

# Exploration and characterization of agriculturally and industrially important haloalkaliphilic bacteria from environmental samples of hypersaline Sambhar lake, India

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**Abstract** Screening of bacteria from Sambhar lake, an extreme hypersaline environment of India, led to the isolation of 93 haloalkaliphilic bacteria growing optimally in media with 2–25 % salt and 6–12 pH. Based on 16S rRNA gene sequences, 93 isolates were further categorized into 32 groups, with each group representing a different taxa belonging to 3 phyla (Firmicutes, Proteobacteria and Actinobacteria). Majority of the isolates (53.12 %) showed similarity with phylum Firmicutes which was followed by Proteobacteria (40.63 %) and Actinobacteria (6.25 %). The isolates belonging to 32 representative groups were further evaluated for the production of extracellular enzymes viz. amylase, cellulase, protease and xylanase, plant growth promoting attributes and BIOLOG<sup>TM</sup> substrate usage. Among all the isolates, xylanase producing isolates were in maximum (68 %) as compared to protease (56 %), cellulase (40 %), and amylase (37 %) producing strains. Similarly, among plant growth promoting activities, ammonia producing isolates were highest (56 %) when compared to

those producing ACC deaminase (53 %), IAA (50 %), hydrogen cyanide (28 %), siderophore (21 %) and solubilizing P (34 %). Isolates showing enzymatic and PGP activities could be further utilized for promoting plant growth in saline affected area.

**Keywords** Bacterial diversity · Extracellular enzymes · Plant growth promoting attributes · Haloalkaliphiles · Saline environment

## Introduction

Soil salinity is one of the most severe environmental factors limiting the productivity of agricultural crops. Evolving efficient, low cost, easily adaptable methods for management of salinity stress is a major challenge (Venkateswarlu and Shanker 2009). Most of the technologies such as development of salinity tolerant varieties, resource management practices, etc. are cost-intensive. Recent studies indicate that microorganisms can help crops to cope with saline stress (Venkateswarlu and Shanker 2009). Halophilic microorganisms present in hypersaline and alkaline aquatic habitats are adapted to high salinity and require a certain concentration of NaCl for their optimum growth. They have been isolated from various saline environment such as low saline marine environment to hypersaline lakes (e.g., the Dead Sea, the Great Salt Lake) and salt mine drainage. These environments are characterized by large amounts of sodium carbonate, or complexes of this salt, formed by evaporative concentration (Tindall et al. 1980). During the past years, special attention has been focused on the study of microbial diversity present in soda lakes (Scholten et al. 2005; Foti et al. 2007). Several workers have reported that soda lakes

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contain representatives of prokaryotic diversity, and that they can be considered as responsible component for nutrients cycling (Zavarzin et al. 1999; Jones et al. 1998). The well-studied Soda lakes in the world are located in East African Rift Valley (Rees et al. 2004), in the Libyan Desert (Imhoff et al. 1979) and in North America, i.e., Mono Lake (Humayoun et al. 2003; Scholten et al. 2005). There are also data available on soda lakes in India (Wani et al. 2006).

The microorganisms isolated from soda and saline lakes have tremendous potential in agriculture for improved crop production under saline soils. Besides importance in agriculture, the halophilic microbes are also good source of industrially important enzymes such as amylases, proteases, nucleases, phosphatases, lipases, DNases, pullulanases and xylanases (Rohban et al. 2009). With increasing emphasis on extremozymes during the recent years, the microbial enzymes are being looked to replace chemical catalysts in transformations and manufacturing processes.

Most of the studies on halophilic bacteria from salt lakes, however, have so far focused on phylogenetic analysis of the organisms and only limited information is available on their agricultural and enzymatic potential. Identification of culturable microbial communities from saline lakes and their characterization will not only strengthen database of salt loving microbes for basic studies, but their potential can also be utilized in agriculture or industries if they possess plant growth promoting attributes or have ability to produce salt/alkali tolerant industrially important enzymes. In the present study, we characterized culturable bacterial diversity from extreme

salt lake through 16S rRNA gene sequencing. The isolates were further screened for plant growth promoting attributes and production of certain important industrial enzymes.

