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IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES STRAINS ISOLATED FROM MARINE SAMPLES IN THE COASTAL AREA OF THANH HOA – QUANG BINH – QUANG TRI

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Abstract. In this study, 46 strains of actinomycetes were isolated from 40 samples including: sediments, sponges, soft corals, echinoderms and starfish collected from three coastal areas of Vietnam: Thanh Hoa – Quang Binh – Quang Tri. The strains were fermented in A^+ medium and fermentation broths were extracted 5 times with ethyl acetate then 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, five strains of actinomycetes exhibited the highest biological activity were chosen (Code: G212, G222, G233, G227 and G241). In particular, strains G222, G233, G227 and G241 were resistant 6/7 strains of microorganisms test, with MIC values from 64 µg/ml to 256 µg/ml; Moreover. All of the five strains were highly resistant to yeast *Candida albicans* ATCC10231. These strains were then subjected to morphological and phylogenetic investigations based on 16S rDNA gene sequences. The results showed that strains G212, G222 andG227 belonged to the genus *Streptomyces*; strains G233 and G241were identified as a member of the genus *Micromonospora*.

Keywords: Micromonospora, *Streptomyces*, antimicrobial activity, MIC, 16S rDNA gene sequences.

Classification numbers: 1.5.3; 3.4.4

1. INTRODUCTION

Actinomycetes are Gram-positive bacteria that grows in various environments, with a filamentous form similar to fungi. They are ubiquitous in freshwater and marine water habitats [1]. Actinomycetes are of biological importance because of their efficiency in antibiotic production. They are considered highly valuable as they can produce 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. Need of new antimicrobial agents is greater than ever because of emergence of multidrug resistance in common pathogens, the rapid emergence of new infections and the use of multidrug resistant pathogens in bioterrorism [2]. Resistance of bacteria to the effects of antibiotics has been a major problem in the treatment of diseases [3].

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 [4], antitumor agents [5], antihelminthic agents [6] and herbicides.

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 new actinomycetes, which can yield novel bioactive compounds and industrially important enzymes [7].

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 [8, 9]. *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* [10].

It is obviously 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 Thanh Hoa – Quang Binh – Quang Tri of Viet Nam and also reported on their antimicrobial activity.

2. MATERIALS AND METHODS

2.1. Material

Chemicals

The genomic DNA isolation kits were purchased from Promega (Madison, WI, USA). The mini-prep and DNA gel extraction kits were purchased from Qiagen (Mannheim, Germany). PCR master mix was purchased from Bioneer. Glucose and all other chemicals (for media) were obtained from Himedia (India), Duc Giang (Viet Nam) and Sigma-Aldrich (St. Louis, MO, USA).

Microorganisms test

Seven standard reference microorganisms were come from ATCC Bacteriology Collection including: Three Gram negative bacteria (*Escherichia coli* ATCC25922, *Pseudomonas aeraginosa* ATCC27853, *Salmonella enterica* ATCC13076), and three Gram positive bacteria (*Enterococcus faecalis* ATCC29212, *Stapphylococus aureus* ATCC25923, *Bacillus cereus* ATCC 13245), one yeast strain *Candida albicans* ATCC10231.

2.2. Samples collection

The marine samples were collected using Ponar from three locations at 4 m-24 m in depth with different geographic coordinates; temperature of water was 26-29 ^{o}C in Thanh Hoa – Quang Binh – Quang Tri. The samples were collected into 15 mL or 50 mL sterile Falcon tubes and preserved in ice-box and processed within 24 h.

2.3. 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 ^{o}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 tenfold dilution series to 10^{-3}. At the final dilution step, aliquots of 50 µL were spread on six different media like: 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/L; $CaCO_3$: 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/L; 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 30 ^{o}C, the colonies of actinomycete strains were transferred onto yeast extract-malt extract agar (ISP2 medium) [11, 12].

2.2. Production of crude 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 and 30 ^{o}C. After 7 days cultivation, the fermentation broths were filtered and then extracted with ethyl acetate (5 times). The extracts were evaporated under reduced pressure to yield crude extracts [13].

2.3. Screening of actinomycetes for antimicrobial activity assessment of antimicrobial activity of the extracts 2.2

Crude extracts were tested against the Gram-positive bacteria (*Bacillus cereus* ATCC13245, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC25923), the Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Salmonella enterica* ATCC13076) and the fungi *Candida albicans* ATCC10231. The positive control was streptomycin for bacteria, cycloheximide for fungi *Candida albicans* ATCC10231. Quantitative assay was performed based on dilution method for determination of MIC (Minimum Inhibition Concentration) values of extracts against 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 30 ºC. 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 plate 96 wells with the lowest concentration of reagents that completely inhibits the growth of microorganisms after 24 hours of incubation and were correctly identified based on data of cell turbidity measured by spectrophotometer Biotek and GraphPad Prism DaTa software [14].

