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Research Article

Halophilic bacteria in salt stressed soils of Aurangabad district (MS) India

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Abstract

The crop plants are sensitive to salinity caused by high concentrations of salts in the soil. Halophilic bacteria play an important role during the development of salt tolerance mechanism in the plants. These microorganisms can adapt to extreme concentrations of salts. During the present investigation, soil samples were collected from Aurangabad district (Maharashtra). Most of the soil samples were of black basalt soil and the samples were collected from rhizosphere. Few samples were of red soils, they were neutral to acidic in nature. Mostly saline and rain fed soils were recorded through-out the district.From these samples halophillic bacteria were isolated and characterization of the bacteria was done by using morphological, biochemical and molecular analysis. On the basis of characterization confirmed strains were *P. aruginosa, P. putida, B. pumilus, B. halodenitrificans, P. rifietoensis, E. coli, R. meliloti.* The identified strains have salt tolerance and are highly useful against salt stressed soils and saline environments.

INTRODUCTION

Abiotic stress factors are responsible for reducing average yields of major crop plants. Drought and salinity are major stress factors causing comparatively extreme loss in the field crops. Salt stress affects germination of the seed and physical development of the seedling which ultimately results in suppressed vegetative and reproductive growth and development of the plant. The crop plants are sensitive to salinity caused by high concentrations of salts in the soil. A considerable amount of land in the world is affected by salinity which is increasing day by day (Hasanuzzaman *et al.,* 2013).In addition, the increased salinity of arable land is expected to have devastating global effects, resulting in up to 50% land loss by the middle of the twenty-first century (Mahajan and Tuteja, 2005).

Halophilic bacteria play an important role during the development of salt tolerance mechanism in the plants. These microorganisms can adapt to extreme concentrations of salts. Both genome sequencing and proteome analysis have shown that they contain an excess proportion of acidic to basic amino acids, a feature likely to be required for protein activity at high salinity (Joo and Kim, 2005; DasSarma, 2004).

Halophilic bacteria are a heterogeneous group of microorganisms, generally observed in the rhizosphere and can improve the quality of plant growth. The direct promotion of plantgrowth by halophiles includes synthesis of compound which facilitates the uptake of nutrients from the environment. For example: N2 fixation, phosphate solubilisation, complexation of insoluble ferric iron by siderophore production, production of phytohormones such as auxins, cytokinins, and

gibberellins and lowering of the ethylene concentration.During the present investigation, soils samples were collected from various parts of Aurangabad district and bacterial strains were isolated for further studies.

MATERIALS AND METHODS

To study the activity of halophilic bacteria in the salt stressed soils, isolation and characterization was done as per the method given below.

A) Isolation and Salt tolerance of the bacterial strains:

Ten samples of salt stressed soils from rhizosphere were collected from different parts of the Aurangabad district and brought to the laboratory for further investigations. The location wise rhizosphere soil samples and some properties of that soil presented in Table 01. Sample were sorted out and preserved in sterile polythene bags and labeled properly.10gm of the soil sample was mixed with 30ml distilled water and mixed well with the help of stirrer for 10 min.Then allowed to settle for another 10 min and pH of the supernatant was recorded.

Plates of LB agar medium with NaCl (10%) were prepared. NaCl was added to create salt stressed environment. The appearance of growth on plates containing NaCl was considered as salt or pH tolerance ability of bacterial colonies (Sevin*et al.*, 2016).Plates were inoculated with the supernatant with the help spreader.Plates kept at 28°C temperature in incubation chamber for 48 hrs after that bacterium colonies selected and re-streaked on LB agar slants till the pure culture was obtained.

Table 01: Rhizosphere soil sample collected from Aurangabad district

Sr. No.	Location	Water holding capacity	pH	Remark
01	Isarwadi, Tq. Paithan, Dist. Aurangabad (Latitude: 19.4776906)	39.32 %	8.6	Water logged soil
02	Dhakephal, Tq. Paithan, Dist. Aurangabad (Latitude: 19.4776906)	37.81 %	8.2	Water logged soil
03	Verul, Tq. Khultabad, Dist. Aurangabad (Latitude: 20.050009)	36.66 %	8.1	Vegetable (Chilly) soil
04	Waregaon, Tq. Fulambri, Dist. Aurangabad (Latitude: 20.092444)	34.86 %	8.0	Black cotton soil
05	Nagad, Near Gautala, Tq. Kannad, Dist. Aurangabad (Latitude: 20.277005)	32.78 %	8.2	Hilly region soil
06	Aland, Tq. Sillod, Dist. Aurangabad (Latitude: 20.307938)	37.48 %	8.0	Soyabean soil
07	Ladsawangi, Tq. & Dist. Aurangabad (Latitude: 19.972848)	35.45 %	8.3	Black cotton soil
08	Kaygaon, Tq. Gangapur, Dist. Aurangabad (Latitude: 19.706031)	33.77 %	8.5	Godavari river side soil
09	Borsar, Tq. Vaijapur, Dist. Aurangabad (Latitude: 19.915317)	34.56 %	8.6	Jowar soil
10	Ajantha, Tq. Soygaon, Dist. Aurangabad (Latitude: 20.580926)	33.76 %	8.3	Wheat soil

B) Morphological Studies of the bacterial cultures: Shape, size, structure of colonies and pigmentation of each bacterial colony was recorded. The Gram's reaction of each isolate was recorded separately.