## Materials and methods

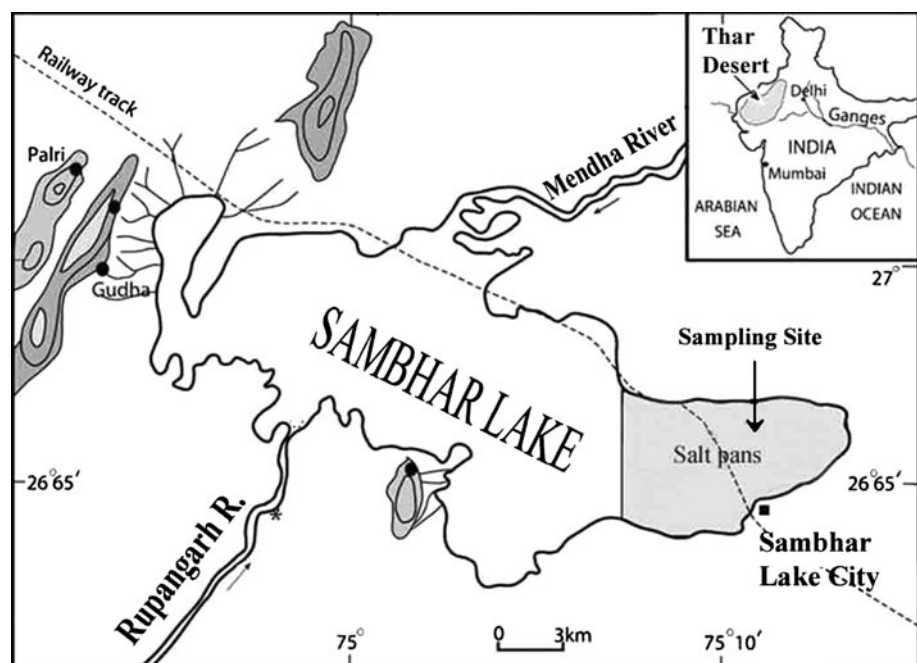
### Sampling site

The salterns (Salt beds) present in the Sambhar Salt Lake, Sambhar, Rajasthan, India were selected for the isolation of microorganisms. Sambhar salt lake is the largest inland saline lake of India. It is located in southwest of Jaipur, Rajasthan, India and its catchment lies within the geo coordinates of  $26^{\circ}28'43''$ – $27^{\circ}33'10''$ N and  $74^{\circ}35'40''$ – $75^{\circ}50'28''$ E, whereas the lake lies within the latitudes of  $26^{\circ}52'$ – $27^{\circ}02'$ N and  $74^{\circ}54'$ – $75^{\circ}14'$ E (Fig. 1).

### Enrichment of samples for isolation of bacteria

Water and sediment samples were collected from four different sampling sites and used for isolation of bacteria following an enrichment strategy. To 10 ml of liquid Horikoshi Medium I with 20 % NaCl and pH 9, 1 g sediment or 1 ml water sample was inoculated and incubated for 7 days at 37 °C. An aliquot of 100  $\mu$ l from each enriched sample was spread plated on three different media viz. Horikoshi I, Horikoshi II (Joshi et al. 2008) and nutrient agar with modifications and incubated at 37 °C for 48 h. The media were modified by incorporating them with 10 and 20 % NaCl and pH was maintained at 9.0 using 1 N

**Fig. 1** Location of Sambhar Salt Lake in Rajasthan, India; Salt pans were the sites for sample collection



NaOH. Following incubation, the isolates were purified through repeated streaking and all purified isolates were preserved in 35 % glycerol for further utilization.

#### DNA isolation and PCR

Extraction of total genomic DNA from 93 isolates was carried out using Gen Elute Bacterial Genomic DNA Kit (Sigma, USA). Amplification of 16S rRNA gene was carried out by using universal primers pA (5'-AGTTTGATCCTGGCTAG-3') and pH (5'-AGGAGGTGATCCAGCCGCA-3') (Edwards et al. 1989). The amplification conditions were used as described earlier (Sahay et al. 2011).

#### Sequencing of 16S rRNA gene and phylogenetic analysis

The PCR amplified 16S rRNA genes were purified with a Quiaquick purification kit (Qiagen). The nucleotide sequences were di-deoxy cycle sequenced with fluorescent terminators (Big Dye, Applied Biosystems) and run in 3130xl Applied Biosystems ABI prism automated DNA sequencer. The rRNA gene sequence was checked by sequencing both strands using primers pA and pH for forward and reverse reaction. The partial 16S rRNA gene sequences of the isolated strains were compared with those available in the databases. Identification to the species level was determined as a 16S rRNA gene sequence similarity of  $\geq 97$  % with that of a prototype strain sequence in the GenBank. The phylogenetic tree was constructed on the aligned datasets using the neighbour-joining method implemented in the program MEGA 4.0.2 (Tamura et al. 2007). Bootstrap analysis was performed on 1,000 random samples taken from the multiple alignments.

#### Nucleotide sequence accession numbers

All 16S rRNA gene sequences of the strains (grouped) were deposited in GenBank and accession numbers (JN645842–JN645873) were obtained.

#### Screening of isolates for tolerance to NaCl and pH

For assessing the tolerance level of NaCl, isolates were inoculated in different wells of micro-titer plates having nutrient broth with different salt concentration (2–30 %) in increment of 1 %. Micro-titer plates were incubated at 37 °C in a temperature-controlled Automated Microbiology Growth Analysis System (Oy Growth Curve Ab Ltd, Finland), where growth in terms of optical density at 600 nm was measured in each well at regular interval. All the observations were taken in triplicate. Similarly, for studying pH tolerance, the isolates were spot inoculated on

nutrient agar (NA) medium with pH ranging from 6 to 13, in an increment of pH 1. The pH in the medium was maintained by addition of 50 mM each of phosphate buffer (pH 6.0–8.0), bicarbonate buffer (pH 9.2–10.6) or sodium hydroxide/potassium chloride buffer (pH 12.0–13.0) to the growth medium.

#### Screening of isolates for extracellular enzyme production

All the thirty two isolates were tested qualitatively for production of four extracellular enzymes. The production of amylase (Manachini et al. 1988), protease (Ramesh et al. 2001), xylanase (Sanghi et al. 2007) and cellulase (Zvereva et al. 2006) by all the isolates at NaCl concentration supporting optimum growth was estimated.

#### Screening of isolates for plant growth promoting (PGP) attributes

Isolates were also screened for their ability to exhibit plant growth promoting attributes through assessing phosphate solubilization and production of siderophore, ammonia, hydrogen cyanide, ACC-deaminase and indolic compounds at their optimum NaCl tolerance concentration. All the isolates were screened for phosphate solubilisation on Pikovskaya's agar plates (Pikovskaya 1948). Pikovskaya's medium containing tri-calcium phosphate was prepared and poured into sterilized petriplate. Isolates of halophilic bacteria were spot inoculated on the plates and incubated at 28 °C for 4–7 days. The plates were observed for clearing or solubilization zones around the colonies. Bacterial isolates were assayed for siderophore production on the Chrome azurol S agar medium as described by Schwyn and Neilands (1987). Chrome azurol S agar plates were spot inoculated with test organism and incubated at 28 °C for 48–72 h. Development of yellow–orange halo around the growth was considered as positive for siderophore production. Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for at 24–72 h. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman 1992). Single isolates were streaked onto KB medium supplemented with 4.4 g glycine L<sup>-1</sup> to screen for cyanide production. Thereafter the Petri dishes were inverted. A piece of filter paper impregnated with 0.5 % picric acid (yellow) and 2.0 % sodium carbonate was placed in the lid of each Petri dish. The Petri dishes were sealed with parafilm and held at 20 °C for 48–96 h. Discoloration of the filter paper to orange-brown after incubation indicates microbial production of cyanide (Bakker

and Schippers 1987). ACC (1-Aminocyclopropane-1-carboxylic) deaminase activity was analyzed by using DF Salt minimal medium with ACC as sole source of nitrogen (Penrose and Glick 2003). Indole-3-acetic acid (IAA) production was estimated colorimetrically (Glickmann and Dessaux 1995).

