Trakia Journal of Sciences, Vol. 10, Suppl. 1, 8-16, 2012

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

COMPARATIVE ANTIOXIDANT PROFILING OF *EXIGUOBACTERIUM* ISOLATED FROM DIFFERENT GEOGRAPHICAL REGIONS

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ABSTRACT

Antioxidants are compounds that are produced by a cell in response to the reactive oxygen species (ROS) during stress conditions. These antioxidant molecules are produced by the many bacteria under various stress conditions. The main aim of this study was to investigate the comparative antioxidant profile of seven *Exiguobacterium* strains isolated from soil and water samples of varied regions from Leh, in the North and Kerala, the south part of India. The biochemical characterization and 16S rDNA sequence of these strains showed 95-99% nucleotide sequence homology with that of *Exiguobacterium strains.* The growth patterns of these seven isolates were studied against salt, pH and radiation. Furthermore, these seven strains were investigated for different antioxidant assays; DPPH, ABTS, Hydrogen Peroxide, Hydroxyl Radical, Nitric Oxide, Fe^{2+} chelation and superoxide anion scavenging. The results showed that overall 70 % higher antioxidant activity was observe in these extremophilic strains. Higher DPPH activity was observed in salt resistant strains while more hydroxyl radical scavenging activity was found in acidophilic strains. The Superoxide anion scavenging activity was observed in all strains except psycrophilic strain. The psycrophilc strain showed good (Fe^{2+}) chelation activity. This study demonstrated that *Exiguobacterium* produces different antioxidant compounds in response to its different extreme survival conditions.

Key words: Antioxidant, *Exiguobacterium*, DPPH, TEAC, SOD.

INTRODUCTION

In the era of 1900s, discovery of microbial life which could survive extremes of salt, temperature, acid, pressure, radiation or oxygen scarcity, which otherwise are detrimental to complex organisms, was made. Such organisms that thrive in extreme physical or geochemical conditions are termed as extremophiles [1] [2]. Extremophiles are broadly categorized into thermophiles, psychrophiles, halophiles, acidophiles, alkaliphiles, and barophiles depending upon their tolerance [3]. Many microorganisms have been profiled according to their specific tolerance such as *B.subtilis* (an anaerobic thermophilic halophilic)[4], *M.jannaschii* (thermophilic, tolerates 230 atm pressure, is a methane producer and utilizes CO_2 as sole carbon source)[5] *and D.radiodurans* for its characteristic property of radiation tolerance till 1.5 million rads [6]. Furthermore, all these stated organisms are also known to produce antimicrobials and/or antioxidants. However, extremophiles like *Nanoarchaeum equitans* and *Exiguobacterium* are yet to be assessed for their potential role in producing these bioactive compounds [7] [8].

Since these organisms grow in extreme conditions, they tend to generate more ROS, leading to cell death. In order to prevent this oxidative damage organisms have developed a complex network of antioxidants [9] [10]. Antioxidants are bio-molecules which can be either enzymes, secondary metabolites or metal ligands. These are known to eliminate free radical species within the cell. Many assays including DPPH, ABTS, Nitric Oxide, Metal

chelation, Hydroxyl Radical Scavenging and enzyme assays for SOD, catalase have been developed for the profiling of these antioxidants [11] [12].

Here, we provide a comparative analysis of the antioxidants from *Exiguobacterium* sp. isolated from different geographical regions.

MATERIALS AND METHODS

Isolation and culture conditions

The strains were isolated from the soil and water samples of different geographical regions of India. The isolates were purified by repetitive streaking onto the Luria Berteni Agar (Himedia) media plates. These were subjected to different temperature range, pH range and salt percentage to check for optimal conditions of growth and survival [13] [15].

Molecular And Biochemical Characterisation

The biochemaical characterisation was done using Bergey's Manual of Determinative Bacteriology [14] [15]. The 16s rRNA gene amplication was performed [16] [15] on the genomic DNA extracted from the strains using the universal primers (fD1 5′AGTTTGATCCTGGCTCA 3′ and rP2 5′ACGGCTACCTTGTTACGACTT 3′). The purification of the gene sequence was done using PCR Purification kit (mdi technologies) and the samples were sequenced at GCC biotech (Pvt.) Ltd. (Kolkata,India). NCBI, BLAST algorithm was used for similarity searches for these purified amplicons.

