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Isolation and Identification of Cytotoxic Potential Bacterium from Marine Sponge *Spirastrella inconstans*

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ABSTRACT

In order to explore the cytotoxic potential of sponge associated marine bacteria, bacterial colonies were obtained from marine sponge *Spirastrella inconstans*. Totally 14 aerobic, heterotropic and morphologically different bacterial strains were purified and cultured in marine broth. Crude metabolite was extracted using ethylacetate as extracting solvent. The metabolites were tested for its antiproliferative activity against A549 lung adenocarcinoma cell line. The colorimetric based MTT assay revealed strain MB13 as potent cytotoxic bacterium. The strain was identified as *Pseudoalteromonas piscicida* using 16s rRNA sequencing and biochemical test.

Keywords: *Spirastrella inconstans, antiproliferative, lung adenocarcinoma, Pseudoalteromonas piscicida.*

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INTRODUCTION

Oceans represent an under-explored environment for microbial diversity and novel secondary metabolites (Magarvey et al., 2004)[1]. Marine bacteria produce chemicals for their own use in diverse array of function including defence, offence and signalling. Also marine microorganisms have wide array of gene source to produce new metabolites with a wide variety of biological applications (Kobayashi and Ishibashi, 1993)[2]. Microorganisms and their isolates represent a major source of unexplored scientific potential. It should be noted that the number of microbial organisms isolated from the vast ocean territories continues to increase each year. Despite the enormous difficulty in isolating and harvesting marine bacteria, microbial metabolites are increasingly attractive to science because of their broad-ranging pharmaceutical applications. The metabolic and physiological capacity of marine microorganism allows itself to adapt the extreme environmental conditions provides an enormous potential for the production of unique compounds. Bioactive natural products originated from bacterial source cover an enormous structural diversity and include multiple compound classes, such as terpenes, polyketides and nonribosomal peptides. These compounds are synthesized by cellular enzymes which are often encoded by genes organized on the same locus in so called biosynthetic pathways.

Two of the classes with a well understood biosynthetic pathway are polyketides (PKs) and nonribosomal peptides (NRPs), which are often isolated from marine microorganisms (Hughes and Fenical, 2010)[3]. Marine bacteria produces secondary metabolites have yielded pharmaceutical products such as novel anti-inflammatory agents (e.g., pseu-dopterosins, topsentins, scytonemin and manoalide), anti- cancer agents (e.g., bryostatins, discodermolide, eleutherobin and sarcodictyin) and antibiotics (e.g., marinone) (Boopathy and Kathiresan, 2010)[4]. Sponge and its symbiotic microbes derived compounds span a wide range of chemical classes with an equally wide range of biotechnologically relevant properties (Matsunaga and Fusetani, 2003)[5].

In many instances, the limited availability of sponge material may preclude the commercial production of bioactive compounds of potential pharmaceutical importance. The isolation of bioactive compounds from symbiotic bacteria could overcome these limitations by providing a consistent yield using large scale laboratory culture, eliminating the need to harvest sponge from natural environment. Also current research aimed to identify a cytotoxic metabolite producing sponge associated bacterium against Non Small Cell Lung Cancer (NSCLC).

MATERIALS AND METHOD

Isolation of sponge associated bacteria

The sponge specimen *Spirastrella inconstans* (Dendy) was collected from the intertidal zone of Palk Bay, Rameshwaram during low tide. Initially, the sponge sample was washed with jets of filtered and autoclaved seawater until they were visibly free of debris. Then the sponge surface was sterilized with rapid swab of 70% ethanol followed by the specimen immersed in autoclaved seawater and then aspirated. One gram of central core of sponge tissue was homogenized with 99 ml of sterilized seawater. The homogenate was serially diluted upto 10^{-5} and spread on the entire surface of 1:10 Marine Agar (peptone, 0.5 g; yeast extract, 0.1 g; FePO4, 0.1 g; agar, 15 g; dissolved in 1 litre of seawater; pH 7.2–7.6) (Zheng et al., 2006)[6]. The plates were incubated at room temperature (approx 30-37ºC) for 5 days and isolation of bacteria with different colony characteristic was carried out from third day onwards till fifth day. On fifth day counts were used for calculation of colony forming unit (CFU). Different types of colonies were isolated and were repeatedly streaked to obtain pure cultures and stored in marine agar slant at 4^{o}C for further studies.

