Vietnam Academy of Science and Technology

Vietnam Journal of Marine Science and Technology

journal homepage: vjs.ac.vn/index.php/jmst

Screening antimicrobial activities of actinomycetes raw extracts from marine ecosystems of Nha Trang and Van Phong Bay, Khanh Hoa Province

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Received: 26 May 2024; Accepted: 2 December 2024

ABSTRACT

Marine actinomyces' enormous genetic and functional diversity of has led scientist to search for potential secondary metabolite. Among the producers of important natural bioactive compounds discovered, actinomycetes are known to be efficient secondary metabolites with a broad spectrum of biological activities, such as antimicrobial, anticancer, and enzymes. The primary purpose of this study was to isolate and screen for antibacterial activity of potential actinomycete from marine ecosystems of Nha Trang and Van Phong Bay, Khanh Hoa Province, located in the middle-south of Vietnam. The marine actinomycete strains were characterized, and the ethyl acetate extracts were screened for antimicrobial activities using the microtiter broth dilution method. Out of which, the best MIC was produced by G112 and C011 strains. The lowest MIC values result in 16 µg/mL. G112 showed a zone of inhibition against four bacteria and *Candida albican*. C011 exhibited three different bacterial pathogens. The morphology and genetic results indicated that strain G112 belongs to *Streptomyces* species, and C011 was identified as a member of *Pseudonocardia* genus.

Keywords: Antimicrobial activity, bioassay, MIC, marine actinomycetes, *Pseudonocardia*.

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https://doi.org/10.15625/1859-3097/22149

INTRODUCTION

Vietnam has 28 coastal provinces and cities, a 3,260 km-long coastline from North to South with nearly 3,000 islands, and 2 archipelagos with over 20 types of ecosystems belonging to 6 different marine biodiversity regions. So, exploring organisms from Vietnam's sea will have a promising future and yield novel metabolites [1].

Many researchers worldwide have been working to find new genera and compounds from the marine habitat. Actinobacteria have clear potential benefits for humans as sources of novel bioactive compounds. Consequently, they are important in biotechnology, pharmaceutical, and other industries.

Some scientists have suggested that Organisms can survive in harsh environment because they are forced to change their genetic and metabolic diversity to withstand living conditions. Actinomycetes living in extremely harsh conditions may enhance the production of secondary metabolites that are diverse in both their structure and biological activity [2, 3].

Marine actinomycetes inhabit ecological niches of the ocean at different coordinates, such as water, sediments, sponges, seawater, corals, mangroves, seaweeds, plankton, plants, vertebrates, invertebrates, and others marine organisms even isolated from the deep ocean [4, 5].

Along with the advancement in science, technology and the development of advanced instruments have facilitated scientists' taking samples from difficult areas of the ocean, and microbial culturing can be carried out, and compounds from marine actinomycetes will continue to be discovered. In recent decades research and results on marine actinomycetes have increased markedly [2].

Research and application of marine microorganisms benefit humans by promoting sustainable development. Overexploiting marine macroorganisms can lead to resource depletion and ecological imbalance, while microorganisms can be cultured using growth medium on a large scale to generate metabolites.

MATERIALS AND METHODS

Chemicals: Chemicals and products used for molecular biology were purchased from Thermo Scientific, Qiagen (USA). Antibiotics (control) were from Sigma (USA). Microbial culture media were purchased from Hi-Media, India.

Cultivation media: A1, M1, ISP1, ISP2, PDA, NZSG were the culture media used to isolate and cultivate marine microorganisms. The pH of the culture was adjusted to 7.0.

Test bacterial strains: Seven different microbial (ATCC) cultures were used for the bioassay. Reference strains: 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 one fungus strain *Candida albicans* ATCC10231.

Marine samples: Marine samples were previously taken by SCUBA from different locations at different depths in the Nha Trang beach area and Van Phong Bay. The specimens were collected in autoclaved bottles with sterilized seawater and 30% glycerol. They were kept at 4 ^{o}C and shipped to the Institute of Marine Biochemistry for study. The samples were washed in sterilized seawater to remove dirty debris.

