned to brownish black to black after sporulation (Figure 2). The morphological characteristics of these isolates were consistent with their classification in the genus (Kawamoto *et al*., 1989).

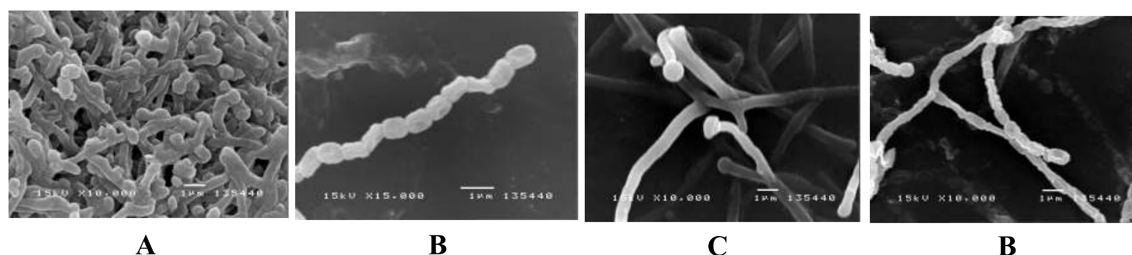

Figure 1. Scanning electron micrographs of the representative strains G244(A); G246 (B); G266 (C), and G290 (D) grown on SCA agar for 2 weeks at 30°C.

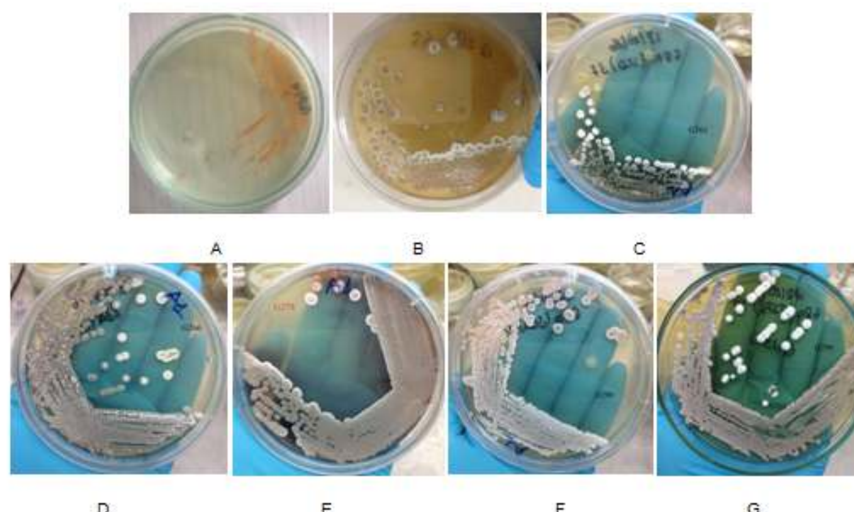

Figure 2. Morphological appearance of isolates. The colors of the substrate mycelium were vivid orange A(G244) and from white turned to brownish after sporulation B(G246), C(G261), D(G266), E(G278), F(G280) and G(G290).

Identification of actinomycetes by phylogenetic based on 16S rRNA gene sequences

Seven potential isolates were selected for identification by 16S rRNA gene sequencing. The obtained sequences were analysed by Bioedit program and compared with those in GenBank database. The obtained results showed that 16S rRNA sequences of G246, G261, G266, G278, G280 and G290 strains exhibited high similarity (99%) with genus *Stretomyces* spp; The strain G244 was identified (99% similarity) of 16S rRNA gene sequence with genus *Micromonospora* in GenBank) (Figure 3).

Streptomyces is a genus of Gram-positive bacteria that grows in various environments, with a filamentous form similar to fungi. The morphological differentiation of *Streptomyces* involves the formation of a layer of hyphae that can differentiate into a chain of spores. The most interesting property of *Streptomyces* is the ability to produce bioactive secondary metabolites such as antifungals, antivirals, antitumoral, anti-hypertensives, and mainly antibiotics and immune suppressives (Patzer *et al*., 2010; Khan 2011). Another characteristic of the genus is complex multicellular development, in which their germinating spores form hyphae. Then, multinuclear aerial mycelium forms septa at regular intervals, creating a chain of spores (Ohnishi *et al*., 2008).