Bacterial culture and crude extract preparation

The aim of this present experiment is to identify a potential bacterium which is capable of producing cytotoxic metabolites. For the production of secondary metabolites, the purified marine bacteria as above were cultured in 300 ml of Marine broth (peptone 5 g, yeast extract 1 g and $FePO_4$ 0.1 g, dissolved in 1 L of seawater, pH 7.2) in 500 ml Erlenmeyer flasks (Zheng *et al.*, 2006)[6]. Flasks were then incubated on a shaker at 220 rev $min^{-1}$for 7 days at 27±3 ^{o}C. On 7th day the broth was centrifuged at 5000 g for 30 min to remove the cell and the supernatant extracted with ethyl acetate (EtoAc) (100 ml x 3). EtoAc was removed under reduced pressure at 37 ^{o}C. Extracts were dissolved in methanol to make a stock solution, which was then diluted to appropriate concentrations with culture medium for cytotoxic analysis.

Cell culture

The lung cancer cell line (A549) was obtained from National center for cell science (NCCS), Pune. The cells were maintained in cultured in Dulbecco's modified Eagle medium (DMEM) medium containing 100 unit/ml penicillin and 100 unit/ml streptomycin and supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere with 5% CO_2. The same media was used in the following A549 cell culturing experimental assays.

Cell viability (MTT) assay

MTT is a colorimetric assay based on the activity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-

yl)-2, 5-diphenyl tetrazolium bromide (MTT) to an insoluble, colored formazan product which is measured spectrophotometrically. A549 cells were inoculated in 96-well plates at the density of 2 x 10^5cells per well. The cultured cells were incubated for 24 h for attachment, prior to addition of crude extract at different concentration 50,100, 150 and 250 µg ml^{-1}, and then cells were incubated for 24 h. 5 µl of methanol alone added without metabolite maintained as control. The cell survival rate was determined with addition of MTT (5 mg ml^{-1} in PBS) 20 µl per well and further incubated for 4 h at 37 ^oC. After pouring out the culture medium, 200 µl of DMSO was added to each well to dissolve the purple formazan product in living cell and measured by the absorbance at 492 nm (Mosmann, 1983)[7]. The percentage of cell viability calculated against drug concentration. The IC_{50} value for potent cytotoxic metabolite was calculated by the procedure of Yang et al. (2000)[8].

Identification of Cytotoxic bacterial Strain

The potential strain MB13 which shows significant cytotoxic activity was identified by biochemical study also using 16s rRNA sequencing. Selected isolate was grown on MacConkey agar media. The shape and colour of the colonies were examined under the microscope after Gram staining. Isolates were biochemically analyzed for the activities of sugar fermentation, oxidase, catalase, V-P test, MR-VP test, starch hydrolysis and gelatin hydrolysis, motility, indole production and citrate utilization. These tests were used to identify the isolates according to Bergey's Manual of Determinative Bacteriology (Buchanan et al., 1974)[9]. The selected strain was further identified by 16S rRNA partial sequencing. The genomic DNA extracted from the marine sponge associated bacterium MB13 was PCR amplified for 16s rRNA genes using the universal bacterial primers Eubac 27F (5'- AGA GTT TGA TCG TGG CTG AG- 3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T- 3'). The partial 16s rRNA gene sequencing was done using Perkin Elmer Applied biosystems and ABI prism software was used to align the sequence and compared sequence were retrived by the queries generated by BLAST of Genbank Database. Phylogenetic analysis was performed with MEGA 4.0 (Tamura et al., 2007)[10]. The tree topologies were inferred using the neighbour-joining method and submitted to NCBI Genbank.

IR spectra

FTIR analysis of the crude cytotoxic metabolite was carried out as follows; the sample (10 mg) was ground with 200 mg of KBr (spectroscopic grade) in a mortar before pressed into 10 mm diameter disks under 6 tons of pressure. FTIR spectra were obtained on a Perkin–Elmer 1600 series, spectrometer. The analysis conditions used were 16 scans at a resolution of 4 cm^{-1} measured between 400 and 4,000 cm^{-1}.

