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ISOLATION, CHARACTERIZATION, AND BIOACTIVITY OF DEEP SEA BACTERIA WITH SPECIAL REFERENCE TO INDUCTION OF ANTIBACTERIAL AND ANTIOXIDANT METABOLITES FOLLOWING GAMMA IRRADIATION

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ABSTRACT

With an objective to find new marine-derived antibiotics and antioxidants, marine bacteria were isolated from sediment samples collected from depths of 556 m and 698 m in Laccadive Sea of Indian Ocean. The antibacterial and antioxidant activity of these bacteria was studied. Preliminary assays revealed that none of the isolates possess antibacterial activity. However, exposure to gamma radiation resulted in synthesis of metabolites in one of the strain leading to the observation of antibacterial effect. Parallel to this, all the strains were evaluated for their antioxidant activity. The same strain, which exhibited antibacterial activity after gamma-irradiation, showed potent antioxidant activity. The extract which showed biological activity was studied by thin layer chromatography studies. The isolated strain was characterised to be *Bacillus sonorensis* based on phylogenetic analyses using molecular biological techniques.

Keywords: Antibacterial activity, antioxidant activity, gamma irradiation, phylogenetic analyses.

INTRODUCTION

The scope of novel metabolites from terrestrial microorganisms is decreasing hence it is suggested to search some new environments. This has encouraged the new strategies to search useful microbes from marine environments. In this quest for new bio-diversity, the exploration of marine micro organisms particularly marine bacteria has proved to be a rich source of secondary metabolites that display antibacterial properties (Burgess *et al*., 1991; Baam *et al*., 1996). The antibacterial compounds isolated from marine bacterial species are inhibitory to terrestrial bacteria and also many bacterial strains, which are of considerable ecological significance (Saz *et al*., 1963). Studies showed that distribution of antibacterial metabolites is high in sediment living marine bacteria as compared to other sources (Kelecom, 2002). Also deep-ocean sediments hold great promise as a source of genetically novel microorganisms producing structurally unique secondary metabolites (Fenical and Jensen, 2006).

Recently most of the bioprospecting programmes were focused on shallow and often tropical marine organisms, mainly due to the ease of collecting them. But the scientific interest is now focused on the potential medical uses of organisms found in the deep sea. The development of methods for sampling, identification and successful culture of deep-sea microorganisms has uncovered a new resource for drug discovery (Skropeta, 2008).

Search for antibacterial agents in the Deep Sea microorganisms has indeed led to the discovery of new and unique antibiotics. These includes a new pluramycin metabolite, g-indomycinone, along with the known metabolites rubiflavinone C-1 and b-indomycinone from *Streptomyces* sp. obtained from 4680 m deep sediment core (Schumacher *et al*., 1995). Another example is an antibacterial aminoglucose of *Bacillus* sp. from deep waters in the western Pacific Ocean (Fusetani et al., 1987). Apart from these Macrolactin A, which showed moderate antibacterial activity was isolated obtained from 1000-m deep sediment sample (Gustafson *et al*., 1989).

Keeping this in view, the objective of present study was to find out novel marine bacteria with metabolites having antioxidant and antibacterial properties. The samples were obtained from the continental shelves and slopes from depths of 500 to 700 meters i.e., beyond SCUBA, using Indian Research vessel ORV (Oceanographic Research Vessel) Sagar Kanya in Laccadive Sea area.

MATERIALS AND METHODS

Study Site

Sediment samples were collected aboard the Indian Research Vessel ORV 'Sagar Kanya' at station depths of 556 m (09°43.57′N, 75°35.06′E) and 698 m (09°44.03′N,

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75°34.63′E) in the north of Laccadive Sea of Indian Ocean during cruise SK253 in December 2008.