Marine environment contains a wide range of distinct *Streptomyces* that are not present in the terrestrial environment. Though some reports are available on antibiotic and enzyme production by marine actinomycetes, the marine environment is still a potential source for isolating new actinomycetes, which can yield novel bioactive compounds and industrially important enzymes (Cai *et al*., 2007).

In addition, *Micromonospora* species – the dominant actinomycetes are possible to be isolated from aquatic habitats such as streams, lake mud, river sediments, beach sands, sponge and marine sediments (Rifaat, 2003; Eccleston *et al.,* 2008). *Micromonospora* species, together with *Streptomyces* species are best known for synthesizing antibiotics, especially aminoglycoside, enediyne, and oligosaccharide antibiotics. Thus, their impact on medicine is considerable. Of common antibiotics in the medical field, gentamicin and netamicin belong to the aminoglycoside antibiotics yielded by *Micromonospora* (Bérdy, 2005).

Research focused on marine environment has been gaining importance in recent years. However, still it has not been fully explored and there is tremendous potential to identify novel organisms with various biological properties. The present investigation showed that actinomycetes tentatively identified as Streptomyces species have strong antimicrobial activities against pathogenic bacteria (Sujatha *et al*., 2005; Ramesh *et al*., 2009).

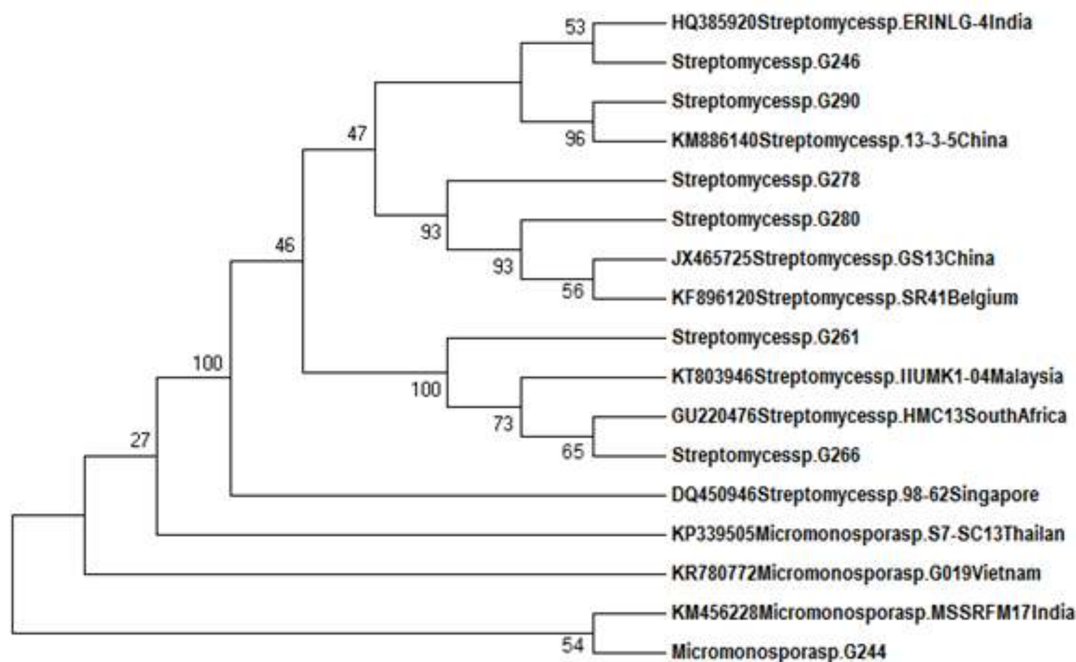

Figure 3. Neighbor-joining tree based on almost-complete 16S rRNA gene sequences showing relationships between the strains in groups and representative members of the genera *Streptomyces* and *Micromonospora* were used as an outgroup. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; Bar, 0.01 substitutions per nucleotide position.