Marine microbial isolation: Marine samples (0.5 g weight or smear pattern) were crushed and diluted in sterilized seawater, homogenized by vortexing for 1 minute, and was treated at 60^{o}C for 6 min. These suspensions were diluted to tree serial, 1/1,000 dilutions. Aliquots of 50 µL were spread on isolation media. The petri plates were incubated at 30^{o}C for about 14 days. Isolated strains were observed every day. The colonies are believed to be actinomycetes and were purified through several rounds of transfer to fresh culture media [6, 7].

Extraction: In this stage, the actinomyces were *inoculated* into 50 L A1 broth medium flasks, pH 7.0, and raised at 30^{o}C for about 14 days, with 150 rpm. After two weeks of cultivation, the culture broths were filtered by filter paper (thickness 0.35–0.5 mm, particle

retention 3 µm) and then extracted with ethyl acetate (5 times × 15 minutes) [8].

Antimicrobial assay: The microtiter broth dilution method determined minimum inhibitory concentration (MIC) values according to the published method. Stock solutions of crude extracts were prepared in dimethylsulphoxide (DMSO) at 1% concentration (10 mg/mL DMSO). Streptomycin and nystatin were used as positive controls. Test microbials were prepared overnight and diluted in LB broth, the turbidity was adjusted to 0.5 Mc Farland standard solution. The antimicrobial activity of extracts or isolated compounds was evaluated against 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* ATCC14579) and one yeast strain *Candida albicans* ATCC10231). The from the American Type Culture Collection (ATCC) obtained the reference strains. Mueller Hinton Broth (MHB) was used to activate of test bacteria, while Luria Broth (LB) was used for the *Candida albicans*.

The microbial solutions were prepared to give a final 2×10^5 CFU/ mL concentration. The antimicrobial assays were carried out in 96-well microtiter plates containing LB broth - supplemented with the crude extracts at different concentrations ranging from 256 µg/mL to 2 µg/mL. After 24 h of incubation, the number of colonies was counted in the first clear well, displaying no visible (This is accomplished by spreading cultures onto each appropriate agar plate). The MIC was recorded as the lowest compound concentration that killed 95–99% of the bacteria/yeast compared to that in the initial well. All experiments were done in three times, in duplicates [9, 10].

Identification of marine actinomyces: Actinomyces strains were grown for about 14 days at 30°C in A1 broth. The sequencing 16S rRNA method was used for identification of potential strains. The 16S rRNA gene with highly conserved sequences is approximately 1.5 kb. Polymerase chain reactions were carried out in 25 µL volumes, containing 2.0 µL of total DNA, 8.5 µL of ddH_2O, 12.5 µL master mix, 1.0 µL (10 pmol/µL) for each primer 16sF: 5'- GAGTTT GATCCTGGCTCAG-3', 16sR: 5'- AAGGAGGTGA TCCAACC-3').

The reaction had been programmed to preheat at 94°C for 3 mins, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 30 seconds and elongation at 72°C for 1 min 20 seconds before a final extension of 72°C for 10 mins. 7 µL of PCR products was visualized by electrophoresis in 1% agarose gels and with ethidium bromide staining. PCR products were purified by QIAquick PCR Purification Kit (Qiagen) and sent for sequencing. DNA Analyzer performed the 16S rRNA gene sequencing (ABI PRISM 3100, Applied Bioscience). The sequencing results were handled by BioEdit 7.2 and compared with bacterial 16S rRNA sequences in the GeneBank database by the NBCI Blast program [6, 11].

