

The Antibacterial and Anticancer Activity of Marine Actinomycete Strain HP411 Isolated in the Northern Coast of Vietnam

Huyen T. Pham, Nhue P. Nguyen, Tien Q. Phi, Phuong T. Dang, Hy G. Le

Abstract—Since the marine environmental conditions are extremely different from the other ones, marine actinomycetes might produce novel bioactive compounds. Therefore, actinomycete strains were screened from marine water and sediment samples collected from the coastal areas of Northern Vietnam. Ninety-nine actinomycete strains were obtained on starch-casein agar media by dilution technique, only seven strains, named HP112, HP12, HP411, HPN11, HP 11, HPT13 and HPX12, showed significant antibacterial activity against both gram-positive and gram-negative bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus epidemidis* ATCC 12228, *Escherichia coli* ATCC 11105). Further studies were carried out with the most active HP411 strain against *Candida albicans* ATCC 10231. This strain could grow rapidly on starch casein agar and other media with high salt containing 7-10% NaCl at 28-30°C. Spore-chain of HP411 showed an elongated and circular shape with 10 to 30 spores/chain. Identification of the strain was carried out by employing the taxonomical studies including the 16S rRNA sequence. Based on phylogenetic and phenotypic evidence it is proposed that HP411 to be belongs to species *Streptomyces variabilis*. The potent of the crude extract of fermentation broth of HP411 that are effective against wide range of pathogens: both gram-positive, gram-negative and fungi. Further studies revealed that the crude extract HP411 could obtain the anticancer activity for cancer cell lines: Hep-G2 (liver cancer cell line); RD (cardiac and skeletal muscle letters cell line); FL (membrane of the uterus cancer cell line). However, the actinomycetes from marine ecosystem will be useful for the discovery of new drugs in the future.

Keywords—Marine actinomycetes, antibacterial, anticancer, *Streptomyces variabilis*.

I. INTRODUCTION

THE development of biotechnology of microorganisms produced thousands of drugs from terrestrial microorganisms, but still not enough to meet the demand for therapy new infection diseases [1], [2]. So, marine ecosystem is a new target of scientists to look for a new source of medicines. Approximately 10,000 metabolites are isolated to search new biopharmaceuticals from marine organisms, many of which showed pharmacological properties [6]. A broad spectrum of biological activities has been detected, such as antibiotic, antifungal, cytotoxic, anticancer and, antiviral drugs

Huyen T. Pham, Nhue P. Nguyen, Tien Q. Phi are with the Institute of Biotechnology, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam (e-mail: sunduongminh@gmail.com, npnhue@ibt.ac.vn, tienpq@ibt.ac.vn).

Phuong T. Dang is with the Institute of Chemistry, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam (dangtuyetphuong@gmail.com).

Hy G. Le is with the Center for High Technology Development, VAST, 18 Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam (corresponding author, phone: 84-4-38363257; fax: 84-4-38363144; e-mail: legiahy@ibt.ac.vn).

[13], [16], [17]. In 2005, Berdy reported the data of produced biological compounds in different groups of marine microorganisms as algae, actinomycetes, fungi, symbiotic microorganisms in sponges. These microorganisms are the pharmaceutical sources of anthracyclines, peptides, mitomycins and others [2], [4]. Among of them, actinomycetes are a valuable source to identify variety bioactive compounds. Approximate 45% of them have been discovered in different actinomycetes species, for example, genus *Streptomyces*, *Micromonospora*, etc... [11]. Representatives of the genera *Streptomyces* are widely abundant in aquatic ecosystems and frequently prevail over other groups of actinomycetes [11], [12]. To date, new antibiotics have been found from marine actinomycetes [5], [9], [15].

The present study deals with screening for the isolation of actinomycetes to produce antibiotics, isolated from marine water and sediment samples collected from the coastal areas of Northern Vietnam and determination of their antibacterial and the anticancer activity.