Phylogenetic Analysis

The 16S rRNA sequence analysis of the isolated and already annotated strains of *Exiguobactreium* sp. was performed using dnapars algorithm of PHYLIP program generating an un-rooted (neighbor joining) phylogenetic tree[17].

Screening For Production Of Antioxidants

The extraction process is initiated by shake flask fermentation of the bacterial strains from which the supernatant was separated by centrifugation. The final extraction was performed using Diaion-HP20 with a slight modification on the culture conditions for each strain [18] [19].

Antioxidant activity

The antioxidant activity was determined by performing a variety of assays; DPPH radical scavenging assay[17], ABTS radical scavenging assay, Hydroxyl radical scavenging assay, Hydrogen peroxide scavenging assay, Nitric oxide scavenging assay etc.

DPPH$^{\cdot}$ Radical scavenging assay

The samples were assayed for various concentrations of 0-10mg/ml for DPPH scavenging [20].

ABTS$^{\cdot+}$ Radical Scavenging Assay

The ABTS activity was analysed for 0-10mg/ml concentrations of the bacterial extracts [21].

Hydrogen Peroxide Scavenging Assay

The scavenging of hydrogen peroxide was assayed by calorimetric estimation at 230nm [22].

Nitric Oxide Scavenging Activity

Nitric oxide scavenging activity was analysed by extent of completion of the diazotization reaction [23].

Hydroxyl Radical Scavenging Activity

The assay for hydroxyl radical scavenging was carried out using EDTA-ascorbate reaction mechanism [24].

Fe^{2+} Chelation

The chelation capability of the bacterial extracts was determined by the standard method with modifications as necessary [25].

Sod Enzyme Assay

The scavenging of superoxide anion by the bacterial extracts was estimated by NBT reaction [26].

RESULTS

Culture Conditions

The bacterial strains isolated showed a growth temperature range of 4°C to 40°C. The pH and salt survival conditions showed that the strains grow in a pH range of 5-9 and sustained salt (NaCl) concentration up to 12% as shown in **Figure 1** and **Table 1**.

Molecular And Biochemical Characterization

The bacteria are non-spore forming, Gram positive bacteria. They are rods with rounded ends and generally occur in chains. They are facultative anaerobes but can actively grow in the presence of oxygen. The DNA sequences obtained after 16s rRNA amplification have been submitted to NCBI and accession numbers have been obtained as in **Table 1.** BLAST algorithm suggested that they belong to the class of Exiguobacterium sp.

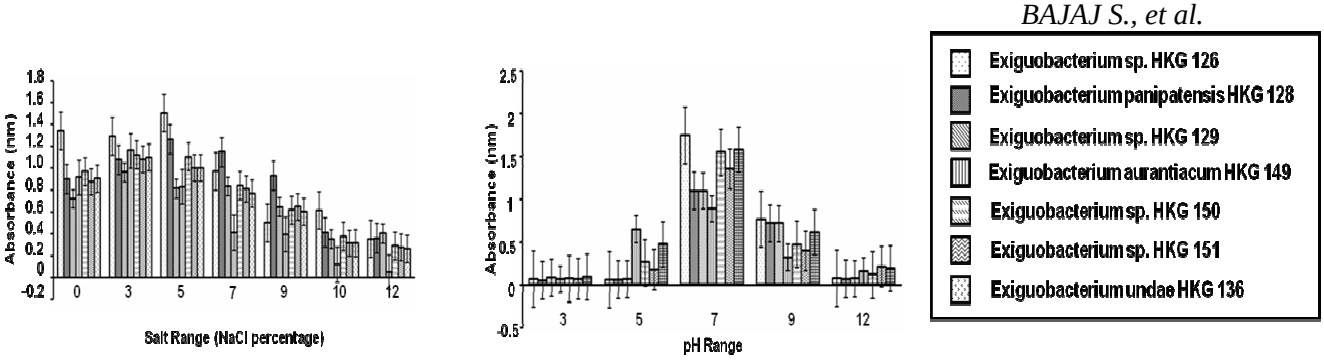

Figure 1. Optimal Growth Conditions for the seven Exiguobacterium strains (a) Salt Survival (b) pH Survival

Table 1. *The Accession Numbers of the isolated strains after 16S rRNA gene sequencing obtained after submission to NCBI.*

