RESEARCH ARTICLE

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF HALOTOLERANT AZOTOBACTER SPECIES FROM SALINE SOIL IN NANDED DISTRICT

Anupama Prabhakarrao Pathak*[1], Mansi Mohan Tak[1], Gautam Tanaji Kamble[1], Babasaheb Surwase[1], B. B. Pendkar[2], Makarand Cherekar[3] and Prashant S. Wakte[4]

[1]School of Life Sciences (DST-FIST Phase-I and UGC-SAP DRS-II Sponsored School), Swami Ramanand Teerth Marathwada University, Nanded-431606, Maharashtra, India

[2]Kfert Lab MIDC CIDCO Nanded, Maharashtra, India

[3]MGM's College of Computer Science & IT, Nanded, Maharashtra, India

[4]D. S. M's College of ACS, Parbhani, Maharashtra, India

*Corresponding author E-mail: anupama.micro@rediffmail.com

DOI: https://doi.org/10.5281/zenodo.17185105

Abstract:

Salinity is one of the major abiotic stresses that adversely affect modern agriculture and constitutes a major problem everywhere in the world. To deal successfully with a difficult matter or situation 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 region of Nanded Maharashtra. In the present study, soil samples Four collected from different region of Nanded Maharashtra. namely Tuppa, Jawahar Nagar, Vishnupuri and MIDC CIDCO Nanded Maharashtra were used. Isolation of Halotolerant *Azotobacter* was carried out on Nitrogen free mannitol Agar and Ashby's mannitol agar plate containing 3% and 5% NaCl concentrations. All the isolates were identified by performing morphological analysis and various enzymatic analysis viz catalase test, nitrate reduction test, phosphate solubilizing test, Gelatinase test, Cellulase test and starch hydrolysis test. 10 halotolerant *Azotobacter* isolates and were found to have cellulase, catalase, and phosphate solubilizing and nitrate reductase enzymatic activities where ten of the Halotolerant *Azotobacter* species was able to tolerate 5% NaCl concentration.

Keyword: Halotolerant *Azotobacter*, Biofertilizers, Nitrogen Fixation.

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". Due to high salt stress the plant growth decreases and structure of soil also gets disturbed. Saline soil contains excessive salt which results in deficiency of nitrogen, phosphorus and other trace elements which are required for plant growth.[1] At high salinity, retardation of germination and growth of seedlings have also been reported by many investigators. 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.[1] Application of nitrogen fixers have been suggested best strategy by bioremediation of saline soil among them *Azotobacter* and *Azospirillium* is of keen importance. *Azotobacter* is a multitasking organism, it fixes nitrogen non-symbiotically, degrades cellulose, phosphates and most importantly it degrades lignin also in trace amount. [1]

Thus, the main aim of the present study was to isolate and characterize halotolerant *Azotobacter* species from different region of Nanded Maharashtra. namely Tuppa, Jawahar Nagar, Vishnupuri and MIDC CIDCO Nanded Maharashtra.

Materials and Methods:

Sample Collection and Isolation

Soil samples were collected from different region of Nanded namely Tuppa, Jawahar Nagar, Vhishnupuri and MIDC, CIDCO Nanded Maharashtra from depth of 10-15 cm. All the samples were Investigated for Halotolerant Azotobacter species. Samples were serially diluted where 0.1 ml sample were taken and spreaded on the Nitrogen free mannitol Agar and Ashby's mannitol agar Plate containing 3% and 5% NaCl concentrations using streak plate technique. The plates were then incubated at room temperature for 24 -72 hours.

Identification

All the 24 Azotobacter isolate were verified for their purity and then studied for colony morphology and pigmentation. The cell shape and gram's Stainig were also recorded as per the standard procedure. All the isolate obtained were then subjected for various morphological and enzymatic analysis.

Morphological Biochemical and Enzymatic Analysis

Selected isolate of Azotobacter 24 were biochemically characterised by IMViC test, carbohydrate fermentation, Oxidase test Catalase test,H_2S production test, Denitrification test, starch Hydrolysis test, Gelatin Liquification test. Various enzymatic properties of the obtained isolate were studied. It includes catalase test, nitrate reduction test, Phosphate solubilizing test, cellulase test and starch hydrolysis test.