RESULTS AND DISCUSSION

Marine microbial world was a potential research object to discover novel bioactive metabolites. The bioactive compounds are chemical compounds isolated/derived from the nature i.e. living organisms such as plants, animals and microorganisms. These compounds may be derived from primary or rather secondary metabolism of these organisms (Berdy, 2005)[11].The area of application of biologically active compounds from bacterial origin is wide, involving agricultural, industrial and pharmaceutical sectors.

Isolation of sponge-endosymbiotic bacteria

About 14 aerobic, heterotropic and morphologically different bacterial strains were isolated from *S. inconstans* tissue and labelled as MB1 to MB14. Different types of pigmented like brown, yellow and pale yellow colonies were isolated. Some milky white colour strain also found. Total count of bacterial colonies observed as $3x10^2$ on fifth day. The water column of the oceans contains approximately 10^6 bacterial cells per ml (Hagstrom et al., 2002)[12]. Marine bacteria and other marine microorganisms develop unique metabolic and physiological capabilities. These capabilities enable them to survive in extreme habitats and to produce compounds that might not be produced by their terrestrial counterparts. Since 1990, the number of bioactive metabolites from marine bacteria has exponentially increased.

Screening and selection of cytotoxic bacterium

The purified bacterial strains (MB1 – MB14) cultured in 300 ml broth and cell free supernatant extracted with equal volume of ethyl acetate on seventh day. Crude metabolites from fourteen different strains were used in evaluation of its antiproliferative effect against A549 cell line. The final product of each concentrated extract was weighed around 400-500 mg. The concentration of crude metabolite for antiproliferative assay was designed based on this yield. Among 14 marine bacteria isolated from sponge *S. inconstans*, the strain MB13 strain showed significant cytotoxic activity against A549 cell line. At 50 µg ml^{-1} concentration of crude extract, it inhibited 76% of cell viability. The metabolite of MB13 inhibited the growth rate of A549 cell in dose dependant manner, upon increasing concentration the viability of the cell decreased (Figure. 1). The cell growth was inhibited up to 50% at the concentration of 150 µg ml^{-1}. The other bacterial extract did not exhibit considerable cytotoxic activity. Also metabolite of other bacterial strain does not show significant variation depend on different concentration. The strain MB13, with brown pigmentation had significant cytotoxic activity with IC_{50} value of 153±3 µg ml^{-1} was selected as cytotoxic bacterium. Zheng et al. (2006)[6] isolated a cytotoxic bacterium NJ6-3-1 with

IC_{50} values of 150 ± 4.6 µg/ml against HeLa cells from marine sponge *Hymeniacidon perleve*. Cytotoxic activity of crude extract obtained from isolated bacterial culture determined by MTT assay. Marine sponges are a rich source of novel microorganisms with potential pharmacological activity (Hentschel et al., 2001)[13].