Isolated strains	Accession Numbers	Growth Temperature	Salt Concentration NaCl	pH range
Exiguobacterium sp. HKG 126	HM012684.1	37°C	12%	N
Exiguobacterium panipatensis strain HKG 128	HM369792.1	37°C	12%	N
Exiguobacterium sp. HKG 129	HM369791.1	37°C	12%	N
Exiguobacterium aurantiacum strain HKG 149	JN571722.1	37°C	0 %	5-9
Exiguobacterium sp. HKG 150	JQ425481.1	37°C	12%	5-9
Exiguobacterium sp. HKG 151	JQ425482.1	37°C	12%	5-9
Exiguobacterium undae strain HKG 126	JF790197.1	4°C	12%	5-9

Phylogenetic Analysis

The phylogenetic analysis of the isolated strains and the annotated strains of Exiguobacterium sp. showed that the isolated strains were close neighbors to each other whereas the already annotated strains were clustered in one group. This revealed that the bacteria that are isolated from stress conditions

would show somewhat similar characteristics. However, within the cluster of the isolated strains E.aurantiacum HKG 149 slightly diverges out therefore, could have slight difference in its properties. Hence, although these strains are close neighbors but a slight difference in the activities may be observed **(Figure 9).**

Antioxidant Activity

The antioxidant activity of the extracts of Exiguobacterium sp. was found to be concentration dependent; increased with the increase in extract concentration. The activity was analyzed till a concentration of 10mg/ml as maxima in these cases.

DPPH• Radical Scavenging Assay

The samples tested at various concentrations of 0-10mg/ml showed about 15-20% activity on an average at the concentration of 2mg/ml, to that of the standard quercetin (which showed a constant 70% activity on all concentrations). The activity increased considerably on increasing the concentration of the extracts from 15% at 2mg/ml to 60% at 10 mg/ml, showing that higher the concentration of the extract taken higher is the antioxidant activity **(Figure 2).**

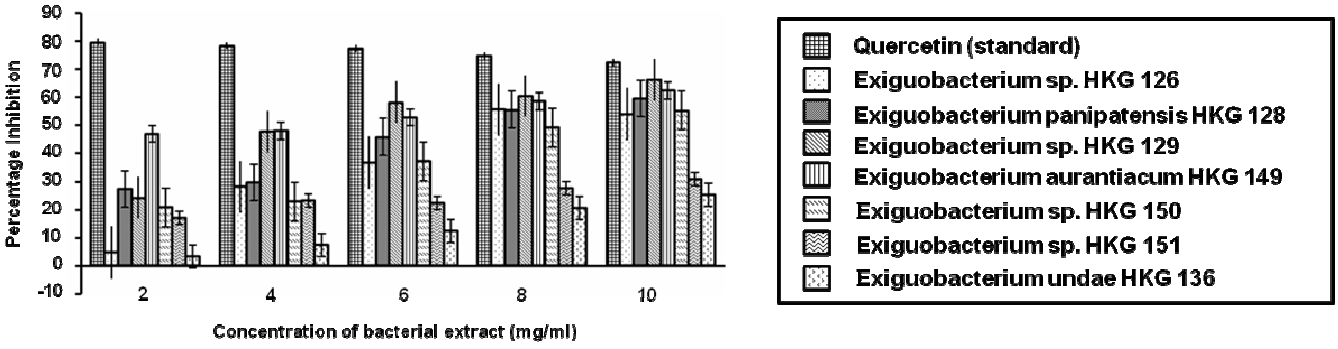

Figure 2. DPPH radical scavenging assay Exiguobacterium extracts

ABTS$^{\bullet+}$ *Radical Scavenging Assay*

The bacterial extracts tested for ABTS$^{\bullet+}$ radical de-colorizations at different time points and at increasing concentrations of 0-10mg/ml were found to show an increase in percentage inhibition after 1 minute of initial mixing and around 5 minutes. Figure 3 shows the time point increase in the values.

The percentage inhibition was tabulated as the antioxidant concentration giving same percentage inhibition of absorbance of the radical cation at 734 nm as 1 mg/ml Trolox was calculated in terms of the Trolox equivalent antioxidant capacity (TEAC) at each specific time-point, in Table 2.

