ANTIMICROBIAL CHARACTERISTICS AND TAXONOMICAL DIVERSITY AMONG ACTINOMYCETES ISOLATED FROM CATBA ISLAND, VIETNAM

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ABSTRACT

In this study, 424 actinomycete strains isolated from soil and litter samples on Catba island (Haiphong, Vietnam) were subjected to the screening for the inhibitory activities against microorganisms, including bacteria (*Micrococcus luteus*, and *Escherichia coli*) and cukaria (*Candida albicans* and *Fusarium oxysporium*) Through two screening steps, 17 strains were selected for their high inhibitory activity against one or more target microorganisms Crude extracts in ethyl acetate from culturing media of the selected strains were analyzed via thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC), in which chloramphenicol, kitasamycin, erythromycin and raw extract of anthracycline were used as standards. The obtained results showed that antibiotic substances produced by the selected strains could not be put in any group of the analyzed standards, except the strain A396 which appeared to produce a chloramphenicol-like antibiotic Taxonomical studies based on the morphology and 16S rDNA sequencing indicated that the collection of actinomycetes isolated from Catba island contained mainly *Streptomyces* species (about 70%) and the group of rare actinomycetes (non-Streptomyces) which made of 30% of the collection was dominated by *Micromonospora*, *Nonomureae* and *Nocardia* genera. Of the 17 selected strains with highest antimicrobial activity, ten strains were affiliated to the genus *Streptomyces* (as based on the morphology) and seven strains belonged to the genus *Nonomuraea* (as based on 16S rDNA sequence analyses) The strains selected in this study could serve as sources for discovering new antibiotic substances in Vietnam.

Keywords: *antibiotics, actinomycetes, Streptomyces, Nonomuraea, TLC, HPLC*

INTRODUCTION

In the context of alarming increase of antibiotic resistance among pathogens, search for new antimicrobial agents with different mechanisms of action is becoming utmost important (Habte-Gabr, 2002; Tenover, 2006). The history of new drug discovery shows that novel skeletons, in the majority of cases, come from natural sources such as microbial and plant extracts. Among microorganisms, actinomycetes present one of the most attractive sources of antibiotics and other biologically active substances of highly commercial value. Currently about 16,500 antibiotics have been discovered from microorganisms, two-thirds of which was produced by actinomycetes (Hopwood, 2007). Various antibiotic substances from actinomycetes have been characterized, including aminoglycosides, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyester, polyketides, (Goodfellow *et al.*, 1988). These substances have been successfully used as herbicides, anticancer agents, drugs, immunoregulators and antiparasitic agents (Thomson, Bialphos, 1995).

Actinomycetes are Gram positive bacteria having high G+C content (>55%) in their DNA. The majority of actinomycetes has free living, saprophytic life form and is widely distributed in soil, water and plant litter. Actinomycetes play an ecologically important role in material recycling in nature, they decompose and utilize difficult-to-degrade organic matters such as humic acid in the soil. At the presence, actinomycetes are defined as the order *Actinomycetales* which is consisted of 13 suborders, 42 families and about 200 genera (Ashutosh, 2008; Duong, Ando, 2010).

This study aimed to investigating biodiversity and to screening actinomycete strains exhibiting high antimicrobial activity among a collection of actinomycetes isolated from Catba island, a national park with rich biodiversity in Vietnam. The selected strains were then subjected to further studies on the antibiotic substances produced as well as their phylogenetic affiliation.

MATERIALS AND METHODS

Actinomycete strains and culture conditions

The 424 actinomycete strains used in this study were isolated from soil and leaf litter samples from Catba island, Vietnam. All of them were maintained as stock cultures frozen at –80°C in 20% glycerol solution at Vietnam Type Culture Collection (VTCC). Before use in screening experiments, the strains were reactivated on YS agar medium (glucose 1%, yeast extract 0.2%, agar 1.7%, pH 7.0) and incubated at 30°C for 3 to 4 days (Duong, Ando, 2010).

For the tests of antibiotic activity, these strains were cultivated in soybean meal liquid medium (soluble starch 2%, glucose 1%, soybean meal 1.5%, peptone 0.5%, $CaCO_3$ 0.3%, pH 7) and incubated at 30°C by shaking at 100 rpm for 3 to 4 days. The centrifuged culture broths were then used in antibiotic screening experiments as well as in chromatography analyses.