Sampling and Strains Isolation

Sediment samples were collected using a gravity corer (Chandralata *et al*., 2004). As soon as the core was brought on board, samples were collected by inserting a sterile plastic bag at the lower end of the core, into which the sediment samples were extruded. Samples from two similar stations were mixed into one composite sample and kept in a freezer on board and after transportation to lab, stored at -70°C in a deep freezer. Standard dilution plating procedure was used to recover bacteria from sediment samples. Approximately 1g of wet sediment was suspended in 9ml of sterile artificial sea water and vortexed for 1min for thorough mixing of the sample. The sample was serially diluted up to 10^{-5} dilutions. Three replicates of 0.1ml sediment suspension from 10^{-3}, 10^{-4} and 10^{-5} dilutions were spread on Zobell Marine Agar (ZMA) medium (HiMedia, India). The plates were incubated at 30°C and monitored daily up to one month for the development of bacterial colonies. The individual colonies were identified on the basis of visual morphology and colour and streaked on to fresh agar plates to make pure cultures. This process was repeated many times till pure isolates were obtained.

The pure cultures were maintained on ZMA plates, sealed with parafilm and stored at 4°C. They were also stored as glycerol stocks (Floodgate and Hayes, 1961) in triplicates, where a 2 ml of pre sterilized vials with 0.15 ml of 100% glycerol, 0.85 ml of logarithmic phase culture of the isolated strains were added and vortexed vigorously for a minute to ensure mixing of microbial culture and the glycerol. These cultures were then stored at -70°C.

Fermentation and Extraction of Metabolites

Strains were grown in 100 ml of modified Zobell Marine Broth (consisting of peptone 5g/l yeast extract 1g/l and KH_2PO_4 0.1g/l) prepared in 100% artificial seawater, at pH 7.5. After incubation on a rotary shaker at 200 rpm for 72 hours at 30°C, the fermentation broth was extracted using Diaion HP-20 (Supelco, Bellefonte, PA, USA) resin by a modified method of Lee *et al*. (2003) and Melissa (2008). The extraction was performed in triplicates for each strain.

Preparation of Adsorbent Resin

The adsorbent resins was soaked and swelled in 100% (v/v) methanol for 12 hours to remove impurities and the solvent was removed completely by washing with distilled water. The wet resin was weighed at 5% level and added to the fermented broth. This broth with resin was kept on a shaker for 3 hours at 150 rpm.

Recovery of Metabolites from Resin

After shaking for 3 hours, the fermented broth with adsorbent resin was centrifuged at 8000 rpm for 15 minutes and the supernatant was discarded. To the pellet containing Diaion HP-20 and cell mass, 100ml of methanol was added and the metabolites extracted. The pellet was re-extracted with acetone following the same procedure. The methanol and acetone fractions were pooled together and concentrated *in vacuo* by rotary evaporator at 32°C and 150 rpm. The crude extract thus obtained by Diaion HP-20 was dissolved in minimum amount of methanol, collected in pre weighed vials and evaporated to dryness. The weight of crude extract was calculated and stored at 4°C.

Antibacterial assay

The influence of gamma-irradiation on microbes was studied for the antibacterial proficiency. The crude extracts were prepared from the isolated strains and tested directly for antibacterial activity. The extracts were also prepared from microbial strains after gamma-irradiation treatment and tested for antibacterial activity.

Gamma Irradiation treatment

For gamma irradiation treatments, strains were grown up to the stationary phase of growth on ZMA plates. Fully developed cultures were exposed to gamma (γ) irradiation at various dose levels of 1 kGy, 2.5 kGy, 5 kGy, 7.5 kGy and 10 kGy using 60Co gamma chamber (Gamma Cell 5000, Bhabha Radiation Isotope Technology, Bombay) installed at Institute of Nuclear Medicine and Allied Sciences (Ministry of Defence), Defence Research and Development Organization, Delhi, India. The dose rate delivered was 1.92 kGy/h using Baldwin Farmer's secondary dosimeter and Fricke's chemical dosimetry method. Immediately after irradiation, a single colony of cultures were streaked onto fresh agar plates and incubated for 2 days at 30°C. Gamma (γ) irradiation survivors at each dose were selected followed by growth on ZMA plates and the highest tolerable dose for each strain was determined and noted. Cultures unexposed to radiation served as controls. Individual colonies of the irradiated strains at their highest tolerable dose were maintained on ZMA plates, sealed with parafilm and stored at 4°C.