2.4. Identification of actinomycetes

The actinomycete strains were incubated for 14 days at 30 ºC on starch casein agar (SCA) and its morphology was examined using scanning electron microscopy (model JSM-5410 LV; JEOL). Samples for scanning electron microscopy (SEM) were prepared as described by Itoh et al [15].

Sequencing 16S rDNA method was used for identification of chosen strains. Amplifications were performed in a 25.0 µL mixture containing 16.3 µL of H_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') primer [16] and 2.0 µL of genomic DNA. The reaction tube was then put into MJ Thermalcycler, which had been programmed to preheat at 94 ^oC for 3 min, followed by 30 cycles of denaturation at 94 ^oC for 1 min, annealing at 60^oC for 30s and elongation at 72 ^oC for 45s before a final extension of 72 ^oC for 10 min. Estimated product size was about 1500 bp. PCR products were purified by DNA purification kit (Invitrogen). The 16S rDNA gene sequencing was carried 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 GenBank database using NBCI Blast programme. 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 programme version 4.1 [17].

3. RESULTS AND DISCUSSION

From 40 marine samples were collected of Thanh Hoa – Quang Binh – Quang Tri areas Vietnam. 46 actinomycete strains were isolated. These actinomycete strains were cultivated in SCA medium broth. The fermentation broths were extracted 5 times with ethyl acetate then the extracts were evaporated under reduced pressure to yield crude extracts. Crude extracts were tested against 7 reference strains. From the results of screening, we chose 5 strains of actinomycetes that have the highest biological activity (Code: G212, G222, G233, G227 and G241) (Table 1).

Table 1 shows the results of the screening of the crude extract of 5 selected strains on antibacterial activity. The results of the current study revealed that 6/7 strains of reference microorganism were against following G222, G233, G227 and G241 with values MICs from 64 µg/mL to 256 µg/mL, whereas G212 showed moderate antibacterial against only with 4/7 reference strains. Specially, all of the five strains had not resistant to *E.coli* ATCC25922. In addition, all crude extract showed a high MIC against *Candida albicans* (ATCC10231), from 64 µg/mL to 256 µg/mL. According to the previuos report, out of 15 strains screened in the North East Coast of Viet Nam, there were only three strains of G115, G119, G120 being resistant to *P. aeruginosa* ATCC27853, and G057 being resistant to *S. enterica* ATCC 13076 [18]. This result shows that the biological activity of the strains depends very much on geographic location

during sample collection. The different sensitivity between Gram positive and Gram negative bacteria could be explained by different in membrane structure of these microorganisms. Gram negative bacteria have an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes. The gram positive bacteria would more susceptible due to having only an outer peptidoglycan layer which is not an effective permeability barrier [19, 20].

*Table 1.*Antimicrobial activity of crude extracts of ethyl acetate extracts from 7strains.

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
1	G212	128	-	128	-	-	64	256
2	G222	256	64	64	-	128	128	128
3	G233	256	128	64	-	128	64	64
4	G227	256	128	128	-	128	128	128
5	G241	256	256	64	-	256	64	64
Streptomycin		*256*	*256*	*128*	*32*	*256*	*128*	-
Cycloheximide		-	-	-	-	-	-	*32*

Units for concentration of crude extracts is MIC (µg/mL)