Table 2. *TEAC values for the bacterial extracts from the ABTS assay.*

Sample / Time	0 min	1 min	2 min	3 min	4 min	5 min
RMS	4.5	7.2	5.6	5.5	5.9	7
RWS	2.1	5.7	4.8	4.7	4.8	5.7
RVS	2.1	5.7	4.8	4.7	4.8	5.7
P9	0.8	4.2	3.3	3.3	3.3	4.3
LS1B	2	4.8	3.8	3.5	3.75	4.8
LS3B	2	3.2	2.8	2.85	2.8	3.5
AM5	1	4.2	2.4	2.35	2.3	3.2

Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging activity was seen to increase from the start point of the reaction (0 minutes) till 20 minutes. The activity then decreased over the time points, showing that the reaction reaches completion within 20 minutes of initial mixing, i.e. on addition of the substrate H_2O_2; later making H_2O_2 as the limiting element of the reaction **(Figure 4).**

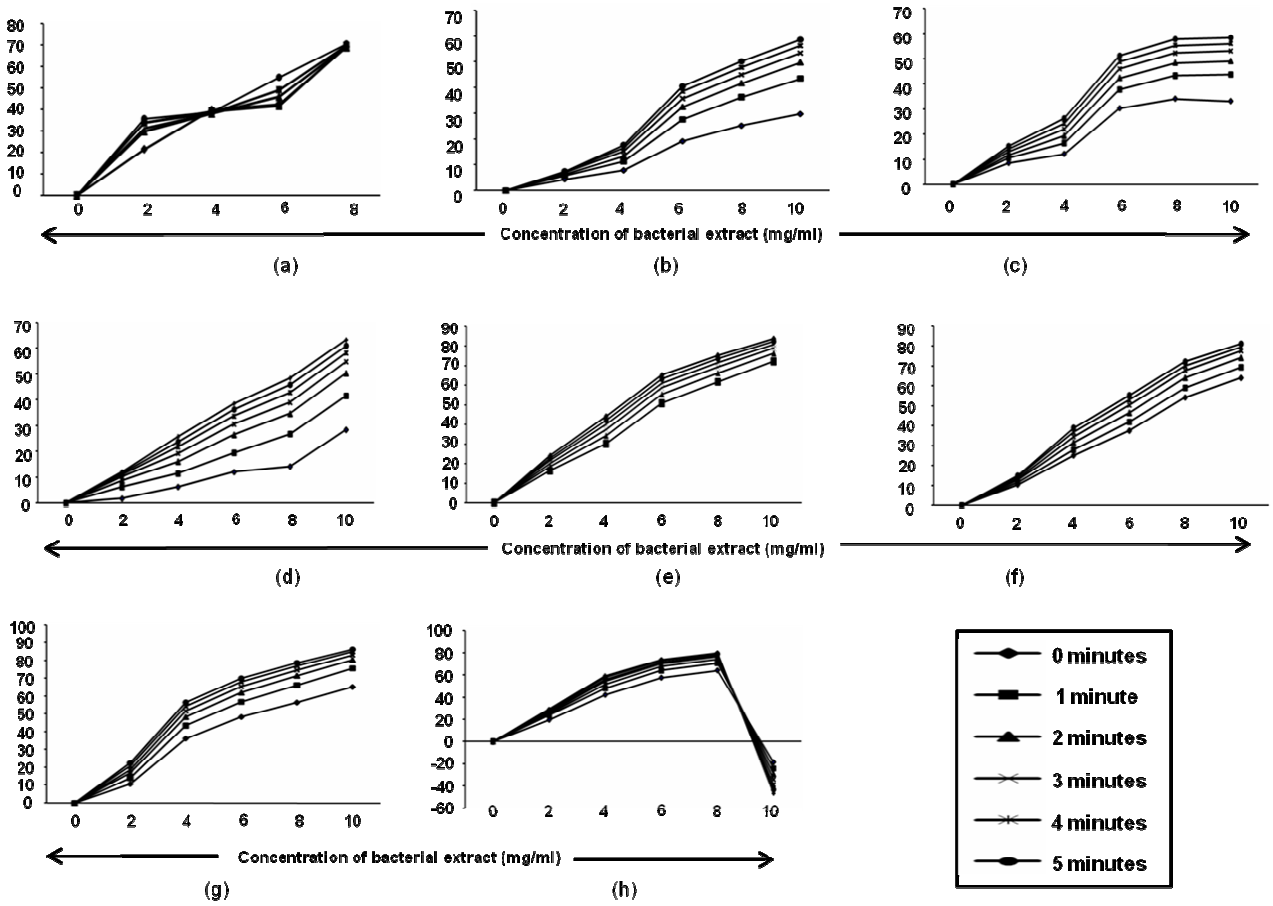

Figure 3. The ABTS.