Antibacterial assay

Antibacterial activity of microbial extract was tested against *Escherichia coli*, *Streptomyces albus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus luteus*, Multi-drug resistant (MDR) *Staphylococcus aureus*, Multi-drug resistant (MDR) *Bacillus cereus* and Multi-drug resistant (MDR) *Salmonella typhi* using standard disc diffusion technique (Bauer *et al*., 1966; Ely *et al*., 2004). Muller Hinton agar No.2 (HiMedia, India) was prepared by dissolving 9.5g in 250ml of distilled water and autoclaved at 15 lbs (121°C) for 15 minutes. The medium was dispensed onto 10 separate pre sterilized petriplates under aseptic conditions. The solidified plates were swabbed with test organisms under aseptic condition.

Discs of size 6 mm diameter were prepared from Whatman's filter paper No. 1 and sterilized at 121°C for 15min. The discs were loaded with 10 µl of crude extract at a concentration of 10 mg/ml and left for drying in the laminar airflow. The discs were placed individually using a sterile forceps on the inoculated agar plates and incubated for 24 hours at 37 °C. After incubation, plates were observed for zones of inhibition and measured with an antibiotic zone scale (Himedia, India) and results recorded in millimeters. Disc of Gentamycin (10 mcg/disc) was used as a positive control and disc loaded with the solvent (Methanol) was used as negative control. The averages of the two repeated discs were taken to evaluate the anti bacterial activity.

Antioxidant Assay

The antioxidant activities of the crude extracts from strains unexposed to gamma radiation and extracts from gamma radiation exposed strains were evaluated using 2,2-Diphenyl-1-picrylhydrazyl (DPPH), where the free radical scavenging capacity of the extract(s) was determined (Chandrasekar *et al*., 2006; Takao *et al*., 1994). DPPH was prepared at a concentration of 80 µg/ml. All the extracts were first tested in qualitative assays and the extracts which showed activity were further tested in quantitative assay. Quercetin, a well-known natural antioxidant, was taken as a positive standard.

This method was found to be efficient for detection of antioxidants produced by marine bacteria (Takao *et al*., 1994).

Quantitative Assay

The extracts showing activity in qualitative assays were taken for quantitative assay. The assay was carried out in 96 well microtitre plates. The crude extracts were reconstituted in methanol to make a stock solution of 10 mg/ml. These were serial diluted and tested at various concentrations of 0.5, $5x10^{-2}$, $5x10^{-3}$, $5x10^{-4}$ and $5x10^{-5}$ mg/ml. 100 µl of extract mixed with 100µL of DPPH and incubated for 30 min at 30 °C. The UV absorbance of these solutions was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the quercetin which was taken as standard.

The % of inhibition was calculated by using the equation:

$$\text{Radical scavenging (\% inhibition)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance of the mixture containing sample.

Thin Layer Chromatography Study

Aluminium sheets coated with silica gel with fluorescent indicator 254 nm (silica gel $60F_{254}$; Merck 1.05554; Darmstadt, Germany) were used for TLC. n-butanol/acetic acid/water (4:1:5, v/v/v) was used as mobile phase to develop the spotted samples on the plate. Once the plates were developed they were taken out and allowed to dry. The developed spots were detected by UV light and by staining with ninhydrin solution (Houssen and Jaspars, 2005). Coloration of spots on plates sprayed with ninhydrin was detected by heating on a hot plate.