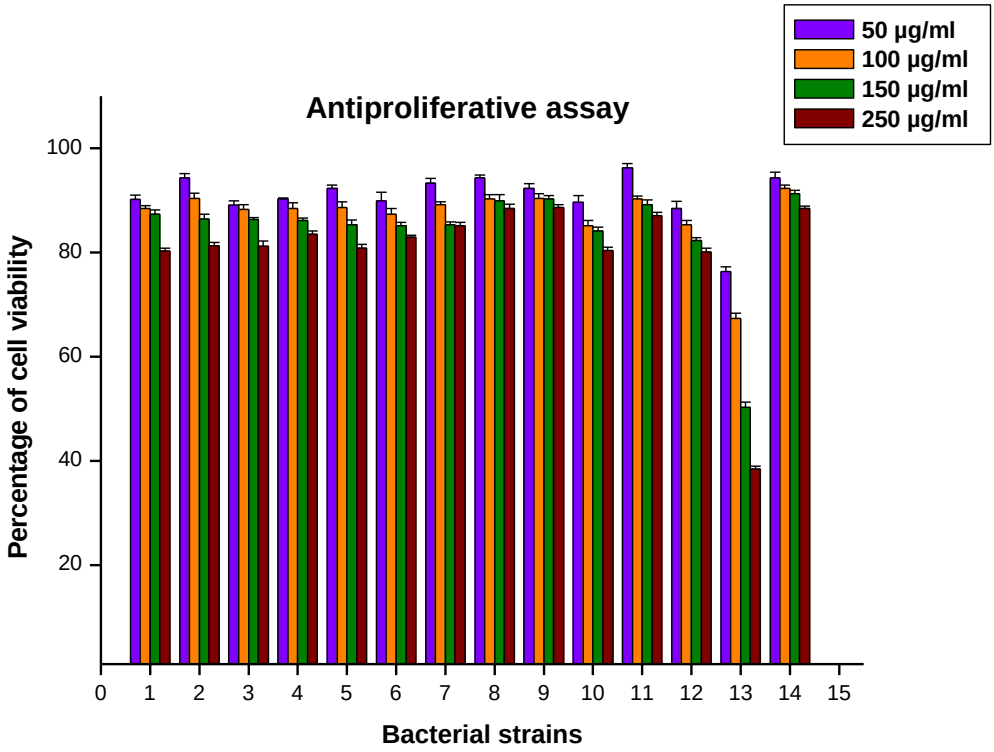

Figure.1. Cytotoxicity of *S. inconstans* associated bacterial metabolites against A549 lung cancer cell line.

Identification of potential bacterium MB13

The selected cytotoxic strain MB13 was identified as *Pseudoalteromonas piscicida* using biochemical tests as per Bergey's manual of systematic bacteriology (Table. 1) and 16S rRNA partial sequencing. The name of the strain was designated as *P. piscicida* MB13. The sequence data was analysed comparatively with already available database and phylogeny based on ClustalX. Figure. 2 clearly indicated that MB13 was close to the member of *P. piscicida.* This sequence had been submitted to Gen Bank (Accession Number: KF113883). The active metabolite producing bacteria *P. piscicida* MB13 was finally selected as the most active strain. Bacteria associated with marine invertebrates are known to display significant antifouling, antibacterial and cytotoxic activities. Especially natural products from sponge have been reported for biomedical potential, pharmaceutical relevance and diverse biotechnological applications (Lee et al., 2001)[14].

Table 1: Biochemical characteristics of strain MB13

Parameter	Character
Cell shape	Rod
Pigmentation	Orange
Gram reaction	Negative
Catalase	+
Oxidase	+
Motility	+
Indole production	-
Citrate utilization	+
Urease	-
Nitrate reduction	+
Gelatin hydrolysis	-
TSI	Alk/Alk
Utilization of	
D-glucose	+
L-arabinose	-
D-mannitol	-
Maltose	+
Xylose	-
D-Mannose	+
Sucrose	+
Glycerol	-
Fumarate	-
Acetate	-
Tween 20	+
Glutamate	+
Pyruvate	-
D-Fructose	+
Galactose	+
Lactose	-
L threonine	-
Ammonia	+
Nitrate	-

Phylogenetic analysis of *Pseudoalteromonas piscicida*

IR spectrum

FTIR spectra were obtained on a FT–IR 8300, Shidmadzu spectrometer. The analysis conditions used were 16 scans at a resolution of 4cm^{-1} measured between 400 and 4,000 cm^{-1} (Figure. 3). The IR spectrum of purified compound displayed an absorption band at ν_{max} 3403 cm^{-1} (hydroxyl). Two strong bands at ν_{max} 2924 and 2854 cm^{-1} are indicative for carboxylic acid O–H stretch. A strong band at ν_{max} 1781 carboxylic acid C=O stretch (Figure. 3).

