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Isolation and characterisation of halotolerant bacteria from saline habitats

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Abstract: *Salinity in the soil was one of the major problems to the agriculture in coastal areas. Suppressed growth and reduced yields are the effects of salinity on agriculture. Microorganisms that live in saline environments adapted to salinity, such halotolerant microorganisms provide a means of cultivation of crops in salinity influenced areas. Halotolerant bacteria which have the plant growth promoting activity, plant growth promoting bacteria (PGPB) may be used to mitigate the effect of salinity in the field. Aim of the present study is to isolate halotolerant bacteria from saline environments and to characterize them morphologically and biochemically. In the current research six bacterial isolates were studied for their morphological and biochemical characteristics. 16s rRNA gene analysis was carried out for one isolate i.e. MGST-02, which showed growth at 8% of NaCl concentration.*

Key Words: *halotolerance, salinity, plant growth promoting bacteria, morphological and biochemical characters.*

1. INTRODUCTION:

Conditions like extreme temperatures, drought, salinity, flooding, pollution, toxicity are unfavourable to plant growth. Worldwide this abiotic stresses greatly affecting the yields of major crops[1].Extreme temperatures, altering rainfall patterns and soil salinization are the resultants of climate change [2] . Over exploitation of irrigation practices used especially in arid and semi-arid regions leading to soil salinization. Approximately 20% of the cultivated and irrigated lands are negatively salt affected with some estimates being as high as 50%[3]. Due to global increase in salt affected area by 1-2% every year, salinity is becoming one of the leading issues in the coming decades.[4]. It is estimated that world's population will reach 9.5 billion by 2050[5]. There is a need to use unproductive saline and barren lands for cultivation to meet the food demand of the growing population[6].Various practices and techniques enhancing crop growth and productivity under salinity stress conditions must be followed. Though molecular breeding programmes and transgenic approaches are useful but have certain limitations in terms of different ethical issues and time requirement[7]. Therefore application of an alternative approach needs to be considered. One such strategy could be the use of bacterial strains tolerant to higher salinity levels with plant growth promoting activity, plant growth promoting bacteria (PGPB), either free living in the soil, rhizosphere, rhizoplane or phyllosphere[8]. Until the discovery of extremophilic microorganisms, for long time it was considered that the extreme environments were free of life[9]. Extremophilic microorganisms can survive in extreme environments such as unusual levels of salt, P^H, pressure and temperature. Those which adapted thrive in hypersaline habitats are considered as halophiles[10].

Previous reports suggest that bacteria belong to genera such as, *Microbacterium, Pantoea, Achromobacter, Rhizobium, Pseudomonas, Bacillus, Paenibacillus, Enterobacter, Burkholderia, Methylobacterium, Azospirillum, and Variovarax* etc. are helpful in tolerance to abiotic stresses[11,12]. To alleviate abiotic stresses these microorganisms are helpful in agricultural lands[13,14,15]. Studies on several microorganisms of the soil reported that these microbes support the plant growth during stress conditions by producing gibberllins, Indole Acetic Acid (IAA) and some other compounds which enhance the root growth and also nutrient content thus improving the plant health under stress[16, 17]. The main aim of this study is to isolate halotolerant bacteria from saline habitats. Selected halotlerant bacterial isolates were evaluated for their morphological, biochemical characters. One isolate i.e. MGST-02 was identified up to species level through 16s rRNA gene analysis.

2. MATERIALS AND METHODS:

Collection of soil sample

Soil sample was collected from provinces coastal area of Visakhapatnam, Andhra Pradesh, 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.

Isolation and Screening of Bacteria:

Soil suspension was prepared with 5g of soil in 20ml of sterile double distilled water and vortexed and serially diluted. 100 µl of 3rd and 4th dilutions were spread on 3% salt amended nutrient agar plates (Himedia) and incubated for 48h at 37°C for isolation of different bacteria.

Salt tolerance assay

All isolated bacteria were subjected to salt tolerance activity, nutrient broths were added with different concentrations of NaCl. The NaCl concentration ranges from 2.5% to 10%. After 24 hours of incubation all bacterial cultures were spectrophotometrically analysed at 660nm.