+ radical scavenging assay for the different bacterial extracts. (a) Trolox, reference (b) Exiguobacterium sp. HKG 126 (c) Exiguobacterium panipatensis HKG 128 (d) Exiguobacterium panipatensis HKG 129 (e) Exiguobacterium aurantiacum HKG 149 (f) Exiguobacterium sp. HKG 150 (g) Exiguobacterium sp. HKG 151 (h) Exiguobacterium undae HKG 136.

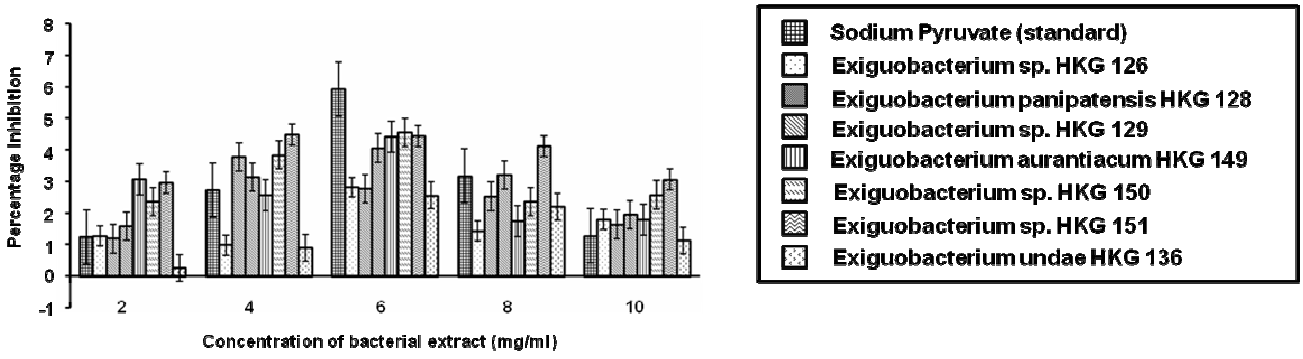

Figure 4. Hydrogen Peroxide Scavenging activity of the *Exiguobacterium sp.* extracts

Nitric Oxide Scavenging Activity

The extracts tend to show an increased power of scavenging the nitric oxide radical as compared to that of curcumin, which is taken as standard. Exiguobacterium sp. HKG 129 tends to show the maximum percentage inhibition on an average, however the percentage inhibition is concentration dependent in all the cases, i.e. increase in concentration leads to an increased inhibition, as shown in **Figure 5**.

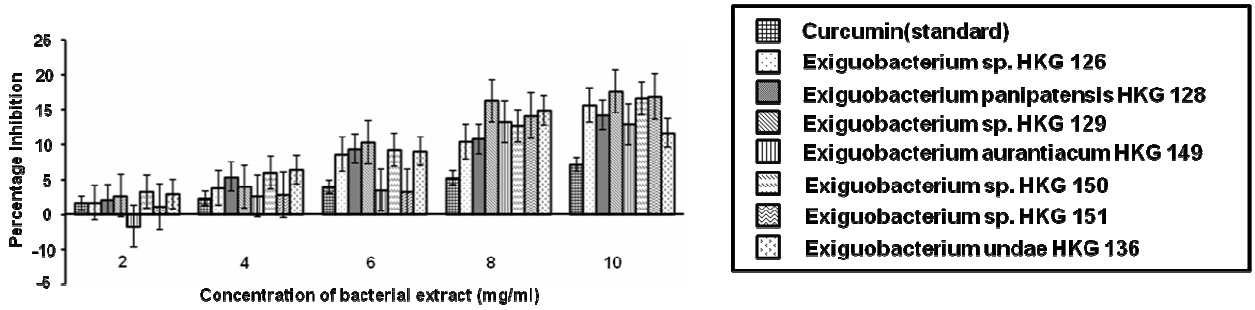

Figure 5. Nitric oxide scavenging activity for different strains of *Exiguobacterium sp.*