Molecular Identification and Phylogenetic Analyses of the Producer

Molecular identification of the active product producer was done by 16S rRNA sequencing. Total Genomic DNA preparation was made to isolate HKG 102 (Takagi *et al*., 1993). Amplification of 16S rRNA gene was performed as described (Weisburg *et al.,* 1991) using universal primers fD1 (5' AGTTTGATCCTGGCTCA 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3'). In a 100µl reaction volume, 20 pmol of each primer was used for 20ng genomic DNA template. Amplification was performed on an automated thermocycler (MJ Research, USA) using 1U Taq polymerase (NEB), and the recommended buffer system. Amplification profile was as follows: 95°C for 5 min followed by 30 cycles of 95°C for 1 min, 55°C for 1min, 72°C for 1min and a final extension of 7min. Amplified DNA was purified using QIAGEN PCR purification kit (QIAGEN, Germany) and was sequenced at TCGA (New Delhi, India). The partial sequence of the 16S rRNA gene of isolate HKG 102 was submitted to NCBI GenBank (accession number: GQ849564).

Sequence similarity searches of the sequences obtained were performed using BLAST algorithm of NCBI (Altschul *et al*., 1997) and multiple alignments of the obtained sequences were performed using Clustal X version 2.0.11 (Larkin *et al*., 2007). Phylogenetic analyses were conducted in MEGA4 (Tamura *et al*., 2007) with bootstrap values calculated based on 1,000 iterations. Evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and presented in the units of the number of base substitutions per site.

RESULTS AND DISCUSSION

Sampling and Strains Isolation

Soil is a vast repository of microorganisms and it is to the soil that search is turned when antibiotics are sought. Thus, our current programme was focused on isolating bacteria from soil derived from an exotic location such as deep marine environment. Bacterial samples were successfully isolated and cultivated from the samples

collected at station depths of 556 m (09°43.57′N, 75°35.06′E) and 698 m (09°44.03′N, 75°34.63′E). Though soil is a preferred source for isolating microorganisms, because of its vast resource of diverse microorganisms, nevertheless the samples collected resulted in very few cultivable bacterial isolates, revealing the need for metagenomic approach to be considered for these kinds of samples.

Study Area

The west coast of India, especially the shelf zones of Laccadive Sea have a wide range of salinity and are a resource for diverse microorganisms. Thus, this ecosystem was selected for search of microorganisms with potent antibacterial and antioxidant properties.

A total of 6 strains were isolated from the sediments collected. All the strains were growing only in the presence of salts in the media showing that the strains are strictly marine thriving. The incubation time, temperature, colony morphology and gram reaction of the isolated strains were noted in table 1.

Gamma Irradiation treatment

In this experiment, strain HKG 102 was found to survive high doses of radiation of 10 kGy. Strain HKG 103 was found to survive doses of 7.5 kGy. Strains HKG 104 and HKG 106 were found tolerating doses of 5kGy. Strains HKG 105 and HKG 107 were only able to survive doses of 1kGy. The results of the survival doses of γ-irradiation are shown in figure 2. It can be noted from these results that the coloured isolates were found tolerating higher doses of irradiation in contrast to non coloured isolates, revealing the possibility of their pigments playing role in protecting them from higher doses of gamma irradiation.

Several studies have shown that gamma-ray radiation could change the genetic diversity of bacteria and fungi, which has an effect on improving the antagonistic potential and production of biocontrol agents (Haggag and Mohamed, 2007; Rugthaworn *et al.*, 2007). Classical strain improvement, when employed for years, has allowed for the selection of strains, which are probably altered in the gene regulation and have increased ability to over produce secondary metabolites. This empirical approach has a long history of success, best exemplified by the improvements achieved for penicillin production (Parekh *et al*., 2000).

Encouraged by this idea all the isolated strains were subjected to gamma irradiation in an empirical approach to improve the antibacterial and antioxidant potential of the isolated strains. The gamma irradiated strains were accessed for their antibacterial activity in secondary antimicrobial assay, and the results compared with preliminary antibacterial assays.

Antibacterial assay

A bioassay based on clearing zone on agar was used to determine the antimicrobial activity of crude extracts against clinical isolates. In the preliminary assay, none of the isolates showed inhibitory activity against the ten different pathogens (data not shown) may be due to small amount of the biologically active compound production or complete absence of capability for secretion of such metabolites by the tested isolates.