RESULTS AND DISCUSSION

Isolation of marine actinomyces

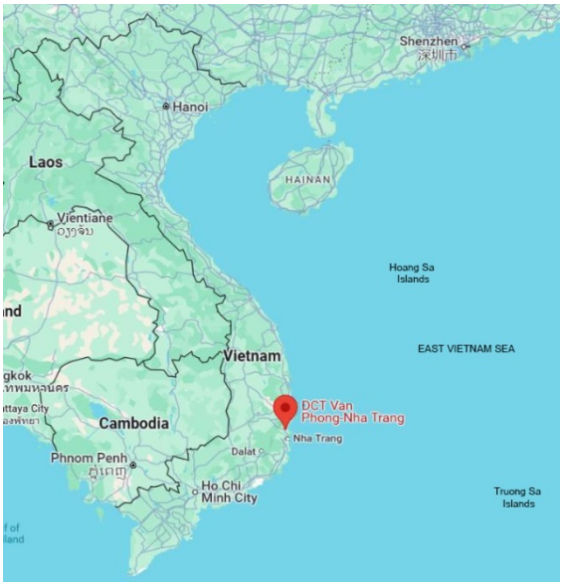


Figure 1. Locations of study area

Initially, on the agar plates, colony types of actinomycete were distinguished based on colony morphology, which can be observed with the naked eye (color, surface, shape, size, texture, effect on growth medium, etc.). Typical

actinomycete colonies adhere to the agar surface with distinct powdery, tough, dry, or fold, often spore-forming, branching filaments or hyphae characteristics [7]. Details of sampling locations and collection parameters are presented in Figures 1, 2 and Table 1.

Figure 2. Some marine samples collection

Table 1. The details of marine samples collection

No.	Latitude (N) - Longitude (E)	Depth	Host	Sample symbol
Nha Trang Bay - Khanh Hoa Province June 15, 2020				
1	12.21596°N - 109.22281°E	26 m	Sediment	7a
2	12.23448°N - 109.23945°E	15.3 m	Sediment	8a
3	12.3589°N - 109.25412°E	16.3 m	Sediment	9a
4	12.23703°N - 109.26930°E	17.4 m	Sediment	10a
5	12.22892°N -109.27676°E	10 m	Sediment	12a
6	12.22898°N - 109.27689°E	7 m	Sediment	13a
7	12.22898°N - 109.27689°E	7 m	Soft coral	17b
8	12.23165°N - 109.24277°E	13 m	Sediment	20c
9	12.23199°N - 109.24356°E	7 m	Sediment	20f
10	12.23199°N - 109.24356°E	7 m	Sponge	20m
11	12.23199°N - 109.24356°E	7 m	Sponge	20q
12	12.23199°N - 109.24356°E	7 m	Sponge	20o
13	12.23199°N - 109.24356°E	7 m	Soft coral	20p
Van Phong Bay - Khanh Hoa Province June 20, 2020				
14	12.67758°N - 109.22717°E	7 m	Crinoid	6n
15	12.65647°N - 109.24252°E	10.5 m	Sediment	23a
16	12.63074°N - 109.26694°E	20 m	Sediment	24a
17	12.63074°N - 109.26694°E	5 m	Sponge	26r
18	12.63074°N - 109.26694°E	5 m	Sediment	26q
19	12.63074°N - 109.26694°E	8 m	Sediment	27q
20	12.63074°N - 109.26694°E	7 m	Sponge	27m

Screening for antimicrobial activity of marine actinomyces

Results for antimicrobial activity of the actinomyces raw extracts are displayed in Table 2.

According to the MIC values in Table 1, we appreciated activity against bacteria and yeast of some strains. The crude extracts had MICs ranging from 8–256 µg/mL on 7 test microbial strains. The isolate extract also demonstrated quite good antibacterial activity against gram (+) and yeast with MIC even equal with Nistatin (for *Candida*). C011 and G112 were the most prominent and showed significant antimicrobial activities. Thus, these two isolates with the best activities were chosen for further studies. The extract of G112 inhibited 5 out of 7 test microbes, including two negative bacteria *Escherichia coli* ATCC25922, *Salmonella enterica* ATCC13076), two positive bacteria (*Enterococcus faecalis*

ATCC29212, *Staphylococcus aureus* ATCC25923, one fungus (*Candida albicans* ATCC10231). The C011 strain also exhibited antibacterial activity on 4 of 7 test microorganisms.

