Report on efficient salt stable *Azospirillum* a Lonar Soda Lake isolate

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ABSTRACT

Lonar Soda Lake located in Buldhana district ($19^0$58' N; $76^0$31' E) Maharashtra, India was selected for isolation of salt stable non symbiotic nitrogen fixing bacteria. Gram negative, rod shaped, motile bacterium was isolated using nitrogen free Jensen's medium from Lonar soda lake sediment sample. Isolate showed luxuriant growth at 30^0C temperature and pH 8. The isolate identified as *Azospirillum lipoferum* using morphological and biochemical characterization. *Azospirillum lipoferum* showed indole acetic acid production at 4% salt concentration and alkaline pH. Effect of isolate on seed germination was also recorded; enhanced germination rate was observed in *Vigna aconitifolia* seeds.

Keywords: Indole acetic acid*, Azospirillum lipoferum*, *Vigna aconitifolia.*

INTRODUCTION

Plants require macro- and micronutrients for their optimal growth and production. Among the different methods of enhancing nutrient quantity and availability for plant utilization is the use of chemical fertilization, which is a fast way of providing plant with necessary macro- and micronutrients. With the rapid growth of world population, the use of chemical fertilization has tremendously increased and hence the probability of environmental pollution. We can use the soil microbes for providing nutrients for plant growth and yield production, which have been proved to be very advantageous (Adesemoye *et al.,* 2008 & 2009).There are a wide range of microbes in the soil, which are able to act in symbiotic like *Rhizobium* (Jadhav RN, 2013) or non-symbiotic association with their host plant (Gray and Smith, 2005). Soil microbes are a great and necessary part of soil ecosystem.

Soil salinity is one of the major problems adversely affecting crop productivity in the arid and semiarid regions. Salt affected soils are of widespread occurrence and have resulted in degradation of more than 2.5 million hactors of otherwise arable lands in the Indo-Gangetic plains (Abrol and Bhumbla, 1971).Therefore; it is of interest to study physiological responses of crop plants and changes in soil biological properties due to salinity stress. The soil microbes are known to play a significant role in mineral nutrition of plants by mediating nutrient transformations in the soil. The influence of salinity on these microbial populations could affect plant growth (Barreto *et al.,* 2011). The present paper describes isolation and identification of salt tolerant *Azospirillum lipoferum* from the Lonar lake situated in Buldhana district of Maharashtra, India (Lat. 19^0 58', long. 76^0 34'). It is the only lake formed in basaltic rock. Production of IAA by the isolated organism and its effect on germination of seeds of *Vigna aconitifolia* was also tested.

MATERIAL AND METHODS

Isolation and characterization of nitrogen-fixing bacteria

Lonar lake sediment soil sample were collected in sterile plastic bags and preserved in laboratory for further processing. Samples of 1.0 g soil were suspended in 9.0 ml saline (pH 7.2) and serial dilutions (1:10) were spread on nitrogen free Jensens medium (Hartmann and Baldani, 2006) and incubated at 30°C for 3 to 5 days. All primary cultures with positive growth were tested for nitrogen fixation in a medium by Neseller's reagent.

Diazotrophic strains were then isolated in pure culture by dilution plating, re-tested, and screened for their salt tolerance For this purpose, nitrogen free medium supplemented with various concentrations of NaCl (ranging from 2-10%) was used for inoculation, nutrient broth supplemented with 0.5% (w/v) NaCl was used as a control and incubation was carried at 30°C, 120 rpm for 24 h. and growth was determined by measuring absorbance at 600 nm. The isolates showing high salt- tolerance were characterized and selected for further study.

Strains were characterized by Gram staining, colony morphology and motility. Biochemical tests were performed as described by Holt et al., (1994).They were also studied qualitatively for their ability to secrete extracellular enzymes (amylase, urease, protease, gelatinase) Indole production, citrate utilization, catalase and oxidase,H2S production,vogus proskauer tests, methyl red and carbohydrate fermentation tests were performed using standerd procedures. Antibiotic susceptibility tests were performed by disc diffusion method using disc of penicillin, streptomycin, tetracycline and chloramphenicol.

pH and temperature tolerance

High salt-tolerant, nitrogen-fixing cultures were further screened for pH and temperature tolerance in Nitrogen free mannitol broth adjusted to pH 5.0-10.0. at 30°C and temperatures ranging from 20°C to 60°C for 24 h and The media were inoculated with overnight grown inoculum (10^5 cells/mL), incubated at 37°C, 120 rpm for 24 h and cell growth determined by measuring absorbance at 600 nm.