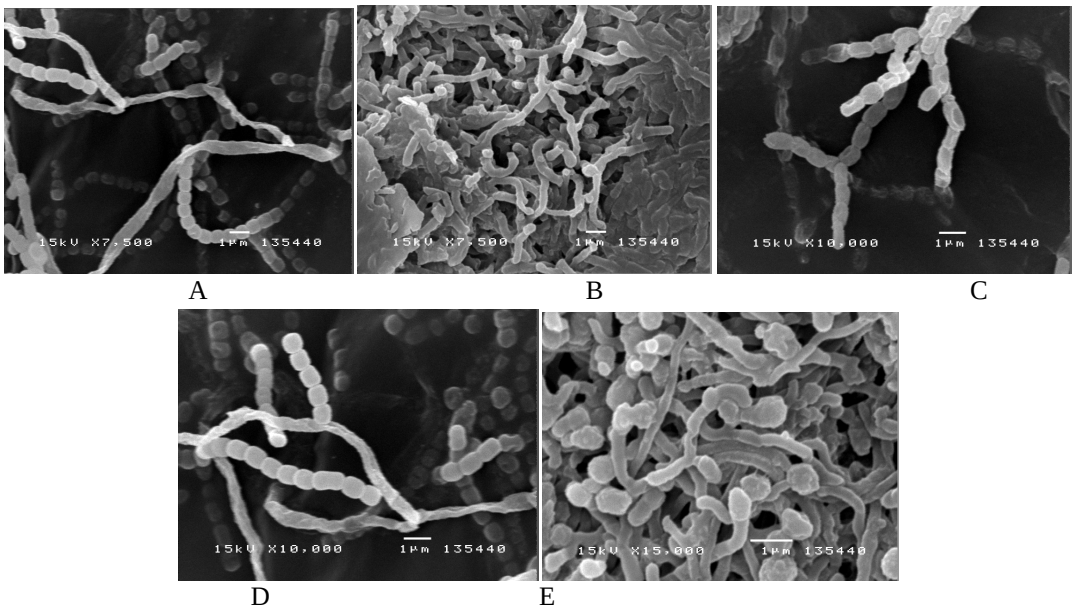

Figure 1. Scanning electron micrographs of the representative strains G212(A); G233 (B); G222 (C); G227 (D) and G241(E) grown on SCA agar for 2 weeks at 30 °C.

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 similar with the reported by Mukherjee and Sen [21] (Figs.1A, 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 motile (Figs.1B, 1E).

The colors of the substrate mycelium were white to vivid orange and turned to brownish black after sporulation (Fig. 2). The morphological characteristics of these isolates were consistent with their classification in the genus [22].

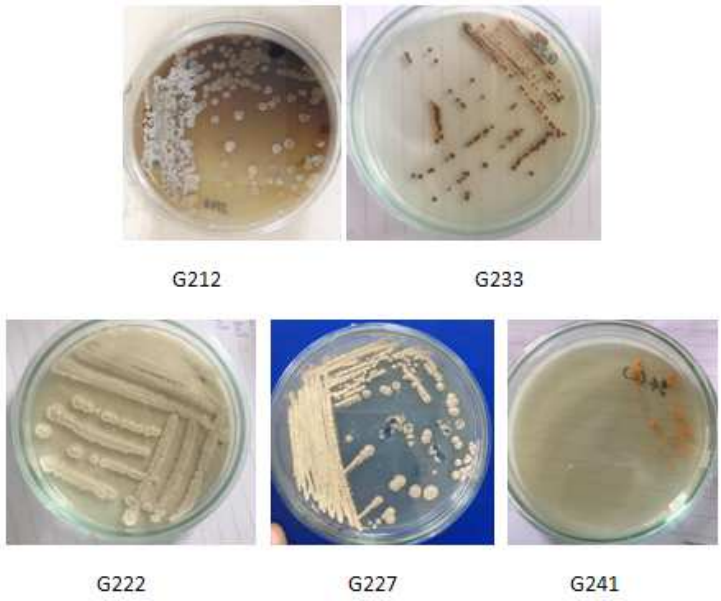

*Figure 2.*Morphological appearance of isolates. The colors of the substrate mycelium were vivid orange (G241) and from white (G222, G227) turned to brownish (G212, G233).

Five potential isolates were selected for identification by 16S rDNAgene sequencing. The 16S rDNA genes were amplified by PCR with compositions as above. PCR products were verified by agarose gel electrophoresis 1 %. The result of electrophoresis image showed a specific band with a size about 1500 bp, corresponding theoretical size 16S rDNA gene of specific primers 9F and 1541R (Fig 3). After analysis of obtained sequences by Bioedit programme, sequences of 5 isolates G212, G222, G227, G233 and G241 were revealed with the size 1443 bp, 1443 bp, 1434 bp, 1436 bp and 1433 bp, respectively. Compare these sequences on GenBank database, the results showed that 16S rDNA sequences of G212, G222 and G227 strains exhibited high similarity (99 %) with Genus *Streptomyces* spp; Strains G233 and G241 were identified (99 % similarity) of 16S rDNA sequences with Genus *Micromonospora* spp in GenBank (Fig. 4).

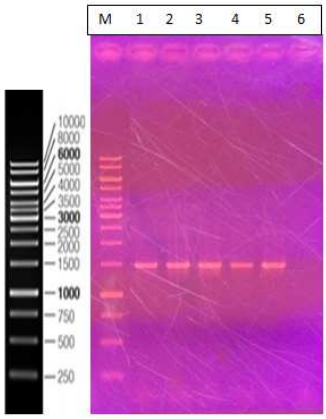

Figure 3. Electrophores is image of PCR products16S rDNA gene of isolates,
M: Marker 1Kb,
Lanes 1-5: PCR products of G212, G222, G227, G233 and G241 isolates,
Lane 6: PCR product control without DNA template.

