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Isolation and Characterization of Halotolerant *Azotobacter* Species from Different Coastal Soils of South Gujarat Region

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Abstract
Salinity is one of the major abiotic stresses that adversely affect modern agriculture and constitutes a major problem everywhere in the world. To cope with increasing demand for agriculture land, the vast wasteland areas of India compromising saline soils need to be put in use. The aim of the present study was to isolate and characterize halotolerant *Azotobacter* species from different coastal region of south Gujarat. In the present study, soil samples were collected from different coastal region of South Gujarat namely Tithal, Danti, Daman and Dumas and were investigated for halotolerant *Azotobacter* species by isolating on Ashby's mannitol agar plate containing 1%, 2% and 3% NaCl concentrations. All the isolates were identified as halotolerant *Azotobacter* species by performing morphological analysis and various enzymatic analysis includes catalase test, nitrate reduction test, phosphate solubilizing test, Cellulase test and starch hydrolysis test. The growth of halotolerant *Azotobacter* species were also studied at different pH and NaCl concentration. Six halotolerant *Azotobacter* isolates were isolated and were found to have cellulase, catalase, and phosphate solubilizing and nitrate reductase enzymatic activities where one of the Halotolerant *Azotobacter* species was able to tolerate 10 % NaCl concentration. The seed germination assay reveals that the inoculation of the halotolerant *Azotobacter* species potentially increase the root and shoot length of the seedling and can serve as a potential source of Biofertilizers that offers an environmentally sustainable approach to boost crop yield under saline condition.

Keywords
Halotolerant *Azotobacter*, biofertilizers, nitrogen fixation, Seed germination.

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INTRODUCTION
The problem of soil being saline is increasing and as a result of which soil fertility is decreasing which is responsible for scarcity of food. The saline soil is "The soil containing sufficient soluble salts to adversely affect the growth of most crop plants" [1]. Due to

high salt stress the plant growth decreases and structure of soil also gets disturbed. Saline soil contain excessive salt which results in deficiency of nitrogen, phosphorus and other trace elements which are required for plant growth. At high salinity, retardation of germination and growth of seedlings have also been reported by many investigators [2].

Various techniques were used for the reclamation of soil such as physical, chemical and biological method. The salt affected soil are unsuitable for crop production and generally cultivated by marginal farmers who grow salt tolerant crops, do not use any chemical fertilizers and hence do not realize the full yield potential of the crop. Biofertilizers are easily affordable alternative to chemical fertilizers for improving crop yields. Microbial bioremediation is suggested by many scientific workers. Application of nitrogen fixers have been suggested best strategy by bioremediation of saline soil [3] among them *Azotobacter* and *Azospirillium* is of keen importance. *Azotobacter* is a multitasking organisms, it fixes nitrogen non-symbiotically, degrades cellulose, phosphates and most importantly it degrades lignin also in trace amount [4].

Thus, the main aim of the present study was to isolate and characterize halotolerant *Azotobacter* species from different coastal region of south Gujarat.

MATERIALS AND METHODS

Sample collection and Isolation

Soil samples were collected from different coastal region of South Gujarat namely Tithal, Danti, Daman and Dumas beach from the depth of 10-15 cm. All the samples were investigated for halotolerant *Azotobacter* species.

Samples were serially diluted where 0.1 ml of sample aliquots were taken and spreaded on the Ashby's mannitol agar plate containing 1%, 2% and 3%NaCl concentrations. The plates were then incubated at room temperature for 24-72 hours. All the isolates were purified by using streak plate technique on Ashby's mannitol salt agar plate.

Identification

All the isolates obtained were then subjected for various morphological and enzymatic analysis.

I Morphological analysis

For morphological analysis, Gram staining and Capsule staining (Maneval's method) was performed.

II Enzymatic analysis

Various enzymatic properties of the obtained isolates were studied. It includes catalase test, nitrate reduction test, phosphate solubilizing test, cellulase test and starch hydrolysis test [1].

- **Catalase test**

Some microbes degrade hydrogen peroxide by producing catalase enzyme. Slide test was carried out for the confirmation of catalase enzyme. The colony isolate was placed on the glass slide, 2-3 drops of hydrogen peroxide was added and the formation of bubbles was observed.

- **Nitrate reduction test**

One of the main characteristics of *Azotobacter* is nitrogen fixation by reducing the nitrate to nitrite. The test is perform to determine the ability of the obtain isolates to convert nitrate to nitrite. The isolates were inoculated in 1% peptone and incubated at 25˚-28˚C for 24 hours and next day result was observed**.**

- **Phosphate solubilizing test**

Pikovskaya's agar medium was used for the phosphate solubilizing test**.** Isolates were checked for their ability to solubilize phosphate by line streaking on the Pikovskaya's agar medium, and was then incubated at 25˚- 28˚C for 36-72 hours. Clear zone around the bacterial colony indicates phosphate solubilizing ability of bacteria [5].

- **Cellulase test**

Isolates were streaked on the Carboxymethylcellulose agar medium to investigate their property of producing cellulase test. The plates were then incubated at 25˚C-28˚C for 7 days.