#### Assessment of bacterial carbon source utilization

Carbon substrate usage by isolates was measured using the GP2 (Gram Positive) and GN2 (Gram negative) Micro-Plate™ (Biolog, Inc., Hayward, California, USA), each 96-well microtiterplate has 95 wells that contain a single substrate per well with one water negative control well, for a total of 96 substrates. Sample preparation and analysis was performed following the directions of the manufacturer (Biolog). Each plate was inoculated with a bacterial suspension (150 µl/well) and incubated for 3–4 days at 37 °C. The resulting utilization patterns were read against a substrate blank well at A590 nm with an automated plate reader (Molecular Devices Corporation, USA). Overall color development in BIOLOG plates was expressed as average well color development (AWCD). AWCD of the plate was calculated using the formula  $(C - R) / \{ [P(C - R) / 95] \}$ , where R is the value of the control well and C is the values of wells containing sole carbon sources.

## Results

#### Isolation of bacteria from enriched samples

The total bacterial count in the sediment samples ranged from 32 to  $59 \times 10^5$  cfu g<sup>-1</sup> whereas in water samples it ranged from 14 to  $38 \times 10^5$  cfu ml<sup>-1</sup>. A total of 140 bacteria were isolated from the water and sediment samples of Sambhar lake. All the isolates were screened for salt tolerance at graded concentrations of NaCl (2–30 %) with regular increment of 1 %. Among 140 culturable isolates that exhibit distinct colony characteristics, a total of 93 isolates showing tolerance to  $\geq 5$  % NaCl were selected for further study. These isolates were purified and used for phylogenetic analysis.

#### Sequencing of 16S rRNA gene and phylogenetic analysis

The 16S rRNA gene of all the 93 isolates was amplified and a single amplicon of about 1,545 bp was obtained for each isolate. Based on near complete sequence analysis of 16S rRNA gene sequences and BLAST search, the 93 isolates were identified and categorized into 32 groups. Taxonomic and phylogenetic analysis of these 32 bacteria

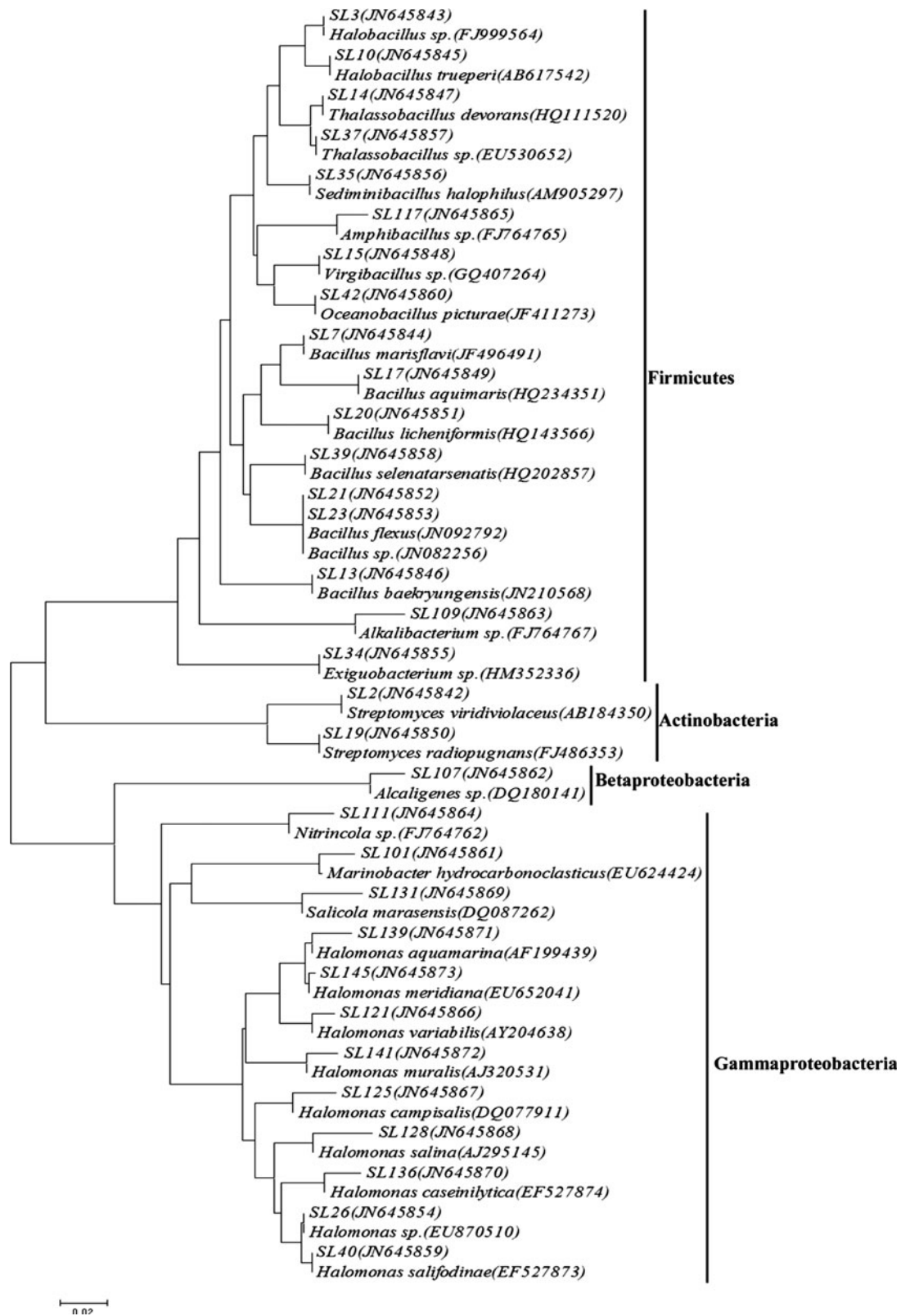
categorized them into three phyla viz. Firmicutes, Actinobacteria and Proteobacteria (Fig. 2; Table 1). Of the 32 groups, 19 were Gram positive and 13 were Gram negative. Amongst the 19 Gram positive isolates, 17 belonged to the phylum Firmicutes (Low G + C) and 2 belonged to phylum Actinobacteria (High G + C) (Table 1). All the 13 Gram negative isolates belonged to the phylum Proteobacteria with predominance of  $\gamma$ -proteobacteria (12) and only one  $\beta$ -proteobacteria (*Alcaligenes*). The phylogenetic tree constructed to determine their affiliations are shown in Fig. 2.

