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Full Length Research Paper

Screening of marine sponge-associated bacteria from *Echinodictyum gorgonoides* and its bioactivity

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This paper describes the evaluation of bacterial strain (MB2) bioactivity isolated from marine sponge. The sponge *Echinodictyum gorgonoides* associated bacterial strain MB2 was tested for its action against various human pathogenic bacterial isolates. The biochemical tests were done to determine the characterization of the bacterial strain and to identify the specific MB2 strain. The cytotoxicity studies of the bacterial strain (MB2) isolated from the sponge was analysed using the Brine shrimp cytotoxicity assay and confirmed against the glioma C6 cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The availability of proteins were analysed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Finally, the isolated bacterial MB2 strain from the marine sponge *E. gorgonoides* was identified through means of 16sRNA sequence analysis. It was then confirmed by means of basic local alignment search tool (BLAST). From these results, it is confirmed that the strain MB2 is *Staphyloccous* sp. Hence it is assumed that the sponge associated proteins and the presence of secondary metabolites could be the source for determining various biological activities.

Key words: *Echinodictyum gorgonoides*, glioma C6 cell lines, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 16s rRNA.

INTRODUCTION

Marine environment have a huge amount of natural sources. The demand of drugs, which can be produced using the effective compounds from marine sources has increased. Bioactive compounds from marine sources (flora and fauna) have extensive past and present use in the treatment of many diseases and serve as compounds of interest both in their natural form and as templates for synthetic modification.

One of the main reasons for studying the marine sources is that oceans cover more than 70% of the world surface and among 36 known living phyla, 34 of them are found in marine environments with more than 300000 and known species of fauna and flora (Jimeno et al., 2004).

Demosponges have the large number bioactive compounds produced from sponge associated microbes. More than 8,000 to 10,000 species of sponges were identified as *Aplysina archeri, Xestospongia muta, Acanthella pulchra, Helicona simulans, Axinella dissimilis, Discodermia dissolute, Raspailia ramosa* (Hooper, 2000). Marine sponges are considered as a gold mine during the past 50 years, with respect to the diversity of secondary

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Abbreviations: PKS, Polyketide synthases; **NRPS,** non-ribosomal peptide synthetases; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **ELISA,** enzyme-linked immunosorbent assay; **PCR,** polymerase chain reaction; **BLAST,** basic local alignment search tool; **TLC,** thin layer chromatography; **EI-MS,** electron ionization mass spectrometry; **NMR,** nuclear magnetic resonance.

metabolites. Sponges are studied because of their wealth in metabolites with a wide variety of biological activities, such as antibacterial, antifungal and antitumor (De Rosa et al., 2002). Marine sponges with the rich source of novel microorganisms have the potential to produce secondary metabolites (Hentschel et al., 2001).

The sponge-associated bacteria cts as a high potential source for the production of antibiotic com-pounds. More than 15,000 species have been described, such as bacteria, archea, fungi and cynobacteria with *Haliclona simulans* having 80 fungal isolates and 100 bacterial isolates. These microbes have the novel genes for producing the bioactive products. The genes like polyketide synthases (PKS) and non-ribosomal peptide synthetases (NRPS) are multifunctional enzymes which are involved in the synthesis of a broad range of structurally diverse natural compounds, many of them are of medical importance (Hutchinson, 2003).

Micro-organisms associated with marine invertebrates are reported to be involved in the production of bioactive molecules. Bioactive compound production in these bacteria could be attributed to the competition among them for space and nutrition (Thakur et al., 2005). Some marine sponge associated bacteria with antimicrobial assets were also detected to have polyketide synthases gene cluster (Jirge and Yogesh, 2010). The objective of the present study is the isolation of a bacterial strain from the marine sponge *Echinodictyum gorgonoides* and its bioactive potential. The strain cultivated on culture flasks were screened biologically and chemically. The culture extracts were analysed for their activity against a set of test organisms, including bacteria using the agar disc diffusion bioassay method, while for the chemical screening, the crude culture extracts were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

MATERIALS AND METHODS

Collection and maintenance of marine sponges

The marine sponge *E. gorgonoides* was collected from the fish nets during the month of December in Kanyakumari at the south east coast of Tamil Nadu, India. The collected sponges were transferred to the laboratory in sterile containers of sea water in the minimum possible time to avoid the external microbial contamination and excessive proliferation. All the adherent fauna were removed by washing sponges with sterile distilled water.