Target microorganisms and culture conditions

Four microorganisms used as targets for the tests of antibiotic activity were *Micrococcus luteus* (a Gram positive bacterium), *Escherichia coli* (a Gram negative bacterium), *Candida albicans* (a yeast) and *Fusarium oxysporium* (a filamentous fungus). The target strains were cultivated in particular nutrient media, i.e. Mueller-Hinton medium (MHA; meat extract 0.3%, hydrolysis casein 1.75%, starch 0.15%, pH 7.4) for *E. coli* and *M. luteus*, yeast/malt extract medium (YM; glucose 1%, peptone 0.5%, yeast extract 0.3%, malt extract 0.3%) for *C. albicans* and *F. oxysporum*. The cultures were incubated under shaking condition at 37°C for *E. coli* and *M.luteus* or 30°C for *C. albicans* and *F. oxysporum*.

Screening for antibiotic producing actinimycetes

Agar disc method

Agar discs (5 mm in diameter) taken from plates of well grown actinomycete cultures were placed onto surface of agar plates previously seeded with one of the target microorganisms and incubated at proper conditions for 2 days. The inhibitory effect was assessed on the basis of the formation of clear zones around the agar discs and the activity was measured by the diameter of these zones (Ichikawa *et al.*, 1971).

Culture broth diffusion method

Small wells were aseptically created (by using hole-borer instrument) on solidified agar plates previously seeded with one of the target microorganisms. Approximately 25 µl of centrifuged culture broths of the isolates were added into the wells and incubated for 2 days at proper condition for the target microorganism. Antibiotic activity was assessed through the inhibitory zones formed around the wells. The experiments were performed in duplicates for all cases, distilled water was used as a negative control (Alex, Hai, 2006).

Chromatography analyses of antibiotics

Ethyl-acetate extraction

Culture broths of the actinomycetes grown on soybean meal liquid medium were centrifuged at 8000 rpm for 15 min at room temperature (RT) and the supernatants were collected for the solvent extraction. To extract the antibiotic substances, equal volume of ethyl-acetate was added to the supernatant and the mixtures were shacked vigorously for 1 h. The solvent was collected by using separation funnels, afterward sodium sulfate was added at 1% (vol/vol). The elimination of solvent was performed in rotary evaporator and the obtained precipitates were dissolved in 1 ml of chloroform (Duong, Ando, 2010). Prior to chromatography, the solutions of crude extracts in chloroform were tested again for the antibiotic activity (with chloroform as the negative control).

Thin Layer Chromatography (TLC)

TLC analyses were performed on Silica Gel G plates (20 x 10 cm) using solvent system chloroform:methanol (90:10). Chloramphenicol, kitasamycin, erythromycin and raw extract of anthracycline were employed as standards. Spots were visualized by UV irradiation (254 and 366 nm) or by spraying with 10% sulfuric acid (H_2SO_4) and fixing at 120°C for 5-10 min (Mokbel, Hashinaga, 2005; Choma, 2010).

High-Performance Liquid Chromatography (HPLC)

Analysis was performed by Agilent 1100 series HPLC (USA), equipped with C18 Synchropak RP-4 column (250 mm × 4.6 mm, ID 11704457 Agilent, USA) and a UV detector. Stock solutions of standard compounds were prepared in methanol at the concentration of 1 $mg.ml^{-1}$ and stored at 4°C in the dark. The solution of 25% acetonitril was used as mobile phase at the flow rate of 1 $ml.min^{-1}$, the column temperature was 30°C. The injection volume

of each sample was 10 µl (Koup *et al.*, 1978; Stubbs *et al.*, 1985; Masakaza *et al.*, 1996; Nollet, 2000; Maudens, 2009).

DNA extraction

The genomic DNA of actinomycete strains was extracted using the method described by Marmur (1961) and Saito (1963) with some modifications. Briefly, cultures of actinomycetes were grown in YG liquid medium for 3 days at 30°C and cells were harvested by centrifugation at 3000 rpm for 5 min. The cells were then homogenized by sterile plastic sticks, washed with 2 ml 1×TE buffer for 2 - 3 times and resuspended in 0.5 ml of 5 mM EDTA (pH 8). Removal of actinomycete cell wall was achieved by treatment with lysozyme (50 µl of 40 $mg.ml^{-1}$) at 37°C overnight, then in the presence of SDS (50 µl of 20%) and proteinase K (50 µl of 4 $mg.ml^{-1}$) at 55°C for 1 h. The extraction was performed by adding an equal volume of phenol:chloroform:isoamine alcohol = 25:24:1 (PCI), mixing and centrifugation at 15000 rpm for 15 min at 4°C. The extraction step was repeated 3 times. Chromosomal DNA was precipitated by addition of 2 volumes of cold 2-propanol, then rinsed with 70% ethanol, dried up at RT and dissolved in 100 µl of distilled water.

