EXPLORING THE ANTIBACTERIAL POTENTIAL OF MARINE ACTINOBACTERIA ISOLATED AT DIFFERENT SEDIMENT DEPTHS IN WESTERN VISAYAS ISLAND

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ABSTRACT

The resistance of opportunistic pathogens to available antibacterial drugs has emerged as a public health threat in a global scale at a terrifying rate, leading to a continuous demand for new antibiotics. Soil dwelling actinobacteria have been known as source of antibiotics. Currently, exploring actinobacteria from marine sediment, particularly the less studied deep marine sediments unravels the mechanisms of adaptation to extreme conditions and may represent untapped reservoirs of unique secondary metabolites and sources of novel natural products with potential antibacterial activities. Thus, this study aimed to evaluate the antibacterial activities of actinobacteria isolated at various depths of marine sediments from Western Visayas island. At ~100 feet depth, marine sediment samples were collected by SCUBA using a sediment core sampler. The marine sediments were categorized into surface, subsurface, middle, sub-bottom, and bottom sediments based on the increasing depth of sediments in the core. In total, 180 actinobacteria isolates were recovered from marine sediments using selective marine minimal media. These isolates were initially screened for antibacterial activities by resazurin reduction assay against Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Staphylococcus aureus* ATCC BAA-44) and Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* Biotech 1754, *Pseudomonas aeruginosa* ATCC 27853 and *Enterobacter aerogenes* ATCC 13048). Thirty-four isolates showed bioactivity out of 180 isolates (39%) initially screened for antibacterial activity. The highest bioactivity were exhibited by isolates recovered from the bottom sediments (~91-110 cm below the seafloor).

To confirm the bioactivity of the 34 active isolates in terms of growth inhibition, microbroth susceptibility assay was performed using the biomass extracts of isolates, followed by the determination of Minimum Inhibitory Concentration (MIC). Eleven isolates showed more than 90% growth inhibition against the test bacteria, with USADSD481 being the most active isolate with MIC values of 19.53 and 4.88 μg/mL against *S. aureus* ATCC 25923 and the multidrug resistant strain *S. aureus* ATCC BAA-44, respectively. Our findings revealed that actinobacteria isolated at different depths of marine sediments from Western Visayas island represent a promising source of antibiotic leads.

Keywords: marine actinobacteria, antibacterial activity, sediment depths, Western Visayas island, multidrug resistant

INTRODUCTION

The search for new microorganisms that produce new or novel secondary metabolites is expected to remain significant in the race against new and emerging diseases and antibiotic resistant pathogens. (1) The spread of multidrug resistant pathogens is producing a global health threat, as we are witnessing the emergence of pathogens resistant to many available antibiotics. In fact, it is estimated that there will be ~10 million deaths globally by 2050 due to infections by antibiotic resistant pathogens. (2) Moreover, the pipeline for the development of new antibiotics is running dangerously low, thus, underscoring the urgent need to find novel and effective antibiotic leads.

Among clinically available antibiotics, 50% of these antibiotics are overused or misused, which led to resistance to clinically available antibiotics. (3) The Philippines is one of the Southeast Asian countries that faces many challenges associated with antibacterial resistance, including increased social and economic costs and rising patient mortality. (4) The Philippine antibacterial resistance data for 2016 indicated that *Klebsiella pneumoniae*, *Escherichia coli,* and *Pseudomonas aeruginosa* were the most common isolated antibiotic resistant bacteria. (5) The resistance to antibiotic agents is achieved through multiple biochemical pathways, and one bacterial cell may be capable of using multiple mechanisms of resistance to survive the effect of an antibiotic.

(6) For example, fluoroquinolone (FQ) resistance can occur due to three different biochemical routes, specifically mutations in genes encoding the FQ target sites, over-expression of efflux pumps and the protection of FQ target sites by protein designated quinolone resistance, all of which may coexist in the same bacteria at a given time. (7) Another example is the production of β-lactamases that is the predominant mechanism of resistance to β-lactams in Gram-negative bacteria, whereas resistance β-lactams in Gram-positive organisms is mostly achieved by modifications of their target site, the penicillin-binding proteins (PBPs) that can be passed both horizontally and vertically to their progeny. (8)

The rising number of multidrug resistant pathogens and the drying up of pipeline for development of new antibiotics clearly demonstrate that a renewed interest in search for new antibiotics is needed. While majority of the clinically available antibiotics were discovered from terrestrial soil dwelling bacteria, bacterial diversity from new environmental niches such as marine ecosystems have yet to be explored. Marine ecosystems are unique environments, characterized by high salinity and pressure, low temperatures, limited nutrients, and variable oxygen concentrations. Moreover, marine sediment-derived microorganisms experiencing these extreme conditions differentiate themselves from their terrestrial counterpart, which is reflected by the diversity of their genetic and metabolic make up. (9) In addition, marine sediments have been shown to harbor billions of diverse microorganisms that could be utilized as source of bioactive natural products. Thus, it is critical that new groups of microbes from unexplored habitats be pursued as sources of novel antibiotics. (10) In general, marine sediment-derived actinobacteria are widely studied for antibacterial, as well as anticancer activities (11), in response to the emerging antibacterial resistance. (12) Sporalactams, a novel ansa macrolide antibiotics isolated from cultures of a *Micromonospora* sp. from a marine sediment, are comprised of unprecedented macrolide with heterocylic core and 14-membered ansa bridge that was shown to be selective and potent inhibitors against *Mycobacterium tuberculosis*. (13) A class of polyketide compounds, nahuoic acids were isolated from marine sediment-derived *Streptomyces* sp. possesses a promising anticancer activity as inhibitor of the histone methyltransferase SETD8. (14) Another novel compound is novobiocin isolated from a *Streptomyces* sp. from the marine sediment sample collected from British Columbia, Canada. (15) Novobiocin is an antibiotic that targets bacterial gyrase by inhibiting the ATP hydrolysis. (16) In this scenario, representatives of marine sediment-derived actinobacteria are prominent resources of biologically active new natural compounds .

The occurrence of the primitive actinomycete was first described after the isolation of large number of strains referred as the MAR 1 from geographically diverse tropical and/or subtropical locations for which the generic epithet '*Salinospora*' was proposed (17), and the species *S. arenicola* and *S. tropica* (18). Tropical studies in marine sediments have reached the Pacific Ocean, specifically Guam, along with Bahamas, US Virgin Islands, Red Sea and Sea of Cortez as sites from where *Salinispora* strains from marine sediments have been recovered. (19) The *Salinispora* sp. has been well studied due to the anticancer metabolite, salinosporamide-A. (20) Aside from salinosporamide-A, other novel bioactive compounds extracted in actinobacteria from marine sediments are abyssomicin-C from *Verrucosispora* sp. (21), rifamycin from *Micromonospora* sp. and marinopyrroles from *Streptomyces* sp. (22)

In the present study, marine sediment-derived actinobacteria in the Philippines was proposed as resource for new antibiotics, specifically from the unexplored marine sediments of Western Visayas island. The extensive coast of Western Visayas is highly rich in marine resources and can be a promising biome for actinobacteria communities that can produce unique secondary metabolites. Currently, exploring actinobacteria in the less studied depths of marine sediments unravels the mechanisms of adaptation to extreme conditions and may represent untapped reservoirs of unique secondary metabolites and sources of novel natural products with potential antibacterial activities. Sediments found below the seafloor have reduced carbon and energy sources available for microorganisms leading to decreased microbial activity and lower cell densities. (23) Microorganisms, including actinobacteria, living in the sediments below the seafloor secrete enzymes to degrade organic matter deposited in the sediments for survival. (24) Thus, this study aimed to evaluate the antibacterial activities of actinobacteria isolated at depths from 0 to -110 centimeters below the seafloor of unexplored marine sediments from Western Visayas island.

