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European Journal of Experimental Biology, 2013, 3(6):351-358

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Isolation and characterization of high salt tolerant bacteria from agricultural soil

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ABSTRACT

Adaptation characteristic of microorganisms in different extreme conditions seeks discoveries in field of pharma, food, & bioenergy sectors. In the present study, new experimental approach was proposed to prove adaptation ability in extreme conditions with growth and development of agricultural soil bacterial species in stepwise adaptation in high salt (NaCl) conditions. Six different bacterial species were isolated from agricultural soil surrounding the Rajkot region. Out of six isolates, two showed salt tolerance up to 10% NaCl concentration. Biochemical and molecular (16S rDNA sequencing) characterization revealed the strains to be Exiguobacterium sp. and Serratia sp. designated as GSD1 and GSD 2 strains. Species were able to adapt upto 10% of NaCl in nutrient broth growth medium. Primary screening for extracellular enzymes (protease, amylase, lipase) secretion unveiled activity of enzymes in nutrient agar plates containing skim milk or starch or mineral oil for protease, amylase and lipase production respectively. This experiment provides the base to link the adaptation capabilities of agricultural soil microorganisms in high salty environment and vice a versa.

Keywords: *Exiguobacterium*, *Serratia*, Salt Adaptation, Extracellular enzymes

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INTRODUCTION

Adaptation is an evolutionary process through which living organisms develop to live in its changed habitats. Lower to higher living organisms are influenced but developed to adjust with different abiotic stresses i.e. changes in salinity of soil and water, temperature, pH, atmospheric humidity, air circulation and radiation [1,2]. Amongst these all soil and water salinity is the major stress impacts on life, and it becomes more prominent with time.

Global warming is the key issue of current world environmental problems, its main emphasis on level and surface temperature of sea. The scientific committee on Antarctic research proposed a rise in mean sea level of up to 1.4 m by 2100 century and other scientists anticipated sea level rise of one to several meters[3-5] in the same phase. Reports year[6].Gujarat has 1663 km, coastal region and rise in 0.5 meter sea level can cover roughly 8452.971 Km^2 land nearby coastal area[6]. This can be even more anti-agricultural if sea level rises about 0.5 to 1.4 meters[7].

Similarly, with global warming, natural disasters like earthquake and tsunami will cost more deadly effects on microorganisms of agricultural soil. Recently, stroked tsunamis in South Asia has affected 11,000 Ha agricultural land [8], with 4067 Ha cropped area and 2260 km of coastal area in India alone[9]. Tsunami contaminated the agricultural soil with salts of Kerala, Andhra Pradesh, Tamilnadu and Pondicherry states of India. In future, natural disasters like tsunami if strike in Gujarat it may contaminate the agricultural soil of Rajkot, Jamnagar, Junagadh, Porbandar, Surat and Kutch districts and it will affects the microorganisms population in agricultural soil. In future high salt contaminated soil, either it will lose some of the microorganisms' species or microorganisms have to adapt to salty soil.

In view of this, we have focused on microorganisms of non-saline soil to adapt it in high salt concentration in artificial synthetic media. We have isolated the total six bacterial species from agricultural garden soil of Saurashtra University campus and experimented to evaluate its adaptability in high salinity medium. Out of total six isolates we have found two isolates *Exiguobacterium* sp. GSD1 (JN020918) and *Serratia* sp. GSD2 (JN020917) with capability to adapt upto 10% of NaCl salt containing medium.

Exiguobacterium sp. have been isolated from markedly diverse sources, including *Greenland* glacial ice, hot springs at *Yellowstone* park, rhizosphere of plant and environment of food processing unit[10].Gram negative bacteria of the genus *Serratia* are opportunistic human, plant and insect pathogens[11]and member of the family *enterobacteriaceae* [12]. Salinity has an effect on microbial cell membrane[13] and synthesis, structure and function of protein [14,15] as well as the growth of the microorganisms [16]. Bacteria develop different defense mechanisms to survive in high salinity environment [17].Present study evaluates the adaptation capabilities of agricultural soil microorganisms in high NaCl containing medium to propose its futuristic adaption in salt contaminated soil, if tsunami like natural disaster strikes or rise in sea level, change the fertility of soil.