PCR amplification, sequencing, and phylogenetic analysis

The 16S rDNA was amplified using primers 27F (AGAGTTTGATCCTGG CTCAG) and 1492R (GGTTACCTTGTTACGACTT). The reaction mixture (50 µl) contained 5 µl of reaction buffer (0.2 M Tris-HCl pH 8.3, 0.25 M KCl, 20 mM $MgCl_2$), 20 nmol of each deoxynucleotide, 50 pmol of each primer, 2. 5 U of *Taq* DNA polymerase, and 1 µl of template DNA. Thermocyles for the PCR included 5 min heat shock at 95°C, followed by 30 cycles of 95°C for 30 second, 52°C for 30 second, and 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were then analyzed by electrophoresis on agarose gel, purified with QIAquick gel extraction kit (Qiagen), and sequenced on ABI 3110 Avant Applied Biosystems sequencer (ABI, USA).

The 16S rDNA sequences were compared with sequences available on the GenBank/EMBL/DDBJ databases by using the BLAST Search tool. The alignment with corresponding sequences was performed by using CLUSTAL_X program, version 1.8 (Thompson *et al.*, 1997) A phylogenetic tree was constructed by the neighbor-joining method (Saitou, Nei, 1987). Topography of the constructed tree was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985)

Morphological characterization

Morphological characteristics of actinomycetes were observed after 2 week incubation at standard conditions. Microscopic characteristics such as fragmentation pattern of substrate mycelia, morphology of aerial mycelia, structure of spore chains and the spore forms were observed under phase contrast microscope (Zeiss) connected with a camera and image controlling software The Atlas-Morphology of actinomycetes (Gernot, 1997) and Identification Manual of Actinomycetes (Miyadoh *et al.*, 2001) were used as references for the taxonomical determination of the isolated strains

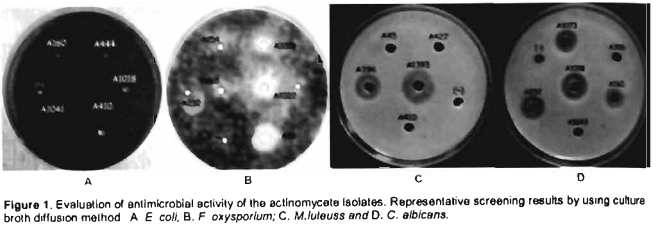
RESULTS AND DISCUSSION

Antibiotic properties of the actinomycete isolates

Among 424 actinomycete isolates, only 115 strains showed noticeable inhibitory activity against at least one of the target microorganisms as shown in the preliminary screening step by using agar disc method. The second step of screening using culture broth diffusion method (Fig. 1), which is more precise, resulted in 17 strains possessing high inhibitory activity against two or more target microorganisms (Table 1). It could be assumed that antibiotic substances produced by these selected strains had broad spectra of activity.

Among the 17 selected actinomycete strains, 14 strains showed inhibitory activity against Gram-negative bacteria (*E. coli*), 14 strains inhibited Gram-positive bacteria (*M. luteus*) and 11 strains had activity against both groups. In the relationship to eukaryotic cells, including fungi (*F. oxysporum*) and yeasts (*C. albicans*), 12 strains possessed antifungal activity and only 5 strains could inhibit yeast cells (Tabble 1).

The obtained results indicated that 9 of the 17 selected strains showed strong activity against both bacterial and fungal groups (diameter of the inhibitory zones > 10 mm). Of special interest were strains A1073 and A1393 which could inhibit all four target microorganisms used in the study (Table 1).



A B C D

Figure 1. Evaluation of antimicrobial activity of the actinomycete isolates. Representative screening results by using culture broth diffusion method A *E. coli*, B. *F. oxysporium*; C. *M.luteuss and* D. *C. albicans.*

Table 1. Antimicrobial activity of the 17 selected actinomycete strains.