Biochemical Characterization:

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

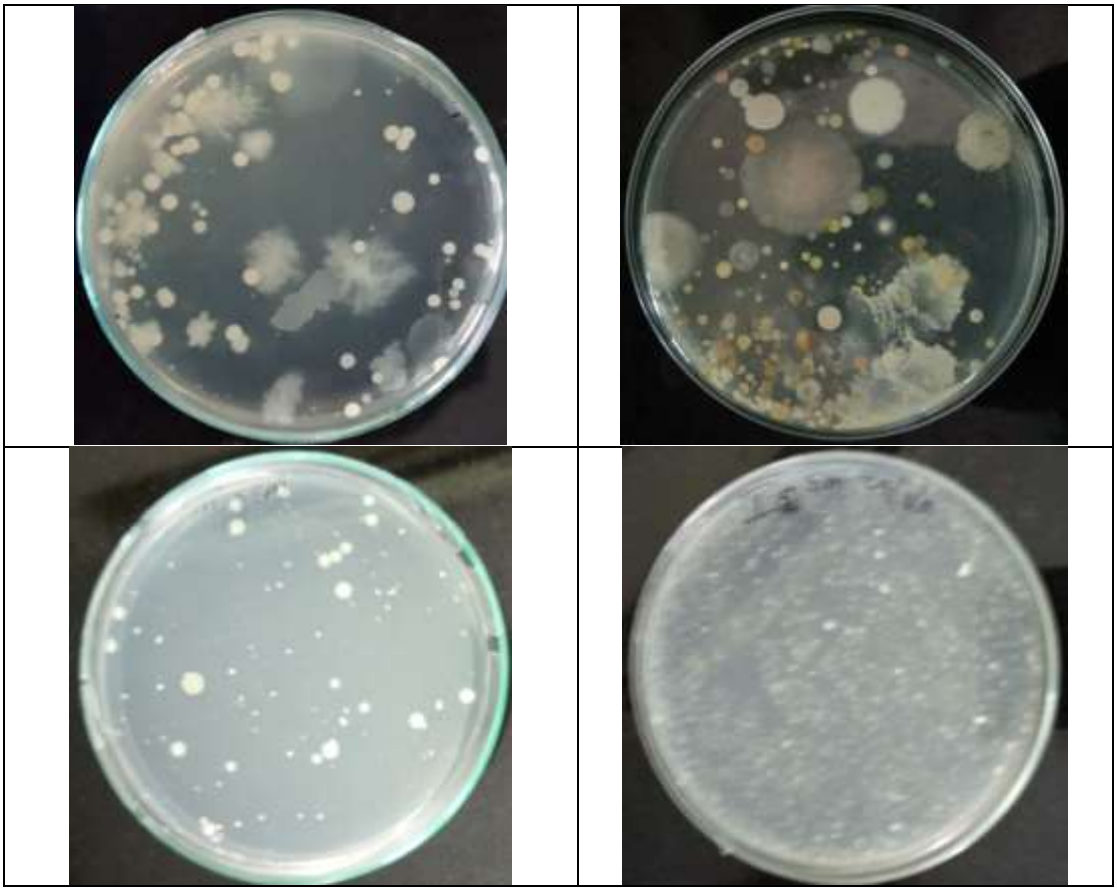
16S rRNA gene sequence analysis

DNA isolation was carried by the SDS extraction method described by Xia et al., (1995)[18] with minor modifications. Two universal primers 27F (5'AGAGTTTGATCMTGGCTCAG 3') and 907R (5'CCGTCAATTCMTTTRAGTTT3') were used to amplify 16S rRNA genes. PCR reaction mixture of 25 µl total volume, containing 1/10 volume 10× *Taq* buffer, 2 mm $MgCl_2$, 1 unit *Taq* DNA polymerase, 0.2 mM dNTP, 20 pmol forward primer, 20 pmol reverse primer and 100 ng DNA. DNA amplification was carried out in a Biorad Mini thermocycler with the following procedure: an initial denaturing step at 94°C for 5 min; 40 cycles for 1 min at 94°C (denature), 1 min at 48°C (annealing), 2 min at 72°C (extension) and a final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide, and photographed. The standard DNA samples (100 bp DNA ladder marker) were used as molecular size marker. The purified PCR products was subjected to Sanger's di-deoxy sequencing, in both forward and reverse direction ns, using Big Dye terminator v3.1 cycle sequencing kit on ABI Prism3700 DNA Analyzer (Applied Biosystems Inc., USA) as per manufacturer's instructions.