Detection of IAA production

For the qualitative determination of IAA production, bacterial cultures were grown at 30^0 C for 48 h on minimal medium (3 g K2HPO4,6g Na2HPO4, 5 g NaCl, 2 g NH4Cl, 0.1 g MgSO4, 8 g glucose in 1 L, pH 7.2) with or without tryptophan (500 mgmL71). Bacterial cells were then removed from the culture medium by centrifugation at 7000 g for 10 min. Approximately 1 mL of the supernatant was mixed vigorously with 4 mL of Salkowski's reagent (150 ml concentrated H_2SO_4, 250 ml H_2O, 7.5 ml 0.5 M $FeCl_3.6H_2O$) and development of a pink color indicated IAA production.

Effect of inoculums on seed germination

Seeds of *Vigna aconitifolia* were used to check effect of isolate. Seed bacterization was done by the method of Weller and Cook (1983). Seeds were surface sterilized with 95 % alcohol for 30 s, followed by 0.1 % sodium hypochlorite for 2–3 min and then washed with sterile distilled water for 5–6 times, left in sterile water for 30 min and germinated on moist sterile filter paper in petridishes. To each petridish, 2 ml sterile water and 9 surface sterilized seeds were added. The seeds were germinated in the dark at 22°C. After 24 h each plate was inoculated with a 1 ml suspension of bacterial cells at 10^8 CFU ml−1) and 1 plate with sterile distilled water kept as control and further incubated in the dark at 25°C for 5 days.

RESULT AND DISCUSSION

Isolation, identification and selection of salt tolerant nitrogen fixing bacteria

Lonar soda lake sediment soil sample were used to isolate salt tolerant, nitrogen-fixing bacteria.We have isolated 8 bacteria, From these only 3 isolates were found to grow very well on nitrogen-free Jenesen's medium indicating that they could fix nitrogen for survival and growth. They are designated as AZ1 to AZ3.Nitrogen fixation ability by the means of accumulation of ammonia in the nitrogen free medium were checked by Nessler's reagent, In the presence NH3 the reagent shows a yellow colouration, it turns to brown if medium were incubated for 48 hrs to 72 hrs.Out of 3 isolates screened for salt- tolerance only 1 isolates tolerated salt concentration above 3%. Isolate 1 is gram negative, motile and slightly curved rod having cell size 0.6 µm. It is catalase positive and utilizes dextrose, mannitol, arabinose, maltose as carbon source. Eckert *et al*, 2001 reported that all these features were very similar to other *Azospirillum spp.* On the basis of morphological and biochemical characteristic presented in Table no.1 it is identified as *Azospirillum lipoferum* (Kanade SN *et al.,* 2011). The higher NaCl tolerent growth of *Azospirillum spp* was seen by Villiana Rivorala (1998).

pH and temperature tolerance

Isolate showed optimum growth at pH 8.0 (Fig. 2), however tolerated awide pH range of 5.0-9.0.It has also tolerated temperature up to 40°C, and could not grow below 30°C (Fig 3). These characteristics indicated their utility in wide spectrum of pH and temperature, which is a major prerequisite as soil inoculants.