#### Salinity and pH tolerance

Of the 32 isolates, 4 isolates (SL26, SL125, SL128, and SL139) could tolerate 25 % NaCl, 8 isolates (SL34, SL141, SL10, SL36, SL145, SL121, SL131 and SL35) were able to tolerate maximum of 20–24 % NaCl concentration whereas the remaining 21 isolates could tolerate maximum of 10–19 % NaCl. Similarly, variation in pH tolerance was also observed. Some of the isolates like SL21, SL26, SL131 and SL19 were able to grow in a pH range of 6.0–12.0 (Table 1). Isolates (SL3, SL13, SL15, SL20, SL35, SL37, SL40, SL101, SL107, SL109, SL111, SL125, SL128, SL136, SL139, SL141, and SL145) capable of growing at higher NaCl concentration of  $>5$  % and pH  $>9$  can be grouped as halo-alkali-tolerant strains (Table 2).

#### Extracellular enzymes and plant growth promotion

The ability to produce four different enzymes was tested among the isolates. Out of 32 isolates 18, 12, 13 and 22 isolates were found to produce amylase, protease, cellulase and xylanase, respectively. Among all the isolates, xylanase producing isolates were highest (68 %) as compared to protease (56 %), cellulase (40 %), and amylase (37 %) producing strains. Four isolates viz., SL10, SL26, SL35 and SL131 showed all four enzyme activities. Highest amylase activity (52.30 IU ml<sup>-1</sup> min<sup>-1</sup>) was presented by isolate SL109, protease activity (58.80 IU ml<sup>-1</sup> min<sup>-1</sup>) by SL23, cellulase activity (52.94 IU ml<sup>-1</sup> min<sup>-1</sup>) by SL136 and xylanase activity (51.83 IU ml<sup>-1</sup> min<sup>-1</sup>) by SL101. Further screening of these isolates for the production of six PGP traits at high salt concentrations revealed that 6 isolates solubilized phosphorus, 16 isolates produced IAA, 7 produced siderophore, 12 produced ACC deaminase, 18 produced ammonia, and 9 showed the production of HCN (Table 2). Similarly, among plant growth promoting activities, ammonia producing strains were highest (56 %) when compared to ACC deaminase (53 %), IAA production (50 %), P-solubilization (34 %), hydrogen cyanide (28 %), and siderophore (21 %) producing strain. Isolate SL35 solubilized highest amount of phosphorus (981.32 µg



**Fig. 2** Phylogenetic analysis of haloalkaliphilic bacterial isolates obtained from Sambhar lake. The 16S rRNA gene sequence based dendrogram representing Sambhar lake isolates were constructed by

neighbor-joining method and reference sequences having  $\geq 97\%$  similarity were used as taxonomic representatives