Strain name	Inhibitory effect against the target microorganisms (diameter of inhibitory zones in mm)			
	E. coli	*M. luteus*	*F. oxysporium*	*C. albicans*
A45	10	0	6	0
A149	8	6	0	0
A154	8	8	0	0
A160	0	6	0	10
A232	6	7	0	12
A390	10	10	18	0
A396	8	12	0	0
A410	24	0	10	0
A427	16	0	12	0
A444	0	7	8	0
A1018	0	40	10	16
A1022	16	30	10	0
A1041	22	28	6	0
A1043	8	32	6	0
A1073	21	30	10	10
A1393	14	18	14	6
A1470	15	26	12	0

Analysis of crude extracts from supernatants of the selected actinomycetes

The 17 selected strains were subjected to analyses of the antibiotic substances in their crude extracts via TLC (Fig. 2).

It was found that most of crude extracts contained several bands in TLC analyses, suggesting that the antibiotic substances produced by the selected strains consisted of two or more components, the activity of which had not yet been identified. Only exception was strains 396 which had band pattern similar to that of chloramphenicol (Fig. 2 A, B and D).

In the HPLC analyses, the antibiotics used as standards in this study appeared on the chromatogram sequentially as erythromycin (at retention time Rt of 3.117 min), chloramphenicol (at Rt of 5.498 min) and kitasamycin (at Rt of 8.597 min). The exception was anthracyclin raw extract which showed several peaks on the chromatogram.

Crude extracts of the 17 selected strains were analyzed on the HPLC under the same conditions. It

appeared that antibiotic substances produced by these strains could not be put into any group of the analyzed standards, except the strain A396 which yielded a significant peak (at Rt of 5.476 min) similar to that of chloramphenicol (Fig. 3). This result was also confirmed by the above TLC analysis (Fig. 2).

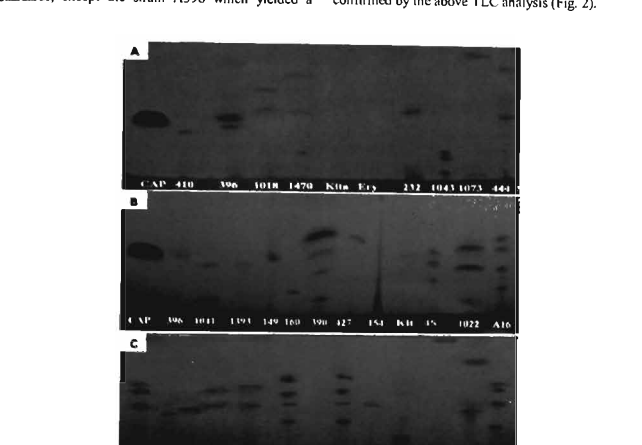


Figure 2. Analyses of crude extracts of the selected actinomycete strains via thin layer chromatography (TLC). A, B & C - observation of TLC plates under UV light. D – schematic illustration of the visualized bands. Abbreviations. CAP- Chloramphenicol; Kita-Kitasamycin; Ery-Erythromycin; and anthracyclin, raw extract A16; Actinomycete strains were designated as numbers on the figures

Taxonomical study of the actinomycete isolates

Some actinomycetes such as the genus *Streptomyces* possess specific morphological characteristics that can be used as basis for taxonomical identification (Miyadoh *et al.*, 2001). In this study, the 424 isolates were first subjected to the morphological classification, allowing to put them into *Streptomyces* and non-*Streptomyces* (rare actinomycetes) groups (Table 2).

The morphological characteristics such as color and aerial mycelium structures (Fig. 4), the form of spore bearing hyphae and spore chains (Fig. 5) were used as the basis for *Streptomyces* identification (Gernot, 1997; Miyadoh *et al.*, 2001). Of the total 424 isolates, 296 strains (making about 70%) were

classified as *Streptomyces*. Ten of the 17 selected isolates, including A390, A410, A427, A1018, A1022, A1041, A1043, A1073, A1393, and A1470 were classified as *Streptomyces* on the basis of their morphological characteristics.

Non-*Streptomyces* actinomycetes included the isolates not having *Streptomyces* morphological characteristics. To identify taxonomical affiliation of these isolates, partial sequences of the 16S rRNA gene (about 900 bp) were used. It was shown that *Micromonospora*, *Nonomuraea* and *Nocardia* were the major non-*Streptomyces* genera in the actinomycete collection obtained from Catba island (Table 2). Species of these three genera were also frequently isolated from other places in Vietnam (Duong, Ando, 2010).