C) Biochemical studies: Biochemical tests *viz.*Starch Hydrolysis, Catalase, IndoleProduction, Urease, Protease, Citrate and Nitrate Reduction were carried out for biochemical characterization of the isolates (Rajput *et al.,* 2013).

D) Molecular analysis:
Molecular analysis was done as per the methods adopted by Chakrabortyet *al*., (2011).
i) Genomic DNA extraction: The broth cultures of bacterial isolates were centrifuged at 10,000 rpm at 28^{o}C for 5 min and the pellets were collected by discarding the supernatant. The pellets were washed thrice with distilled water and re-suspended in 0.5ml of lysis solution (100mM TrisHCl, pH 7.5, 20mM EDTA, 250mM NaCl, 2% SDS, 1mg/ml lysozyme). To it 5 µl of RNase (50mg/ml) was added and incubated at 37^{o}C for 3 hrs. Then 10 µl proteinase K solution(20mg/ml) was added and it was incubated at 65^{o}C for 3min. The lysate was extracted with equal volume of Trisand water saturated phenol: chloroform: isoamylalcohol(25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube and 2 vol of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 rpm for 5 min at 4^{o}C, the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4^{o}C.
ii) Quantification of genomic DNA by gel electrophoresis: The quality of the genomic DNA, isolated from ten different isolates was checked on 0.8% agarose gel electrophoresis. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA. Quantification was done by taking absorbance at 260 & 280 nm in UV-VIS Spectrophotometer.
iii) PCR amplification: All isolates were taken up for RAPD-PCR amplification. Genomic DNA was amplified by mixing the template DNA, with the polymerase reaction buffer, dNTP mix, primers and Taqpolymerase. Polymerase chain reaction was performed in a total volume of 100µl, containing 78µl deionized water, 10µl 10X taq polymerase buffer, 1µl of 1U Taq polymerase enzyme, 6µl 2mM dNTPs, 1.5µl of 100mM RAPD primers and 1µl of template DNA. Two randomdecamers (OPA1- CAGGCCCTTC and OPA4- AATCGGGCTG) were used to determine the RAPD profiles of the isolates. PCR was programmed with aninitial denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 59°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. After RAPD-PCR amplifications, all amplified DNA products were resolved by electrophoresis on agarosegel (2%) in TAE (1X) buffer, stained with ethidium bromide and photographed. After that, all reproducible polymorphic bands were scored and analyzed following UPGMA cluster analysis protocol and computed in to similarity matrix using NTSYS computer program to prepare a dendrogram.
E) Selection and identification of the bacterial strain: Selection and identification of the bacterial strains was done by microscopic and biochemical tests,which were later confirmed by 16s r-DNAanalysis.
F)Chemicals:All chemicals were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.) at the highest availablepurity (typically >95%) unless indicated differently.

RESULTS AND DISCUSSION

During the present investigation, the soil samples were collected from Aurangabad district of Maharashtra. Most of the soil samples were of black basalt soil and the samples were collected from rhizosphere. The soil of the district is commonly known as the black cotton soil because it is best suited for the cultivation of cotton. Few samples were of red soils, they were neutral to acidic in nature. Mostly saline and rain fed soils were recorded through-out the district.North part of the district showed shallow and poor soils while south part particularly in the Godavari valley, soils were deep and fertile as the average crop production was good in the area. Bacterial samples were brought to the laboratory to perform morphological and biochemical tests. Morphological tests shows colony morphology, cell morphology and Gram's staining while Biochemical tests include starch hydrolysis, catalase, indole production, urease, protease, citrate and nitrate reduction. The observations were recorded in Table 02. Following bacterial strains were identified on the basis of morphological, biochemical and molecular analysis.
1. *Pseudomonas aruginosa*(Strain 1): Colonies were creamy white, circular, medium to large sized, opaque with rough margins. Bacteria cells were gram negative, rod shaped and abundant in rhizosphere. In biochemical test they found to be positive in citrate, catalase, indoleproduction , urease and nitrate reduction; while negative response was recorded with starch hydrolysis and protease.
2. ***Pseudomonas putida*(Strain 2):** Colonies were creamy white, circular, small sized, fluorescent with rough margins. Bacteria cells were gram negative, rod shaped and actively present in rhizosphere. Bichemically only citrate reaction was negative