Identification of bacteria was also carried out using ABIS Online: The identification results are only informative and are not intended to represent an official statement. This software is a simplified version of "ABIS online," a non-commercial laboratory tool for bacterial identification, hosted on the REGNUM PROKARYOTAE website at www.tgw1916.net. The desktop version offers enhanced data

analysis and additional user options, including analytics, result printing, identification maps, status tracking, help, and contact features. Metabolic characters of the microorganisms may vary inside species, therefore not fully matching the identification patterns. This have developed applications that will enable users to identify the organisms based on the results of their tests. The software uses probability matrix for identification and the results are expressed in percentage probabilities. The matrix table can also be used to view the properties of these organisms and to compare their properties. Accuracy of the results are dependent on the accuracy of the test results. Databases used here have been created from the matrices/tables published elsewhere.

Biofertilizers Production

The selective and optimized mediums used for mass culturing of Azotobacter biofertilizers are as follows K_2HPO_4, $MgSO_4,7H_2O$ $FeSO_4,7H_2O$ $CaCl_2,2H_2O$ $Na_2MoO_4.2H_2O$ Glucose

Materials and Methods:

For mass production of Azotobacter bacterial strain isolated from various regions and grown on slants for preservation as per need culture from slant were transferred to 100 ml liquid broth of selective as well as optimized medium in the rotary shaker for 48 hrs to prepare starter culture.

Optimization of Biofertilizer Production Using Different Parameter

Effect of pH on growth of inoculum was assessed by incubating inoculated media at 8.5 pH. Effect of temperature on growth of inoculum was assessed by incubating inoculated media at 37^O c temperature.

Production of Biofertilizer

Later on the starter cultures is transferred to the 1 litre flask containing 500 ml media. after incubating for 48 hrs at optimum temperature and pH on 48 hour incubation fermented broth was centrifuged and used for seed treatment. Experimental Setup for Seed Germination Assay Seed Germination Assay 100 ml of the biofertilizer inoculum was used for seed germination. Growth parameters such as shoot and root length, biomass, and phosphorus uptake were measured to assess the effectiveness of the PSB formulation.

a. Seed selection Seeds of wheat (*Triticum*), mung (*Vigna radiata*), moth beans (*Vigna aconitifolia*), peanut (*Arachis hypogaea*), pigeon pea (*Cajanus cajan*), chickpeas (*Cicer arietinum*), brown chickpeas (*Cicer*), and green peas (*Pisum sativum*) were selected for the assay.

b. Treatment Preparation

- Control: Seeds were soaked in sterile distilled water for 30 min.
- Biofertilizer treatment: Seeds were soaked in PSB biofertilizer suspension for 30 min before germination.

c. Germination setup Treated seeds were placed in sterile petri plates lined with moist filter paper. Plates were incubated at room temperature for 4 days.

Result and Discussion:

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

Morphological Characteristics

All the isolate were found with small, circular, convex and mucoid colonies with entire margin,when streaked on fresh same nitrogen free mannitol agar plate. The Halotolerant Azotobacter isolate were microscopically studied by performing Gram staining and special staining i.e. capsule staining. All the isolate were negative short rods, capsule former and motile.

Enzymatic Properties

All the isolate were further studied for their enzymatic activity (Table 1). In the present study all the isolate were found positive for nitrate reductase test, phosphates test and catalase test.

Colony Count

Table 1: Colony count

Time In Hours	Colony count 10^{-1}	Colony count 10^{-2}	Colony count 10^{-3}	Colony count 10^{-4}	Colony count 10^{-5}	Colony count 10^{-6}	Colony count 10^{-7}	Colony count 10^{-8}	Colony count 10^{-9}	Colony count 10^{-10}
24hrs	No growth	No growth	No Growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth
48hrs	Mat growth	Mat growth	Mat growth	665	545	510	476	296	243	124
72hrs	Mat growth	Mat growth	May growth	680	577	533	501	317	268	139

The colony count results at different incubation times are summarized in Table 1. At 24 hours, no visible growth was observed across all dilutions. After 48 hours, mat growth was noted in the first three dilutions (10^{-1} to 10^{-3}), while countable colonies were observed from 10^{-4} to 10^{-10}. The maximum colony counts were recorded at 10^{-4} (665 colonies) and gradually decreased with higher dilutions, reaching 124 colonies at 10^{-10}. At 72 hours, continued growth was observed, with slight increases in colony numbers compared to 48 hours. The highest counts were observed at 10^{-4} (680 colonies), with progressive reduction in subsequent dilutions (139 colonies at 10^{-10}). Overall, growth became evident after 48 hours, and colony numbers increased marginally by 72 hours.