MATERIALS AND METHODS

Environmental Sampling. Assessment of the possible sampling sites was done prior to sampling. The sampling sites must be away from the anthropogenic activities and populated area, no river run-offs and accessible by motor boat and SCUBA. Considering these criteria, two sampling sites in Western Visayas island were identified. At -100 feet depth from sea surface, sediments were collected by SCUBA using a 110-centimeter long sediment core sampler. The samples were placed in sterile tubes, properly labeled and categorized into 5 layers according to the depth of sediments that have accumulated in the core. Tubes 1 to 3 contained sediments from ~ 91-110 cm below the seafloor (bottom); Tubes 4-6 contained sediments from ~76-90 cm below the seafloor (sub-bottom); Tubes 7-9 contained sediments from ~51-75 cm below the seafloor (middle); Tubes 10-12 contained sediments from ~ 26-50 cm below the seafloor sediments (sub-surface) and; Tubes 13-15 contained sediments from ~ 0-25 cm below the seafloor (surface). A total of 30 marine sediment samples were collected in two different sampling sites with 15 samples from each sampling site, in Western Visayas island. The samples in tubes were secured in a zip lock container, and maintained temporarily on ice. The marine sediments were transported immediately to the laboratory and kept at 4°C prior to processing by two culture dependent techniques (15), dry stamp method (DSM) and heat shock method (HSM).

Isolation of Marine Sediment-Derived Actinobacteria. Samples were inoculated in five selective marine minimal (MM) media, with different carbon sources (Mannitol for MM3, Glucose for MM11, Trehalose for MM13, Raffinose for MM51, and Starch for ISP4) in artificial seawater (15), and autoclave for 15 min at 121˚C and 15 psi. The plates were incubated at room temperature (25 to 28°C) for 3 to 8 weeks and checked periodically for the growth of actinobacteria as indicated by the presence of aerial spores and a substratum mycelium, either with or without diffusible pigmentations. Actinobacteria growing on the isolation plates were transferred to marine medium 1 (MM1) (15), using sterile inoculating loops until they are pure. Pure cultures were maintained in glycerol at -80°C for long-term storage. (15)

ANTIBACTERIAL SCREENING

Initial Screening by Agar Overlay Assay using Resazurin Reduction Test. Each pure Actinobacteria isolate was incubated for 14 days in each well of a 96-well microplate containing MM1 broth that served as the template of seed cultures. One hundred microliters (μL) of actinobacteria isolate seed culture was transferred and grown in a separate 96-well microplate containing MM1 agar. The negative control was MM1 agar with no actinobacteria and the positive control was MM1 agar with 20 μL of tetracycline from the stock solution of 10 mg/mL. After 14 days of incubation, tetracycline was added to the positive control wells and all wells with actinobacteria isolates were overlaid with the test organism grown in 1% tryptic soy agar with an optical density of 0.0005 at 620 nm using a microplate reader. The actinobacterial isolates were screened for antibacterial activities, in triplicates, against *Staphylococcus aureus* ATCC 25923, multidrug resistant strain (MDR^+) *Staphylococcus aureus* ATCC BAA-44, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* Biotech1754, *Pseudomonas aeruginosa* ATCC 27853 and *Enterobacter aerogenes* ATCC 13048. The overlaid 96-well microplates were incubated for 24 hrs at 37˚C. After 24 hrs of incubation, resazurin (alamarBlue®) with a concentration of 0.2 mg/mL was added to each well of the 96-well microplates overlaid with the test organism. The wells that retained the blue color of rezasurin indicated dead cells while the reduction of resazurin to resorufin on pink wells indicated live cells. (25)

Confirmatory Assay using Microbroth Susceptibility Assay Testing (**15**). The biomass of active actinobacteria isolates was extracted in ethyl acetate for three days and concentrated *in vacuo* to dryness. The dried extracts were dissolved in dimethyl sulfoxide (DMSO) to create a stock solution with a final concentration of 100 mg/mL. The actinobacteria isolates were screened against the test organisms based on their initial antibacterial activities. The optical density of the bacterial suspension was adjusted to 0.0005 read at 620 nm using a microplate reader. Five μL of actinobacteria extract and 195 μL of bacterial suspension were loaded to a 96-well microplate. Dimethyl sulfoxide (DMSO) was used as negative control and tetracycline as the positive control. The experiments were done in three trials and three replicates. The final concentrations of treatment well and tetracycline were 2.5 mg/mL and 0.25 mg/mL, respectively. The 96-well microplates were incubated overnight for 18-24 hrs at 37˚C and growth inhibitions were measured by the absorbance at 620 nm using a microplate reader. The actinobacteria extracts that showed more than 50% growth inhibition against the test bacteria were considered active. The % growth inhibition was calculated using the equation,

% Growth Inhibition = ((OD negative control- OD treatment)/ OD negative control) * 100.

Minimum Inhibitory Concentration (MIC_{90}) Determination of Active Actinobacteria Extracts. Actinobacteria extracts with at least 90% growth inhibition were tested for the determination of their Minimum Inhibitory Concentration (MIC_{90}). The minimum inhibitory concentration (MIC_{90}) values were determined by the broth microdilution method against the test bacteria. Briefly, 2-fold serial dilutions of extracts were prepared in 96-well microplates from stock solutions in dimethyl sulfoxide (DMSO) to a final volume of 5 µL. The final concentrations tested were from 2500 to 4.88 µg/mL. Bacterial inoculum was prepared from 24 hrs cultures on tryptic soy agar (TSA) at 37°C. The inoculum was diluted into tryptic soy broth (TSB) medium to yield a final inoculum with an optical density of 0.0005 at 620 nm. The microdilution wells, which contained 5 µL of the serially diluted extracts, were inoculated with 195 µL of the resulting bacterial suspension. In the first column, 200 µL of TSB medium was delivered that served as the blank. The next column containing DMSO was used as controls, followed by the dilution of extracts. The assay was performed in three replicates and in three trials. The inoculated plates were incubated at 37°C for 18-24 hrs. The growth was determined by measuring the OD at 620 nm using a microplate reader. The MIC end point was defined as the lowest concentration with complete (90%) growth inhibition as previously reported by Dalisay *et al*. (15).