Isolation and characterization of sponge-associated bacteria

20 g of sponge tissue was excised from the middle of the whole sponge *E. gorgonoides* using a sterile scissor.

The excised portion was washed with sterile sea water to remove any bacteria within the current canals and then the tissue was homogenized using a mortar and pestle. The resultant homogenate of 5 ml was serially diluted with sterile seawater/saline. Then the serially diluted samples were plated on nutrient agar (HiMedia). The plates were incubated at 30°C for 24 h. After incubation, the isolated colony was taken and sub cultured for its maintenance and work. Further, the microbes were characterized by gram staining and biochemical methods to confirm whether it is gram negative or gram positive bacteria.

Antibacterial activity

Antibacterial activity of sponge associated bacteria MB2 from the sponge *E. gorgonoides* were tested against the different bacteria (*Escherichia coli, Pseudomonas, Bacillus*, and *Klebsiella*) by disc diffusion method (What man No.1 filter paper). The cultures of sponges were then centrifuged (Remi) for 15 to 20 min. The supernatant were used for testing extracellular antimicrobial activity. The paper disc (6 mm in diameter) was placed over the medium previously inoculated with the test organism. Then different concentrations (50, 75, 100 and 150 µL) of culture supernatant was added. Methanol was used as the control, the plates were incubated for 24 h at 37°C (Li et al., 2005).

Brine shrimp lethality assay

Dried cysts of *Artemia salina* were incubated in natural sea water at 28 to 30°C under constant aeration for 48 h. After hatching, active *naupli* free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Amount of 20 *Artemia naupli* were added into each concentration of culture extract in 24 micro titre well plates. Control was maintained with dimethyl sulfoxide (DMSO) (HiMedia) instead of extract. After 24 h, dead shrimp was counted using a microscope (Nikon). Larvae that did not exhibit any internal or external movement during several seconds of observation was calculated as dead and the percentage of mortality was calculated to determine the LC_{50} values of the extract using the probity scale analysis.

Cytotoxicity assay

Glioma C6 cancer cell lines was obtained from the National Centre for Cell Science, Pune, India. Cells were grown as a monolayer culture in RPMI (HI Media) medium and incubated at 37°C in a 5% of CO_2 atmosphere. Glioma C6 cells (100 µL) were seeded in 96 well plates. Then it was kept for incubation at 37°C in CO_2 incubator. After that, the medium was refreshed with 100 µL of serum free medium (RPMI-1640, sodium bicarbonate, without L-Glutamine) and 20 µL of MTT (5 $mg.ml^{-1}$ of (3,4,5-dimethylthiozol-2yl)-2,5-diphenyltetrazoliumbromide) was added. The micro titre plates were incubated for three hours in the dark. The developed colour was measured with enzyme-linked immunosorbent assay (ELISA) (Analytical Technologies Limited) reader at 570 nm. The extracts of sponge associated bacterial strains were maintained in triplicates for the assay and the results were plotted in graphs by measuring the optical density (OD) values (Sundaram et al., 2010).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The protein compounds were purified form the culture extract by the ammonium sulphate precipitation method, dialysis and column chromatography. Then the purified protein was separated by SDS-PAGE analysis. The stacking gel of 5% and separating gel 12%

Table 1. Antibacterial activity of the sponge *Echinodictyum gorgonoides* associated bacteria by Agar disc diffusion method (MB2)

Bacterial pathogen	Zone inhibition in different concentration (µl) and Zone diameter (mm)			
	50 µl	75 µl	100 µl	150 µl
E. coli	5	7	7.2	8
Proteus spp	5	8	10	11.5
S. aureus	0	1	2.2	1
Pseudomonas	4	3.2	5.1	7
B. subtilis	10	10.2	11	15.6

Table 2. LC_{50} values for 24 h with their 95% fiducial (lower and Upper) limits, regression equation, Chi-Square (x^2) and P-levels of certain marine sponge *Echinodictyum gorgonoides* associated bacteria against *Artemia salina.*

Sample	LC_{50} with fiducial limits		Regression equation	X^2 (df)
	Lower limit	Upper limit	Y = (Y-bx) + b $\log_{10}$ conc.	
MB-2	1.5307	1.7751	Y = 2.49x + 0.88	5.47(8)

were used. The protein bands were separated based on their molecular weight.