MATERIALS AND METHODS

Collection of soil sample and physico-chemical analysis: Soil sample was collected from provinces of "Van-Vibhag" (agricultural soil) Saurashtra University campus, Rajkot, India. Soil sample was collected from the depth of 10-12 inch in sterile polythene bag and samples were kept at room temperature until used. Soil analysis was conducted to measure electro conductivity, pH, total nitrogen, K_2O, P_2O_5 content at the government soil analysis laboratory, Rajkot.

Isolation and Screening of Bacteria: Soil suspension was prepared with 5g of soil in 20ml of sterile double distilled water and vortexed. Loop full of soil suspension was streaked on N-Agar plates (Himedia) and incubated for 24h at 37°C for isolation of different bacteria. Total six different isolates were found from primary screening and further grown on N-agar plate containing 2%, 4%, 6%, 8% & 10% NaCl separately for 72h at 37°C.

Biochemical Characterization: Biochemical analysis of two isolates were carried out according to Bergey's Manual of Determinative Bacteriology[18] and classified primarily through morphological, physiological and biochemical observation.

Amplification and sequencing of 16S rDNA encoding genes: 16S rDNA genes, from the purified genomic DNA of the 2 isolates were amplified by PCR with the following set of primers: F (5´-AGAGTTTGATCCTGGCTCAG-3´) and R (5´-GGTTACCTTGTTACGACTT-3´). Each 50-µl reaction mixture contained 30 mM Tris (pH 8.4), 50mM KCl, 1.5 mM $MgCl_2$, 50mM concentrations of each dNTPs, 10 pmol of each primer, and 1U of *Taq* polymerase. The PCR was performed with following specification: first step of denaturation for 5 min at 95°C, 30 cycles were performed, denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and polymerization for 1 min at 72°C. The amplified genes were sequenced using sequence scanner v1.0 (Inst Model/Name: 3730XI/ABI3730XL-1414-008) (Applied Biosystems).

Phylogenetic analysis: The 16S rDNA gene sequences of two isolates (*Exiguobacterium* sp. GSD1, *Serratia* sp.GSD2) were compared to references of 16S rDNA gene sequences retrieved from GenBank database. BLASTN 2.2.26+ used to retrieve similar reference sequences and aligned for phylogenetic relationship with our isolated strain by neighbor-joining method using treedyn online software.

GeneBank Submission and nucleotide accession number: 16S rDNA gene sequences of *Exiguobacterium* and *Serratia* were deposited in the NCBI GenBank under accession numbers JN020918 and JN020917 respectively.

Growth Curve: Vegetative cells of *Exiguobacterium sp.* GSD1 and *Serratia sp.* GSD2 were grown in a nutrient broth containing 2%, 4%, 6%, 8% & 10% of NaCl. Control tubes were served without addition of NaCl. 0.1 ml of fresh culture (O.D near about 1.000 at 660nm) was inoculated in medium. O.D was measured of the sample at 660nm with (UV-1800, SHIMADZU) at duration of 1h for 33h.

Extracellular enzyme production at different salt concentration

Amylase production: Amylase production was analyzed by starch hydrolyzing method. Starch agar plate was prepared containing 4% and 8% NaCl. Organisms were inoculated on starch agar plate and incubated at 37°C for 24h. Amylase production was detected as a colorless zone on surrounding of colony on addition of iodine.

Proteolysis production: Skim milk agar plates were prepared containing 4% and 8% NaCl, organisms were inoculated. Dissolved casein surrounding the colony resulted on secretion of protease enzyme.