Of the 17 selected isolates showing the highest antimicrobial activity, 7 strains were classified into non-*Streptomycete* actinomycetes. Sequencing and comparison of the 16S rDNA from these showed that, all 7 strains were affiliated to the genus *Nonomuraea* and could make five different genetic groups within this genus (Fig. 6).

Thus, the actinomycete collection from Catba island had relatively high taxonomical diversity, however the selected strains exhibiting the highest antimicrobial activity were affiliated to only two genera *Streptomyces* and *Nonomuraea*. Many strains of these two genera have been known for antibiotic producing properties (Watve *et al.*, 2001).

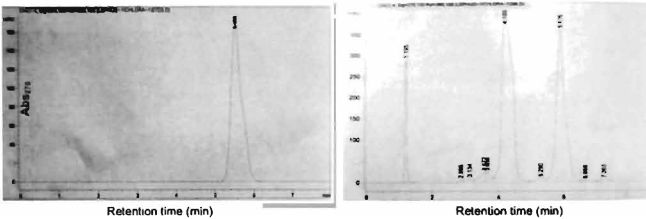


Figure 3. HPLC analysis of crude extract from actinomycetes. A - strain A396, B - chloramphenicol standard

Table 2. Taxonomical grouping of the actinomycete isolates

No	*Streptomyces*	Non-*Streptomyces*	Number of Isolates	%
1	*Streptomyces*		296	69.81
2		*Micromonospora*	27	6.36
3		*Nonomuraea*	17	4.00
4		*Nocardia*	12	2.83
5		*Kineosporia*	8	1.9
6		*Microbispora*	7	1.65
7		*Actinomadura*	7	1.65
8		*Pseudonocardia*	6	1.42
9		*Nocardiopsis*	6	1.42
10		*Micrococcus*	5	1.18
11		*Streptosporangium*	5	1.18
12		Others (each less than 1%)	28	6.6
Sum			**424**	**100.00**

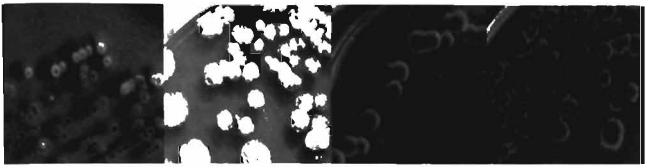


Figure 4. Colony morphology of the representative *Streptomycete* strains.A. A1018; B A390; C A1073 and D. A1043

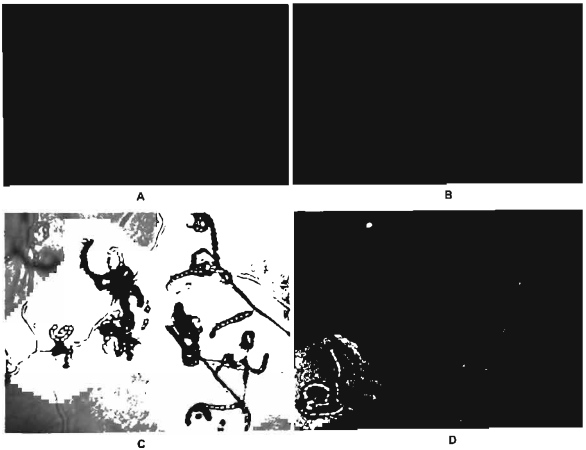


Figure 5. Spore-bearing aerial hyphae of the representative *Streptomyces* strains. A A1018; B. A390; C. A1073 and D. A1043.

More interestingly, besides the four strains of microorganisms used in this study as targets for antibiotic screening, several human cancer cell lines have also been used for testing the inhibitory effects. The preliminary results showed that 3 of the 17 selected strains (A1018, A1022 and A1073) had inhibitory effect on the tested cancer cell lines. In the tests against microorganisms, these strains showed high activity against eukaryotic cells (i.e. *F. oxysporium* and *C. albicans)* that might indicate the correlation between antimicrobial activity (especially against eukaryotes) and activity against human cells. Such kind of correlation however has not yet been elucidated in previous studies.