- **Starch hydrolysis test**

Organisms capable of hydrolyzing starch to maltose possess the enzyme amylase. By this test the presence or absence of this enzyme was ascertained. The isolates were streaked on the starch nutrient agar plate then incubated at 25˚C-28˚C for 24 hours.

III Physiological characteristics

- **Effect of varying NaCl concentration**

The growth of *Azotobacter* species were also studied at different NaCl concentration by streaking the isolates on Ashby's mannitol agar plate with 1%-11% NaCl concentrations and incubated at room temperature for 24 - 72 hours.

- **Effect of varying pH**

The growth of *Azotobacter* species were also studied at different pH by streaking the isolates on Ashby's mannitol agar plate with 5-11 pH and incubated at room temperature for 24 - 72 hours.

Seed germination experiment:

Wheat seed germination assay was done by applying cultures of obtained isolates. The cultures were in sterile Ashby's mannitol broth with 3 % of NaCl to 1.0 OD at 530 nm. Seeds were surface sterilized with 0.1% $HgCl_2$ for 3 minutes and washing with distilled

water 6 times. 20 surface sterilized seeds of wheat were bacterized with inoculums for 30 minutes. The seeds were then transferred on moist sterilized filter paper in Petri plates and were incubated at room temperature and left undisrupted. Seeds soaked in sterile distilled water were used as negative control. The specified distilled water solution containing 3% NaCl addition was followed every day until 7th day. The root and shoot lengths were measured and were then compared [5].

RESULTS AND DISCUSSION

In the present study, six halotolerant *Azotobacter* isolates were obtained and were studied for their morphological, biochemical characteristics and enzymatic activities.

Morphological characteristics:

All the isolates were found with small, round, convex and mucoid colonies with an entire margin, when streaked on fresh same Ashby's mannitol agar plate (Bergey's Manual of Determinative Bacteriology).

The Halotolerant *Azotobacter* isolates were microscopically studied by performing Gram staining and special staining i.e. capsule staining. All the isolates were Gram negative short rods, capsule former and motile. The similar findings were also obtained by Akhter *et al*., (2012) and Nawadkar *et al* (2015) [6, 1].

Enzymatic properties:

All the isolates were further studied for their enzymatic activity (Table 1). Isolates A5 and A6 had not shown starch hydrolysis zone and were negative for amylase production. Akhter *et al* (2012), in their study, found all the isolates were amylase producers [6]. In the present study, all the isolates were found positive for nitrate reductase test, phosphatase test and catalase test, while Nawadkar *et al* (2015), in their study, have found one of isolate was phosphatase and nitrate reductase negative. All the isolates were found to have potential characteristics of producing Cellulase enzyme which was similar to the result obtained by Nawadkar *et al* (2015) [1].

Table 1: Enzymatic properties

Enzyme Assay	Isolates					
	A1	A2	A3	A4	A5	A6
Catalase test	+	+	+	+	+	+
Nitrate reduction test	+	+	+	+	+	+
Phosphate solubilizing test	+	+	+	+	+	+
Cellulase test	+	+	+	+	+	+
Starch hydrolysis test	+	+	+	+	-	-

Physiological characteristics

- **Effect of varying NaCl concentration**

Table 2: Effect of varying NaCl concentration on the growth of the halotolerant *Azotobacter* species

NaCl Concentration	Isolates					
	A1	A2	A3	A4	A5	A6
1%	+	+	+	+	+	+
2%	+	+	+	+	+	+
3%	+	+	+	+	+	+
4%	+	+	+	+	+	+
5%	+	+	+	+	+	+
6%	+	+	+	+	+	+
7%	+	+	+	+	-	+
8%	+	+	+	+	-	-
9%	+	+	+	+	-	-
10%	-	-	-	+	-	-
11%	-	-	-	-	-	-

In present experiment A4 is the more salt resistant among the isolates obtained as it could tolerate up to 10% NaCl concentrations, whereas A1, A2 and A3 can tolerate up to 9% NaCl concentration. The growth of A5 isolate was inhibited at 7% NaCl concentration, A6 showed growth up to 7% and

inhibited in 8% NaCl concentration (Table 2) similar findings were also obtained by Islam *et al*. (2008) [7]. According to Bergey's manual of Systematic Bacteriology (Holt, 1994) at more than 1% NaCl concentration only *A. chroococuum, A. Vinelandii* and *A. armeniacus* can survive [8].

- **Effect of varying pH**

In the present study A1-A6 growth was observed from 5-9 pH and growth of A1, A3 and A5 was inhibited at pH 10 (Table 3). These results were resembles to that with Nawadkar *et al*. (2015) [1]. In 2005, Tejera *et al.* has assessed growth rates at different initial pH values ranging from 4-9 and showed that a lower number of isolates grew on N-free media at pH value as high as 8.7 [9].