Characterization of the Most Bioactive Isolates by Scanning Electron Microscopy. After the qualitative and quantitative approaches of antibacterial screening, the spore morphology of the most bioactive isolates with more than 90% growth inhibition against the test bacteria were characterized by scanning electron microscopy. The 7-day actinobacteria culture was withdrawn from the MM1 medium. This was then followed by fixation with 25% glutaraldehyde solution, and stored in 4°C for 2 hrs. The sample was then precipitated using poly-L-lycine, oven dried for 10 min and washed with PBS. The sample was dehydrated by a series of gradient exchange using 30%, 50%, 85%, 95% and absolute alcohol, and stored again in 4°C for 10 min. Before mounting to the specimen metal stab for viewing, the sample was freeze dried for 30 min to remove the ice crystals, sublime the sample and avoid damage of cells. Once the sample was mounted, the metal stub was viewed on the Scanning Electron Microscope (JEOL JSM-5510LV) at an accelerating voltage of 25 kV and intact spore structures were then selected for examination at 12,000x magnification.

RESULTS

Isolation of Actinobacteria. After 6 weeks of incubation, a total of 180 actinobacteria isolates were recovered from 30 Western Visayas island sediment samples using DSM and HSM isolation techniques (Fig. 1) in five selective marine media (Fig. 2). The number of actinobacteria isolates recovered using DSM and HSM were relatively the same, with 89 and 91 isolates, respectively. Meanwhile, the actinobacteria isolates were recovered in five selective marine media (MM3, MM11, MM13, MM51 and ISP4), with varying carbon sources namely: mannitol for MM3; glucose for MM11; trehalose for MM13; raffinose for MM51 and; starch for ISP4. Most of the isolates recovered were grown in MM11 with 111 isolates (61%), followed by MM3 with 23 isolates (13%), MM13 with 21 isolates (12%), MM51 with 18 isolates (10%) and only 7 isolates (4%) from ISP4.

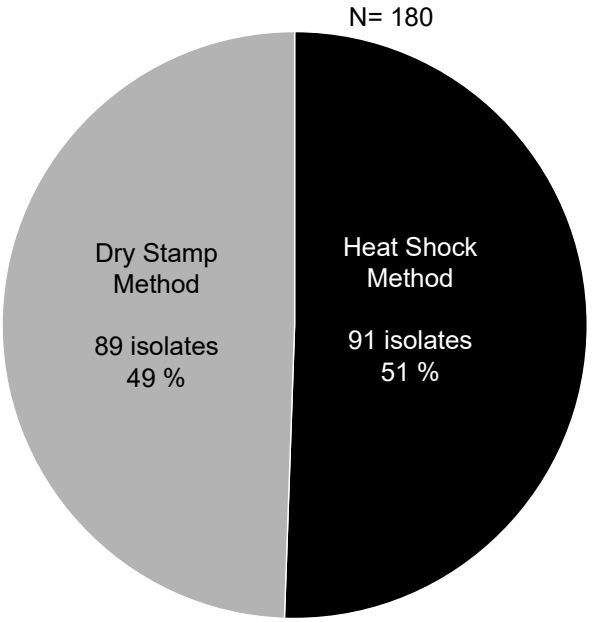

Figure 1. Recovery Profile of Marine Sediment-Derived Actinobacteria Using Two Culture-Dependent Techniques. Actinobacteria were isolated from marine sediments by dry stamp method (DSM) and heat shock method (HSM) in five selective marine media. From the 180 actinobacteria isolates recovered in 30 marine sediment samples from Western Visayas island, 91 isolates (51%) were obtained through HSM and 89 isolates (49%) were processed using DSM.

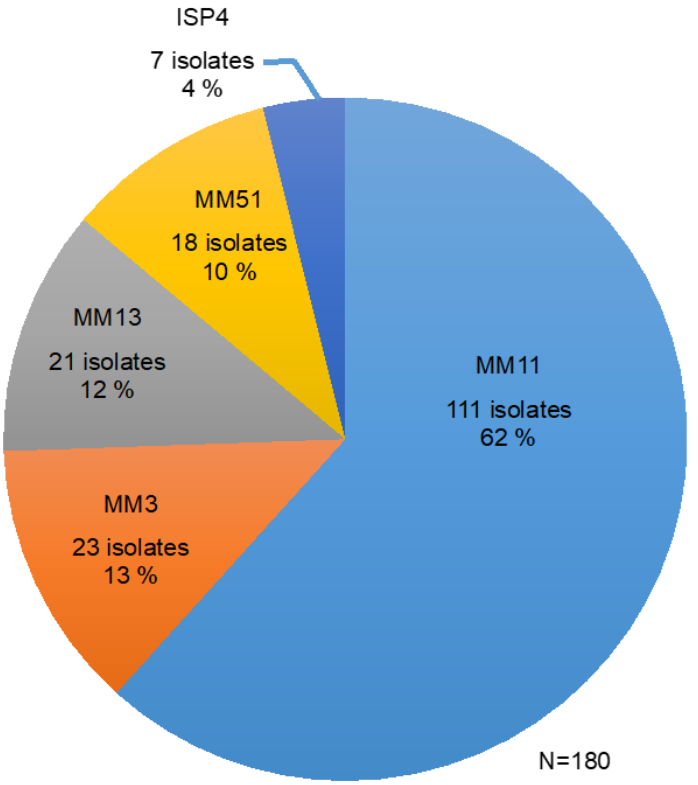

Figure 2. Recovery Profile of Marine Sediment-Derived Actinobacteria from Five Selective Marine Media. The isolation of actinobacteria from marine sediments was performed using DSM and HSM culture-dependent techniques in five selective marine media with varying carbon sources: mannitol for MM3; glucose for MM11; trehalose for MM13; raffinose for MM51 and; starch for ISP4. Out of the 180 actinobacteria isolates, most of the isolates were recovered from MM11 with 111 isolates (61%), followed by MM3 with 23 isolates (13%), MM13 with 21 isolates (12%), MM51 with 18 isolates (10%) and only 7 isolates (4%) from ISP4.

Based on the depth of the sediments (Fig. 3) that accumulated in the core, sub-bottom (76-90 cm below the seafloor) sediments had the highest yield of recovery with 68 isolates (37%), followed by the subsurface (26-50 cm below the seafloor) sediments with 42 isolates (23%); surface (0-25 cm below the seafloor) sediments with 35 isolates (19%); middle (51-75 cm below the seafloor) sediments with 21 isolates (11%) and; bottom (91-110 cm below the seafloor) sediments with 18 isolates (10%).