3. RESULT:

From the different soil samples collected from the coastal area six bacterial isolates were isolated based on the morphological characters of colonies. All six isolates can tolerate NaCl concentration up to 3%. Later these isolates were proceeded to calculate the maximum NaCl tolerance and biochemical and molecular characterization.

Figure: 1 Salt tolerant bacterial isolation plates showing the diversified colonies.



Morphological characterization

A total of six isolates obtained as a result of spreading technique followed by streaking. The isolates were coded as MGST-01, MGST-02, MGST-03, MGST-04,MGST- 05 and MGST-06. The colonies of isolates MGST-01, MGST-02, MGST-04 and MGST-06 were circular and the colonies of isolates MGST-03 and MGST-05 were irregular. All the colonies except MGST-04 which was yellow were white in colour. Colonies of isolates MGST-02 and MGST-06 were flat; colonies of isolates MGST-01, MGST-03, MGST-4 and MGST-05 were raised. Isolates MGST-02, MGST-03, MGST-04,MGST- 05 and MGST-06 were spherical in shape (cocci), MGST-01 was rod shaped (bacillus).Isolates MGST-01 was motile, remaining five isolates MGST-02,MGST-03,MGST-04 ,MGST- 05 and MGST-06 were non motile.

Table 1: showing morphological characteristics of the bacterial isolates

Biochemical tests	Bacterial isolates					
	MGST-01	MGST-02	MGST-03	MGST-04	MGST-05	MGST-06
Colony Colour	White	White	White	yellow	White	White
Colony shape	Circular	Circular	Irregular	Circular	Irregular	Circular
Elevation	Raised	Flat	Raised	Raised	Raised	Flat
Shape	bacilli	cocci	Cocci	Cocci	Cocci	Micrococci
Motility	+	-	-	-	-	-

Biochemical characterization

It was inferred that three bacterial isolates MGST-01, MGST-02 and MGST-03 were gram +ve, other isolates MGST-04, MGST- 05 and MGST-06 were gram –ve. Citrate utilization test indicates the capacity of bacteria to utilize citrate as a source of carbon and energy. CO_2 and nitrogen were released which later formed carbonate and hydroxide respectively. Blue colour indicated +ve for this test, isolates MGST-01, MGST-02 MGST-04 and MGST-06 were +ve and isolates MGST-03 and MGST-05 were –ve. Starch hydrolysis test was based on colour reaction of non-hydrolysed starch with iodine, it provides deep blue colour but its breakdown products progressively become violet, brownish red and finally colourless. Isolates MGST-01, MGST-02, MGST-03 and MGST-04 were +ve for starch hydrolysis test, isolates MGST-05 and MGST-06 were –ve for the test. Isolates MGST-04 and MGST-05 were +ve for Methyl Red test, four isolates MGST-01, MGST-02 MGST-03 and MGST-06 were –ve for MR test. Voges Proskauer test (VP test) determines the capability of some microorganisms to produce non-acidic or neutral end products, such as acetyl methyl carbinol, from the organic acids that results from glucose metabolism. Pink colour in the medium indicates +ve result. Isolates MGST-02 MGST-05 and MGST-06 were +ve as pink colour was found in the medium, isolates MGST-01, MGST-03 and MGST-04 were –ve for VP test. When bacteria grown in a medium containing nitrate as the only source of nitrogen, if the bacteria possess nitrate reductase then they convert nitrate into nitrite. This can be observed by adding sulphonic acid which forms diazonium salts, which in turn respond with α-naphylamine, results in formation of red azo dye. Three isolates MGST-02, MGST-05 and MGST-06 were +ve for nitrate reductae test. Isolates MGST-01, MGST-03 and MGST-04 were –ve for nitrate reductase test. Formation of bubbles on addition of $H_2O_2$to bacteria indicates presence of catalase. Isolates MGST-01, MGST-02 and MGST-06 were +ve for catalase test and isolates MGST-03, MGST-04 and MGST-05 were –ve for catalase test.