Lipase production: Nutrient agar plates containing vegetable oil prepared to study extracellular lipase activity. Test organisms were inoculated and incubated for 24h at 37°C. Addition of $CuSO_4$ in plates on next day develops bluish green appearance surrounding the colony, confirms the hydrolysis of fat in glycerol & fatty acid.

RESULTS

Soil Analysis: Soil analysis data showed in (Table 1) confirms quality as on agricultural soil.

Table 1 Analysis for different physicochemical parameter of soil

Sample	E.C. mS/M	pH	% Nitrogen	P_2O_5 Kg/ha	K_2O Kg/ha
B	0.62	8.7	0.38	28	400

Bacterial Identification, Screening and Biochemical test: Gram staining revealed *Exiguobacterium* sp. as a grampositive aerobic microorganism with orange, nontransparent, circular colony characteristic grown on N-agar after48h of incubation. *Exiguobacterium sp.* exhibited negative test for indole production, citrate utilization, ammonia and urea reduction as well as acid production, whereas nitrate reduction and carbohydrate fermentation was found to be positive (Table 2). However, the other strain (*Serratia* sp.) is gram negative aerobic bacteria exhibiting off-white, non-transparent and irregular shape colony. Biochemical test for *Serratia* sp. showed negative indole production, ammonia and urea reduction as well as acid production test, whereas citrate utilization and carbohydrate fermentation result confirmed positive test (Table 2).

Table 2 Biochemical Characterization

Tests	*Exiguobacterium* sp. GSD1	*Serratia* sp. GSD2
Indol production	-	-
Urease test	-	-
Methyl red test	+	+
Voges proskauer	-	-
Simmon Citrate test	-	-
Ammonia reduction	-	-
Nitrate reduction	+	+
Triple sugar iron agar test	Slant yellow, Butt yellow, No H_2S production Fermentation observed	Slant yellow, Butt yellowish red, No H_2S production Fermentation observed

** + indicates positive results, − indicates negative results.*

Phylogenetic analysis on the basis of 16s rDNA analysis: Distance phylogenetic trees for two isolates were constructed by the neighbor-joining method using TreeDyn 198.3 and the topology of the trees was evaluated by bootstrapping score over 1,000. Alignment positions with gaps were excluded from the calculations (Figure 1 & 2). Phylogenetic tree showed the position of *Exiguobacterium* GSD1 and *Serratia* sp. GSD2 with respect to other GenBank 16S rDNA sequences. The tree was constructed by Neighbor joining method and minimum possibility with alignment of 812 and 1172 base pairs, with bootstrap support values greater than 90% and 95% (Figure 1 & 2).