Hydroxyl Radical Scavenging Activity

The hydroxyl radical-mediated deoxyribose degradation was inhibited by the bacterial extracts in a Fe^{2+} EDTA ascorbate-H_2O_2 reaction mixture at a minimum concentration of 0.5 mg/ml. On comparing with the standard, the activity was almost 50% on average at this concentration **(Figure 6).**

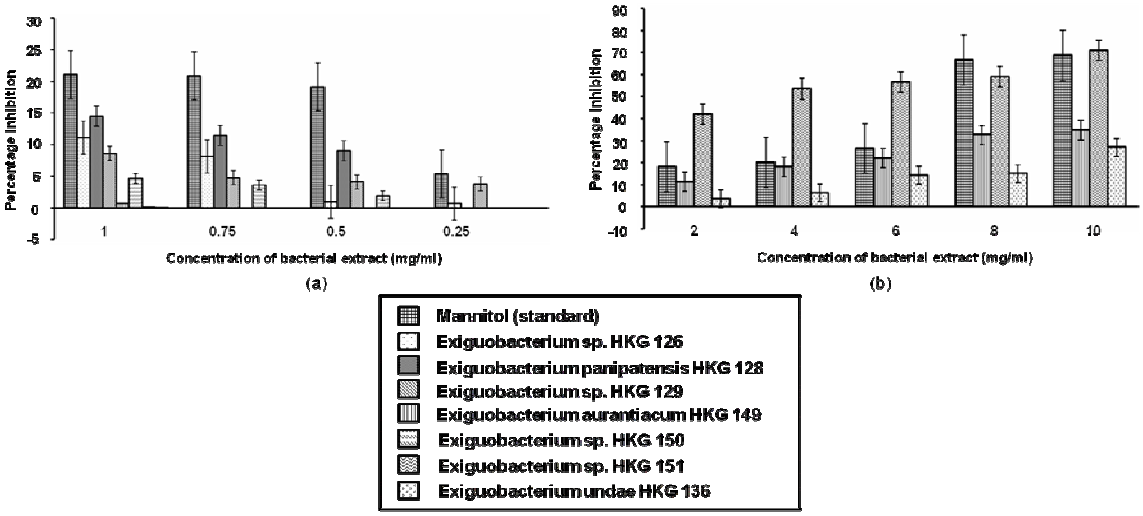

Figure 6. Hydroxyl radical scavenging activity (a) Scavenging of all the seven strains at lower concentrations (b) Scavenging of three strains *E. aurantiacum* HKG149, *Exiguobactreium* sp. HKG151 and *E.undae* HKG136 at increased concentrations.

The assay was repeated with higher concentrations (0-10 mg/ml) for the samples that showed negligible activity. On increasing the concentration, the extracts showed increased inhibition of the nitric oxide radical as shown in fig. 6b. Hence it is stated that the antioxidant activity is concentration dependent.

Fe^{2+} chelation

The metal chelation activity was studied for the bacterial extracts, for their ability to interfere with the formation of Fe^{2+} ions and ferrozine complex. This means that the extracts tend to capture the Fe^{2+} ion before ferrozine can form a complex with it. As found in most of the assays for antioxidants the percentage inhibition is dose dependent and is higher than that of the standard known. A 0.5 mg/ml concentration shows around 18% inhibition on average for all samples compared to 2% for the standard curcumin, hence, highly potent for metal chelation (**Figure 7**).

Sod Enzyme Assay

The scavenging assay for superoxide anion is carried out by the enzyme superoxide dismutase. The assay results show that the starins that survive at 37°C show about 40% scavenging of superoxide radical on comparing with a control that shows 55% activity. However, the psychrophilic strain showed no scavenging activity for superoxide anion which could be because of the fact that eventhough SOD enzyme helps resist cold temperatures but it also denatures during severe stress conditions **(Figure 8)**.