The results indicate that microbial colonies required more than 24 hours for visible growth, with substantial development occurring between 48 and 72 hours. The progressive decline in colony count with increasing dilution is consistent with the principle of serial dilution, where fewer viable cells are expected at higher dilutions. Similar findings have been reported where bacterial growth was minimal during the lag phase but increased exponentially after 48 hours of incubation (2, 3). The stable colony counts between 48 and 72 hours suggest that the organisms had reached their exponential phase and were approaching the stationary phase. This pattern reflects typical microbial growth kinetics and validates the reliability of the dilution plate method in estimating viable counts.

Colony Character

The five bacterial isolates (APP1–APP5) showed similar colony morphology with minor variation in size. Colonies ranged from 2 mm (APP1) to 5 mm (APP4 and APP5). All isolates were

circular, white in color, with entire margins, smooth surface, flat elevation, sticky consistency, and opaque appearance. Microscopic characterization revealed that all isolates were motile and Gram-negative (table 2).

Table 2: Colony character

Sr. No.	Colony characters	APP 1	APP2	APP3	APP4	APP5
1.	Size	2mm	4mm	4mm	5mm	5mm
2.	Shape	Circular	Circular	Circular	Circular	Circular
3.	Color	White	White	White	White	White
4.	Margin	Entire	Entire	Entire	Entire	Entire
5.	Surface	Smooth	Smooth	Smooth	Smooth	Smooth
6.	Elevation	Flat	Flat	Flat	Flat	Flat
7.	Consistency	Sticky	Sticky	Sticky	Sticky	Sticky
8.	Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
9.	Motility	motile	motile	motile	motile	motile
10.	Gram nature	-ve	-ve	-ve	-ve	-ve

The colony characteristics of the isolates (APP1–APP5) revealed a high degree of similarity, with all colonies being circular, white, smooth, and opaque with entire margins. Such features are commonly reported for phosphate solubilizing bacteria (PSB), particularly members of *Pseudomonas* and *Enterobacter* species (4). The motility and Gram-negative nature observed in all isolates are also in agreement with previous findings, where PSB were predominantly Gram-negative and motile rods (2). Furthermore, similar morphological and biochemical traits were reported by Gupta *et al.* (3), suggesting that these characteristics may be typical identifiers for PSB isolates. Overall, the present results support earlier reports and indicate that the isolates share common morphological attributes associated with efficient phosphate solubilizers.

Enzyme Profile

Table 3: Enzyme profile

Test	APP1	APP2	APP3	APP4	APP5	APP6	APP7	APP8	APP9	APP10
Amylase test	-ve	-ve	+ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve
Proteases test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Gelatin test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Citrate utilization test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Catalase test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Nitrate Reduction test	-ve	+ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

The enzymatic profile of the isolates (APP1–APP10) demonstrated considerable variation. Amylase activity was detected in APP3, APP6, APP8, and APP10, while the remaining isolates tested negative. All isolates were positive for protease production, citrate utilization, and catalase activity, indicating strong metabolic and enzymatic potential. In contrast, none of the isolates showed gelatin hydrolysis. Nitrate reduction was observed only in APP2, while the other isolates were negative for this test.

The enzyme activity pattern of the isolates suggests that protease, citrate utilization, and catalase activity are common characteristics among phosphate solubilizing bacteria (PSB). Similar findings were reported by Sridevi *et al.* (5), who noted consistent protease and catalase activity in Rhizobium isolates. The limited amylase activity observed (40% of isolates) is in contrast with earlier reports where amylase production was a common trait among PSB (6). The absence of gelatin hydrolysis in all isolates aligns with Gupta *et al.* (3), who reported variability in gelatinase activity among bacterial strains. Overall, these results confirm that the isolates possess diverse enzymatic traits that may contribute to their survival and functional efficiency in soil ecosystems.

IMViC Test

The IMViC test results for all ten isolates (APP1–APP10) showed negative reactions for both the Methyl Red (MR) and Voges-Proskauer (VP) tests. None of the isolates produced stable acid end products from glucose fermentation (MR test), and none showed acetoin production (VP test).

Table 4: IMViC Test

Test	APP1	APP2	APP3	APP4	APP5	APP6	APP7	APP8	APP9	APP10
Methyl - Red	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Voges Proskauer	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

All isolates tested negative for Methyl Red and Voges-Proskauer, indicating absence of stable acid or acetoin production. Such results are common in soil and plant-associated bacteria with alternative metabolic pathways. Similar observations were reported by Nautiyal (2), Gupta *et al.* (3), and Madigan *et al.* (7), highlighting their metabolic versatility.