II. MATERIALS AND METHODS

A. Materials

The marine water and sediments were collected by using core sampler from the coastal regions of Northern Vietnam.

The test organisms have been used for determines of antibacterial activity: *Staphylococcus epidemidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 11105 and *Candida albicans* ATCC 10231. These strains belonged to the strains collection of Department of Fermentation Technology, Institute of Biotechnology, Vietnam Academy of Science and Technology.

The cancer cell lines: Hep-G2 (liver cancer cell line); MCF7 (breast adenocarcinoma cell line); RD (cardiac and skeletal muscle letters cell line); FL (membrane of the uterus cancer cell lines) has been used for *in vitro* screening antitumor activity. These experiments were performed in Department of Experimental Biology, Institute of Chemistry of Natural Products.

B. Isolation of Marine Actinomycetes

Actinomycetes were isolated by continuous dilution plate technique on starch casein agar (SCA) medium (g/l): Starch 10, casein 10, KH₂PO₄ 0.5, MgSO₄ 0.5, NaCl 30, distilled water 500 ml, sea water 500 ml, agar 18.0, pH 7.0. The plates were incubated at 28°C and the numbers of colonies were determined after 7 days. The selected colonies were picked up

and further purified by streak plate technique over starch casein agar slants.

The antimicrobial activity was examined preliminarily by agar block and well diffusion method against bacteria and fungi [3]. After this preliminary testing the most active strains were selected for further study.

C. Taxonomical Characterization of Actinomycetes

The selected strains were determined morphological characteristics on the specific medium: Oat meal agar (ISP3), malt extract agar (ISP2), inorganic salt starch agar (ISP4), glycerol asparagine base agar (ISP5), tyrosine base agar (ISP7) and SCA. The color of actinomycetes was determined by Tresner HD, Backus EJ, 1963. Pigment is determined on peptone yeast extract iron agar (ISP6), tryptone-yeast extract broth (ISP1) and ISP7. Spore-chains and spore surface was observed under electronic microscope after 14 days incubation. The physiological and biochemical characterizations of actinomycetes were examined the ability of nitrogen utilization in a basal medium ISP8 (%w/v): D-glucose (0.1), MgSO₄.7H₂O (0.05), NaCl (0.05), FeSO₄.7H₂O (0.001), K₂HPO₄ (0.1), agar (1.2) and with or without L-asparagine (0.1), and carbon utilization in the medium ISP9 with or without D-glucose 1% (w/v). Results were determined after 15 days incubation [19], [21].

D. DNA Extraction, Amplification and Sequencing

Genomic DNA of HP411 was extracted from cultures grown on SCA using the method proposed by Moller, 1998 [14]. Primers, FC27 (5'-AGAGTTTGATCCTGGCTCAG-3') and RC1492 (5'-TACGGCTACCTTGTACGACTT-3'), were used to amplified 16S rDNA gene. The reaction conditions were as following the initial denaturation at 94°C for min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min; reaction products carried out electrophoresis on a 1% agarose gel and checked with ethidium bromide under UV light, and then purified and sequenced directly using a Taq DyeDeoxy Terminator Cycle Sequencing Kit by the ABI Prism 3730 automated DNA sequencer (Applied Biosystems). Both strands were sequenced as a cross-check by using forward and reverse sequencing primers. The generated sequences were aligned using ClustalX software. The consensus sequence was compared with 16S rDNA gene sequence database GenBank (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic tree species is established on the basis of genetic distances by Kimura M, 1980 [8], using the Neighbor-joining method [18].

E. Preparation of HP411 Extract

Fermentation broth of HP411 was centrifuged at 8000 rpm/min at 4°C for 10 minutes. The supernatant were mixed with different solvent to extract bioactive compounds. The suspension was shaking for 6-8 hours at 4°C with ethyl acetate (1:1), then the solvent was extracted by Büchner funnel and dried under diminished pressure to collect crude antibiotic. Actinomycete biomass was washed with water, and then added methanol (1g/5 ml methanol). The solution was shaken at 4°C,

200 rpm for 2 hours and then extracted similarly as the above protocol. The precipitated compound was used for minimum bactericidal concentration and cytotoxicity assay.

