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INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI:10.21474/IJAR01/14873
DOI URL: http://dx.doi.org/10.21474/IJAR01/14873

RESEARCH ARTICLE

ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING HALOTOLERANT BACTERIA FROM SALINE RHIZOSPHERIC ENVIRONMENTS

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Manuscript Info

Manuscript History
Received: 10 April 2022
Final Accepted: 14 May 2022
Published: June 2022

Abstract

Among the abiotic stress parameters salinity is the major problems to the agriculture in coastal areas. Salinity leads to stunted growthand reduced yields.Bacteria isolated from saline environmentswere physiologically adapted to saline conditions, such tolerant microorganisms provide a means of cultivation of crops in salinity influencedareas. 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. Soil samples were collected from the high saline environments from Visakhapatnam and Kakinada coast. A total 7 morphologically different bacteria were isolated, among them isolate-07 was found to be high salt tolerant and plant growth promoting rich bacteria. All bacteria were biochemically characterized and isolate-07 was characterized by 16s rRNA gene sequencing and confirmed as Bacillus megaterium.

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Introduction:-

Green gram (Vignaradiata) is one of the most important grain legume among pulses in India. It is widely cultivated in the worldwide for high protein in its seeds. It is highly nutritious and the green pods are eaten as vegetable. Being a legume, it enriches soil health through biological N fixation with rhizobia and it can also break disease cycles and encourage mycorrhizae (Hedley, 2001). As the price of nitrogen fertilizer increases, it is considered increasingly profitable crops, because of the lower input requirements. It is the cheapest source of dietary protein for human and livestock. It is extensively used in various culinary preparations and recommended for diabetes. In India, about 378251 MT pulses are produced from 918261 acres of land, which is very low as compared the total requirement (BBS, 2017). To meet up the demand for consumption of pulses, we are losing huge amount of currency during the importation of pulses. Therefore, increment of green gram production is crucial importance. It is unfortunate that, as legumes, Green gram tends to grow in more marginal environments, under various biotic and abiotic stresses, especially saline and drought conditions, and as such their yield is often much below their potential (Turner et al., 2003). Soil salinity is a major, and the most persistent, threat to irrigated agriculture in India. The effect of salinity on the growth of green gram plants has been reported sporadically (Islam, 2001; Faruquei, 2002). In India, more than 30% of the net cultivable land is in the coastal area. The salt affected area in the coastal zone of the country was about 0.83 Mha in 1975-76, which expanded to 3.1 Mha over the last three decades (Haque, 2006).

Salt stress hampers the agricultural productivity by lowering the yield of various crops in arid and semi-arid regions of the world (Kapoor and Srivastava, 2010; Abd El-Wahedet al., 2015;Hasanet al., 2017). Seed germination is one of the most critical periods in the life cycle of plants. It is a biological process that demands as pre-requirement the

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seed viability, which needs physiologic pathway ready to active the metabolism, salt stress can change the metabolic activity during the imbibitions process of the seed. Salt stress greatly influenced the germination of green gram seed. The germination rate determines the crop productivity by optimizing germination factors. A fruitful crop production largely depends on the adequate seed germination, as well as the proper seedling establishment (Almansouriet al., 2001; Bhattacharjee, 2008). The highest germination percentage was observed in the control among all of the treatment combinations, as reported by Naher and Alam (2010). The plant growth and seed germination, as well as final germination percentage, were exponentially reduced by salt stress (Rahmanet al., 2000). However, with increasing (0 to 180 mM of NaCl), salinity decreases germination by 50% in species of the genus Phaseolus, reported by Bayuelo-Jimenez et al. (2002). The germination of seed, plant growth and development were adversely affected by salinity (Dash & Panda, 2001). Moreover, salinity stress significantly reduced the seed germination by producing an osmotic potential that avoids water uptake or due to toxic effects of Na+ and Cl- ions (Khajeh-Hosseiniet al., 2003) Isolation of the salt tolerant plant growth promoting bacteria and their molecular characterization were the major objectives of the work.