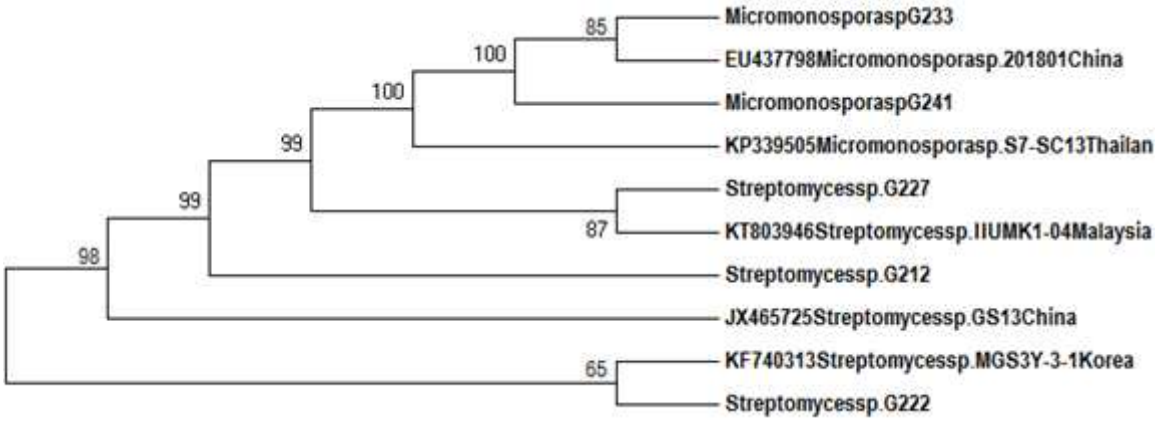

Figure 4. Neighbor-joining tree based on almost-complete 16S rDNA gene sequences showing relationships between the strains in groups and representative members of the genera *Streptomyces* and *Micromonospora* were used as an outgroup. The numbers on the branches indicate the percentage bootstrap values of 1,000 replicates; Bar, 0.01 substitutions per nucleotide position.

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 [23, 24, 25]. Another characteristic is of the genus is complex multicellular development, in which their germinating spores form hyphae, with multinuclear aerial mycelium, which forms septa at regular intervals, creating a chain of spores [26].

Micromonospora species are best known for synthesizing antibiotics, especially aminoglycoside gentamicin and netamicin), enediyne, and oligocharide antibiotics which contribute their impact medicinal usage [27]. Furthermore, *Micromonospora* species has been intensively investigated and isolated anticancer antibiotics such as anthraquinones, hracyclines, alkaloids, and macrolides [28, 29].

Several *Micromonospora* isolates were found to produce the bioactive compounds for example: The extract of *Micromonospora aurantiaca* with doxorubicin (DOX) were treated on human carcinoma of nasopharynx (KB cells). The result showed this combination caused 10-fold enhanced cell death at concentrations that each agent alone is poorly effective. The enhanced cytotoxicity of the combined treatment may result from augmentation of DOX-induced apoptosis by *Micromonospora aurantiaca* extract [30]. Diazepinomicin was isolated from the marine sponge-associated strain *Micromonospora* sp. RV115. Results showed that diazepinomicin exhibited antioxidant capacity using two different strategies including cell-free and cell-based assays. Diazepinomicin was able to protect cells from toxicity and genomic damage induced by the strong oxidant H_2O_2. This antioxidant activity will add a new perspective on the use of diazepinomicin in chemoprevention therapy for different types of cancer [31]. The results presented above confirmed that *Micromonospora*, especially marine *Micromonospora* have the potential to make an important contribution to fight against the pathogen.

4. CONCLUSION

From 40 samples including: sediments, sponges, soft corals, echinoderms and starfish collected from three sea areas of Vietnam: Thanh Hoa – Quang Binh – Quang Tri, we isolated 46 strains of actinomycetes. All most of the isolates exhibited antimicrobial activity.We chose 5 strains of actinomycetes that have the highest biological activity (Code: G212, G222, G233, G227 and G241). Specificly, strains G222, G233, G227 and G241 were resistant 6/7 strains of microorganisms test, with MICs values from 64 µg/ml to 256 µg/ml. In addition, all of the five strains were highly resistant to yeast *Candida albicans* ATCC10231. The morphological and phylogenetic investigations based on 16S rDNA gene sequences showed that: strains G212, G222 and G227 belonged to Genus *Streptomyces*; strains G233 and G241 were identified as Genus *Micromonospora*.

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