F. Cytotoxic Activity

The precipitated HP411 extract was dissolved in dimethyl sulfoxide (DMSO) (10 mg/ml) to determine the anticancer activity. This assay performed using the method described by Likhitayawuid *et al.*, 1993 [10]. Ellipithine and 0.5% DMSO is used as a positive and negative control, respectively. Dose response curves were drawn from 6 concentrations of 2 fold serial diluted concentration to determine that inhibition of cell growth by 50% (IC₅₀ value).

G. Determination of Minimum Bactericidal Concentration (MBC)

MCB was used to determine the lowest concentration of HP411 extract required to kill bacterium test. Ten organism test cultivated in LB medium until reach OD=1. And then, 5 µl diluted cultivated medium from 10⁻¹ to 10⁻⁶ was used to incubate in the LB medium containing 50 µg/ml extract. The result of optical density was obtained after 24 h incubation at 37°C [7].

III. RESULTS AND DISCUSSION

A. Isolation and Assessment of Antibacterial Activity of Marine Actinomycete

From 200 samples, collected in the Northern coast of Vietnam, were isolated 99 actinomycete strains have antibacterial activity. However, there are seven actinomycetes that show wide broad range of antimicrobial activity (Table I). Among of them, strain HP411, isolated from sediment sample, could obtain the highest antibiotic activity with positive Gram and negative Gram bacteria and pathogen yeast.

TABLE I
THE ANTIBACTERIAL ACTIVITY OF THE ISOLATED STRAINS OF ACTINOMYCETES

Actinomycete strains	Inhibition zone (D-d, mm)			
	<i>S. epidemidis</i> ATCC 12228	<i>E. coli</i> ATCC 11105	<i>B. subtilis</i> ATCC 6633	<i>C. albicans</i> ATCC 10231
HP112	15	15	23	6
HP12	0	12	22	0
HP411	22	25	22	14
HPN11	20	24	20	16
HP 11	0	11	20	0
HPT13	10	20	10	0
HPX12	18	19	20	10

B. Taxonomical Characterization of Strain HP411

The morphological characteristics of strain HP411 is described in Fig. 1 and Table III. Branched aerial hyphae were formed on short sporophore and these aerial hyphae were straight type or coiling ends with slightly wavy hooks (RA). The number of spore in chain was up to about 30 spores (Fig. 1 (B)). Spiny spores with oval shape developed on branched substrate hyphae.

The color of the aerial hyphae is one character that uses to classify *Streptomyces* HP411. The aerial hyphae mass appeared as light gray, light grayish reddish brown, light grayish olive light brownish yellowish gray and whitish gray on all the used media, therefore it could be assigned to the grey color (Table II). No melanin or light brown pigment produced on the ISP2 with edge white colonies, white to gray and dark gray (Table II) [19], [20]. In ISP2 medium, soluble yellow to brownish pigments were produced. The soluble pigments were not affected by the change of pH.

TABLE II
COLOR CHARACTERISTICS OF STRAIN HP411 ON THE ISP MEDIUM

Medium	Aerial mass color	Reverse side pigment	Soluble pigment	Melanoid pigment
Tryptone malt extract agar (ISP-1)	Light gray	Grayish purple	Negative	Negative
Yeast malt extract agar (ISP-2)	Light grayish reddish brown	Grayish purple	Light brown	
Oat-meal agar (ISP-3)	Whitish gray	White	Negative	
Inorganic salts starch agar (ISP-4)	Light grayish olive	White	Negative	
Glycerol-Asparagine agar (ISP-5)	Whitish gray	White	Negative	
Peptone yeast extract iron agar (ISP-6)	Light brownish gray	Light brownish yellowish gray	Negative	Negative
Tyrosine agar (ISP-7)	Light brownish yellowish gray	Brownish gray	Negative	Negative