CONCLUSION

From 145 samples including sediments, sponges, soft corals, echinoderms and starfish collected from three sea areas of Vietnam: Hue, Da Nang, and Quang Nam, 81 strains of actinomycetes were isolated. Most of the isolates exhibited antimicrobial activity, seven strains of actinomycetes that have the highest biological activity were chosen to be identified by morphological and phylogenetic investigations based on 16S rRNA gene sequences. The strains G246, G261, G266, G278, G280, and G290 belonged to genus *Stretomyces*; strain G244 were identified as genus *Micromonospora*. Specifically, All of the seven strains were resistant from 4 to 5 out of 7 strains of microorganisms test, with values MICs from 2 µg/mL to 256 µg/mL. In addition, six of the seven strains selected were highly resistant to yeast *C Albicans* ATCC10231with MIC values from 2 µg/mL to 256 µg/mL. Research results have shown that marine actinomycetes isolated from the marine environment of Vietnam promise to be a rich source of materials for secondary bioactive compounds.

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ĐỊNH DANH VÀ HOẠT TÍNH KHÁNG KHUẨN CỦA CÁC CHỦNG XẠ KHUẨN ĐƯỢC PHÂN LẬP TỪ CÁC MẪU THU THẬP Ở VÙNG VEN BIỂN HUẾ, ĐÀ NẴNG VÀ QUẢNG NAM

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TÓM TẮT

Vi sinh vật được quan tâm đặc biệt bởi khả năng sinh tổng hợp các hợp chất thứ cấp có giá trị cao và cung cấp cho chúng ta các cấu trúc hóa học mới lạ và đa dạng. Xạ khuẩn là nguồn sản xuất phổ biến nhất các chất kháng sinh, khoảng 2/3 loại kháng sinh được phát hiện trong tự nhiên là từ xạ khuẩn. Trong nghiên cứu này, chúng tôi phân lập được 81 chủng xạ khuẩn từ 145 mẫu gồm: trầm tích, hải miên, san hô mềm, da gai và sao

biển thu được từ 3 vùng biển của Việt Nam: Huế, Đà Nẵng và Quảng Nam. Các chủng đã được lên men trong môi trường A^+ và môi trường lên men được chiết xuất 5 lần với ethyl acetate. Các chất chiết xuất đã bay hơi dưới áp suất giảm để tạo ra các cặn chiết thô. Phương pháp định lượng được sử dụng để xác định MIC (nồng độ ức chế tối thiểu) của cặn chiết đối với 7 chủng vi sinh vật kiểm định. Từ kết quả sàng lọc, Từ các kết quả sàng lọc, bảy chủng actinomycetes có hoạt tính sinh học cao nhất (Mã số: G244, G246, G261, G266, G278, G280 và G290) được lựa chọn để định danh bằng hình thái học và phát sinh loài dựa trên trình tự gen 16S rRNA. Kết quả cho thấy 6 chủng G246, G261, G266, G278, G280 và G290 thuộc về chi *Streptomyces*; và chủng G244 thuộc chi *Micromonospora*. Đặc biệt, chủng G244, G278, G280 đã kháng được 5/7 chủng vi sinh vật, với giá trị MICs từ 2 µg/mL đến 256 µg/mL; và ba chủng G261, G266, G290 cho thấy tác dụng ức chế đối với 4/7 chủng vi sinh vật kiểm định, với giá trị tương ứng MICs từ 2 µg/mL đến 256 µg/mL. Ngoài ra, sáu trong số bảy chủng được lựa chọn có hoạt tính ức chế nấm *Candida albicans* ATCC10231 rất cao với giá trị MICs từ 2µg/mL đến 256 µg/mL. Những kết quả thu được cho thấy rằng các chủng xạ khuẩn biển ở Việt Nam cũng là nguồn nguyên liệu tiềm năng để tìm kiếm các chất có hoạt tính sinh học.