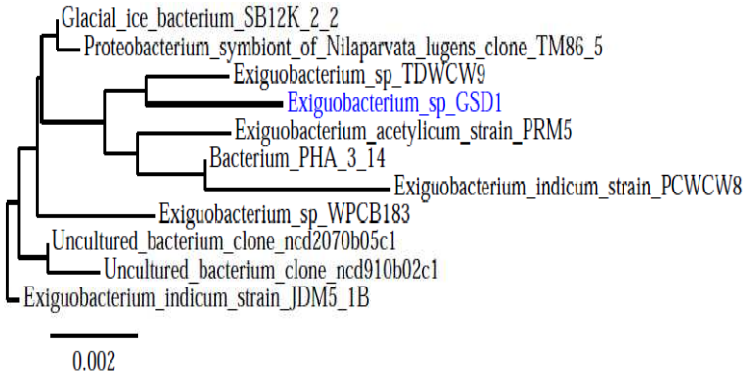

Figure 1 Phylogenetic tree analysis of *Exiguobacterium* sp. GSD1

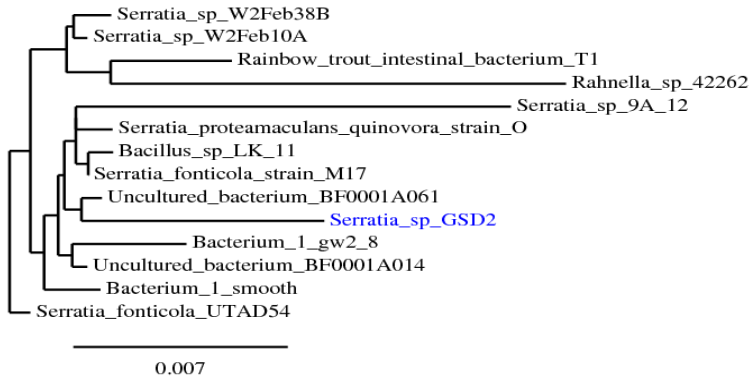

Figure 2 Phylogenetic tree analysis of *Serratia* sp. GSD2

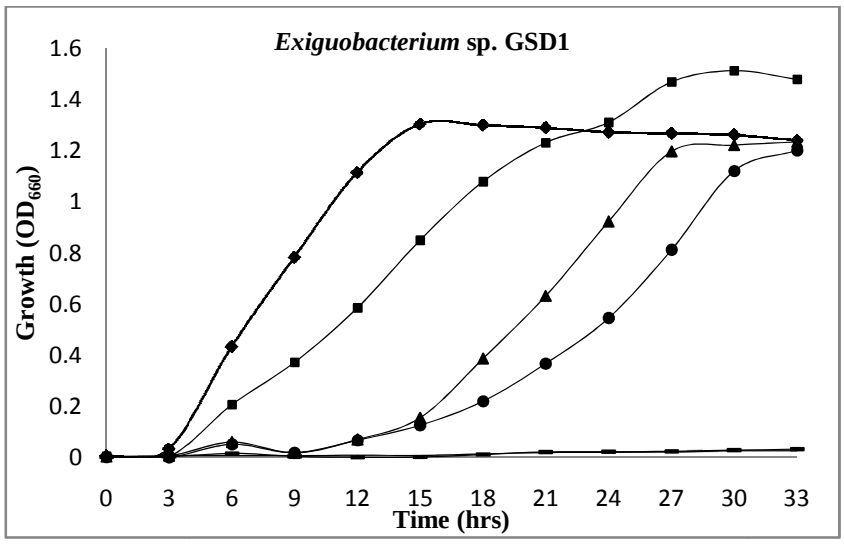

Figure 3 Growth curve of *Exiguobacterium* sp. GSD1 incubated under various NaCl concentrations
♦0% NaCl, ■2% NaCl, ▲4% NaCl, ●6% NaCl, —8% NaCl, □10% NaCl

Growth curve:

As Observed in Figure 3, *Exiguobacterium* sp. GSD1 showed log phase after the three to four hours of incubation time periods grow in 6% NaCl concentration, where as culture grown in 0%, 2% and 4% NaCl showed log phase entry earlier then 6%, 8% and 10% NaCl, In case of organism grown in 8% and10% NaCl showed no growth even

after the 32h incubation. Similar observation has been noticed with *Serratia* sp. GSD2 grown in 0% to 10% NaCl (Figure 4).