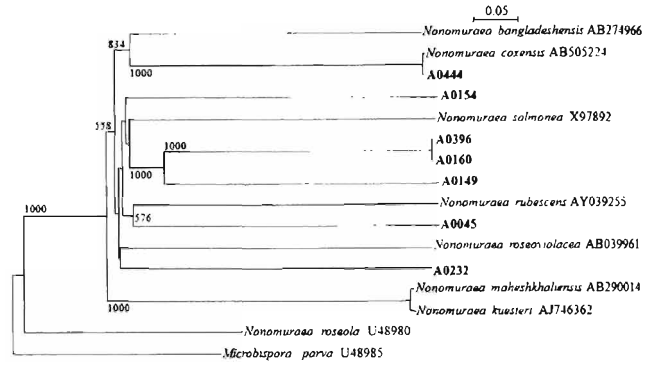


Figure 6. Neighbor-joining tree of 16S rDNA partial sequences showing phylogenetic positions of the 7 actinomycete strains in the relationship to type strains of the genus *Nonomuraea*. Bootstrap values of more than 500 are given at branching points. Bar = 0.05 nucleotide divergence in the DNA sequences. *Microbispora parva* was chosen as outgroup

CONCLUSION

Screening a relatively high number of actinomycetes isolated from Catba island yielded high proportion of antibiotic producing strains (115 of 424, about 27%), among those, 17 strains were selected according to their significant inhibitory activity against at least one of four target microorganisms (including *E. coli*, *M. luteus*, *F oxysporium*, *C. albicans*). TLC and HPLC analyses revealed that antibiotic substances produced by the selected strains were diverse in chemical nature, therefore could serve as potential sources for searching new metabolites for drug development.

Taxonomically, about 70% of the actinomycetes strains isolated from Catba island belonged to the genus *Streptomyces*, the rest belonged to non-*Streptomyces* group which was dominated by *Micromonospora*, *Nonomureae* and *Nocardia* genera. The 17 selected strains having the highest antimicrobial activity were affiliated exclusively to *Streptomyces* and *Nonomurea* genera. The positive effects against human cancer cell lines observed in three strains (A1018, A1022 and A1073) among the 17 selected strains would also be quite promising for discovering antitumor substances.

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TÍNH KHÁNG KHUẨN VÀ ĐA DẠNG SINH HỌC CỦA XẠ KHUẨN PHÂN LẬP TỪ ĐẢO CÁT BÀ, VIỆT NAM

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TÓM TẮT

Tổng số 424 chủng xạ khuẩn phân lập từ mẫu đất và lá mục thu thập ở đảo Cát Bà (Hải Phòng, Việt Nam) được sử dụng để sàng lọc hoạt tính kháng đối với cả vi khuẩn (*Micrococcus luteus* và *Escherichia coli*) và vi sinh vật nhân thực (*Candida albicans* và *Fusarium oxysporum*). Qua hai bước sàng lọc, 17 chủng đã được chọn lọc dựa trên hoạt tính ức chế đối với một hoặc nhiều vi sinh vật kiểm định. Chiết xuất thô trong ethyl

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acetate của các chủng lựa chọn được phân tích bằng sắc ký bản mỏng và sắc ký lỏng hiệu năng cao, trong đó chloramphenicol, kitasamycin, erythromycin và chiết xuất thô của chủng vi sinh vật sinh anthracyclin được sử dụng làm chất chuẩn. Kết quả thu được cho thấy các chất kháng sinh do các chủng lựa chọn tạo ra không nằm trong nhóm của các chất kháng sinh sử dụng làm chất chuẩn. Trường hợp ngoại lệ duy nhất là chủng A396 tạo chất kháng sinh cùng nhóm với chloramphenicol. Nghiên cứu phân loại dựa trên các đặc điểm hình thái và trình tự 16S rDNA cho thấy xạ khuẩn phân lập từ Cát Bà tương đối đa dạng, trong đó nhóm *Streptomyces* chiếm đa số (70%) và nhóm xạ khuẩn hiếm (non-*Streptomyces*) chiếm 30%, gồm các chi chính là *Micromonospora*, *Nonomuraea* và *Nocardia*. Ngoài ra, 10 trong số 17 chủng lựa chọn có hoạt tính kháng khuẩn cao được xếp vào chi *Streptomyces* (dựa vào các đặc điểm hình thái), 7 chủng còn lại được xếp vào chi *Nonomuraea* (dựa trên so sánh trình tự 16S rDNA). Các chủng lựa chọn từ nghiên cứu này có thể sử dụng làm đối tượng để nghiên cứu phát hiện các chất kháng sinh mới ở Việt Nam.

Từ khóa: *Chất kháng sinh, xạ khuẩn, Streptomyces, Nonomuraea, TLC, HPLC*