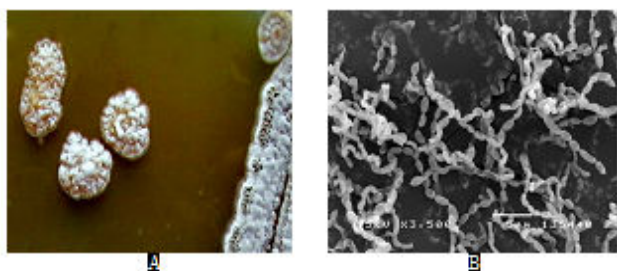


Fig. 1 Photomicrographs of HP411 colonies on ISP3 after 21 days (A). Scanning electron micrograph showing spore chain and spore surface (B)

The aerial hyphae of HP411 have spore chains of *Retinaculiaperti* (RA) type with oval spores of spinous surface (Fig. 1 (B)). The physiological and biochemical characteristics of HP411 were presented in Table IV. Strain HP411 belonged to mesophilic microorganism, the optimal temperature is between 25-37°C. It grew well on light acidic and alkaphilic medium pH of 5-10. In addition, the results showed that HP411 could grow on the high NaCl concentration (up to 10% NaCl).

Strain HP411 could grow well on nitrogen sources containing medium such as amino acid: L-phenylalanine, L-leucine, L-asparagine monohydrate, L-tryptophan, L-isoleucine, L-lysine, L-threonine and 2-Amino-2 hydroxy methyl 1,3 promo. However, it poorly grows on medium ISP8. Strain HP411 could utilize D-glucose, mannitol, D-xylose and mannose as carbon sources. But it cannot grow on the ISP9 and saccharose containing medium. Moreover, the results

showed that HP411 has high capacity to hydrolyze starch, casein and cellulose.

TABLE III
MORPHOLOGICAL CHARACTERISTICS OF STREPTOMYCES HP411

Characteristics	HP411
Spore chain	<i>Retinaculiaperti</i> (RA)
Spore surface	Spiny (Sp)
Number of spores in chain	10-30
Catalase activity	+
Oxidase activity	+
Ure activity	+
Casein hydrolysis	+
Starch hydrolysis	+
CMC hydrolysis	+
Soluble pigments	+
Growth at	
4°C	-
15°C	±
25°C	+
30°C	++
37°C	+
45°C	-
pH	
5	±
7	++
8	++
10	+
11	-
NaCl (%w/v)	
2%	+
5%	+
7%	+
10%	±

TABLE IV
PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF STREPTOMYCES HP411

Characteristics	HP411
Growth on sole carbon sources (1.0%w/v):	
D-Glucose (Positive control)	+
Arabinose	+
Saccharose	-
D-xylose	+
Myo-Inositol	+
Mannitol	+
D-fructose	+
L-rhamnose	+
Raffinose	±
Mannose	+
Cellobiose	+
ISP9 (Negative control)	-
Growth on sole nitrogen sources (0.1%w/v):	
L-Asparagine monohydrat (Positive control)	+
L-Histidine monohydrat	-
L- Phenylalanine	++
L-Leucine	++
L-Tryptophan	+
L-Arginine monohydrat	±
L-Isoleucine	+
L-Valine	-
L-Methionine	±
L-Lysine	+
L-Threonine	+
L-Cysteine	-
2 Amino 2 hydroxy-methyl 1,3 promo	+
ISP8 (Negative control)	±

The morphological, physiological and biochemical characteristics of strain HP411 were compared with other actinomycetes is the classification and showed that it has, strain HP411 has characteristics similar to the species *Streptomyces variabilis* [19], [21]. To clarify the genus and species of strain HP411, 16S rDNA gene sequence need to be analyzed.