16S Ribosomal ribonucleic acid (rRNA) analysis

The isolated strains of bacteria (MB-2) from the sponge *E. gorgonoides* showed a significant antimicrobial activity. Hence the species was identified by polymerase chain reaction (PCR) amplification of the 16S rRNA gene, Basic Local Alignment Search Tool (BLAST) analysis and comparison of the sequences in the Genbank nucleotide database. The 16s rRNA gene was amplified with specific primers, 16s forward primer (5'-AGAGTRTGATCMTYGCTWAC-3') 16s reverse primer (5'-GYTAMCTTWTTACGRCT-3') obtained from (Genei, Bangalore). The reaction mixture was DNA: 1 µl, 16s forward primer 400 ng, 16s reverse primer 400 ng, dNTPs (2.5 mM each) 4 µl 10X Taq DNA polymerase assay buffer 10 µl, Taq DNA polymerase enzyme (3U/ µl) 1 µl Water X µl, total reaction volume: 100 µl. The complete reaction mixture was incubated in a gradient thermocycler, (Eppendorf) PCR temperature Profile: Initial denaturation: 94 for 5 min; denaturation: 94 for 30 s; annealing: 55 for 30 s; extension: 72 for 2 min; final extension: 72 for 15 min; $Mgcl_2$: 1.5 mM final concentration; number of cycles: 35. PCR products were analysed by electrophoresis on agarose gel electrophoresis. PCR products were purified and sequenced. The sequences were compared with known sequences in the genbank nucleotide database or NCBI mega BLAST and the species level was identified as the nearest phylogenetic neighbour with 99% sequence similarity.

RESULTS

In the present investigation, the bioactivity of the sponge associated bacteria (MB2) showed antimicrobial and anticancer properties. The isolated bacterial strain from the sponges *E. gorgonoides* was assayed for their antimicrobial activity against the 5 different pathogens such as *E. coli, Pseudomonas, Staphyloccous aureus*, *Bacillus subtilis* and *Proteus* spp. The marine bacteria were active aganist the four different pathogens such as *E. coli, Pseudomonas, S. aureus and B. subtilis* except *Proteus* spp (Table 1). The zone inhibition was measured with various concentrations of extracts such as, 50, 75, 100, 150 µl.

The cytotoxicity of the isolated strain MB2 from the sponge *E. gorgonoides* was tested against the *A. salina* (Table 2). It showed that increase in concentration of the culture extract have high mortality rate and the LC_{50} values were calculated using Probit scale analysis. The culture of the isolated strain MB2 was tested against the glioma C6 cell line for their anti-cancer activity in 24 well microtitre plate (Table 3). The absorbance was measured at 575 nm by ELISA reader (Figure 1). The sponge associated bacterial strain MB2 from the sponge *E. gorgonoides* act as the source of proteins for producing the secondary metabolites. The sample MB2 had the protein with the molecular weight of 29.35 kDa (Figure 2). The bacterial strain MB2 isolated from the sponge *E. gorgonoides,* was identified by 16s rRNA analysis. It was confirmed that the strain MB2 is the sequence of *Staphyloccous* spp.

DISCUSSION

The present study deals with the isolation of bacterial strains from the marine sponge and the assessment of its bioactive potential. Sponges have been the focus of much recent interest due to the following two main factors: (1) They form close associations with a wide variety of microorganisms and (2) they are a rich source of biologically active secondary metabolites.

Sponges form close associations with a wide variety of

Table 3. MTT assay for MB2 strain of *Echinodictyum gorgonoides* using glioma C6 cell lines.