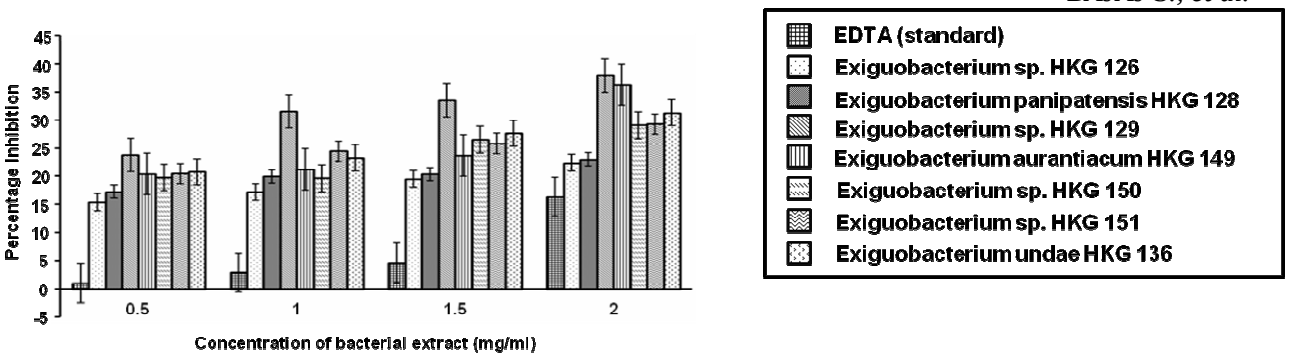

Figure 7. The metal (Fe^{2+}) chelation activity is shown for the extracts of *Exiguobacterium sp.*

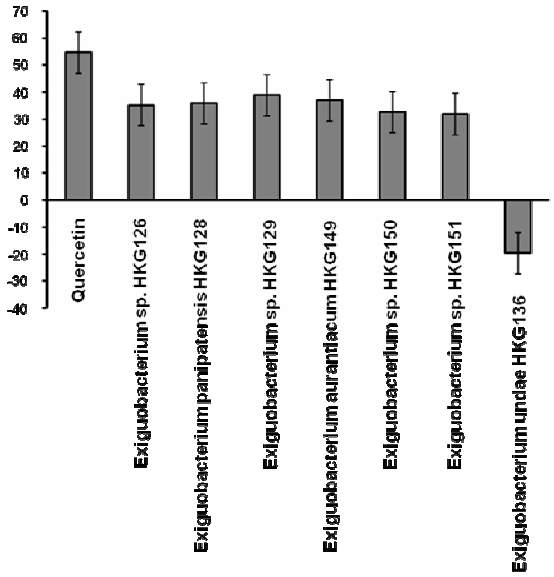

Figure 8 Superoxide anion scavenging activity of *Exiguobacterium* extracts.