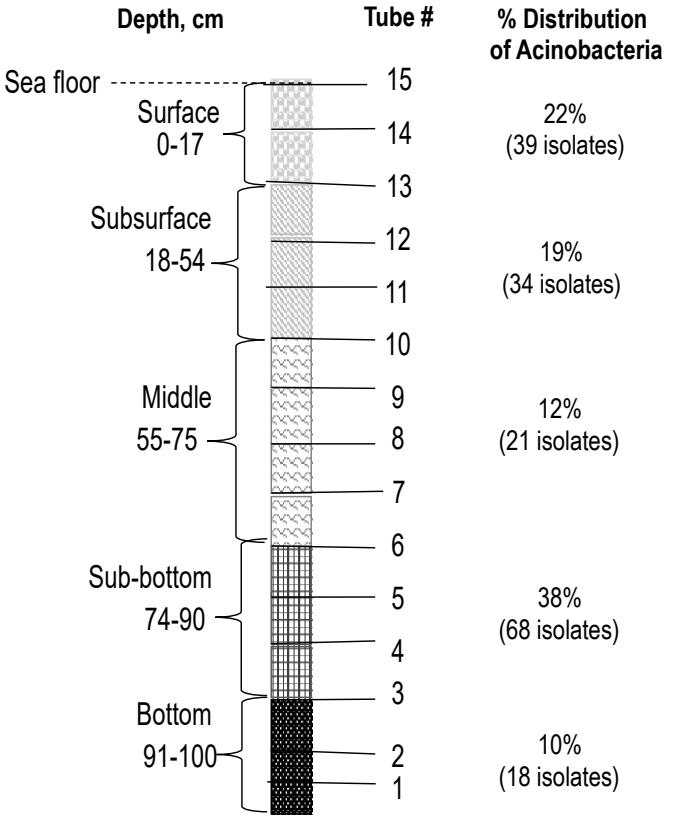

Figure 3. Actinobacteria Distribution Isolated at Different Marine Sediment Depths from Western Visayas Island. After 6 weeks of incubation and regular checking for actinobacteria growth, 180 actinobacteria isolates were recovered from marine sediment samples. The sub-bottom sediments (~-74-90 cm) yielded the highest number of Actinobacteria with 68 isolates (38%).

ANTIBACTERIAL SCREENING

Agar Overlay Assay using Resazurin Reduction Test. The first approach of evaluating the antibacterial activities of the actinobacteria isolates was the initial screening by agar overlay assay using resazurin reduction test. The isolates were screened against six test bacteria: two Gram-positive bacteria, *S. aureus* ATCC 25923 and *S. aureus* ATCC BAA-44 (MDR^+); and four Gram-negative bacteria, *E. coli* ATCC 25922, *K. pneumoniae* Biotech 1754, *P. aeruginosa* ATCC 27853 and *E. aerogenes* ATCC 13048. Our findings indicated that actinobacteria isolates recovered from the bottom layer (91-110 cm below the seafloor) had the highest number of active isolates (7) (Fig. 4) with a hit rate of 39% against the test bacteria, followed by 5 active isolates from middle (51-75 cm below the seafloor) sediments with a hit rate of 24%, then 8 isolates from the subsurface (26-50 cm below the seafloor) sediments with a hit rate of 21%, and a number of active isolates from sub-bottom (76-90 cm below the seafloor) and surface (0-25 cm below the seafloor) sediments with 10 (15%) and 4 (12%) isolates, respectively. Moreover, most of actinobacteria isolates have shown antibacterial activities against *S. aureus* ATCC 25923 (19 isolates) and the multidrug resistant strain *S. aureus* ATCC BAA-44 (17 isolates) (Fig. 5). Furthermore, 14 of the bioactive isolates against Gram-negative bacteria have shown activity against *E. coli* ATCC 25922.

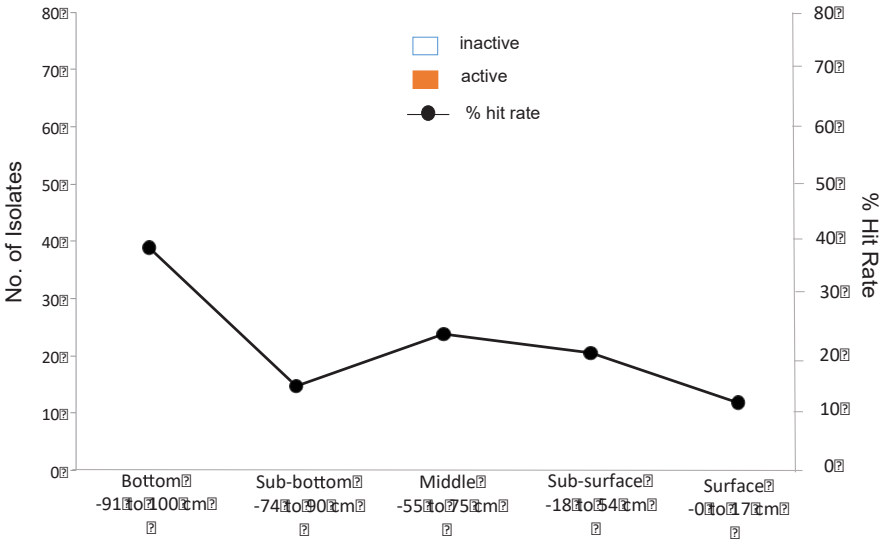

Figure 4. Marine Actinobacteria Isolates Evaluated for Antibacterial Activities Based on Sediment Depths. A total of 180 marine sediment-derived actinobacteria isolates from two sampling sites in Western Visayas Island were initially screened using agar overlay assay by resazurin reduction test against six test bacteria. Thirty-four isolates exhibited antibacterial activities and the highest number of bioactive isolates (7) were recovered from the bottom (91-110 cm below the seafloor) with a hit rate of 39%.

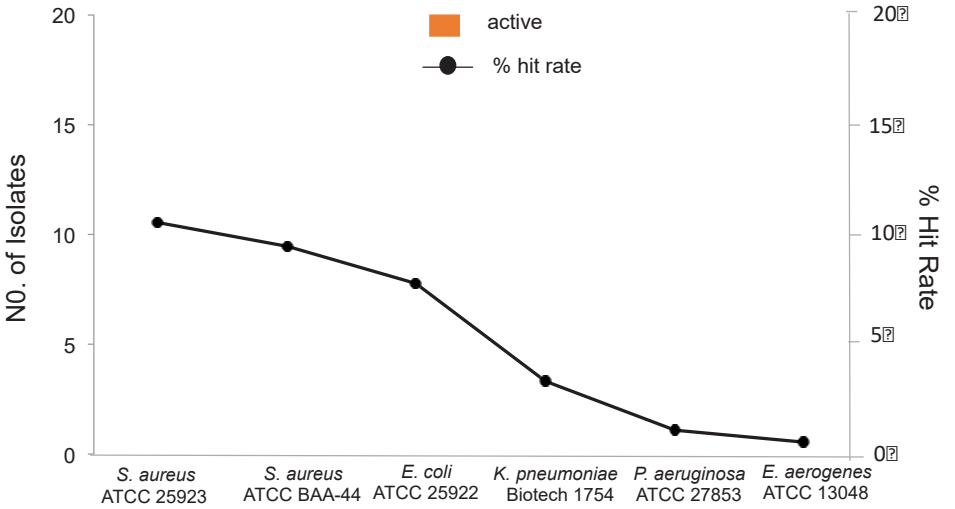

Figure 5. Antibacterial Activity and Hit Rate Profile of Actinobacteria Isolates Against Six Test Bacteria. The 180 actinobacteria isolates were initially screened for antibacterial activities by agar overlay assay using resazurin reduction test. The Gram-positive bacteria, *S. aureus* ATCC 25923 (19 isolates) and *S. aureus* ATCC BAA-44 (17 isolates) were more susceptible to the bioactive isolates with hit rates of 11% and 9%, respectively. For the Gram-negative bacteria, most of the bioactive isolates (14) have shown antibacterial activity against *E. coli* ATCC 25922 with a hit rate of 8%.