Concentration of the sample MB2 (µl)	OD at 570 (nm)
5	0.082
10	0.083
15	0.091
20	0.096
25	0.108
30	0.083
35	0.087
40	0.087

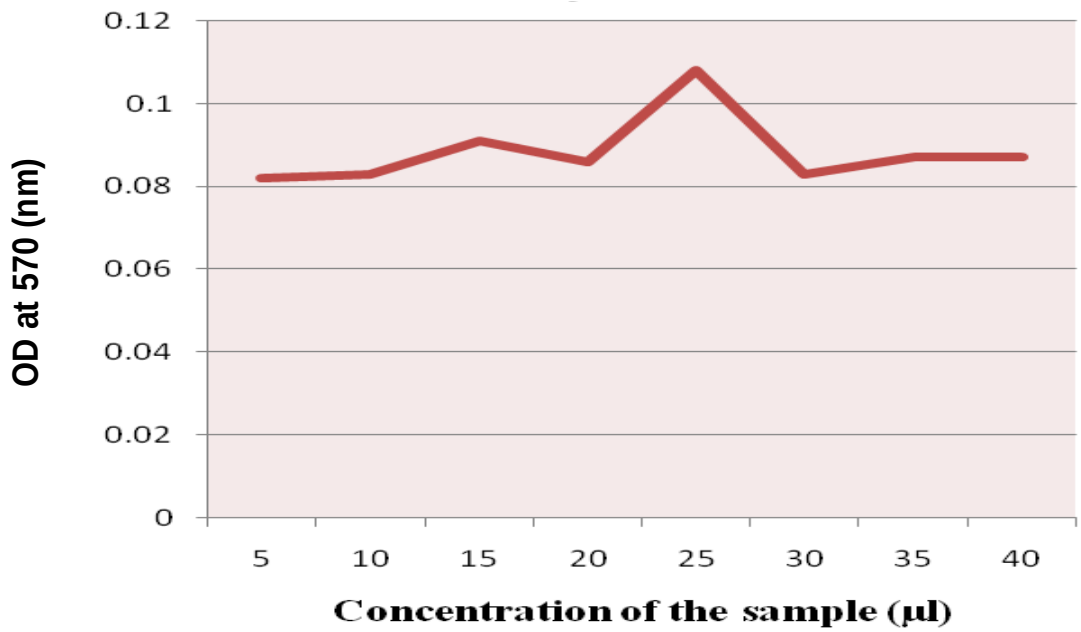

Figure 1. MTT assay for *Echinodictyum gorgonoides* associated bacterial strain against glioma C6 cell lines.

microorganisms which act as a rich source of biologically active secondary metabolites (Hooper and Vansoest, 2002). (Imhoff and Stohr, 2003) reported that sponges due to filter feeding mechanism which leads to passage of microbes inside the sponges results in the deposition of secondary metabolites.

Sponges associated with *Streptomyces* are cultivable; to develop marine-derived products are a promising source for the discovery of novel bioactive compounds (Manilal et al., 2010). Nearly of 45 marine fungi sp. isolated from the two sponges *Fasciospongia cavernosa and Dendrilla nigra* were screened for antimicrobial activity against pathogenic bacteria and fungi. The bioactivity of the strains isolated from the marine sponges. Initial screening revealed that 58 strains showed antibacterial activity against *B. subtilis, S. aureus*, *Vibrio cholerae,* and *E. coli* and 36 strains had antifungal activity against *Aspergillus niger, Saccharomyces cerevisiae* and *Candida albicans*. Earlier, 26 marine endosymbiotic actinomycete strains from *Callyspongia diffusa*, isolated from the Bay of Bengal (coast of India), were screened for antagonistic and antimicrobial activity against pathogenic bacteria and fungi (Gandhimathi et al., 2008).