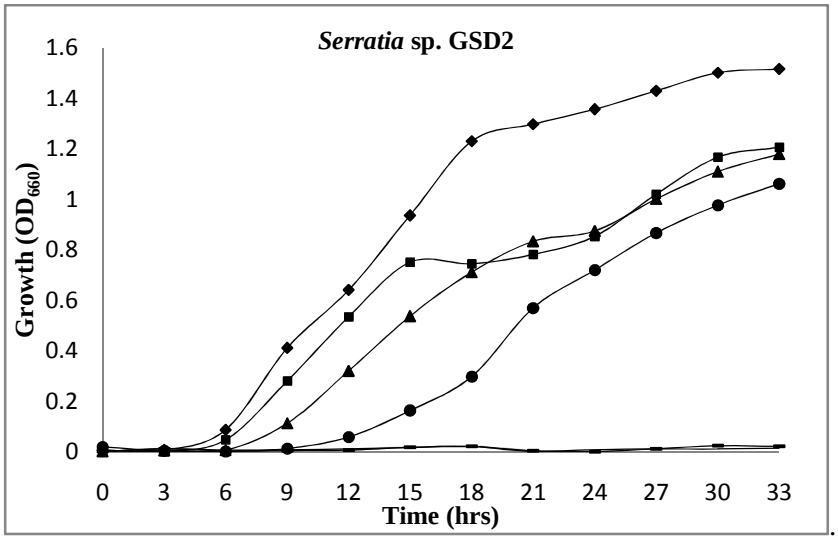

Figure 4 Growth curve of *Serratia* sp. GSD2 incubated under various NaCl concentrations
♦0% NaCl, ■2% NaCl, ▲4% NaCl, ●6% NaCl, —8% NaCl, □10% NaCl

Screening for Extracellular enzyme production on agar plates:
Exiguobacterium sp. GSD1 and *Serratia* sp. GSD2 exhibited protease, amylase and lipase activity in 0% and 4% NaCl containing medium, whereas *Serratia* sp. GSD2 exhibited amylase and lipase activity in 8% of NaCl medium (Table 3).

Table 3 Qualitative enzyme production test for Caseinase, Amylase and Lipase

Enzyme production	*Exiguobacterium* sp. GSD1			*Serratia* sp. GSD2		
	0%	4%	8%	0%	4%	8%
Caseinase	+	+	-	+	-	-
Amylase	+	+	+	+	+	+
Lipase	+	+	-	+	+	+

** + indicates positive results, − indicates negative results*

DISCUSSION

The present study was designed to evaluate the adaptation properties of agricultural soil microorganism under the high salt (NaCl) growth medium to establish the adaptability characteristics of non-saline agricultural soil microorganism in saline medium. *Exiguobacterium* sp. GSD1 *strain* and *Serratia* sp. GSD2 were the isolates utilized for experiments.

Exiguobacterium sp. has been found from much wide range of habitats of all over the world. *Exiguobacterium* sp. exhibits in wide range of habitats including cold and hot environments with temperature range from -12°C to 55°C[19]. This diversification in habitats of *Exiguobacterium* sp. is responsible of its adaptation in diverse conditions and beneficial to survive against extreme environmental factors. *Exiguobacterium* genus comprises psychrotrophic, mesophilic, and moderate thermophilic species[20,21], with pronounced morphological diversity (ovoid, rods, double rods, and chains) depending on species, strain, and environmental conditions[22]. Our experimental results of growth curve of *Exiguobacterium* sp. GSD1, suggest the down-regulation of growth under successive increase of NaCl concentration. As shown in figure 3, without additional NaCl concentration in growth medium it did not affect the growth of organism but as the concentration of NaCl increases, growth of organisms decreased with maximum tolerance upto 10% NaCl concentration. Several *Exiguobacterium* strains possess unique properties of interest for application in biotechnology, bioremediation, industry and agriculture. *Exiguobacterium* sp. are capable of neutralizing highly alkaline textile industry wastewater[23], high potential for pesticide removal[24]

and reducing arsenate to arsenite[25]. Furthermore, several enzymes (alkaline protease, EKTA catalase, guanosine kinase, ATPases, dehydrogenase, esterase) with stability at a broad range of temperatures were purified from different *Exiguobacterium* strains [26-31]. Our isolates *Exiguobacterium* sp.GSD1 showed extracellular enzyme production of protease, lipase and amylase on N-agar plate with additional food material respectively casein, mineral oil and starch. Plate results reveal that enzyme secretion was decreased with increase in salt concentration with 4% and 8% NaCl in comparison to 0% NaCl, this observation was made on the basis of zone of enzyme production. In view of this we can assume that enzymes would be secreting in extracellular environment but because of high salt concentration in medium, enzymes could not be active fully or decrease in growth of microorganism directly affects the production of extracellular enzymes. From the observation on production of enzyme, we can deduce that as salt concentration increases, it decreases the activity of enzyme, but high salt concentration could not inhibit the enzyme activity completely. These results are in hope and favor to develop and upgrade strategies for enzyme production sustainable in high saline medium. Report on *Exiguobacterium* 255-15 isolated from *Siberian permafrost* showed potential stress responses with changing growth rate, cellular morphology, cell membrane composition, polysaccharide composition, and carbon utilization under different temperature conditions and wide range of salt concentration makes *Exiguobacterium* dynamic organism to survive and study for evolutionary linkages[32].