C. 16S rRNA Analysis of Strain HP411

Amplified 16S rRNA gene obtained with 1,200 bp in size, was compared directly by basic local alignment search tool (BLAST). The phylogenetic tree revealed that strain HP411 has an identity of 99% with *Streptomyces variabilis* NBC5 (GQ268022.1) (Fig. 2). The result was quite in agreement with the morphological and physiological characteristic comparison of strain HP411 with *S. variabilis*.

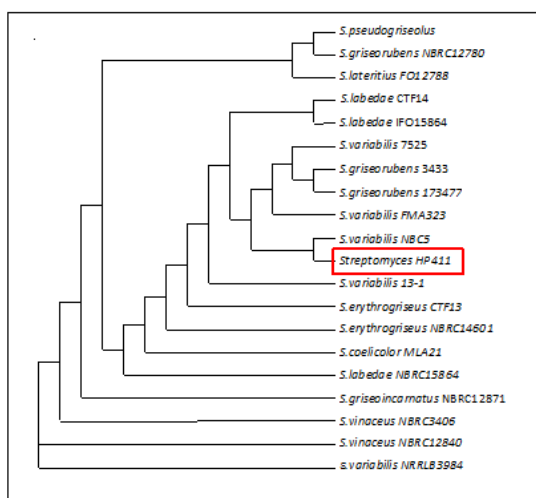


Fig. 2 The genetic relationships of strain HP411 based on the 1,200 bp 16S rDNA gene sequence.

D. Antimicrobial Activity of Strain HP411

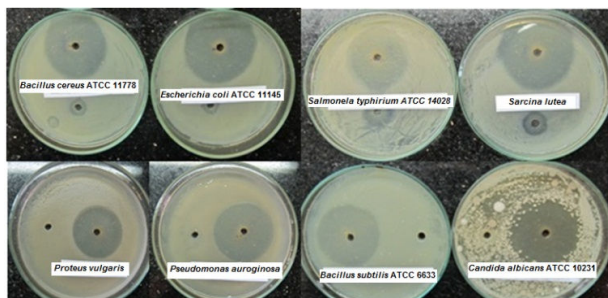


Fig. 3 Inhibition zone of HP411 extract against microorganism test (50µg/ml-37- 42 mm)

After optimizing the extraction step, the ethyl acetate is selected for collecting bioactive compound of HP411. Strain HP411 extract that was containing ethyl acetate was added into the hole on LB agar medium to determine the antibacterial activities that reduces the viability of the initial bacterial inoculum by 99.9%. The results on Fig. 3 showed that all organism tests sensitive with HP411 extract. The ethyl

acetate as positive control does not show significantly different MCB value with organism test (Figs. 3 and 4).

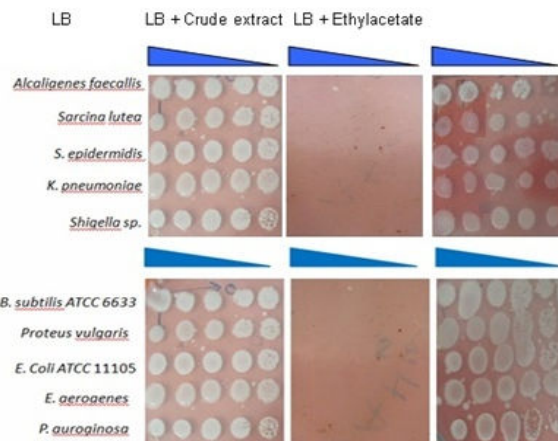


Fig. 4 The antimicrobial activity of HP411 extract with different Gram-negative and Gram-positive bacteria

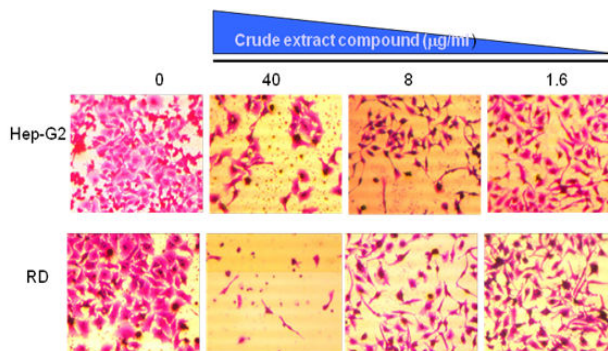


Fig. 5 Image of cytotoxic test Hep G2 cell line and RD cell line with crude extract