From a total of six strains, which were all tested for antibacterial production after gamma-irradiation against ten different pathogens including three multi-drug resistant pathogens, only one strain HKG 102 showed satisfactory results i.e., secondary metabolite induction after gamma irradiation, which may be due to mutations at the operator site or other regulatory sites on the gene, relieving end product repression and allowing over-production of biologically active compound or bringing in a new function, to produce the active secondary metabolite. Several other workers have shown the induction of novel bioactive compounds by UV, X- and γ-radiation in various models, including bacteria and plants (Hung and Johnson, 2008; Vilenskaya *et al*., 1972). A plethora of theories advocate that marine bacteria produce the secondary metabolites to protect themselves against the harmful effects of UV radiation (Karentz, 2001). *Bacillus* strain (HKG 102) discovered by us, inhibited considerably the development of all test cultures, except multi-drug resistant *Salmonella typhi*. The inactivity towards multi-drug resistant *Salmonella typhi* may be

Table 1. Colony morphological characteristics of the isolates.

Strain	Medium	Incubation period	Colour	Form	Elevation	Margin	Shape	Gram reaction
HKG 102	ZMA	3-4 days	Orange	Circular	Raised	Entire	Bacilli	+
HKG 103	ZMA	3-4 days	Dark yellow	Circular	Raised	Entire	Spirilla	–
HKG 104	ZMA	3-4 days	Chalk white	Rhizoid	Flat	Lobate	Cocci	–
HKG 105	ZMA	3-4 days	White	Circular	Raised	Entire	Cocci	–
HKG 106	ZMA	3 days	Bright yellow	Circular	Raised	Entire	Cocci	–
HKG 107	ZMA	3 days	Cream	Circular	Raised	Entire	Cocci	–

attributed to the inability of the strain to synthesize molecules that have a bacteriostatic or bactericidal effect on the *Salmonella* strain. The inhibitory activity was observed against both gram positive and gram negative bacteria. The *Bacillus* strain showed good spectra of inhibitory activity against *Streptomyces albus, Bacillus subtilis, Pseudomonas aeruginosa, Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Dietzia* sp., multi-drug resistant *Staphylococcus aureus* and multi-drug resistant *Bacillus cereus* (Table 2). It is worth mentioning that this strain showed inhibitory activity against multi-drug resistant *Staphylococcus aureus* and multi-drug resistant *Bacillus cereus*. The inhibitory activity was against both gram positive and gram negative bacteria. It is clear that, methanol which was used as a negative control, did not show any inhibitory activity and the fact also indicated that methanol has completely evaporated from the assay discs. Maximum inhibition zone, 18mm was noted against multi-drug resistant *Staphylococcus aureus* (Fig. 1), whereas minimum inhibition zone, 13mm was against *Streptomyces albus*.

Table 2. Antibacterial activity (inhibition zone measured in mm) of crude extract from HKG 102 strain after gamma irradiation against clinical isolates following disc diffusion technique.

Strain tested	Size of zone of inhibition (mm)[a]
Streptomyces albus	13
Bacillus subtilis	15
Pseudomonas aeruginosa	15
Staphylococcus aureus	16
Micrococcus luteus	14
Escherichia coli	14
Dietzia sp.	15
Multi drug resistant *Staphylococcus aureus*	18
Multi drug resistant *Bacillus cereus*	17
Multi drug resistant *Salmonella typhi*	–

[a]Key: – negative

Despite the fact that the inhibition zones were smaller, when compared to the positive control Gentamycin, the activity demonstrated was remarkable for the detail that the crude extract was tested at low concentration of 10 µl/disc and may show significant activity when the crude extract is purified.

As was demonstrated earlier, representatives of the genus *Bacillus* are capable of producing several antibiotic substances (Motta *et al*., 2004; Rosenfeld and Zobell, 1947). Presumably, this is the reason underlying the wide range of antibacterial activity of the bacteria tested. Studies on the antibacterial property of these Laccadive sea bacteria towards opportunistic microorganisms demonstrated that the representatives of the genera *Bacillus* were strong antagonists of microorganisms that cause various human diseases.