Table 2. Antimicrobial activity of crude ethyl acetate extracts from 35 actinomyces strains

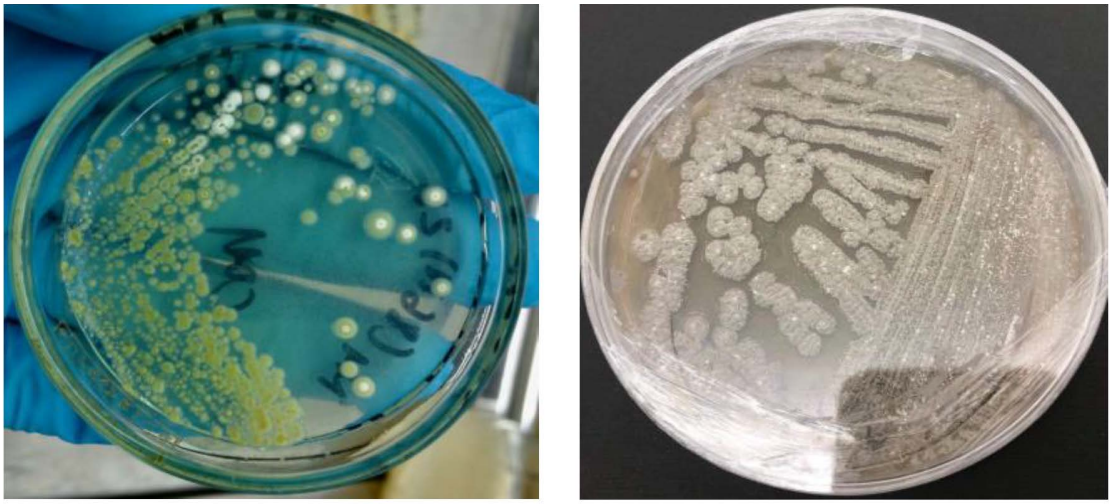
No.	Sample	MIC (µg/mL)						
		Gram +			Gram -			Yeast
		E. faecalis	*S. aureus*	*B. cereus*	*E. coli*	*P. aeruginosa*	*S. enterica*	*C. albicans*
Samples from Nha Trang beach area								
1	G042	-	256	-	-	256	-	-
2	G046	256	-	128	-	-	-	128
3	G048	-	-	-	-	-	-	-
4	G054		-	256	-	-	-	-
5	G058	256	-	-	-	-	-	-
6	G059	128	-	128	-	-	-	32
7	G063	-	-	-	-	-	-	-
8	G066	-	-	-	-	-	-	-
9	G069	-	-	-	-	-	-	-
10	G080	-	-	256	-	-	-	-
11	G085	256	-	-	-	-	-	-
12	G086	-	-	-	-	-	-	-
13	G088	-	-	-	-	-	-	-
14	G089	-	-	-	-	-	-	-
15	G090	-	-	-	-	-	-	-
16	G092	256	256	128	-	-	-	-
17	G093	128	256	-	-	-	-	64
18	G095	-	-	-	-	-	-	-
19	G096	256	256	128	-	-	-	256
20	G097	-	-	-	-	-	-	
21	G102	-	128	-	-	-	-	-
22	G103	-	-	-	-	-	-	-
23	G106	-	-	-	-	-	-	-
24	G109	64	-	256	-	-	-	16
25	G111	-	-	-	-	-	-	
26	G112	128	64	-	64	-	128	64
Samples from Van Phong Bay								
27	C010	-	-	-	-	-	-	8
28	C011	64	128	64				32
29	C012	32	-	64	-	-	-	16
30	C013	128	256	-	-	-	-	64
31	C014	-	-	-	-	-	-	-
32	C015	-	256	-	-	-	-	
33	C016	-	-	-	128	-	-	-
34	C017	-	64	-	-	-	-	-
35	C018	-	-	-	-	-	-	-
Streptomycin		256	256	128	32	256	128	-
Nistatin		-	-	-	-	-	-	8

Notes: MIC: values are the means of three trials which did not show any variation; (-): not active. Streptomycin is used as reference antibiotics for bacteria and nystatin for yeast.

Identification of isolates

The Gram-positive cells of strains G112 and C011 grew well on the A1 agar medium. C011 tends to form small spherical colonies, about 2 mm in diameter, deeply embedded in the agar.

G112 forms grey-brownish to brown-blackish colonies on A1 agar, with branched substate and short aerial mycelia. The colonies are medium sized (2–3 mm in diameter). Brown diffusible pigment was observed on the G112 agar plate. Their colony image was shown in Figure 3.