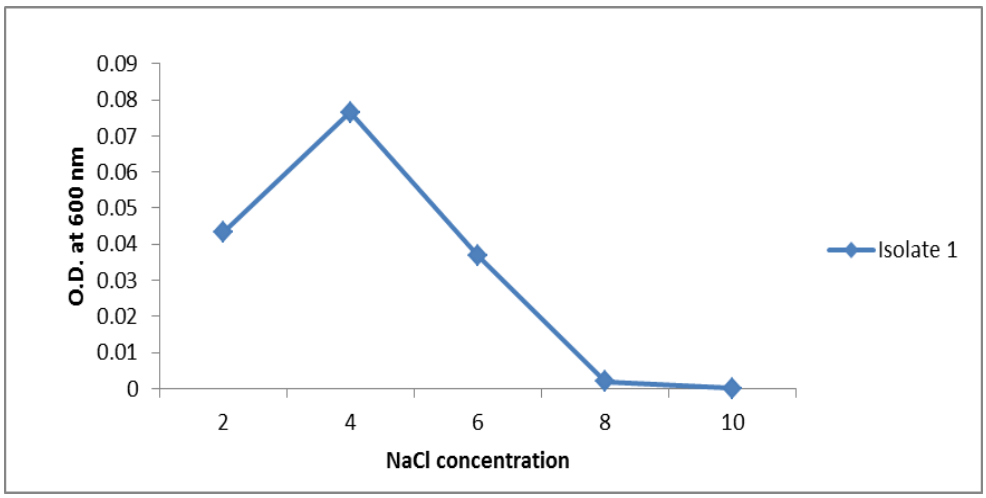

Fig. 1: Effect of NaCl on growth

Table no.1 Morphological and biochemical characteristics

Tests	Results	Tests	Results
Morphology	Rod	Sucrose	+
Grams nature	Negative	Maltose	+
motility	motile	Fructose	+
Catalase	+	Dextrose	+
NaCl range for growth (%)	2-4%	Mannitol	+
Temp.optimum ^{0}C	30^{0}C	Indole production	+
pH optimum	8	Methyl red	+
Utilization of		Vogus proskur	-
Melliboise	-	Citrate utilization	-
Rhamnose	+	Enzyme profile	
Inositol	-	Amylase	-
Ribose	-	Gelatinase	-
Raffinose	-	Urease	-
Trehalose	-	Protease	-
Adonitol	-	Cellulose	-
Lactose	+	Antibiotic suspetibility	
Arabinose	+	Penicillin	R
Cellobiose	-	Streptomycin	S
Galactose	-	Tetracycline	S
Sorbitol	+	Chloramphenicol	R
Identified as *Azospirillum lipoferum*			

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Detection of IAA production

Mehnaz S (2006) reported that *Azospirillum lipoferum* has the highest nitrogenase activity and IAA production activity. Our isolate also showed remarkable Indole acetic acid production.

Effect on Seed germination

Enhanced seed germination was noticed in test seed samples. Beneficial effects of inoculation with Azospirillum on wheat yields in both greenhouse and field conditions have been reported by Ganguly *et al.* (1999). In the present study salt resistant *Azospirillum lipoferum* producing IAA was isolated which showed significant effect on seed germination. Hence the said strains could be effectively used as Biofertilizer.

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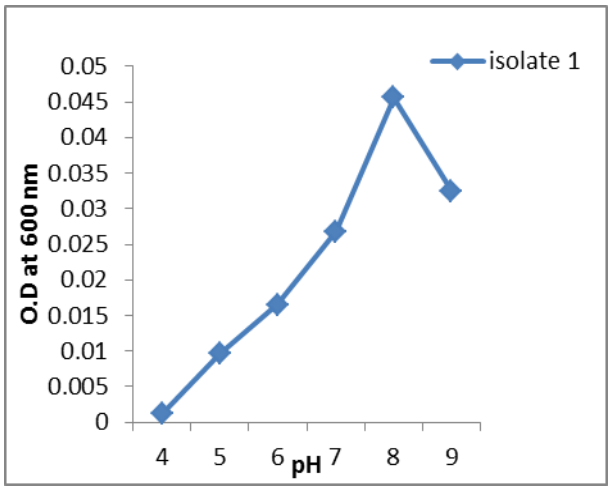

Fig:2 Effect of pH on growth

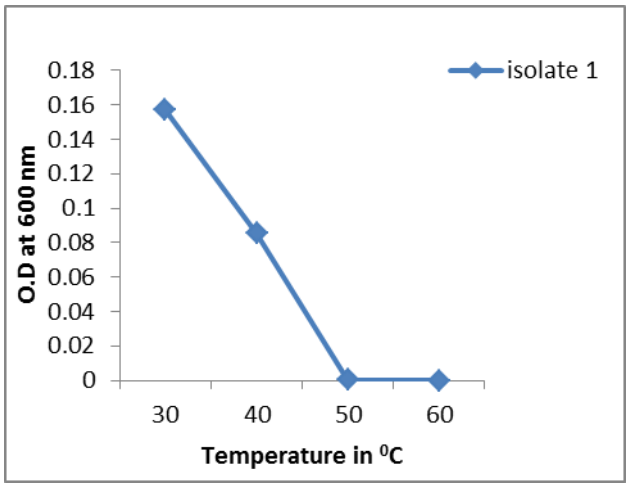

Fig:3 Effect of temperature on growth

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