Fig. 1. Bioassay plate showing antibacterial activity against multi-drug resistant *Staphylococcus aureus* Negative and blank control: methanol (1), crude extract from HKG 102 strain before gamma-irradiation treatment at 10 µl/disc (2), crude extract from HKG 102 *Bacillus sonorensis* after gamma-irradiation treatment 10 µl/disc showing 18 mm clear zone (3), positive control: gentamicin at 10 mcg/disc showing 31 mm clear zone (4).

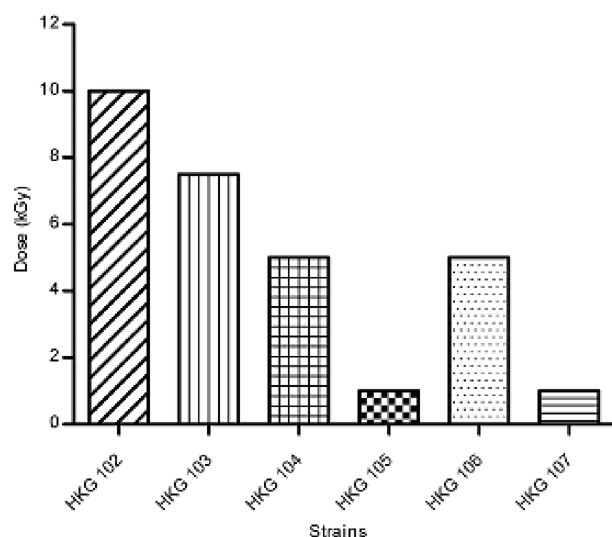

Fig. 2. Survival doses of γ-irradiation on the isolated strains.

Furthermore, the encouraging biological activities seen in this study show that the deep sea bacteria in the Indian shelf zone is a potential source of marine microorganisms and demands further investigation.

Antioxidant Assay

The antioxidant effects of the crude extracts, prepared from the isolated strains before irradiation treatment, were compared with a well known antioxidant quercetin by determining the % of free radical scavenged which is generated by DPPH.

As seen in figure 3, crude extract of isolate HKG 102 exhibited remarkable antioxidant activity when compared with the quercetin. A dose-dependent inhibition was observed; at lower concentrations ($5x10^{-5}$ and $5x10^{-4}$ mg/ml) crude extract showed strong antioxidant property than quercetin but showed comparable activity with that of quercetin with the increase in concentration. The antioxidant test with DPPH used in this experiment is based on a proton radical scavenging action, which is one of the various mechanisms of antioxidation. This result also indicates that the antioxidants produced by the isolate HKG 102, play a role in protecting cells by quenching singlet oxygen generated by gamma radiation making them to survive at high doses of radiation of 10 kGy.

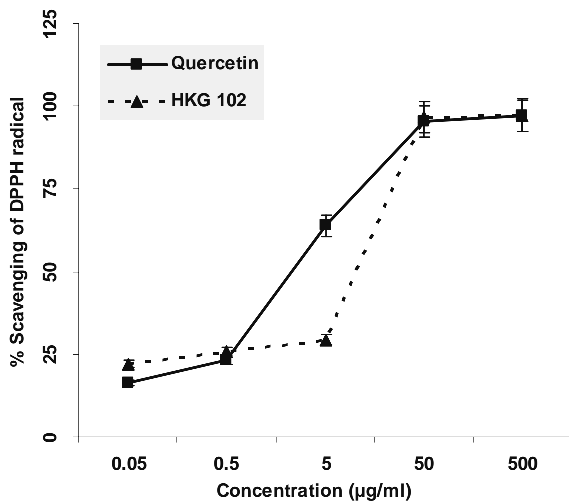

Fig. 3. Inhibitory activity of the HKG 102 crude extract in the DPPH assay. The experiment was done in triplicate and values are expressed as mean ± S.D.