E. Cytotoxic Activity of Strain HP411

The HP411 extract was used to study cytotoxic analysis for four cancer cell lines. The results in Fig 5 show that Hep-G2 and RD cells were changed shape and lost cell contacts. In particularly, cells lost their surface morphology, but cytotoxicity was not found when dealing with DMSO. Among four cancer cell lines, MCF7 (breast carcinoma) showed higher tolerance with HP411 extract than other cell lines (Tables V and VI). Moreover the IC₅₀ value of HP411 extract was 13.7, 40, 4.41 and 12.6 µg/ml against liver cancer, skeletal muscle, cardiac RD, cervical carcinoma FL cell which was incubated in 5x10⁴ cells/ml with various concentration of HP411 extract.

TABLE V
CYTOTOXIC ACTIVITY OF *STREPTOMECES* HP411 EXTRACT ON CANCER CELL LINES

Sample	Concentration (µg/ml)	Cell survival (%)			
		Hep-G2	MCF7	RD	FL
DMSO (-)		100.0	100.0	100.0	100.0
Positive control (+)	5	0.3±0.04	0.5±0.1	1.1±0.2	2.5±0.07
HP411	40	16.6±0.5	60.8±0.9	6.2±0.4	2.8±0.2

TABLE VI
IC₅₀ VALUES OF HP411 EXTRACT ON THE CANCER CELL LINES

Sample	Concentration of sample (µg/ml)	IC ₅₀ for cancer cell line (µg/ml)			
		Hep-G2	MCF7	RD	FL
Positive control (+)	5	0.19	0.15	0.22	0.18
HP411	40	13.73	>40	4.41	12.6

IV. CONCLUSION

From ninety-nine actinomycete strains isolated from the coastal areas of Northern Vietnam were screened only seven strains exhibited significant antibacterial activities against both gram-positive and gram-negative bacteria. Further studies were carried out with the most active HP411 strain against *Candida albicans*. This strain was identified as *Streptomyces variabilis* (GQ268022.1). The HP411 strain was cultured and extracted to collect crude antibiotic substance, which showed potent activity *in vitro* against Gram negative, Gram positive and fungi. Moreover, the results showed that the cancer cells were more sensitive to these compounds. The IC₅₀ value of HP411 extract was of 13.7, 40, 4.41 and 12.6 µg/ml against liver cancer Hep-G2, skeletal muscle MCF, cardiac RD and cervical carcinoma FL cell which were incubated in 5x10⁴ cells/ml concentration.

ACKNOWLEDGMENT

This research project was supported by Vietnam Academy of Science and Technology (VAST 02.04/1112).