**Table 1** Phylogenetic characterization of isolates with salt and pH tolerance levels

Isolate no.	Accession number	Identification	NaCl		pH		Similarity to nearest neighbor (%)	Class affiliation
			Tolerance range (%)	Optimum (%)	Tolerance range	Optimum		
SL3	JN645843	<i>Halobacillus</i> sp.	2–16 ± 0.10	8	9–11 ± 0.20	9.70	99	Firmicutes
SL7	JN645844	<i>Bacillus marisflavi</i>	2–11 ± 0.10	7	8–12 ± 0.20	9.80	99	Firmicutes
SL10	JN645845	<i>Halobacillus trueperi</i>	2–23 ± 0.10	14	8–11 ± 0.20	9.00	99	Firmicutes
SL13	JN645846	<i>Bacillus baekryungensis</i>	2–10 ± 0.10	5	9–11 ± 0.20	10.00	99	Firmicutes
SL14	JN645847	<i>Thalassobacillus devorans</i>	2–12 ± 0.10	7	6–11 ± 0.20	10.00	99	Firmicutes
SL15	JN645848	<i>Virgibacillus</i> sp.	2–12 ± 0.10	6	9–12 ± 0.20	10.00	99	Firmicutes
SL17	JN645849	<i>Bacillus aquimaris</i>	2–11 ± 0.10	5	6–11 ± 0.20	10.00	99	Firmicutes
SL20	JN645851	<i>Bacillus licheniformis</i>	2.7–11 ± 0.10	6	9–10 ± 0.20	8.80	100	Firmicutes
SL21	JN645852	<i>Bacillus flexus</i>	2–12 ± 0.10	6	6–12 ± 0.20	9.90	100	Firmicutes
SL23	JN645853	<i>Bacillus</i> sp.	2.5–14 ± 0.10	8	8–12 ± 0.20	10.20	100	Firmicutes
SL34	JN645855	<i>Exiguobacterium</i> sp.	2–24 ± 0.10	15	6–12 ± 0.20	10.80	99	Firmicutes
SL35	JN645856	<i>Sediminibacillus halophilus</i>	2–22 ± 0.10	11	9–12 ± 0.20	10.80	100	Firmicutes
SL37	JN645857	<i>Thalassobacillus</i> sp.	2–17 ± 0.10	9	9–11 ± 0.20	10.50	99	Firmicutes
SL39	JN645858	<i>Bacillus selenatarsenatis</i>	2–16 ± 0.10	9	8–11 ± 0.20	10.80	99	Firmicutes
SL42	JN645860	<i>Oceanobacillus picturae</i>	2–10 ± 0.10	6	6–10 ± 0.20	8.80	99	Firmicutes
SL109	JN645863	<i>Alkalibacterium</i> sp.	2–19 ± 0.10	9	9–12 ± 0.20	9.80	98	Firmicutes
SL117	JN645865	<i>Amphibacillus</i> sp.	2–13 ± 0.10	6	8–11 ± 0.20	8.90	98	Firmicutes
SL26	JN645854	<i>Halomonas</i> sp.	2–25 ± 0.10	14	6–12 ± 0.20	11.00	100	Proteobacteria
SL40	JN645859	<i>Halomonas salifodinae</i>	2–18 ± 0.10	9	9–11 ± 0.20	8.90	99	Proteobacteria
SL101	JN645861	<i>Marinobacter hydrocarbonoclasticus</i>	2–17 ± 0.10	7	9–10 ± 0.20	8.90	98	Proteobacteria
SL111	JN645864	<i>Nitrincola</i> sp.	2–14 ± 0.10	8	9–11 ± 0.20	9.60	98	Proteobacteria
SL121	JN645866	<i>Halomonas variabilis</i>	2–20 ± 0.10	11	8–11 ± 0.20	8.90	99	Gammaproteobacteria
SL125	JN645867	<i>Halomonas campisalis</i>	2–25 ± 0.10	16	9–11 ± 0.20	8.70	98	Gammaproteobacteria
SL128	JN645868	<i>Halomonas salina</i>	2–25 ± 0.10	16	9–11 ± 0.20	8.60	97	Gammaproteobacteria
SL131	JN645869	<i>Salicola marasensis</i>	2–23 ± 0.10	11	6–12 ± 0.20	10.80	98	Gammaproteobacteria
SL136	JN645870	<i>Halomonas caseinilytica</i>	2–21 ± 0.10	14	9–11 ± 0.20	9.20	98	Gammaproteobacteria
SL139	JN645871	<i>Halomonas aquamarina</i>	2–25 ± 0.10	17	9–12 ± 0.20	9.80	98	Gammaproteobacteria
SL141	JN645872	<i>Halomonas muralis</i>	2–24 ± 0.10	14	9–12 ± 0.20	9.60	98	Gammaproteobacteria
SL145	JN645873	<i>Halomonas meridiana</i>	2–23 ± 0.10	15	9–12 ± 0.20	10.90	99	Gammaproteobacteria
SL107	JN645862	<i>Alcaligenes</i> sp.	2–17 ± 0.10	7	9–11 ± 0.20	9.20	98	Betaproteobacteria
SL2	JN645842	<i>Streptomyces viridiviolaceus</i>	2–12 ± 0.10	7	8–10 ± 0.20	9.00	99	Actinobacteria
SL19	JN645850	<i>Streptomyces radiopugnans</i>	2.5–11 ± 0.10	6	6–12 ± 0.20	10.50	99	Actinobacteria

$\text{PO}_4 \text{ ml}^{-1}$ ) and isolate SL109 showed highest IAA production of  $570.98 \mu\text{g mg}^{-1}$ protein. Interestingly, one isolates SL23 exhibited all six PGP activities along with three enzyme activities viz., amylase, protease and xylanase.

#### Carbon utilization pattern

A total of 32 representative isolates were selected for carbon substrate utilization at 5 % NaCl concentration. The BIOLOG metabolic profiles of thirty two bacteria (19 Gram-positive and 13 Gram-negative) belonging to

Firmicutes, Actinomycetes and Proteobacteria based on use/non-use of 95 substrates is shown in Fig. 3a, b. At 52 % similarity, 13 clusters could be made for Gram-positive. Isolate SL17 could utilize highest number of the sugars tested (48 %) whereas SL19 utilized the lowest number of sugars (11 out of 95 sugars). Gram negative bacteria produced 11 clusters at 46 % similarity level and isolate SL121 could utilize highest number of the sugars tested (67/95; 65.26 %) whereas SL125 utilized the lowest number of sugars (12 out of 95 sugars). The variability in utilization of carbon sources by *Halomonas* strains was

**Table 2** Plant growth promoting attributes and extracellular enzyme production by identified isolates at their optimum salt and pH levels