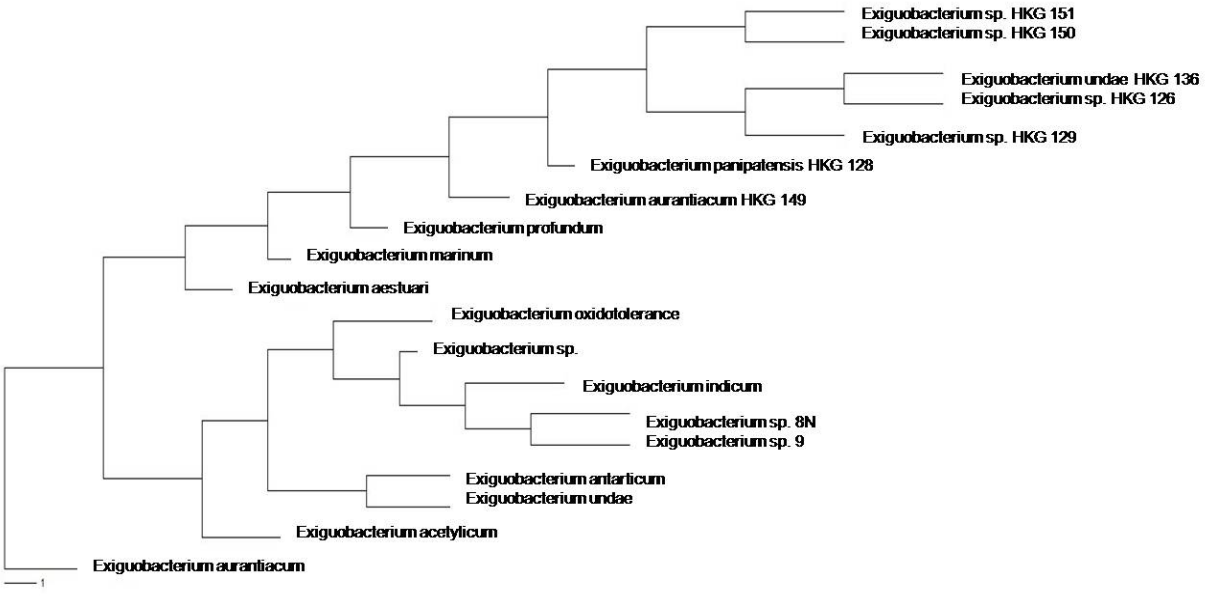

Figure 9. The phylogenetic tree for the isolated strains and well annotated strains of *Exiguobacterium* sp.

CONCLUSION

In this paper, we have isolated seven extremophilic sp. of *Exiguobacterium* from different geographical regions of India. These species were analyzed for phylogeny which showed that the isolated strains were close neighbors to each other therefore, predicted to show somewhat similar properties. The biochemical characterization showed that the *Exiguobacterium* sp. appear to be rod like in the form of chains and are gram-positive but, in appearance may resemble gram-negative bacterium. This is because some bacteria loose the thickness of the peptidoglycan layer during their growth period leading to loss of stain. Certain bacteria, especially extremophiles, display variable response to stain due to their growth in stress conditions like lack of nutrients, pH, salt, temperature etc. In such stress conditions, the oxygen required for growth in turn produces reactive oxygen species (ROS). These ROS start a cascade of reactions, ultimately leading to cell death. In order to prevent this, the cells have developed some special scavenging molecules for these ROS, called antioxidants.

Hence, the extremophile *Exiguobacterium* was checked for various antioxidants by a series of assays; DPPH, ABTS, Hydrogen Peroxide, Hydroxyl Radical, Nitric Oxide, Fe^{2+} chelation assays and superoxide enzyme inhibition. The results suggested that *Exiguobacterium* shows about 70% antioxidant activity, relative to the standards used. Also, it was inferred that the antioxidant activity is concentration dependent; increase in concentration of the extract lead to an increased antioxidant activity.

On a comparative analysis between the sensitive and resistant strains for salt, pH and temperature, the salt sensitive bacterium *Exiguobacterium aurantiacum* HKG 149 which appears to diverge out from the cluster, showed higher ability of scavenging DPPH radical ~50% even at a very low concentration (2 mg/ml) of the extract on comparing with the salt resistant bacteria (*Exiguobacterium* sp. HKG 126, *Exiguobacterium panipatensis* HKG 128, *Exiguobacterium* sp. HKG 129, *Exiguobacterium* sp. HKG 150, *Exiguobacterium* sp. HKG 151, *Exiguobacterium undae* HKG 136). This activity remained almost constant even with the increase in concentration. The hydrogen peroxide inhibition reaction was seen to begin immediately on the addition of the substrate but dropped down immediately as compared to the rest which followed a reaction completion within 20 minutes. This bacterium did not show hydroxyl radical inhibition at low concentration as compared to the remaining 6 bacterium. Similarly, the 4 bacterial strains (*Exiguobacterium aurantiacum* HKG 149, *Exiguobacterium* sp. HKG 150, *Exiguobacterium* sp. HKG 151, *Exiguobacterium undae* HKG 136) under acid stress showed hydroxyl radical scavenging activity only at higher concentration (2-10 mg/ml) of the extracts on comparison to the 3 strains (*Exiguobacterium* sp. HKG 126, *Exiguobacterium panipatensis* HKG 128, *Exiguobacterium* sp. HKG 129) which show scavenging at lower concentrations (0-2 mg/ml) also. Also, the psychrophile (*Exiguobacterium undae* HKG 136) showed no inhibition of superoxide ion as compared to the rest of the 6 bacteria. The Trolox equivalent antioxidant capacity (TEAC) for the salt sensitive and psychrophilc bacteria showed a high response within 1 min of the reaction. These all had a high ability of Fe^{2+} chelation.
Since antioxidants work against free radicals produced during stress and are important in cell survival therefore, these can be used in various drug delivery pathways. They help in preventing food rancidity and spoilage due to oxidation. These can also be used to help prevent diseases caused by free radicals release like Alzheimer's and cancer. This study shows that the *Exiguobacterium* sp. is positive for antioxidants hence can be used as a source for their extraction.

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