A total of 180 actinobacteria isolated at different marine sediment depths were screened for antibacterial activities by agar overlay assay using resazurin reduction test against the six test bacteria (Fig. 6). Thirty-four isolates displayed antibacterial activities against the Gram-positive bacteria (14), Gram-negative bacteria (20), and to both (7), which can be indicative of the potential of actinobacteria as antibacterial agents that can be derived from the marine sediments of Western Visayas island.

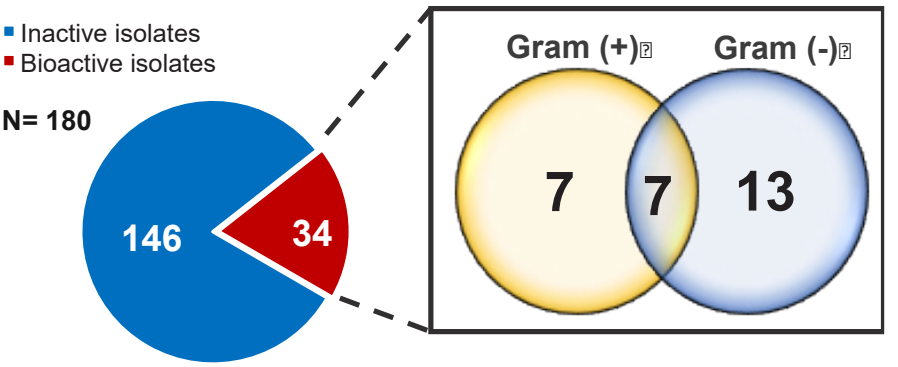

Figure 6. Initial Screening: Agar Overlay Assay Using Resazurin Reduction Test (3 trials). A total of 180 actinobacteria isolates were initially screened using agar overlay assay by resazurin reduction test. Thirty-four isolates retained the blue resazurin dye and were found active against the test bacteria; the Venn diagram shows that seven isolates were active only against Gram-positive bacteria, 13 isolates for Gram-negative bacteria only, and 7 were active to both Gram-positive and Gram-negative bacteria. This assay was performed in three replicates.

Microbroth Susceptibility Assay of the Initially Screened Active Isolates in the Resazurin Reduction Test. The next approach of evaluating the antibacterial activities of bioactive actinobacteria isolates is to quantify their antibacterial activities by microbroth susceptibility assay (15). The biomass of the 34 initially screened active isolates were extracted with ethyl acetate, concentrated to dryness *in vacuo*. The dried extracts were reconstituted with DMSO to make a final concentration of 100 mg/mL as stock culture. Actinobacteria extracts (2.5 mg/mL) were considered active if they exhibit more than 50% growth inhibition against the test bacteria. Out of the 34 initially screened active isolates, 21 (61%) actinobacteria isolates have confirmed antibacterial activities (Table 1). The isolates were only screened against the test organisms where they exhibited antibacterial activities in the initial screening. Bioactivity was exhibited by 19 isolates against the Gram-positive bacteria, *S. aureus* ATCC 25923 and *S. aureus* ATCC BAA-44, whereas five isolates were active against the Gram-negative bacteria, *E. coli* ATCC 25922. Interestingly, 12 isolates have shown more than 50% growth inhibition against the multidrug-resistant test bacteria, *S. aureus* ATCC BAA-44. Moreover, three isolates were found active to both Gram-positive and Gram-negative bacteria, *E. coli* ATCC 25922. None of the isolates showed antibacterial activities against *P. aeruginosa* ATCC 27853, *K. pneumoniae* Biotech 1754 and *E. aerogenes* ATCC 13048.

Regarding the depth on where the actinobacteria was isolated, most of the bioactive isolates were from the subsurface (26-50 cm below the seafloor) and bottom (91-110 cm below the seafloor) sediments, as shown also in their antibacterial activities in the initial screening. Further screening was done to the isolates that showed more than 90% growth inhibition by the determination of Minimum Inhibitory Concentration (MIC_{90}) values.

Table 1. Summary of Antibacterial Activities of the Bioactive Actinobacteria Isolates.

Isolate Code (N=21)	Sediment Depth, cm	% Growth Inhibition[#]		
		S. aureus ATCC 25923	*S. aureus* ATCC BAA-44	*E. coli* ATCC 25922
USADSD353	Bottom, 91-110	87 ± 2	*	*
USADSD357	Bottom, 91-110	*	94 ± 3	*
USADSD360	Bottom, 91-110	*	95 ± 1	86 ± 0.9
USADSD364	Bottom, 91-110	88 ± 1	*	*
USADSD484	Bottom, 91-110	67 ± 5	*	*
USADSD261	Sub-bottom, 76-90	90 ± 1	*	*
USADSD176	Sub-bottom, 76-90	*	92 ± 1	*
USADSD249	Sub-bottom, 76-90	52 ± 5	*	*
USADSD262	Middle, 51-75	86 ± 1	*	*
USADSD264	Middle, 51-75	*	*	82 ± 2
USADSD250	Subsurface, 26-50	57 ± 5	60 ± 5	59 ± 6
USADSD243	Subsurface, 26-50	80 ± 3	86 ± 2	-
USADSD251	Subsurface, 26-50	59 ± 8	59 ± 3	*
USADSD268	Subsurface, 26-50	90 ± 1	*	*
USADSD476	Subsurface, 26-50	90 ± 0.3	91 ± 0.1	*
USADSD481	Subsurface, 26-50	90 ± 0.7	92 ± 0.7	80 ± 1
USADSD757	Subsurface, 26-50	*	95 ± 0.2	*
USADSD292	Subsurface, 26-50	*	*	75 ± 2
USADSD164	Surface, 0-25	96 ± 1	92 ± 0.5	*
USADSD165	Surface, 0-25	*	96 ± 0.5	*
USADSD323	Surface, 0-25	96 ± 0.1	89 ± 0.3	*
Tetracycline		92 ± 8	100 ± 0.1	102 ± 0.4

Concentration of actinobacteria extracts = 2.5 mg/mL; positive control Tetracycline = 0.25 mg/mL

Performed in three trials and three replicates;

*, not tested because these isolates were inactive in the initial screening -, not active

[#], No actinobacteria isolates were found to be active against *P. aeruginosa* ATCC 27853, *K. pneumoniae* Biotech 1754 and *E. aerogenes* ATCC 13048.

Minimum Inhibitory Concentration (MIC_{90}) Determination of Active Actinobacteria Extracts. Five of the most bioactive actinobacteria isolates (USADSD164, USADSD176, USADSD261, USADSD357, USADSD481) with more than 90% growth inhibition were screened further by the determination of Minimum Inhibitory Concentration (MIC_{90}) values (Fig. 7). Gram-positive test bacteria were susceptible to the actinobacteria crude extracts. Notably, USADSD481 isolated from the subsurface (26-50 cm below the seafloor) sediments was tested for the determination of MIC_{90} values for the two Gram-postive bacteria, having the MIC values of 19.53 µg/mL and 4.88 µg/mL against *S. aureus* ATCC 25923 and the multidrug-resistant test bacteria, *S. aureus* ATCC BAA-44, respectively (Table 2). Two isolates from the sub-bottom (76-90 cm below the seafloor) sediments, USADSD176 and USADSD261 had MIC values of 39.06 µg/mL and 312.5 µg/mL against *S. aureus* ATCC BAA-44 (MDR^+) and *S. aureus* ATCC 25923, respectively. In addition, an isolate from the bottom (91-110 cm below the seafloor) sediments, USADSD357 has an MIC value of 625 µg/mL against the multidrug resistant test bacteria. Moreover, the colonies of these bioactive isolates were grayish in terms of aerial spores and from light to dark yellow diffusible pigmentations after 6 weeks of incubation.