It was found that the sponge associated bacteria from *E. gorgonoides* have showed high antibacterial activities. Antimicrobial activities of marine bacteria associated with sponges at the south east coast of India showed variety of secondary metabolites such as amino acids, sugars, fatty acids, terpenes, etc. Recently, it was reported that sponge associated *Streptomyces* synthesizes food grade pigments (Selvakumar et al., 2009). In our present study,

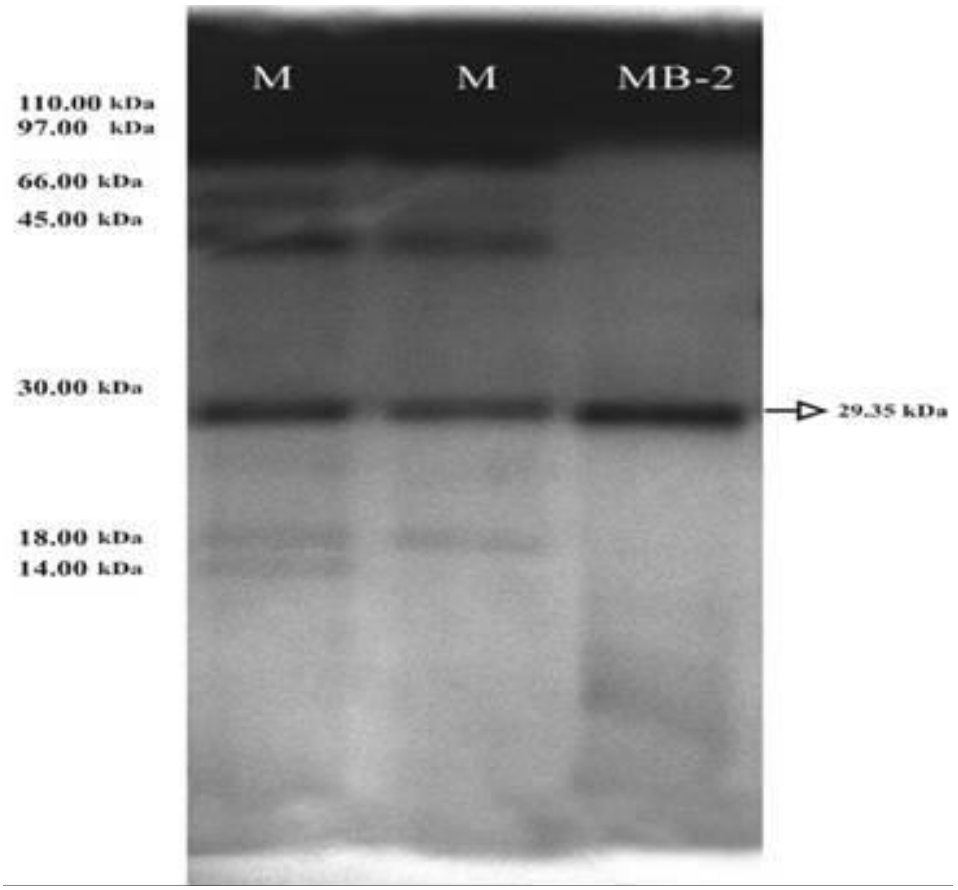

Figure 2. Sodium dodecyl sulphate (SDS) profile of *Echinodictym gorgonoides* associated bacteria (MB2).

the bacterial strain MB2 (*Staphyloccous* spp) from the sponge *E. gorgonoides* collected from kanyakumari showed high antimicrobial activity against the bacterial pathogenic organisms (Figure 3).

Marine bacteria associated with sponge *Aaptos* sp. showed strong growth inhibition indicator microorganisms. The prevalence of antimicrobial resistance in Indonesia shows possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria especially to handle the occurrence of multi drugs resistant (MDR) strains. The major antimicrobial metabolite, isolated from sponges through bioassay-guide fractionation of thin layer chromatography (TLC) bioautography overlay assay, was identified as norharman (a beta-carboline alkaloid) by electron ionization mass spectrometric (EI-MS) and nuclear magnetic resonance (NMR) (Takaizawa et al., 1993).