Table 2: Biochemical characters of bacterial isolates

Biochemical tests	Bacterial isolates					
	MGST-01	MGST-02	MGST-03	MGST-04	MGST-05	MGST-06
GramStaining	+ve	+ve	+ve	-ve	-Ve	-ve
Citrate	+	+	-	+	-	+
Starch Hydrolysis	+	+	+	+	-	-
MR	-	-	-	+	+	-
VP	-	+	-	-	+	+
Nitrate Reductase	-	+	-	-	+	+
Catalase	+	+	-	-	-	+

Positive (+), Negative (-)

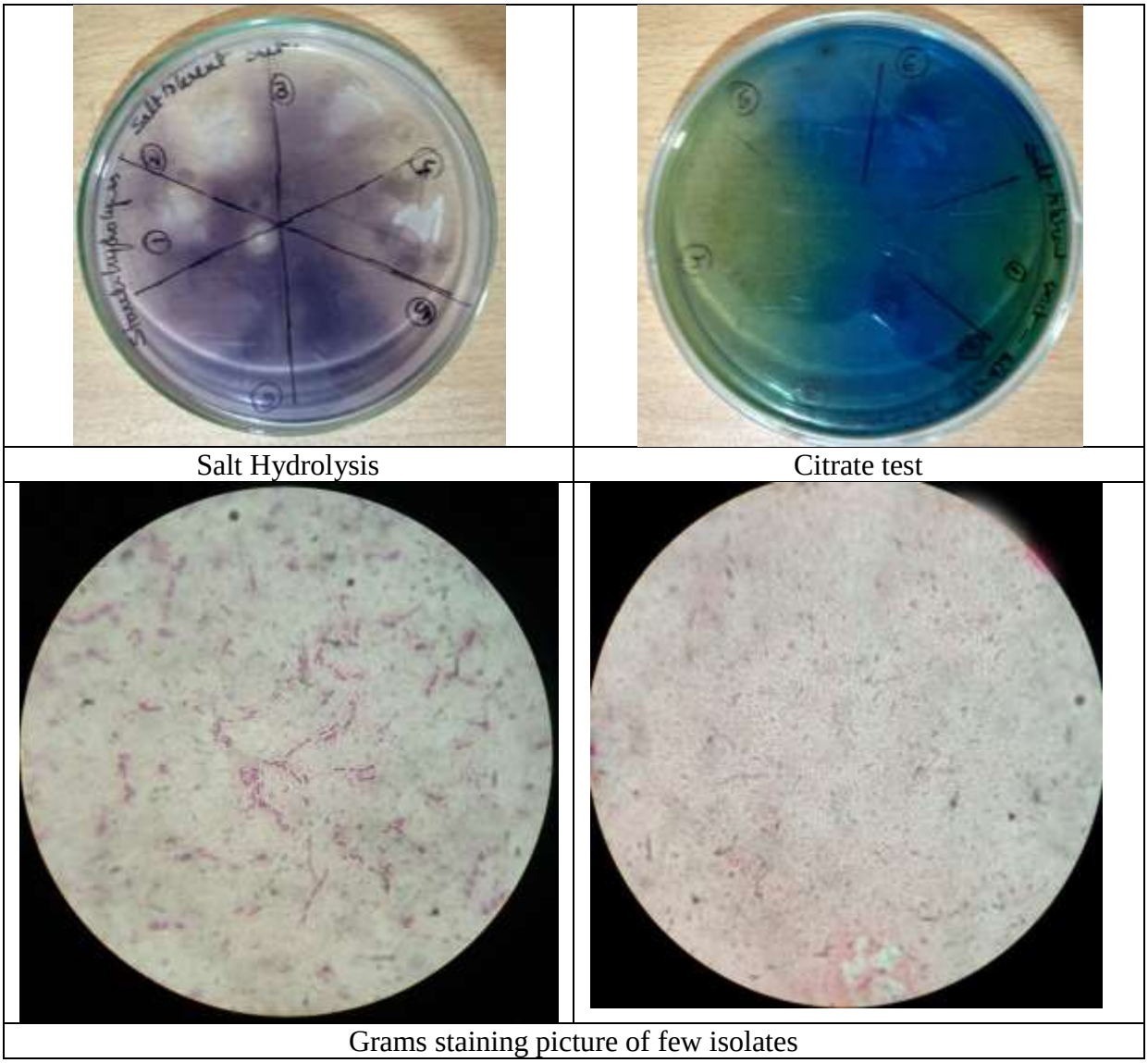


Figure 2: Biochemical characters of bacterial isolates

Salt tolerance assay

The six bacteria studies in this experiment exposed to gradient concentration of NaCl from 2.5 to 10 %. Each bacterium had their own tolerance level. It is evident that highest OD value was shown by MGST-02 followed by MGST-05, MGST-02 and MGST-03, MGST-01 and MGST-04 under unstressed condition. OD values gradually decreased with increased NaCl concentrations. It is clear that bacterial growth was shown to be decreased as the concentration of NaCl increased in the medium. Highest growth was recorded for the isolate MGST-02 the isolate showed growth up to 8% of NaCl concentration. No growth was observed in the medium exceeding 8% of NaCl concentration. Least growth was observed in isolates MGST-01 and MGST-04 for these isolates growth was observed up to 3.5% of NaCl concentration in the medium.

Table 3: Bacterial growth kinetics under salt stress.

Bacterial Isolate	OD at 660 nm													
	Control	NaCl concentration												
		2.5%	3%	3.5%	4%	4.5%	5%	5.5%	6%	6.5%	7%	7.5%	8%	8.5%	9%
MGST-01	1.5	1.1	0.58	0.41	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MGST-02	2.20	2.11	1.97	1.48	1.33	1.19	0.67	0.44	0.38	0.33	0.29	0.23	0.12	0.0	0.0
MGST-03	1.5	1.32	1.26	0.64	0.36	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MGST-04	1.29	1.12	0.56	0.27	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MGST-05	1.93	1.88	1.54	1.27	1.09	0.96	0.74	0.31	0.18	0.0	0.0	0.0	0.0	0.0	0.0
MGST-06	1.80	1.17	0.62	0.48	0.31	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