Sugar Fermentation

Table 5: Sugar fermentation

Test	APP1	APP2	APP3	APP4	APP5	APP6	APP7	APP8	APP9	APP10
Glucose	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Maltose	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve
Mannitol	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Sucrose	-ve	-ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve
Lactose	-ve	+ve	-ve	-ve	+ve	-ve	-ve	+ve	+ve	+ve

The enzymatic and biochemical profiling of the isolates revealed variable activity. Amylase was detected in APP3, APP6, APP8, and APP10, whereas protease, citrate utilization, and catalase were positive in all isolates. Gelatin hydrolysis was absent in all cases. Nitrate reduction was observed only

in APP2. IMViC tests showed negative reactions for both Methyl Red and Voges-Proskauer in all isolates, indicating limited acid or acetoin production.

The present results indicate that the isolates possess diverse enzymatic and biochemical capabilities, characteristic of halotolerant plant growth-promoting bacteria. Protease, catalase, and citrate utilization are consistent with metabolic versatility reported in halotolerant Azospirillum and Azotobacter species (8, 9). Limited amylase and nitrate reduction suggest strain-specific variation, similar to findings in salt-tolerant bacteria from saline soils (10). Negative MR and VP reactions align with observations in other halotolerant PGPR, reflecting adaptation to environmental stress while maintaining key growth-promoting functions. These traits support their potential use as bioinoculants under saline conditions.

Optimization of biofertilizer production using different parameter

Maximum growth of Azotobacter was observed at 37^0C temp and pH 9.

Conclusion:

In the present study, halotolerant Azotobacter species were isolated from the from different region of Nanded Maharashtra. namely Tuppa, Jawahar Nagar, Vishnupuri and MIDC CIDCO Nanded Maharashtra and were found Gram negative rods motile capsules formers and were found to have cellulase, Amylase catalase, nitrate reductase and Phosphate solubilizing enzymatic Activities. In our study one of the Halotolerant Azotobacter species found was able to tolerate 6% concentration. The current study reveals that the inoculation of the halotolerant Azotobacter species potentially increases 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 environment sustainable approach to boost crop yield under saline condition.

Acknowledgment:

Authors are thankful to Hon. Dr. Manohar Chaskar, Vice-Chancellor of Swami Ramanand Teerth Marathwada University, Nanded for providing infrastructure and necessary facilities to perform research work.

References

1. Bhavna, B., Shruti, S., & Sangeeta, S. (2019). Isolation and Characterization of halotolerant Azotobacter species from different coastal soils of South Gujarat Region. *International Journal of Pharmacy and Biological Sciences*, *9*(2), 774-779.
2. Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters, 170*(1), 265–270.
3. Gupta, R., Singal, R., Shanker, A., Kuhad, R. C., & Saxena, R. K. (2012). A modified plate assay for screening phosphate solubilizing microorganisms. *Journal of General and Applied Microbiology, 58*(2), 69–81.
4. Pikovskaya, R. I. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Microbiology, 17,* 362–370.

5. Sridevi, M., Mallaiah, K. V., & Yadav, N. C. S. (2012). Phosphate solubilization by *Rhizobium* isolates from *Crotalaria* species. *Journal of Microbiology and Biotechnology Research, 2*(3), 475–481.
6. Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. A. (2013). Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus, 2,* 587.
7. Madigan, M. T., Martinko, J. M., Bender, K., Buckley, D., & Stahl, D. (2018). *Brock biology of microorganisms* (15th ed.). Pearson.
8. Sarkar, R., Ray, P., & Toppo, J. (2023). Evaluation of plant growth promoting potential and nitrogen fixing efficiency of Halotolerant *Azospirillum* sp. isolated from coastal paddy fields. *Journal of Indian Society of Coastal Agricultural Research, 41*(2), 15–27.
9. Abdel Latef, A. A. H., Omer, A. M., Badawy, A. A., Osman, M. S., & Ragaey, M. M. (2021). Strategy of salt tolerance and interactive impact of *Azotobacter chroococcum* and/or *Alcaligenes faecalis* inoculation on canola (*Brassica napus* L.) plants grown in saline soil. *Plants, 10*(1), 110.
10. El-Beltagi, H. S., Ahmad, I., Basit, A., Abd El-Lateef, H. M., Yasir, M., Tanveer Shah, S., Ikram, M. Z. (2022). Effect of *Azospirillum* and *Azotobacter* species on the performance of cherry tomato under different salinity levels. *Gesunde Pflanzen, 74*(2), 487–499.