Materials and Methods:-

Collection of soil sample

Soil sample was collected from coastal area of Visakhapatnam and Kakinada, Andhra Pradesh, India. Soil samplewas collected from the depth of 10-12 inch in sterile polythene bag and samples were kept at room temperature untilused.

Isolation and Screening of Bacteria

Soil suspension was prepared with 1g of soil in 10ml of sterile double distilled water and vortexed and seriallydiluted. 100 µl of 3rd and 4th dilutions were spread on 3% salt amended nutrient agar plates (Himedia) and incubated for48h at 37°C for isolation of different bacteria.

Phenotypic characterization of the bacterial isolates

Physiological and biochemical characters of the bacterial isolates were examined according to methods described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The isolates were characterized for the following traits: color, pigment, form, elevation, margin, diameter, surface, opacity and texture.

Salt tolerance assay

All isolated bacteria were subjected to salt tolerance activity, nutrient broths were added with differentconcentrations of NaCl. The NaCl concentration ranges from 2.5% to 10%. After 24 hours of incubation all bacterialcultures were spectrophotometricallyanalysed at 660nm.

Estimation of ammonia liberation

100 ml Jensen's broth was inoculated individually selected seven isolated pure bacterial cultures and incubated in rotatory shaker at 30^{o}C.1ml broth culture of each the tube was aseptically transferred to the separate microcentrifuge tubes and centrifuged for 5min at 10,000rpm.100 µl of each test supernatantwas transferred to clean microcentrifuge tubes.100 µl of Nessler's reagent was added to the supernatant including blank.800 µl distilled water was added to the sample and incubated for 30min at room temperature.Absorbance was measured spectrophotomerically at 450nm.

Estimation of IAA production

The ability of the bacterial isolates to produce IAA was determined qualitatively and on Yeast Extract Mannitol broth medium.All pure isolates were inoculated onto 250ml conical flasks containing 50ml YEM broth media along with 0.1% L-tryptophan in triplicates. The cultures wereincubated in orbital shaker at 28 ± 2ºCand 120 rpm for 9 days. Cultures were centrifuged at 12000rpm for 5min and 500µl of supernatant from liquid cultures are taken into 1.5ml tube, 1ml of salkowaski reagent was added. Salkowski reagent was prepared by dissolving 2% of 0.5M $FeCl_3$ in 35% perchloric acid. Reaction tubes were incubated for 30 min in dark at room temperature. And the development of pink colour indicates the IAA production. Optical absorbance was measured at 530 nm. IAA concentrations were determined by using standard graph of known concentrations of IAA ranged between 1.25-100µg/ml. By extrapolating the obtained OD value onto standard graph, the quantity of IAA concentration produced by the bacterial isolates was obtained. (Dawwamet al., 2013).

16S rRNA gene sequence analysis

DNA isolation was carried by the SDS extraction method described by Xia et al., (1995)18 with minormodifications. 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× Taqbuffer, 2 mm MgCl2, 1 unit TaqDNA polymerase, 0.2 mMdNTP, 20 pmolforwardprimer, 20 pmol reverse primer and 100 ng DNA. DNA amplification was carried out in a Biorad Mini thermocyclerwith the following procedure: an initial denaturing step at 94°C for 5 min; 40 cycles for 1 min at 94°C (denature), 1 minat 48°C (annealing), 2 min at 72°C (extension) and a final elongation step at 72°C for 5 min. PCR products wereseparated by electrophoresis on 1.5 % agarose gel containing 0.5 µg/ml ethidium bromide, and photographed. Thestandard DNA samples (100 bp DNA ladder marker) were used as molecular size marker. The purified PCR productswas subjected to Sanger's di-deoxy sequencing, in both forward and reverse direction ns, using Big Dye terminator v3.1cycle sequencing kit on ABI Prism3700 DNA Analyzer (Applied Biosystems Inc., USA) as per manufacturer's

Results and Discussion:-

Total 10 soil samples were used for this bacterial isolation study. With that samples total 7 different bacteria was isolated which were minimum salt tolerance at 3% NaCl. All these bacteria were physiologically and biochemically characterized further.