Thin Layer Chromatography Study

The crude extract from HKG 102 was separated and gave positive reaction when sprayed with ninhydrin when acidic solvent system (4:1:5 n-butanol/acetic acid/water) was used as a mobile phase. The results suggest that the extract is more polar in chemical solubility. The crude extract showed a purple spot and pink-red spots when sprayed with ninhydrin, suggesting that the extract contained amines. Clues to the composition of the bioactive compound were found in this study.

16S rDNA Sequencing and Analyses

The 16S ribosomal DNA sequence of the isolate HKG 102 which was showing antimicrobial activity was determined and the partial sequence of the 16S rRNA gene was submitted to NCBI GenBank (accession number: GQ849564).

A BLAST analysis of the isolate HKG 102 showed that it was highly similar to several species of the genus *Bacillus* in Genbank, including *Bacillus sonorensis* (maximum identity: 99%), *Bacillaceae bacterium* (maximum identity: 99%), *Bacillus licheniformis* (maximum identity: 99%) and *Bacillus* sp. (maximum identity: 99%). The phylogenetic tree also exhibited similar genetic relationship. The phylogenetic analysis showed clear homology of the isolate with respective reference species and their evolutionary relationship with other species in the same genus (Fig. 4).

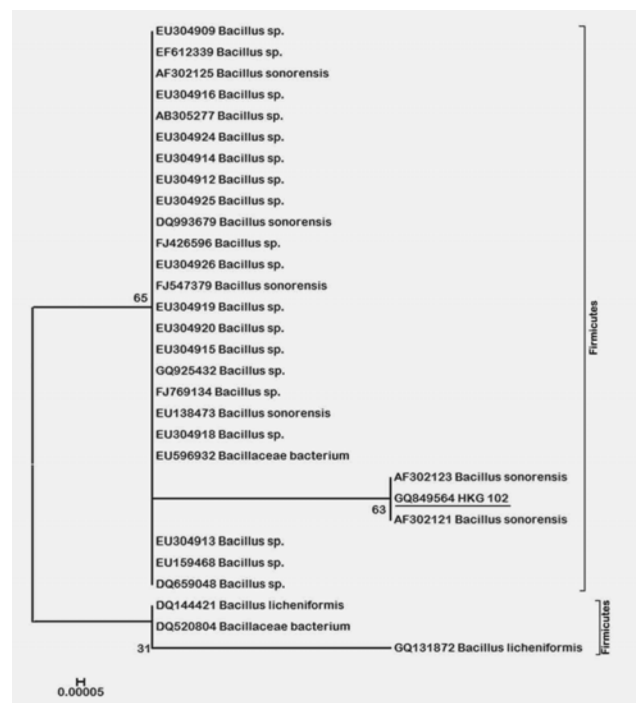

Fig 4. Phylogenetic tree showing evolutionary relationship of HKG 102 16S ribosomal RNA gene with reference sequences obtained through BLAST analysis. Sequences obtained from this study are underlined. The optimal tree with the sum of branch length = 0.00516888 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985).

In the phylogenetic tree constructed by Neighbor-Joining method the isolate HKG 102 was placed on the same clade with the *Bacillus sonorensis* (Fig. 4) supported by the 1000 bootstrap replicates, where associated taxa were clustered together.

By the above molecular and phylogenetic analysis the active bacterial strain HKG 102 can be concluded as *Bacillus sonorensis* and the complete lineage of the bacterial isolate can be written as Phylum: Firmicutes, Class: Bacilli, Order: Bacillales, Family: Bacillaceae, Genus: *Bacillus*, Species: *sonorensis.*

CONCLUSIONS

This study confirmed that the frequency of antibacterial activities displayed by the marine bacteria were superfluous as compare to terrestrial samples and may be expected to be new potential producers of new antibacterial and antioxidants. This study also suggested that the induction of antibacterial metabolites upon irradiation could be lead to a variety of novel bioactivities molecules.

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