Our isolated *Serratia* sp. GSD2showed growth upto 10% NaCl in growth medium, with supporting results same on agar plate at 10% NaCl. Previously, *Serratia* species has been isolated from water, soil, skin of animals (including man) and from the surfaces of plants. *Serratia sp.* grows at temperature range from 5–40° and pH range from 5 to 9[33,34]. Strains of this species produce plant-growth-promoting chemicals, have anti-fungal and anti parasitic properties, encourage the establishment of nitrogen-fixing symbionts and act as insect pathogens [35-38]. These observations revealed that in future if any agricultural soil area covered with saline environment by any natural disaster, our isolated *Serratia* sp. will be a natural remedy for removal of NaCl to keep agricultural soil fertile. A subspecies of *Serratia marcescens* (*S. marcescens* subsp. *sakuensis*) and a urea-dissolving species (*Serratia ureilytica*) have been described previously [39-40]. In our isolated S*erratia* sp.GSD2 species, we have found biofilm production[41], which increase with increase in salt concentration from 0 % to 10 % NaCl salt concentration. In this present experiment *Serratia* sp. GSD2 also produces extracellular enzymes amylase, protease and lipase. We have found decrease in extracellular enzyme activity as increase in salt concentration ranging from 0%, 4%, 8% NaCl.

Protein content in terms of total gene expression was increased with increase in salt concentration as shown in *Exiguobacterium* sp. GSD1 *&* *Serratia* sp. GSD2, as well as gradual decline in extracellular enzyme activity was also observed in proteinase, lipase and amylase upto 8% NaCl in solid medium. Biochemical and biophysical data have been postulated for several salt-tolerant enzymes, including glutaminase from *Lactobacillus rhamnosus*[42] α-amylase from *Bacillus dipsosauri*[43], protease from *Aspergillus* sp. FC-10[44], α-type carbonic anhydrase from *Dunaliella Salina*[45]and thermolysin from *B. thermoproteolyticus*[46], Lipase from *Pichia anamola*[47]. Halophilic and halotolerant microorganisms have various mechanisms to adapt against high salt concentration i.e. synthesis of betaine, Ectoine and hydroxyectoine, β-Glutamate, trehalose etc.[48,49], any of or all of these mechanism would be followed in our isolated microoorganisms under high salt media. Diminished extracellular enzyme activity on increase in salt concentration in our experiments are in support that halophilic enzymes are found to have multilayered hydration shells that are of considerably of greater size and order compared to non-halophilic enzymes[50].

In conclusion, this study provides the futuristic aspect of agricultural soil microorganisms on adaptation and use as a natural fertilizer, whenever any nature disaster like tsunami strikes the coastal region of *Saurashtra* (Gujarat). The above studied microorganism *Exiguobacterium* and *Serratia* will able to survive in high salt condition and could be the possible factor to revive fertility of agricultural soil and removal of salt contamination. Further, extracellular enzyme production by *Exiguobacterium* and *Serratia* opens the doors for its application in food, pharma and bioenergy industries. In view of the above, present study provides the possible natural bioremediation approach for famers of *Saurashtra* region of Gujarat state as well as possible microbiological survivor in future under any natural disaster like tsunami or earthquake.

Acknowledgement
Authors are thankful to Dr. Navin R, Sheth, Head, Department of Biochemistry for his valuable guidance and support throughout the experimental work.

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