Isolates no.	Amylase (IU ml <sup>-1</sup> min <sup>-1</sup> )	Protease (IU ml <sup>-1</sup> min <sup>-1</sup> )	Cellulase (IU ml <sup>-1</sup> min <sup>-1</sup> )	Xylanase (IU ml <sup>-1</sup> min <sup>-1</sup> )	P-solubilization (PO <sub>4</sub> µg ml <sup>-1</sup> )	IAA (µg mg <sup>-1</sup> protein)	Siderophore	ACC deaminase	NH <sub>3</sub>	HCN
<i>Halobacillus</i> sp.	–	40.67 ± 0.46	–	–	102.18 ± 01.16	225.70 ± 02.57	–	+	+	+
<i>Bacillus marisflavi</i>	–	–	–	–	–	215.61 ± 02.45	–	+	+	–
<i>Halobacillus trueperi</i>	48.16 ± 0.55	44.04 ± 0.50	14.35 ± 0.16	22.38 ± 0.25	–	–	+	+	–	+
<i>Bacillus baekryungensis</i>	–	–	–	32.83 ± 0.37	–	–	–	+	+	–
<i>Thalassobacillus devorans</i>	–	–	–	26.75 ± 0.30	–	–	+	+	+	–
<i>Virgibacillus</i> sp.	–	–	–	34.52 ± 0.39	–	–	–	+	++	–
<i>Bacillus aquimaris</i>	–	40.88 ± 0.46	27.51 ± 0.31	35.55 ± 0.40	–	349.26 ± 03.97	–	–	+	–
<i>Bacillus licheniformis</i>	–	30.60 ± 0.35	34.53 ± 0.39	32.78 ± 0.37	–	–	+	+	–	–
<i>Bacillus flexus</i>	21.35 ± 0.24	–	35.55 ± 0.40	–	–	–	–	–	+	–
<i>Bacillus</i> sp.	53.11 ± 0.60	58.80 ± 0.67	–	29.46 ± 0.33	662.64 ± 07.53	325.05 ± 03.70	+	+	+	+
<i>Exiguobacterium</i> sp.	25.43 ± 0.29	39.64 ± 0.45	–	–	–	338.02 ± 03.84	–	+	+	+
<i>Sedimentibacillus halophilus</i>	52.00 ± 0.59	32.54 ± 0.37	32.51 ± 0.37	51.83 ± 0.59	981.32 ± 11.16	–	–	–	–	–
<i>Thalassobacillus</i> sp.	33.18 ± 0.38	–	52.94 ± 0.60	–	475.48 ± 05.41	–	–	+	–	–
<i>Bacillus selenatarsenatis</i>	36.72 ± 0.42	38.57 ± 0.44	42.64 ± 0.48	33.81 ± 0.38	222.57 ± 02.53	448.27 ± 05.10	–	+	+	–
<i>Oceanobacillus picturae</i>	38.54 ± 0.44	–	–	34.58 ± 0.39	–	327.88 ± 03.73	–	+	+	–
<i>Alkalibacterium</i> sp.	52.30 ± 0.59	–	36.88 ± 0.42	41.68 ± 0.47	–	–	–	+	–	–
<i>Amphibacillus</i> sp.	28.43 ± 0.32	–	–	–	–	270.32 ± 03.07	+	+	–	–
<i>Halomonas</i> sp.	38.24 ± 0.43	32.89 ± 0.37	39.80 ± 0.45	28.77 ± 0.33	–	–	–	–	+	–
<i>Halomonas salifodinae</i>	–	–	–	36.84 ± 0.42	–	–	–	–	–	–
<i>Marinobacter hydrocarbonoclasticus</i>	22.96 ± 0.26	–	–	45.91 ± 0.52	–	570.98 ± 06.49	+	+	–	–
<i>Nitriicola</i> sp.	–	–	–	32.49 ± 0.37	–	450.90 ± 05.13	–	–	–	–
<i>Halomonas variabilis</i>	29.44 ± 0.33	–	–	–	–	457.27 ± 05.20	–	–	–	++
<i>Halomonas campisalis</i>	–	–	–	–	–	–	+	–	+	–
<i>Halomonas salina</i>	35.71 ± 0.41	42.61 ± 0.48	–	–	–	214.78 ± 02.44	–	+	+	+
<i>Salicola marasensis</i>	32.47 ± 0.37	28.46 ± 0.32	47.77 ± 0.54	46.29 ± 0.53	242.80 ± 02.76	–	–	–	+	++
<i>Halomonas caseinilytica</i>	22.36 ± 0.25	–	44.64 ± 0.51	33.50 ± 0.38	–	122.72 ± 01.40	–	–	–	–
<i>Halomonas aquamarina</i>	–	–	–	29.49 ± 0.34	–	178.17 ± 02.03	–	–	–	–
<i>Halomonas muralis</i>	21.85 ± 0.25	–	22.58 ± 0.26	34.65 ± 0.39	–	–	–	–	–	–
<i>Halomonas meridiana</i>	–	–	–	34.64 ± 0.39	–	190.29 ± 02.16	–	–	–	–
<i>Alcaligenes</i> sp.	34.90 ± 0.40	34.51 ± 0.39	–	–	–	402.99 ± 04.58	–	–	+	++
<i>Streptomyces viridiviolaceus</i>	–	–	10.35 ± 0.12	11.45 ± 0.13	–	–	–	–	+	–
<i>Streptomyces radiopugnans</i>	–	–	–	27.49 ± 0.31	–	–	–	–	+	+

Data expressed as mean of average of three replicates, along with standard deviation, (–) not detected, (+) positive for the attributes