The marine invertebrates and their symbionts are continuosly exposed to a broad array of potentially deleterious microorganisms, it is reasonable that the production of bioactive secondary metabolites could act as fundamental mechanism of antimicrobial defense (Engel et al., 2002). Marine bacterium associated with sponge *Haliclona* sp. showed strong growth inhibition against MDR strains. This offers the possibility to use sponge bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria in particular among the member of MDR strains. *Pseudomonas putida* strain isolated from the sponge *Mycale microsigmatosa* produced a powerful antimicrobial substance active against multidrug-resistant bacteria (Radjasa, 2008).

It is suggested that the Brine shrimp lethality assay is considered to be one of the most useful tools for the preliminary assessment of biotoxicity and bioassay with cytotoxic activity against some human solid tumors. The antitumour activity of cell free extracts from sponge associate actinomycetes might be due to the presence of the active secondary metabolites alkaloids and guninine (Selvin et al., 2004). In this present study, the brine shrimp lethality assay, the MB2 (*Staphyloccous* spp) strain shows high cytotoxicity. The culture extracts of MB2 showed effective action against the *A. salina*. The cytotoxocity assay is one of the most useful tools

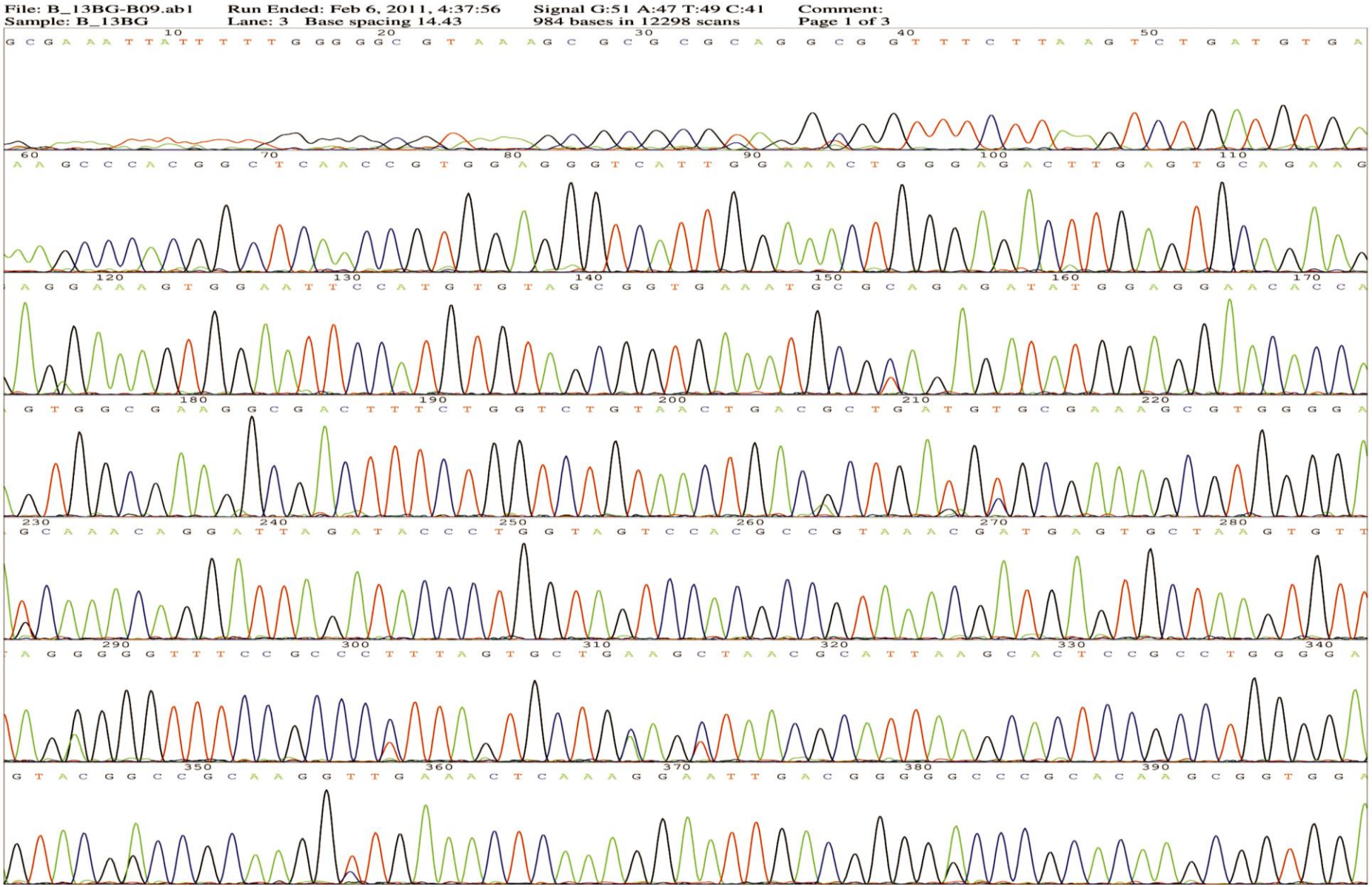

Figure 3. Chromatogram of sponge *Echinodictym gorgonoides associated bacteria Staphylococcus* (MB-2).