Table 02: Morphological and Biochemical tests of the bacterial strains

Sr. No.	Morphological and Biochemical Tests	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7
01	**Colony Morphology**							
	Color	Creamy white	Creamy white	Slightly yellowish	Cream to pale orange	orange	Greyish white	pink
	Shape	Circular	Circular	Irregular	Circular	Irregular	Circular	Circular
	Size	Medium	Small	Large	Large	Medium	Large	Small
	Appearance	Opaque	Fluore-scent	Opaque	Opaque	Shiny	Shiny	Raised
	Margins	Rough	Rough	Rough	Rough	Entire	Smooth	Smooth
02	**Cell Morphology**	Rod	Rod	Rod	Rod	Rod	Rod(Cocco-bacilli)	Rod
03	**Gram Staining**	- ve	- ve	+ ve	+ ve	+ ve	- ve	- ve
04	**Biochemical Tests**							
	Starch Hydrolysis	- ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve
	Catalase	+ ve	+ ve	+ ve	- ve	+ ve	- ve	+ ve
	IndoleProduction	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve
	Urease	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	- ve
	Protease	- ve	+ ve	+ ve	- ve	- ve	- ve	+ ve
	Citrate	+ve	- ve	+ ve	- ve	- ve	- ve	- ve
	Nitrate Reduction	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve

Table03: Location wise distribution of bacterial strains

Strain	Bacterial strain	Observed at Location									
		01	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**
01	*Pseudomonas aruginosa*	-	+	+	+	-	+	+	+	+	+
02	*Pseudomonas putida*	-	+	+	+	-	+	+	+	-	+
03	*Bacillus pumilus*	+	-	+	+	-	-	+	-	+	+
04	*Bacillus halodenitrificans*	+	+	-	-	+	-	+	-	-	+
05	*Planococcusrifietoensis*	-	-	-	-	+	+	-	-	-	+
06	*Escherichia coli*	-	+	+	+	-	+	+	+	+	+
07	*Rhizobium meliloti*	-	-	-	-	+	+	+	+	+	-

while most of the biochemical test response was found positive.

3. *Bacillus pumilus*(Strain 3): Colonies were slightly yellowish in color, irregular, medium to large sized, opaque with rough margins. Bacteria cells were gram positive, rod shaped and rarely present in rhizosphere. Bacterial samples were hydrolyzing starch, producing indole, reducing nitrate, and positive in urease, protease, citrate and catalase.

***4. Bacillus halodenitrificans*(Strain 4):** Colonies were cream to pale orange in color, circular, medium to large in size, opaque with rough margins. Bacteria cells were gram positive, rod shaped and found abundant in rhizosphere. Bacterial samples were positive for starch hydrolysis, indole production, urease and nitrate reduction. Whereas negative response was recorded for catalase, protease and citrate.

***5. Planococcusrifietoensis*(Strain 5):**Orange colored colonies were irregular, medium sized with entire margins. Bacteria were found to be gram positive and rod shaped. Biochemically positive response was recorded for nitrate reduction, urease, indole production and catalase; while negative response was recorded for starch hydrolysis, protease and citrate.

***6. Escherichia coli* (Strain 6):** Colonies were grayish white, circular, large sized, shining with smooth margins. Bacteria cells were gram negative, rod shaped (Coccobacillli) and abundant in rhizosphere. In biochemical test they found to be positive in starch hydrolysis, indole production, urease and nitrate reduction; while negative response was recorded with catalase, protease and citrate.

***7. Rhizobium meliloti*(Strain 7):** Colonies were pale orange to pink in color, circular, small in size, raised with rough margins. Bacteria cells were gram negative, rod shaped and found abundant in rhizosphere. Bacterial samples were positive for starch hydrolysis, catalase, protease and nitrate reduction. Negative response was recorded for indole production, urease and citrate.

Location wise presence or absence of identified bacterial strains is reported in Table 03. It shows that *P. aruginosa*and*E. coli* were found at maximum locations (08 Locations) followed by *P. putida*(07 Locations); while *P. rifietoensis*wasrecorded at only 03 locations in the study area.

Edbeib *et al.*, (2016) explored the biology, adaptaion and role of halophiles in decontamination of hypersaline environments. They highlighted the various strategies adopted by halophiles to compensate for their saline surroundings and include descriptions of recent studies that have used these microorganisms for bioremediation of environments contaminated by petroleum hydrocarbons. Qureshi and Sabri (2012) observed stimulation of chickpea growth and soil aggregation under salt stress by bacterial exopolysaccharide and biofilm formation.

Ramados*set al.*, (2013) concluded that halo-tolerant bacteria isolated from saline environments have potential to enhance plant growth under saline stress through direct or indirect mechanisms and would be most appropriate as bio-inoculants under such conditions.

During the present investigation the halophiles recorded were also reported various workers (Glick *et al.*, 1995; Miller & Wood, 1996; Egamberdievaand Lugtenberg, 2014; Sevin*et al*, 2016). Jha*et al.*, (2012) reported that Pseudomonas sp. are significantly involved in plant growth-promoting activities in Salicornia in salt stress conditions.Siddikee*et al*., (2010) reported 36 halo-tolerant strains belong to 10 different bacterial genera *viz.* Bacillus, Brevibacterium, Planococcus, Zhihengliuella, Halomonas, Exiguobacterium, Oceanimonas, Corynebacterium and Arthrobacter.

The present study concludes that identified strains have salt tolerance. These strains may have future prospects in agriculture and many more industrial applications. They can be highly useful against salt stressed soils and saline environments.

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