A B

Figure 3. Colonies after culturing for 14 days at 30°C. (A) Colonies of strain C011 are white yellowish to yellow, hard and adhere very firmly to the agar. (B) Slowly growing, brown G112 colonies with powdery surface on A1 agar plate

Two candidate actinomyces strains were identified based on amplification and sequencing of the 16s region of the rRNA gene.

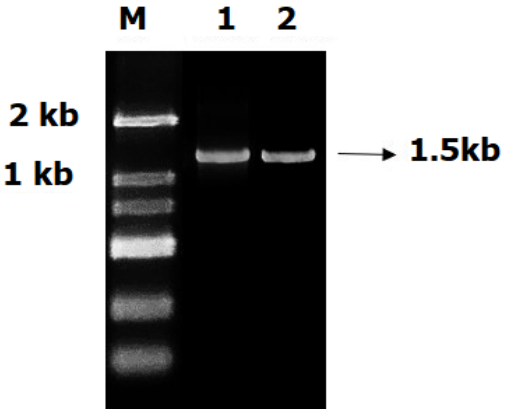


Figure 4. Agarose gel electrophoresis analysis of 16S rRNA genes amplified from two isolates Lane M: the DNA ladder; Lane 1: Strain G112; Lane 2: Strain C011

The PCR products on agarose gel showed DNA bands with approximate sizes of 1,500 bp (Fig. 4). Whole DNA extracts were purified and sequenced. Gene sequences were analyzed and processed using Bioedit software. The nearly complete 16S rRNA gene sequence of strains G112 (1,366 bp) and C011 (1,430 bp) were determined and compared with corresponding sequences in Genbank databases. The isolate G112 belongs to the genus *Pseudonocardia*, showing the highest levels of similarity concerning *Pseudonocardia kunmingensis* strain YIM 63158, Accession NR_108391.1 (99.56%), *Pseudonocardia sichuanensis* strain KLBMP 1115 NR_117801.1 (99.34%). A comparative study of the C011 gene sequence revealed 99.09% identical to *Streptomyces griseobrunneus* strain NBRC 12775, Accession NR_112577.1, *Streptomyces cavourensis* strain NBRC 13026, NR_112345.1 (99.09%). 16S rRNA gene sequences of bacterial isolates identified in this

study were submitted to GenBank with the following accession numbers: OR686186.1 (*Pseudonocardia* sp. strain G112) and OR686180.1 (*Streptomyces* sp. strain C011). The phylogenetic tree was created based on the 16s rRNA gene sequences by MEGAX (Fig. 5).