REFERENCES

- [1] A. L. Demain, S. Sánchez S, "Microbial drug discovery: 80 years of progress", *J. Antibiot.* vol. 62, pp 5-16, 2009.
- [2] J. Berdy, "Bioactive microbial metabolites", *J. Antibiototechnol.* (Tokyo), vol. 58, pp. 1-26, 2005.
- [3] N. S. Egorov, O. Z. Popova, M. V. Bitteeva, V. G. Bulgakova, K. Gofman "Influence of bacterial metabolites on growth and antibiotic properties of some actinomycetes", *Mikrobiologia*, vol. 29, pp. 269-275, 1960 (in Russian)
- [4] H. P. Fiedler, B. Christina, T. B. Alan, C. W. Alan, G. Michael, P. Olivier, P. Carsten, H. Gerhard, H. "Marine Actinomycetes as a source of novel secondary metabolites", *Antonie Leeuwenhoek*, vol. 87, pp. 37-42, 2005.
- [5] B. N. Ghanem, A. S. Soraya, M. E. Zeinab, A. A. E. Gehan, "Isolation and enumeration of marine Actinomycetes from seawater and sediments in Alexandria", *J. Gen. Appl. Microbiol.* vol. 46(45), pp. 105-111, 2000.
- [6] H. Harada, Y. Kamei, "Selective cytotoxicity of marine algae extracts to several human leukemic cell lines", *Cytotechnology*, vol. 25, pp. 213-219, 1997.
- [7] M. Jennifer, "Determination of minimum inhibitory concentration", *J. Antimicrobial Chemotherapy*, vol. 48, Suppl. S1, pp. 5-16, 2001.
- [8] M. Kimura, "A Simple Method for Estimating Evolutionary Rates of Base Substitutions Through Comparative Studies of Nucleotide Sequences", *J. Molecular Evolution*, vol. 16, pp. 111-120, 1980.
- [9] K. S. Lam, "Discovery of novel metabolites from marine actinomycetes", *Curr Opin Microbiol.*, vol. 12, pp. 245-251, 2006.
- [10] K. Likhitwitayawuid, C. K. Angerhofer, G. A. Cordell, J. M. Pezzuto, N. Ruangrungsri, "Cytotoxic and antimalarial bisbenzylisoquinoline alkaloids from *Stephania erecta*", *J. Natural Products*, vol. 56, pp. 30-38, 1993.
- [11] T. J. Mincer, P. R. Jensen, C. A. Kauffman, W. Fenical, "Widespread and persistent populations of a major new marine actinomycete taxon in ocean sediments", *J. Appl. Environ. Microbiol.*, vol. 68, pp. 2005-5011, 2002.
- [12] G. Mohanraj, T. Sekar, "Antagonistic activity of marine *Streptomyces* sp LCJ94 against the shrimp pathogens", *Annals of Biological Research*, vol. 4 (4), pp. 224-227, 2013.
- [13] T. F. Molinski, D. S. Dalisay, S. L. Lievens, "Drug development from marine natural products", *Nat Rev Drug Discov*, vol. 8, pp. 69-85, 2009.
- [14] K. Moller, T. K. Jensen, S. E. Jorsal, T. D. Leser, B. Cartensen, *Veterinary Microbiology*, vol. 62, pp. 59-72, 1998.
- [15] M. Nakagawas, Y. Hayakawa, K. Adachi, H. Seto, "A new depsipeptide antibiotic, variapeptin" *Agric. Biol. Chem.*, vol. 54(3), pp. 791-794, 1990.
- [16] D. J. Newman, G. M. Cragg, "Natural Products As Sources of New Drugs over the 30 Years from 1981 to 2010", *J. Nat. Prod.*, vol. 75(3), pp. 311-335, 2012.
- [17] C. Olano, C. Méndez, J. A. Salas, "Antitumor compounds from actinomycetes: from gene clusters to new derivatives by combinatorial biosynthesis", *J. Nat. Prod. Rep.*, vol. 26, pp. 628-660, 2009.
- [18] N. Saitou, M. Nei, "The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees", *Mol. Biol. Evol.*, 4: 406-425, 1987.
- [19] E. B. Shirling, D. Gottlieb, "Methods for Characterization of *Streptomyces* species" *Intern. J. Systematic Bacteriology*, vol. 16 (3), pp. 313-340, 1966.
- [20] H. D. Tresner, E. J. Backus, "System of color wheels for *Streptomyces* taxonomy" *Appl. Microbiol.*, vol. 11, pp. 335-338, 1963.
- [21] S. T. Williams, M. Goodfellow, G. Alderson, "Genus *Streptomyces*". In: *Bergey's Manual of Systematic Bacteriology*, (S.T. Williams; M. E. Sharpe; J. Holt, Edts.) Baltimore, Williams and Wilkins. vol. 4, pp. 2452-2468, 1989.