Table3: Effect of varying pH on the growth of the halotolerant *Azotobacter* isolates.

pH	Isolates					
	A1	A2	A3	A4	A5	A6
5%	+	+	+	+	+	+
6%	+	+	+	+	+	+
7%	+	+	+	+	+	+
8%	+	+	+	+	+	+
9%	+	+	+	+	+	+
10%	+	+	+	+	+	+
11%	-	+	-	+	-	+

Results showed that *Azotobacter* have variability in pH tolerance. Jimenez *et al.* (2011) also expressed similar views and mentioned that the genus *Azotobacter* is ubiquitous in nature and they can grow with pH ranging from 6.0 to 9.0 of different climatic temperatures [10]. These observations are in agreement with those observed by Peterson and Olsen, 2001 [11], Ramos, 2003 [12] and Knowles, 2008 [13] who studied the growth of *Azotobacter* species in acid and alkali condition

Seed germination assay:

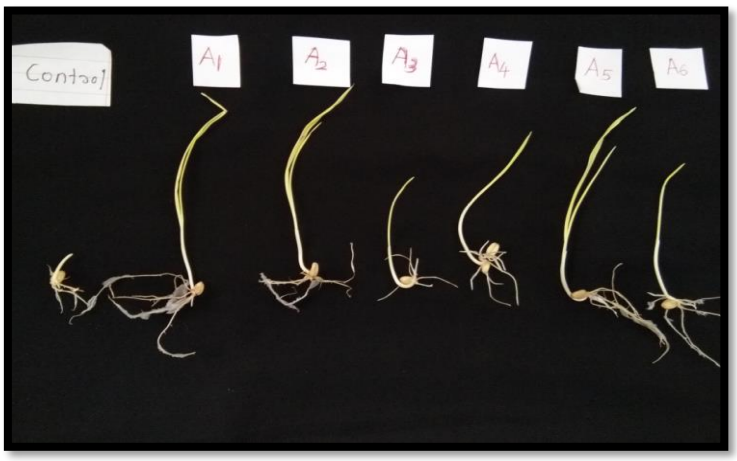

Figure 1: Comparison among the root and shoot length of the seed bacterized with isolates

Table 4: Root and shoot length after 7 days of incubation

Isolates	Root Length (cm)	Shoot Length (cm)
Control	2.2	2.4
A1	4.3	9.3
A2	4.5	10
A3	3.7	6.1
A4	4.1	9.1

A5	**4.6**	**10.5**
A6	3.5	6.4

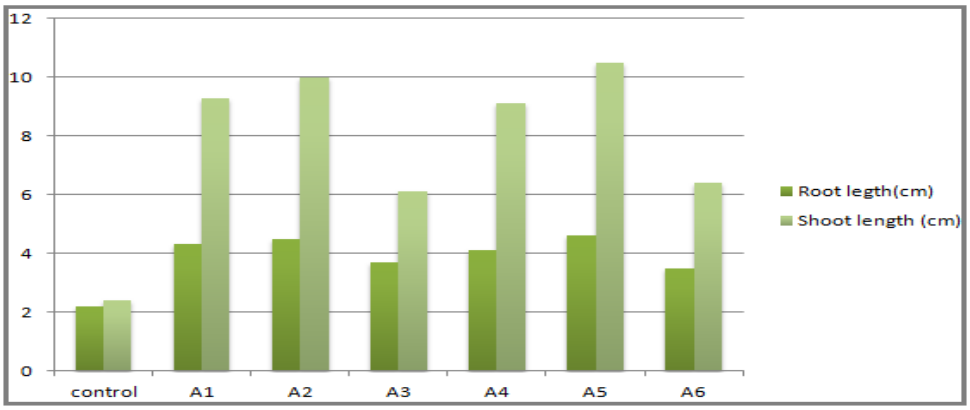

Figure 2: Root and shoot length of the seedlings

Seed germination experiment was performed by the process mentioned by Rodge *et al.* (2016) [5]. Here compared to control which is devoid of any culture all the seeds bacterized by the isolates had flourished very well. However comparing among the bacterized seeds, maximum growth was observed in the seeds bacterized with A5, which shows its ability to act as biofertilizer**.**

Plants exhibit a reduced leaf growth rate owing to decreased water uptake, which restricts photosynthetic capacity. Rhizospheric microorganism, particularly beneficial bacteria can improve plant performances under environmental stress and consequently enhance the yield [14]. The major limitation to a more widespread use of seed inoculation has generally been the variability in effects in both field and laboratory studies [15, 16].

The effects of *Azotobacter* on early seedling development influenced by inoculation and vary with inoculums concentration [17] and age of the culture [18]. Barley is a suitable cereal for study because the processes involved in germination have been studied in detail [19].

CONCLUSIONS

In the present study, halotolerant *Azotobacter* species were isolated from the coastal region of South Gujarat and were found Gram negative rods, motile, capsules formers and were found to have cellulase, catalase, and phosphate solubilizing and nitrate reductase enzymatic activities. In our study, one of the Halotolerant *Azotobacter* species found was able to tolerate 10 % NaCl concentration. The current study reveals that the inoculation of the halotolerant *Azotobacter* species potentially increase the root and shoot length of the seedling by performing the seed germination assay. The halotolerant *Azotobacter* species can serve as a potential source of Biofertilizers that offers an environmentally sustainable approach to boost crop yield under saline condition.

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