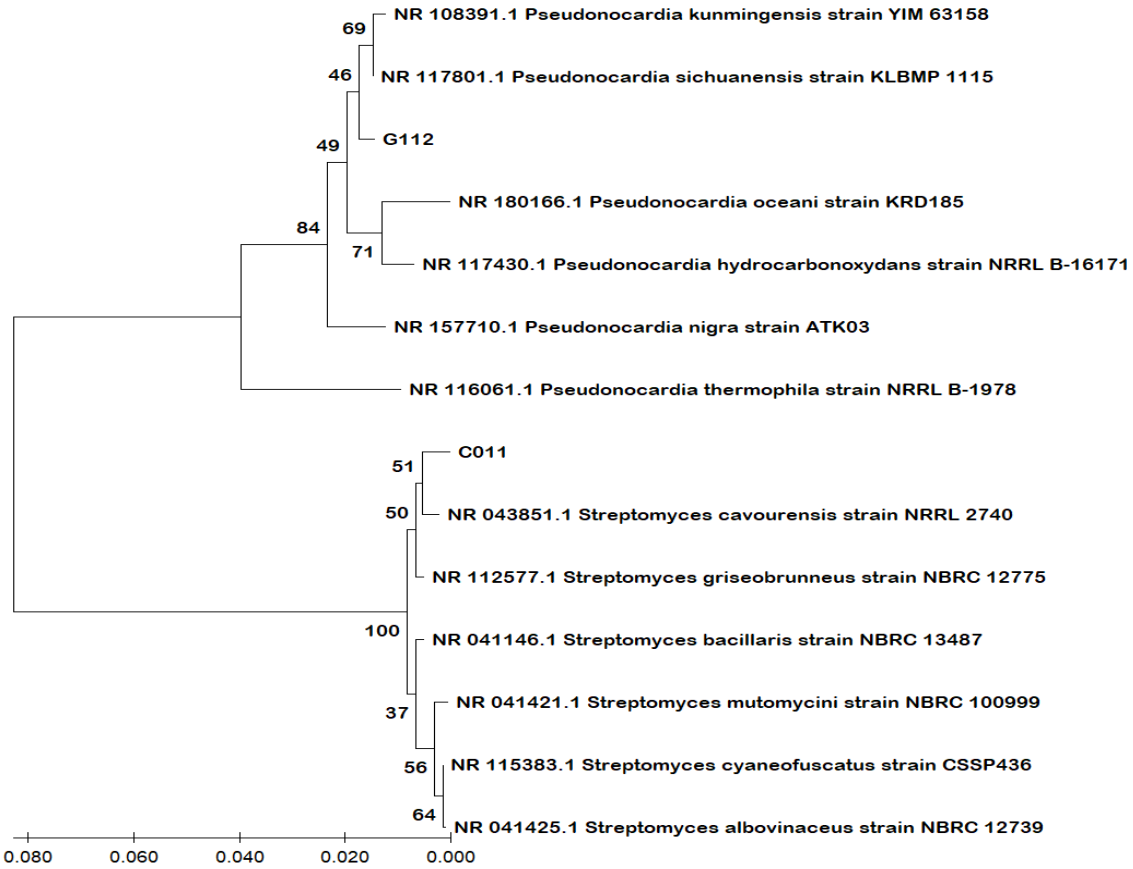


Figure 5. Phylogenetic tree of strains C011 and G112 and related type strains within *Streptomyces* and *Pseudonocardia* based on partial 16S rRNA gene sequences

The evolutionary history was inferred using the Neighbor-Joining method. 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 tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. 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 14 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 480 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [11].

The marine *Streptomyces* genus has long been considered a precious source of marine medicinal compounds and has been exploited for many years. Marine streptomyces are proficient in producing a large of bioactive compounds with essential activities. EtOAc-Ex of *Streptomyces* sp. KS20 from marine ecosystems of Devbagh and Tilmati beaches, Karwar expressed antioxidant activity, significant antimicrobial, and against A549 and PC-3 cell lines with low MIC values that could be developed into medical treatments [6].

Although the genus *Pseudonocardia* has not been widely reported for its medicinal

applications, however, it has been found to produce some of secondary metabolites with anti-bacterial, anti-fungal and anti-tumor properties. Several antibiotics were produced by some genera in the family *Pseudonocardiaceae* (e.g., rifamycin [13], erythromycin [13], and vancomycin [14]). The deep-sea water-derived actinomyces *Pseudonocardia carboxydivorans* M-227, produced Branimycins B and C. These antibiotics exhibit perfect antibacterial activities against a panel of Gram-positive and Gram-negative bacteria, even drug-resistant bacteria [15].

CONCLUSION

We have been exploring actinomyces from the different marine ecosystems of Khanh Hoa province to discover bioactive compounds. Out of 35 strains, isolated from Van Phong Bay, crude extracts of 20 strains exhibited some antagonistic activities against at least one tested strain of pathogenic microorganisms. Some strains even exhibited the lower MIC values than the control.

Amongst the strains investigated in this study, the C011 and G112 displayed superior activity than the whole strains; their ethyl acetate crude extracts had good potential against some ATCC pathogenic microbes (*E. coli*, *B. cereus*, *S. enterica*, *E. faecalis*, *S. aureus*, and *C. albicans*). A phylogenetic tree based on 16S rRNA gene sequences showed the relationship of strain G112 with members of the genus *Pseudonocardia*. At the same time, the isolate C011, with white yellowish to yellow colonies, belonged to the genus *Streptomyces*.

Acknowledgements: This research was funded by the Fogarty International Center of the National Institutes of Health under Award Number D43TW010530.

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