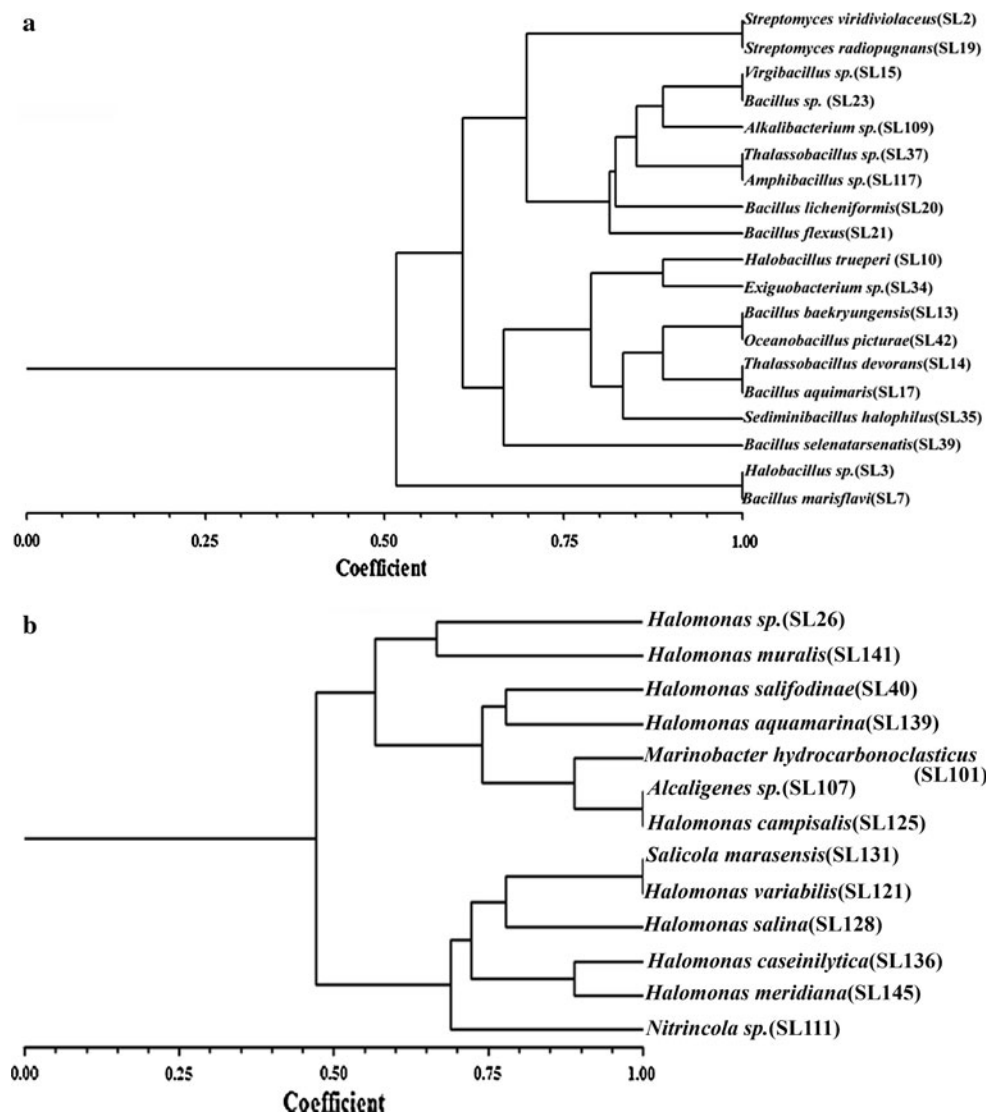
very high (67/95) as compared to Firmicutes and Actinobacteria (46/95) and shows elevated carbon utilization unevenness throughout UPGMA dendrogram. The utilization of L-arabinose, D-mannose, pyruvic acid methyl ester, succinic acid mono-methyl-ester, D-glucuronic acid,  $\beta$ -hydroxybutyric acid were different significantly among *halomonas* strains.

## Discussion

The isolation and characterization of halotolerant and halophilic bacteria from hypersaline environment is of practical importance because these bacteria have biotechnological potential with regard to the production of useful biomolecules, such as osmolytes (compatible solutes), hydrolytic enzymes and exopolysaccharides (Margesin and Schinner 2001). In the present investigation, we have

isolated 93 bacteria from hypersaline condition of Sambhar Lake, India. Phylogenetic analysis of 16S rRNA gene sequences categorized them into 32 groups belonging to three phyla i.e., Firmicutes, Proteobacteria and Actinobacteria. In terms of diversity, the majority of isolates were from the *Bacillus* group within the firmicutes (53 %) and gamma-proteobacteria (40 %). Similar results were reported from the haloalkaline lake Elmenteita, Kenya but the abundance of gamma-proteobacteria was higher as compared to firmicutes (Mwirichia et al. 2010). Deshmukh et al. (2011) also reported majority of bacteria isolated from Lonar soda lake of Maharashtra state, India belonging to phylum firmicutes and few to proteobacteria. In another study on the bacterial diversity of Zabuye salt lake in Tibet region using culture independent approach, the phylogenetic tree showed that some clones (57.14 % of total clones) belonged to 23 genera in gamma-proteobacteria, alpha-proteobacteria, delta-proteobacteria, bacteroidetes,

**Fig. 3** Dendrogram showing the clustering of 19 Gram positive (a) and 13 Gram negative bacteria (b) on the basis of use/non-use of 95 substrates studied by the BIOLOG™ system). The simple matching (SSM) coefficient and unweighted-pair group method with average (UPGMA) clustering were used