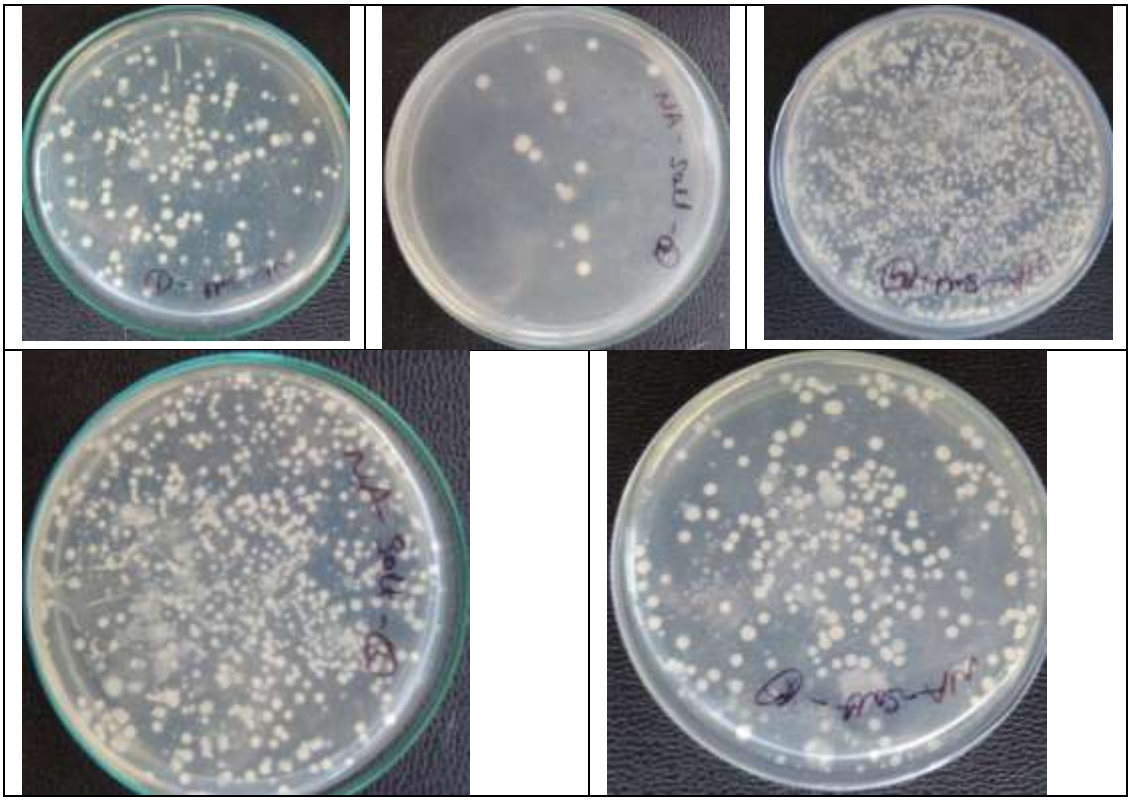

Figure 1:- Isolation of salt tolerant bacteria from soil samples.