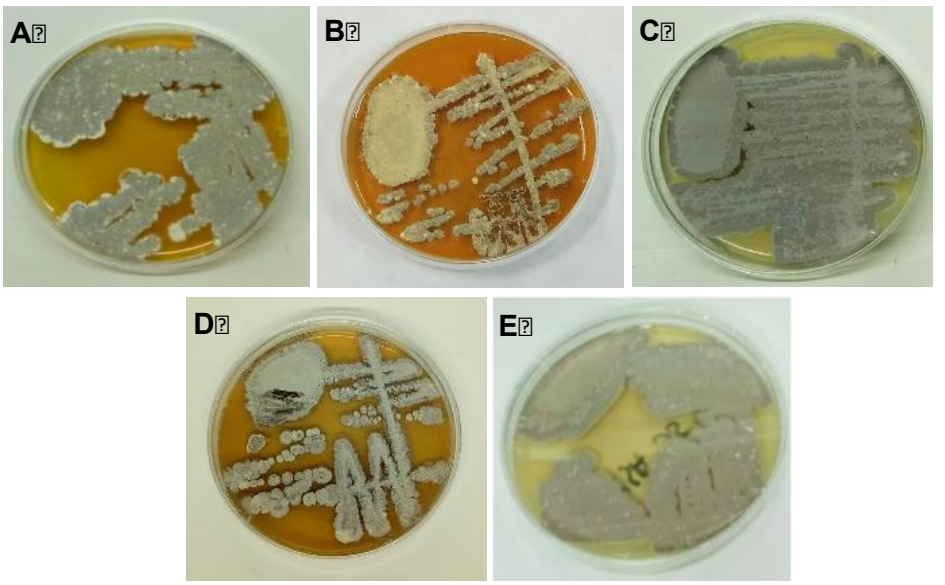

Figure 7. Bioactive Actinobacteria against *S. aureus* ATCC 25923 (A-B) and *S. aureus* ATCC BAA-44 (B-E) and their MIC values (µg/ mL). **A**) USADSD261, 312.5 µg/mL; **B**) USADSD481, 19.53 µg/mL; 4.88 µg/mL **C**) USADSD164, 625 µg/mL; **D**) USADSD176, 39.06 µg/mL; **E**) USADSD357, 625 µg/mL

Table 2. Bioactive Actinobacteria Isolated from Different Sediment Depths from Western Visayas Island and their MIC Values.

Isolate Code	Sediment Depth, cm	Isolation Technique Used	Isolation Media Used	Description of the Colonies	MIC Value (µg/mL)	
					S. aureus ATCC 25923	*S. aureus* ATCC BAA-44
USADSD481	Subsurface, 26-50	HSM	MM11	Grayish spores with dark yellow diffusible pigment	19.53	4.88
USADSD176	Sub-bottom, 76-90	HSM	MM3	Grayish white spores with dark yellow diffusible pigment	*	39.06
USADSD261	Sub-bottom, 76-90	DSM	MM13	Grayish spores with dark yellow diffusible pigment	312.5	*
USADSD357	Bottom, 91-110	HSM	MM11	Grayish spores with light yellow diffusible pigment	*	625
USADSD164	Surface, 0-25	DSM	MM11	Grayish spores with light yellow diffusible pigment	*	625

Performed in three trials and three replicates

*, not tested because these isolates were inactive in the initial screening

Characterization of the Most Bioactive Isolates by Scanning Electron Microscopy. Scanning electron microscopy was performed to study spore morphology of one of the most bioactive isolates, USADSD176, with an MIC value of 39.06 µg/mL against the multidrug-resistant strain *S. aureus* ATCC BAA-44. The 7-day old USADSD176 was observed to be approximately 0.7 µm in size with a smooth spore surface morphology and occurred in chains under 12,000x magnification (Fig. 8).

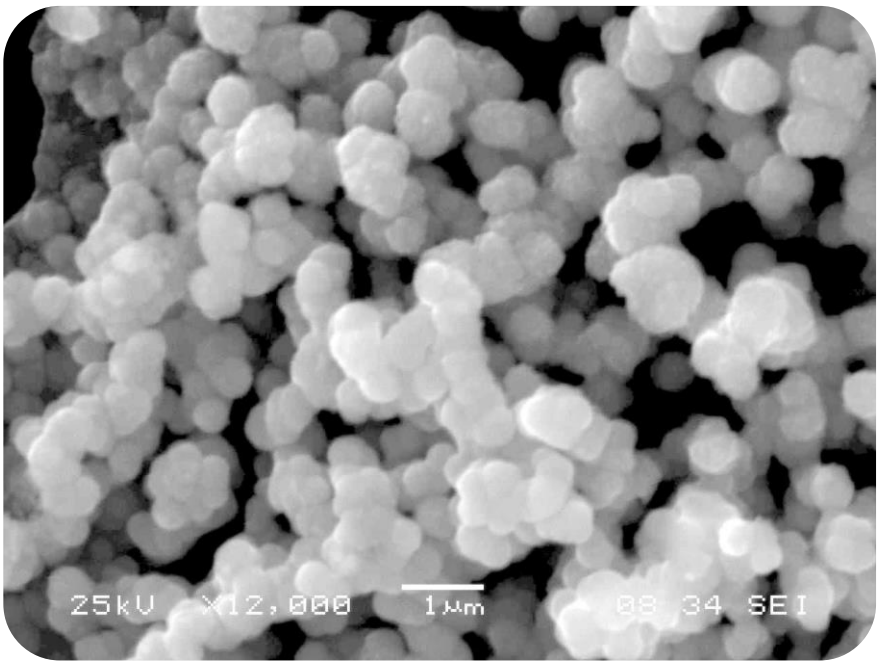

Figure 8. Scanning Electron Microscopic (SEM) Examination of USADSD176. At 7 days of incubation, USADSD176 was viewed in SEM under 12,000x magnification and shown to have smooth spore surface morphology, a common characteristic between *Streptomyces sp.*

DISCUSSION

Actinobacteria are significant source of secondary metabolites accounting to around 10,000 secondary metabolites representing 45% of bioactive compounds synthesized for drug discovery and development. (26) The antibacterial compounds from actinobacteria isolated from the terrestrial environment has been proven to be efficient in the past decades; however, majority of the strains of actinobacteria and their produced secondary metabolites have been exhausted and proven to be totally inefficient in searching for novel molecules as drug candidates.