firmicutes, and verrucomicrobia and the rest clones were quite different (Zhang and Kong 2010). Several workers have reported the abundance of firmicutes in different lakes such as Wadi An Natrun Mono Lake, CA, USA (Mesbah et al. 2007), Mongolian Baer Soda Lake (Ma et al. 2004), Kenyan Soda Lake (Vargas et al. 2004) and brackish water Pulicat lake (Sahay et al. 2011), Chaka lake Tibetan plateau (Jiang et al. 2007). In the present study, the firmicutes were represented mostly by members of the diverse *Bacillus* spectrum and included *Bacillus*, *Halobacillus*, *Thalassobacillus*, *Virgibacillus*, *Sediminibacillus*, *Oceanobacillus* and *Amphibacillus*. Other genera included were *Exiguobacterium* and *Alkalibacterium*. In general the abundance of alkaliphilic, endospore forming Gram positive bacteria belonging to the genus *Bacillus* and other *Bacillus* derived genera have been reported from haloalkaline lakes (Mwirichia et al. 2010) and brackish water lake (Sahay et al. 2011). Of the 13 members of the proteobacteria, 12 belonged to gamma-proteobacteria and only 1 belonged to beta-proteobacteria (*Alcaligenes* sp.). Dominance of proteobacteria was also reported earlier by various workers while working on marine sediments solar salterns (Yeon et al. 2005), brackish water (Sahay et al. 2011) and saline-alkaline lakes (Joshi et al. 2008). Amongst the gamma-proteobacteria, the halomonads were most dominant as has been reported from other soda lake (Mwirichia et al. 2010). The other 3 isolates identified as *Marinobacter hydrocarbonoclasticus*, *Salicola marasensis* and *Nitrincola* sp. have also been isolated from saline habitats. The abundance of *Halomonas* in these habitats is due to their ability to tolerate or require high salt concentration for growth (Jiang et al. 2007).

The variations in the diversity pattern obtained from Sambhar lake and other salt or soda lakes could be due to edaphic or environmental factors; and flora of the region. Most of the permanent vegetation around the Sambhar lake is xerophytic in nature. The main tree species growing in the catchment area are *Acacia senegal*, *A. nilotica*, *Prosopis cineraria*. The main grasses are *Cenchrus pennisetiformis*, *C. ciliaris* and *Chloris dolichostachya*. Another soda lake from India, Lonar lake is surrounded by forest where principal tree species are teak, tamarind and babul.

The distribution and abundance of microbial population to a permanently saline environment includes optimization of basic cell processes, enzyme modulation, nutrient transport and cell membrane function. The bacterial isolates from hypersaline condition also showed the ability to tolerate a wide range of salinity, pH and temperatures, and presented combined hydrolytic activity, which provides the advantage of uses in various industrial processes (Mellado and Ventosa 2003). In the present investigation, xylanase producing isolates were dominant as compared to amylase, cellulase and protease. The observed discrepancy could be

attributed to the fact that extremely halophilic/halotolerant and Gram-positive halophilic bacteria produced more extracellular enzymes such as (amylase, protease, cellulase and xylanase (Table 2) in comparison to moderate halophilic and Gram-negative isolates. Similar variations in the production of industrial enzymes were reported among the isolates from Howz Soltan lake, Iran and Pulicat lake, India (Rohban et al. 2009; Sahay et al. 2011).

We have also evaluated halophilic bacteria for six plant growth promoting attributes. In our study, we found that out of thirty two, six isolates were positive for P-solubilization and isolate SL35 showed highest solubilization of phosphorous (981.32  $\mu\text{g PO}_4 \text{ ml}^{-1}$ ). Phosphate-solubilizing (PSB)  $\gamma$ -proteobacteria brings about mobilization of insoluble phosphates in the soil and increase plant growth under conditions of poor phosphorus availability (Rodriguez and Fraga 1999). Use of these PSB as bioinoculants will increase the available P in soil, helps to minimize the P-fertilizer application, reduces environmental pollution and promotes sustainable agriculture in future.

It has been reported that indolic compounds (indole-3-acetic acid, indolepyruvic acid, and indoleacetamide) have positive effect on root growth and causing rapid establishment of roots beneficial for young seedlings as it increase their capacity to anchor themselves to the soil and to obtain water and nutrients from their environment (Vessey 2003). We have also tested the ability of isolates to produce IAA. Of the total, 16 (50 %) isolates were tested positive. Similarly, good proportions of isolates (53.12 %) were also found positive for ACC deaminase. Cheng et al. (2007) reported that ACC deaminase bacteria conferred salt tolerance onto plants by lowering the synthesis of salt induced stress and promoted the growth of canola in saline environment. One of the most important strain (*Bacillus* sp. SL23) which showed three enzyme activity and all six PGP attributes can be exploited biotechnologically and as well as agriculturally. The strain can be used in various industrial areas such as the detergent industry, leather industry (Birbir and Ilgaz 1996) and food preservation (Mellado and Ventosa 2003; Ventosa and Nieto 1995). The enzymes derived from halophiles display optimal activity in the presence of NaCl and maintain stability over a wide pH range (pH 6–10). This makes them excellent additives for laundry detergent, baking industries and for biopolishing of fabrics and production of stonewashed denims (Setati 2010). PGP attributes can be utilized as bioinoculant to improve the production of crop under saline condition.

The Biolog metabolic profiling analysis demonstrated to be highly effective method for differentiating among the species groups by cluster analysis (Dawson et al. 2002). However, this method has also been adapted and used to characterize the functional potential of the bacteria (Garland and Mills 1991). No positive correlations between

taxonomic and metabolic diversity were found, which is in accordance with previous study by O'Donnell et al. (1993). They suggested that factors other than community structure, such as soil pH, were more important in regulating metabolic activity. In our study it was observed that high salt and pH tolerating bacteria utilized more carbon as compared to low salt tolerating bacteria. The possible reason behind this may be attributed to the fact that bacteria require a sufficient supply of carbon to feed their metabolic pathways under stress condition.

In saline environment, organisms encounter limited amounts of complex mixtures of carbon sources that are often present at low concentrations. As a result, microbial cells have developed multiple systems to utilize a wide array of different substrates as carbon sources resulting increase in metabolic activity.

## Conclusion

The above study has demonstrated that Sambhar lake harbors a wealth of diverse microorganisms, especially high salt tolerant bacteria, having multiple PGP attributes and different enzyme activity. The enzymes are generally haloalkaliphilic which make them suitable to be employed in different industrial processes. These bacteria can also be exploited in developing microbe based formulations to improve the productivity of crop plants grown in saline soils.

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