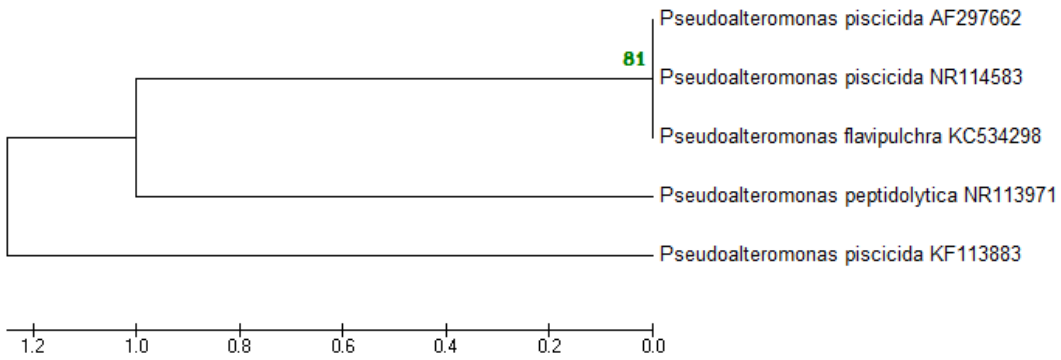

Figure. 2. Phylogenetic tree constructed with MEGA 6 software using neighbour joining method

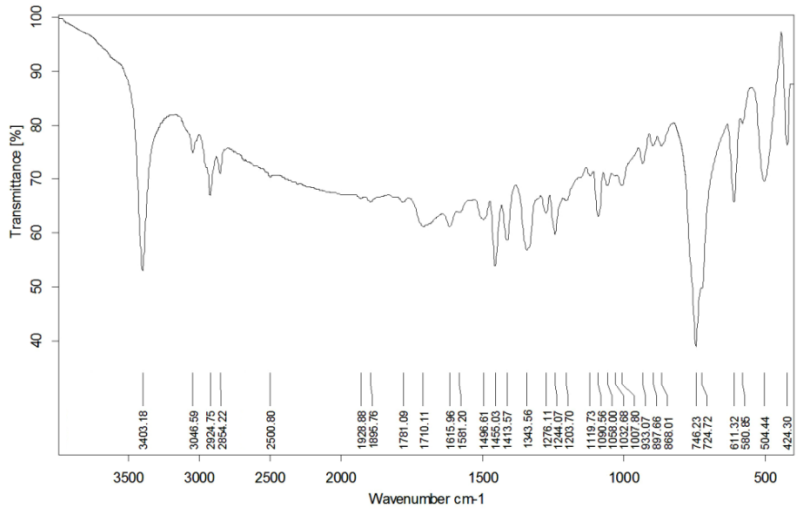

Figure.3. FTIR spectrum for crude metabolite obtained from culture of cytotoxic bacterium *P. piscicida* MB13.

The selected bacterial strain indicated as MB13 and it was identified as *Pseudoalteromonas piscicida* MB13 using biochemical tests as per Bergey's manual of systematic bacteriology and 16s rRNA partial sequencing. Cells were Gram-negative, rod-shaped and motile. Colonies were brown in colour, growth observed under aerobic conditions. *Pseudoalteromonas* consists of Gram negative marine bacteria belonging to the γ-proteobacteria and is present globally in marine waters where they constitute 0.5% to 6% of the total bacterioplankton (Wietz et al., 2010)[15]. Bowman (2007)[16] reported that pigmented species of *Pseudoalteromonas* are often producers of bioactive secondary metabolites displaying cytotoxic (Zheng et al., 2006)[6], antibacterial (McCarthy et al., 1994)[17] and antifungal (Franks et al., 2005)[18] effects. The majority of non- pigmented of *Pseudoalteromonas* indentified as non-antibacterial. The same concept was observed here that brown pigmented bacterium has significant cytotoxic activity than non-pigmented bacteria. Isnanetyo and Kamei, (2003)[19] explored a brown colour brominated

biphenyl compound, 3,3',5,5'-tetrabromo-2,2'-diphenyldiol from *P. phenolica* which is having inhibitory to MRSA.

CONCLUSION

The increasing need for drugs to overcome resistant mechanism resulted in exploring the ocean by numerous scientists in the field of natural products. In the present investigation *P. piscicida* MB13 discovered as potent cytotoxic metabolite exerting microorganism and further investigation is necessary to find out a novel chemical molecule.

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