(27) In the present investigation, actinobacteria isolated in marine sediments from Western Visayas island were screened for antibacterial activities. Moreover, the marine sediments were collected up to -110 cm below the seafloor and were classified into bottom (91-110 cm below the seafloor), sub-bottom (76-90 cm below the seafloor), middle (51-75 cm below the seafloor), subsurface (26-50 cm below the seafloor) and surface (0-25) sediments. This is the first study conducted in the Philippines with the aim of evaluating the antibacterial activities of marine sediment-derived actinobacteria in relation to the sediment depth where the actinobacteria were isolated.

The isolation technique and the carbon source available for marine sediment-derived actinobacteria under standard laboratory conditions are the major determinants of the actinobacteria growth and recovery in this study. The two culture-dependent techniques, dry stamp method and heat shock method, in five selective marine minimal media resulted to 180 actinobacteria isolates from the unexplored marine sediments of Western Visayas island. Dry stamp method (DSM) and heat shock method (HSM) have shown relatively the same number of isolates from marine sediments. DSM is the direct stamping and inoculation of fully dried marine sediments to the medium. It is an isolation technique used to eliminate or minimize the growth of other undesirable microorganisms such as fungi and Gram-negative bacteria. Meanwhile, heat shock method (HSM) involves a series of sonication, vortex mixing and exposure to heat to kill or reduce undesirable fungal and bacterial growth. In a study by Jensen *et al.* (28), DSM has shown good recovery of actinomycetes from tropical Pacific oceans with 44% recovery. In addition, Ulanova and Goo (29) has found that majority of the actinomycete colonies were isolated using DSM from the subseafloor at Nankai and Okinawa Troughs. On the other hand, the heat shock method (HSM) is an effective isolation technique in terms of eliminating undesirable bacterial and fungal growth with the aid of heat. Although HSM can diminish or prevent the growth of bacteria and fungi on isolation plates, the number of actinobacteria may also be reduced. (30) Having a slight edge in terms number of isolates cultivated using HSM than DSM signifies that the heat-sensitive isolation technique was executed properly preventing the reduction of actinobacteria growth in the media plates. Actinobacteria isolates were recovered from selective marine media with varying carbon sources, specifically mannitol for MM3, glucose for MM11, trehalose for MM13, raffinose for MM51 and starch for ISP4. The findings indicated that 61% (111) of the actinobacteria isolates were recovered from MM11 plates with glucose as the carbon source.

The high recovery in MM11 plates is due to the rapid utilization of carbohydrates and readily available for actinobacteria, since glucose is a monosaccharide and a rapidly assimilated carbon source. However, the high Actinobacteria recovery rates from glucose as carbon source is associated to suppress and interfere the formation of many antibiotics (31, 32) and sometimes repress the catabolism of glucose itself. (33) Thus, the depletion of carbon and other nutrient sources is resolved by promoting idiolite formation (34) through the transfer of actinobacteria growth to MM1 which is an enriched medium. In fact, three of the most bioactive isolates (USADSD481, USADSD357 and USADSD164) with more than 90% growth inhibitions were recovered from MM11. The 180 actinobacteria isolates recovered from the two isolation techniques and in five selective media were purified several times to get rid of any contaminations or carry-overs. The pure actinobacteria cultures were maintained in glycerol before storing in -80°C freezer.

It should be emphasized that the 180 actinobacteria isolates reported in this study represent only a portion of the total population of actinobacteria in the microbial communities in marine sediments of Western Visayas island. There were isolates recovered from the surface (0-25 cm below the seafloor) and subsurface (26-50 cm below the seafloor) sediments, but it is noteworthy to highlight that most of the isolates were from the sub-bottom (76-90 cm below the seafloor) sediments and a number of isolates from the bottom (91-110 cm below the seafloor) sediments. The presence of actinobacteria at these sediment depths supports the claim of Bull *et al*. (35) that actinobacteria also occur in deep below seafloor sediment, which are sometimes recognized as dominant populations. Actinobacteria that can be found in deeper marine sediments depths exist in dormant states and are well-adapted microorganisms to these adverse conditions. Aside from their ability to degrade biomolecules from sedimentary deposits, they can obtain nutrients from dead cells by releasing extracellular enzymes to degrade the cell walls of fungi. (36) These adaptations may indicate that the actinobacteria and other microorganisms present in the marine sediments below the seafloor can also secrete enzymes and novel molecules to compete for nutrients against other bacteria and can be a promising source of secondary metabolites.

There were no direct correlations in the number of actinobacteria isolates to the sediment depth collected. Most of the isolates were recovered from sub-bottom (76-90 cm below the seafloor), but it is not conclusive of the distribution of actinobacteria at different sediment depths. This claim is supported by Goodfellow and Hanes (37) were they did not find any correlation between depth and the number of Actinomycetes recovered in marine sediments.

The factors that can affect the number of actinobacteria that can be isolated in marine sediments include media composition (38), isolation techniques (39), and incubation time (40) and temperature (41) of the isolates. However, it is evident that actinobacteria are present at different marine sediment depths. Increasing the depth of marine sediments during sample collection, along with the appropriate isolation media and culture-dependent techniques to be used can provide a more extensive distribution data of actinobacteria occurrence in sediments of marine ecosystems. Actinobacteria isolated from the marine environment have currently received considerable attention due to the production of structural diverse and novel secondary metabolites with antibacterial properties (42), such as N-acetyl-N-demethylmayamycin (43), marinopyrroles (44), and essramycin (45). The main objective of determining novel secondary metabolites is to resolve the problem of resistant pathogens, which has short susceptibility to the presently used drugs. (46)

Streptomyces and other members under the phylum *Actinobacteriaceae* have shapes resembling filamentous fungi with a layer of hyphae that can differentiate into chain of spores. Spore chains are observed under a scanning electron microscope with high magnifications to visualize the spore morphology of actinobacteria in high resolution. As shown by results of SEM examination on one of the most bioactive isolate, USADSD176 has smooth spore surface and approximately 0.7 μm in size which are the common features of *Streptomyces sp.* (47) The sporulation of actinobacteria induces secondary metabolite production which is linked to be their adaptive mechanisms against stresses such as the exhaustion of available nutrients. Furthermore, the ability of the spores to survive in the marine environment must have been increased due to the pigment and aroma present in the spores in some species (48), which stimulates cell development and enhances the secondary metabolite production. (49)

To avoid reoccurrence of actinobacteria, two sampling points were chosen considering that these sites were away from the population, no river run offs present and accessible by the research team. The isolates were evaluated for antibacterial activities against two Gram-positive (*S. aureus* ATCC 25923 and *S. aureus* ATCC BAA-44) bacteria and four Gram-negative (*E. coli* ATCC 25922, *K. pneumoniae* Biotech 1754, *P. aeruginosa* ATCC 27853, and *E. aerogenes* ATCC 13048) bacteria. The first approach of antibacterial screening was the agar overlay assay using the resazurin reduction test with the principle related to live/dead cell evaluation of antibacterial activities.

After 24 hrs of incubation at 37°C, the wells that retained the blue resazurin dye indicated dead cells or positive antibacterial activity, while the reduction of resazurin to resorufin in the pink wells indicates live cells or inactivity of the isolate against the test bacteria.