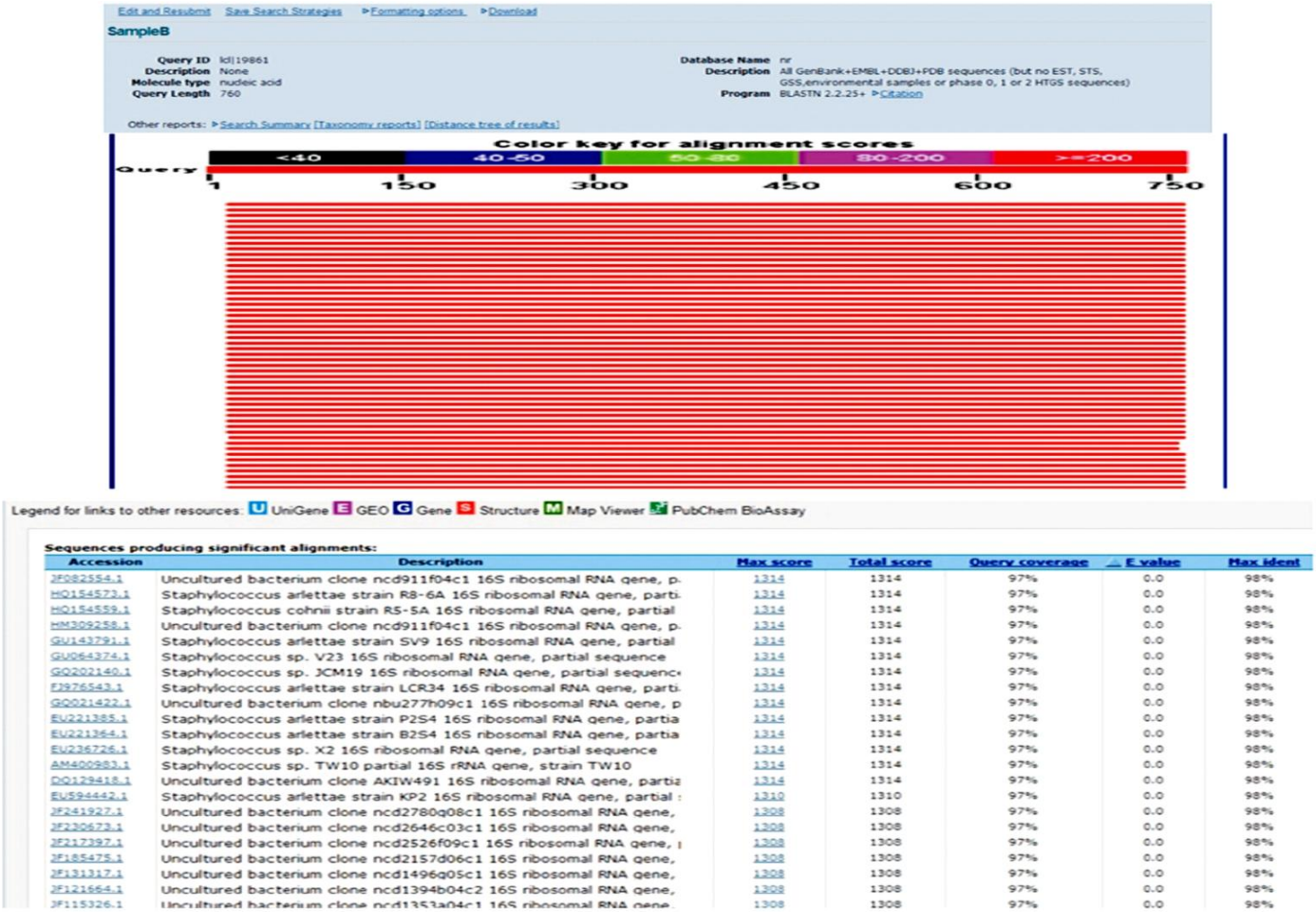

Figure 4. Sequence analysis of sponge *Echinodictym gorgonoides associated bacteria* MB2 identified as *Staphylococcus* submitted in Genbank

for the preliminary assessment of biotoxicity and bioassay with cytotoxic activity of samples against breast cancer cell line (MCF). The antitumour activity of cell free extracts from sponge associate actinomycetes might be due to the presence of the active secondary metabolites (Sundaram et al., 2010). It is determined that the MB2 (*Staphyloccous* spp) strain showed high cytotoxic effect against the Glioma C6 cancer cell line.

It is assumed that TLC autobiographic overlay assay is performed to detect the antimicrobial activity of a large number of sponge samples. This technique tracks the biological activity through the separation of active compounds (Sukarmi and Radjasa, 2007). The antimicrobial ability of the strain could be due to the availability of proteins. The proteins separated by SDS-PAGE analysis also may act as the enhancers for the production of secondary metabolites.