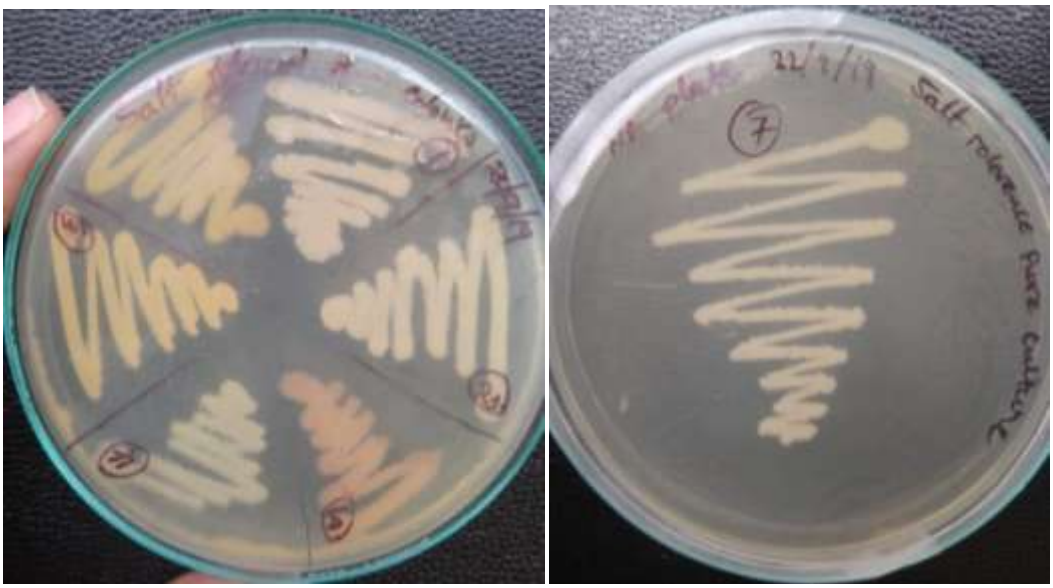

Figure 2:- Pure colonies of the salt tolerant bacteria.

Biochemical characterization

All these seven isolates were characterized using biochemical techniques. MGS-01, MGS-02 and MGS-06 isolate showed negative result for nitrate reduction remaining isolates were found positive. MGS-05 and MGS-06 isolate showed negative result for urea hydrolysis and remaining isolates were found positive. All isolates showed negative result for H_2S production. Isolate MGS-02, MGS-05 and MGS-06 showed positive result for oxidase activity and the remaining isolates showed negative result. Isolates MGS-02 and MGS-07 showed positive result for citrate and remaining isolated showed negative result. Isolates MGS-01, MGS-02 and MGS-03 showed positive result for MR rest and the remaining showed negative result. MGS-02 and MGS-05 showed negative result for VP test and the remaining were showed positive results. MGS-01, MGS-05 MGS-11 isolated showed positive results for catalase hydrolysis and the remaining isolates showed negative result. MGS-04 and MGS-06 isolates showed negative results for starch hydrolysis and the remaining isolates showed positive results. MGS-04 isolates showed positive result for lipid hydrolysis and the remaining isolates showed negative result. All isolates showed positive result for IAA production. Only isolate MGS-04 showed negative result for motility and spore production remaining isolates were showed positive result for them.

Table 1:- Morphological and biochemical characteristics of isolated bacteria.

Test Name	Bacterial isolate names						
	MGS-01	MGS-02	MGS-03	MGS-04	MGS-05	MGS-06	MGS-07
Reduction of nitrate	-	-	+	+	+	-	+
Urea Hydrolysis	-	-	-	-	+	+	-
H2S Production	-	-	-	-	-	-	-
Oxidase	-	+	-	-	+	+	-
Gelatinase production	+	-	+	-	-	+	-
Citrate	-	+	-	-	-	-	+
Catalase	+	-	+	+	-	-	-
MR	+	+	+	-	-	-	-
VP	+	-	+	+	-	+	+
Casein Hydrolysis	-	-	+	+	+	-	-
Starch Hydrolysis	+	+	+	-	+	-	+
Lipid Hydrolysis	-	-	-	+	-	-	-
IAA production	+	+	+	+	+	+	+

Spore formation	+	+	+	-	+	+	+
Motility	+	+	+	-	+	+	+
Colony colour	Cream	Cream	Brown	White	Yellow	Yellow	White
Bacteria shape	Rod	Rod	Cocci	Cocci	Rod	Rod	Rod
Grams staining	Positive	Positive	Positive	Positive	Negative	Positive	Positive

Plant growth promoting factors

Plant growth promoting activities like ammonia production, IAA production and Phosphate solubilization were studied for the selected bacteria after one week of incubation in the certain medium. All seven bacteria were showing these three activities. Ammonia production ranges between 89.5 µg/mlto 395.5 µg/ml, MGS-07 showed maximum activity. IAA production ranges between 53 µg/mlto 319.2 µg/ml, MGS-07 showed maximum activity. Phosphate solubilization activity ranges between 38.5 µg/mlto 189.5 µg/ml, MGS-07 showed maximum activity. In the present research MGS-07 showed dominant in all plant growth promoting activities.