The qualitative approach of antibacterial screening resulted to 34 active isolates as indicated by the retained blue resazurin dye in the wells. Furthermore, most of the bioactive isolates were recovered from the bottom (91-110 cm below the seafloor) sediments with a hit rate of 39% against the six test bacteria, followed by the actinobacteria isolated from the middle (51-75 cm below the seafloor) with 24% hit rate, then subsurface (26-50 cm below the seafloor) sediments with 21%, and sub-bottom (76-90 cm below the seafloor) and surface (0-25 cm below the seafloor) sediments with 15% and 11%, respectively. The results presented (*see* Fig. 4 above) for the initial antibacterial screening of the actinobacteria isolates is the summarized data of two sampling sites. Looking at the number of bioactive isolates from each sampling site, still, the bottom (91-110 cm below the seafloor) sediments followed by the middle (51-75 cm below the seafloor) and subsurface (26-50 cm below the seafloor) sediments yielded the highest % hit rates consistently despite of the difference in distribution of actinobacteria isolates in two sites (data not shown). Interestingly, the large number of actinobacteria isolates recovered from the sub-bottom (76-90 cm below the seafloor) sediments and the promising bioactivity of the isolates from the bottom (91-110 cm below the seafloor) sediments can be implications of the high distribution of actinobacteria isolates at increasing sediment depth below the seafloor, with potent antibacterial activities.

The next approach of antibacterial screening was to confirm and quantify the bioactivity of the initially screened actinobacteria isolates in terms of growth inhibitions against the target bacteria using microbroth susceptibility assay. Bacterial growth inhibition is a complex process that starts with the physical interaction of the molecule and its specific targets and involves biochemical, molecular, and structural changes, acting on multiple cellular targets such as DNA replication, RNA synthesis, cell wall synthesis, and protein synthesis. (50) The secondary metabolites produced by actinobacteria are not only responsible for their bioactivity, but also important ecological features in order to compete with other microorganisms and to survive in the marine microbial community.

From the 34 bioactive isolates in the initial screening, 21 (61%) have been confirmed to have antibacterial activity with more than 50% growth inhibition against the test bacteria. Interestingly, three isolates (USADSD250, USADSD360 and USADSD481) have shown antibacterial activities to both Gram-positive and Gram-negative bacteria indicating a wide range of antibacterial activities of actinobacteria isolated and that could be recovered in the marine sediments of Western Visayas island.

Notably, 12 isolates have shown promising antibacterial activity against the multidrug-resistant test bacteria, *S. aureus* ATCC BAA-44, an Iberian clone of Methicillin-resistant *S. aureus* (MRSA). (51) It has a wide range of resistance including ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, cephalothin, doxycycline, gentamicin, erythromycin, imipenem, methicillin, penicillin, tetracycline, oxacillin azithromycin, clindamycin, ceftriaxone, rifampin, amikacin and tobramycin; however, it is known susceptible to teicoplanin, chloramphenicol, fosfomycin, mupirocin, netilmicin, streptomycin, trimethoprim/sulfamethoxazole, and vancomycin. The multidrug resistance of *S. aureus* ATCC BAA-44 is due to a modified penicillin binding protein (PBP2' or PBP2a) encoded by the *mec*A gene, since *S. aureus* ATCC BAA-44 is one of the *S. aureus* strains that is confirmed to carry the *mec*A gene conferring resistance to methicillin. (52) The presence of PBP2a confers resistance towards all β-lactam antibiotics apart from ceftobiprole, a pyrrolidinone-3-ylidene-methyl cephalosporin hydrolysed by extended-spectrum ß-lactamases and AmpC beta-lactamases, and demonstrated *in vitro* activity against MRSA. (53) As β-lactam antibiotics are the first-line compounds for treatment of staphylococcal infections, diverse marine sediment-derived actinobacteria can be a new and promising source of secondary metabolites with antibacterial properties in response to the increasing resistance of multidrug resistant pathogens. Tetracycline was used as the positive control in all of the antibacterial screenings. Tetracycline is a broad-spectrum naphthacene antibiotic produced semi-synthetically from chlortetracycline, an antibiotic isolated from the bacterium *Streptomyces aureofaciens* that inhibits the binding of aminoacyl-tRNA to the mRNA-ribosome complex during protein synthesis. (54) In spite of the previous reports (55, 56) stating the scope of drug resistance of *S. aureus* ATCC BAA-44 and other multidrug resistant strains to tetracycline, most tetracycline-resistant *Staphylococci* remain normally sensitive to minocycline. (57) In fact, *S. aureus* ATCC BAA-44 has an intermediate resistance to minocycline.

On the other hand, only five isolates (USADSD250, USADSD264, USADSD292, USADSD360, and USADSD481) have bioactivity against *E. coli* ATCC 25922 and none on the rest of the Gram-negative (*K. pneumoniae* Biotech 1754, *P. aeruginosa* ATCC 27853 and *E. aerogenes* ATCC 13048) test bacteria. The low number of actinobacteria isolates with antibacterial activities against Gram-negative bacteria are associated to the outer membrane of Gram-negative bacteria which is an important barrier that provides protection against toxic compounds, including antibiotics and host innate immune molecules such as cationic antimicrobial peptides. (58) The outer membrane is a largely asymmetric bilayer composed of glycolipid lipopolysaccharides (LPS) and glycerol phospholipids that serves as a barrier for protection against toxic compounds, including antibiotics, whose targets are largely beyond this surface layer. (59)

Further evaluation of antibacterial activities was done on selected actinobacteria isolates with more than 90% growth inhibition against the test bacteria by the determination of Minimum Inhibitory Concentration (MIC_{90}) values. The goal of this antibacterial susceptibility testing is to determine the lowest concentration of actinobacteria crude extracts (2500 to 4.88 µg/mL) that could inhibit 90% of the growth of target bacteria after overnight incubation. The isolates screened for the determination of MIC values showed consistent 90% growth inhibitions at different MIC breakpoints against the Gram-positive test bacteria. The most bioactive actinobacteria isolates with more than 90% growth inhibition, including USADSD164, USADSD176, USADSD261, USADSD357 and USADSD481 screened for their MIC values, demonstrates the antibacterial potential of actinobacteria present at different sediment depths of Western Visayas island, specifically against multidrug- resistant strains and pathogenic bacteria. The phylogenetic analysis of the bioactive actinobacteria isolate will be conducted in the future to provide molecular (16s rRNA) information of the actinobacteria isolate species. Moreover, chemical analyses of the crude extract will be pursued to reveal the structural diversity of the secondary metabolites produced by actinobacteria and their mechanisms of action as antibacterial agents.

CONCLUSIONS

In closing, the findings in this study demonstrated the widespread distribution of actinobacteria in a range of depths below the seafloor in Western Visayas island. The resulting 180 marine sediment-derived actinobacteria recovered from this niche is an implication of diverse distribution of actinobacteria at increasing sediment depth below the seafloor, with potent antibacterial activities. The qualitative and quantitative screening of their antibacterial activities revealed the occurrence of actinobacteria in marine sediments below the seafloor, with promising antibacterial activities against multidrug-resistant bacterial pathogen. Our data provides useful insights for natural product research and urges further probing of the Philippines remote islands for possible discovery of novel actinobacterial strains as potential source of bioactive compounds.

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