16s rRNA gene sequencing analysis

In order to screen useful microorganisms inhabiting the rhizospheric soil near coastal areas of Visakhapatnam, six strains were isolated. One isolate i.e. MGST-02 which showed growth at 8% NaCl concentration was characterised phylogenetically by sequencing PCR-amplified 16S rRNA gene. The sequence identity was searched by the BLAST analysis using NCBI database. Phylogenetic analysis using 16S rRNA revealed that the isolate MGST-02 was 99.05% similarity with *Acinetobacter calcoaceticus*. The phylogenetic relationship between the isolated 16S rRNA gene sequence compared to those of representative species were illustrated in the figure 4.

MGST-02

TAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGGGTGAT
GGTGCTTGCACTATCACTTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGAC
AACATTTCGAAAGGAATGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGC
GCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTA
GCGGGTCTGAGAGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTATGG
TTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTCTAGTTAATACCTAGAGATAGTGGACGTTACTCGCA
GAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGATTT
ACTGGGCGTAAAGCGCGCGTAGGCGGCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAAT
TGCATTCGATACTGGTTAGCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCG
TAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGTGCGAAA
GCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGG
GCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGACTA
AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA
AGAACCTTACCTGGCCTTGACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACAT
ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTTTTCCTTATTTGCCAGCGAGTAATGTCGGGAACTTTAAGGATACTGCCAGTGACAAACTGGAGGAA
GGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTAC
AAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCA
ACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTT

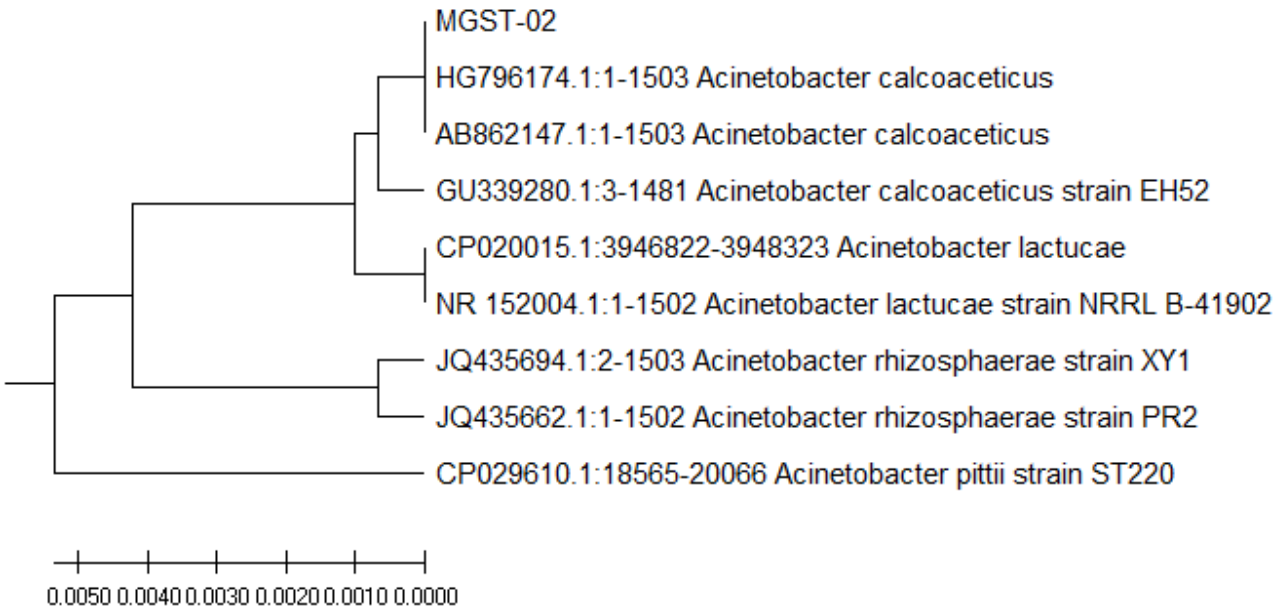


Figure: 4 UPGMA phylogenetic tree

4. Discussion:

The organisms isolated during this study were unique having moderate salt tolerance capacity. However animal pathogens viz. *E.coli, Micrococcus, Staphlococcus, Vibrio* etc. were also known to be naturally halotolerant microbes[19,20,21]. Extremophiles have been isolated from environments in which they are not expected to grow actively[22]. Our current research has shown that bacteria isolated from sample collected from coastal area of Visakhapatnam, Andhra Pradesh were halotolerant bacteria with different characteristics. During the past decades, the studies on ecology, physiology and taxonomy halophilic and halotolerant organisms revealed an impressive diversity[23]. In this study all the six isolates were grown on medium supplemented with different NaCl concentrations (i.e. from 2.5-10%). No growth was observed on medium containing above 8% NaCl concentration. This indicates that the isolates were halotolerant organisms which are in agreement with those reported by Bowers et al., (2009)[24] who reported that halotlerant bacteria can grow over a wide variety of salt concentrations. Verma, (1993)[21] was also reported growth of *Staphylococcus* in nutrient medium containing 10% NaCl which is also a normal salt concentration for halotolerant organisms. It was reported that *Micrococcus luteus* a halotolerant bacterium could tolerate up to 25% NaCl. Halotolerant bacteria grow best at the temperatures range 28-37^{0}C on medium supplemented with 3-4% NaCl.

5. Conclusion:

Six organisms were successfully isolated from coastal area of Visakhapatnam, that could grow in salinity range from 2.5-4% NaCl. From the study it is evident that the bacterial isolate MGST-02 was salt tolerant it can tolerate up to 8% of NaCl concentration. The results revealed that, the isolated MGST-02 is *Acinetobacter calcoaceticus* and is moderately salt tolerant. The ability to thrive in salty environments suggests their possible use for bioremediation purpose. The bacteria to be tested for plant growth promoting activity. Halotolerant plant growth promoting bacteria enhance crop growth and productivity. In future, these can be utilized as biofertilizer to ameliorate salt stress and increase crop production in an economically sustainable manner.

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