Table 2:- Plant growth promoting factors like Ammonia production, IAA production and Phosphate solubilization activities of selected bacteria.

S.No	Bacterial Isolate	Ammonia (µg/ml)	Indole Acetic Acid (µg/ml)	Phosphate Solubilization (µg/ml)
1	MGS-01	89.5±0.43	112.5±0.09	38.5±0.44
2	MGS-02	150.5±0.91	184.9±0.21	109.4±0.30
3	MGS-03	352.1±1.04	103.2±0.05	56.4±0.45
4	MGS-04	228.0±0.33	53.0±0.33	39.0±0.33
5	MGS-05	103.8±0.61	220.4±0.45	98.4±0.98
6	MGS-06	285.1±0.89	89.3±0.05	128.4±1.29
7	MGS-07	395.5±0.63	319.2±0.08	189.5±1.04

Salt tolerance activity

The major attribute studied was effect of salinity on the isolated bacteria and to identify the high salt tolerant bacteria. In this study we have studied from 3.5% to 10% salt concentration and the OD was observed at 660 nm for the bacterial growth. all seven bacteria showed observed growth at 5% salt concentration also. After that MGS-01 didn't show growth at 5.5% NaCl concentration. At 7.5% NaCl concentration only MGS-02 and MGS-07 showed observed growth. finally at 10% NaCl concentration MGS-07 is the only bacteria showed observed growth (Table 3)

Table 3:- Effect of different concentrations of NaCl on bacterial growth.

Isolate	**OD at 660 nm**														
	Control	**NaCl Concentration**													
		3.5 %	**4 %**	**4.5 %**	**5 %**	**5.5 %**	**6 %**	**6.5 %**	**7 %**	**7.5 %**	**8 %**	**8.5 %**	**9 %**	**9.5 %**	**10 %**
MGS-01	**1.40**	**1.13**	**0.85**	**0.49**	**0.20**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
MGS-02	**1.20**	**0.86**	**0.83**	**0.73**	**0.58**	**0.51**	**0.45**	**0.43**	**0.36**	**0.21**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
MGS-03	**1.09**	**0.78**	**0.63**	**0.34**	**0.31**	**0.25**	**0.20**	**0.15**	**0.11**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
MGS-04	**1.54**	**1.21**	**0.83**	**0.41**	**0.28**	**0.19**	**0.06**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
MGS-05	**0.95**	**0.81**	**0.76**	**0.71**	**0.70**	**0.62**	**0.58**	**0.38**	**0.21**	**0..18**	**0.08**	**0.0**	**0.0**	**0.0**	**0.0**
MGS-06	**0.74**	**0.60**	**0.57**	**0.55**	**0.51**	**0.44**	**0.40**	**0.28**	**0.14**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
MGS-07	**1.28**	**1.19**	**1.16**	**1.09**	**1.02**	**0.87**	**0.85**	**0.81**	**0.76**	**0.74**	**0.59**	**0.56**	**0.51**	**0.49**	**0.32**

Conclusion:-

Soil samples were collected for the 10 different areas of coastal belt of Visakhapatnam and Kakinada. Among the soil samples 7 different bacterial isolates were able to find at 3% NaCl concentration. All the seven bacteria having the Ammonia production, IAA production and Phosphate solubilization at different levels.Among the seven bacteria MGS-07 showing the maximum values for the three activities i.e. 395.5µg/ml ammonia liberation, 319.2 IAA production and 189.5µg/ml phosphate solubilization. And fortunately the same bacteria having high salt tolerance activity, at 10% NaCl concentration it showed 0.32 OD at 660 nm.

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