The strain NJ6-3-1 from which wide antimicrobial spectrum was identified as *Pseudoalteromonas piscicida* was based on its 16S rRNA sequence analysis (Figure 4). Antimicrobial activity was found in several isolates, two of which were identified as *Rhodococcus* sp. and *Pseudomonas* sp. by partial 16S rRNA gene sequencing. *P. putida* Mm3 was identified on the basis of 16S rRNA gene sequencing and phenotypic tests. Molecular typing for Mm3 was performed by RAPD-PCR and comparison of the results to other *Pseudomonas* strains (Li et al., 2005). In the present study using the 16sRNA analysis the strain MB2 was identified (Figure 5). Using BLAST, the sequence similarity was identified. The sample MB2 is *Staphyloccous* spp.

Conclusion

In this present work, it is revealed that the marine sponge associated bacteria *Staphylococcus* sp. have the ability to synthesize various secondary metabolites. It could act

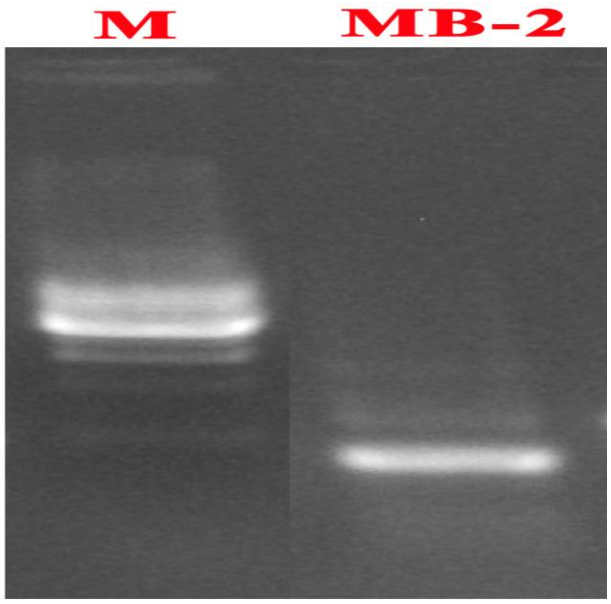

Figure 5. 16s RNA analysis of *Echinodictym gorgonoides* associated bacteria (MB2) and marker M.

as the vital source for promoting various antimicrobial and antitumor effects. Hence the sponge *E. gorgonoides* associated bacteria is considered as a good source for the isolation of bioactive compounds and development of new drugs.

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