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Study of Siderophore Production in Salt Tolerant *Azotobacter salinestris* Species for Sustainable Approach under Saline Soil

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Abstract: *Azotobacter is a non-symbiotic nitrogen-fixing bacteriawell known for siderophore production. Siderophores are small, high-affinity iron-chelating compounds that play a vital role in augmenting iron availability in soil, thereby promoting plant growth by sequestering free iron molecules and aiding in their transportation. Its adaptability to salt stress conditions will have significance as the salinity problem is prevailing due to various reasons.This study investigates the siderophore production under salt stressed condition by Azotobacter speciesisolated from salt pan area of Mumbai region. The isolate was identified as Azotobacter salinestris species using 16S rRNA sequencin gand showed tolerance upto 8% NaCl concentration.Siderophore production was confirmed through a CAS assay and identified as Hydroxamate type by Csaky's assay.The effect of different salt concentration on siderophore production was studied and the production was found to increase with increase in salt concentration showing maximum production at 4% NaCl (8.6 x 10^{-1}mM). The growth stimulant property of the siderophore was assessed by bioassaymethod against various bacterial strains was evaluated.The combined biostimulant and iron-chelating properties of the siderophore under salt stress suggest that this approach could enhance biofertilizer efficiency, providing a natural alternative to chemical agents in agriculture.*

Keywords: Azotobacter salinestris; siderophore; salinity; biostimulant; biofertilizer

I. INTRODUCTION

Iron is an essential micronutrient for plants, playing an important role in numerous biological activities, including electron transport, oxygen metabolism, nitrogen fixation, etc. Although it is the fourth most abundant metal in the earth's crust, the bioavailability of iron is very low (Schalk I.J.; 2008). The content of soluble (available) iron is extremely low in comparison with the total iron content leading to deficiency of iron. Insoluble Fe^{3+} may be used more easily in the environment when siderophores are present, enhancing the bioavailability of Fe^{3+}.Siderophores are among the phytohormones produced by microbes. Siderophores (Greek for "iron carrier") are low-molecular-weight, high-affinity iron-chelating compounds that are produced by organisms to solubilize Fe^{3+} for uptake. The production of siderophores and their detection have been documented in many research studies.

Various environmental stresses like high temperatures, soil salinity, drought and flood have affected the production and cultivation of agricultural crops, among these soil salinity is one of the most devastating environmental stresses, which causes major reductions in cultivated land area, crop productivity and quality (Yamaguchi and Blumwald, 2005; Shahbaz and Ashraf, 2013). Microorganisms could play a significant role in this respect, if we exploit their unique properties such as tolerance to saline conditions and siderophore production under salt stressed conditions.

The benefit of PGPR are yet to be maximized and its role in nutrient uptake and stress management are emerging areas in agriculture that is not yet well understood. Among PGPR, *Azotobacter* species are of particular interest due to their high respiratory and metabolic rates. They are known for fixing atmospheric nitrogen, producing growth hormones like indole acetic acid (IAA), and synthesizing important metabolites such as siderophores which scavenge iron from the soil and make it available to plants. The production of hydrogen cyanide (HCN) plays a role in the biological control of

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pathogens (Ahmad *et al.* 2008). Furthermore, *Azotobacter* can solubilize phosphorus, which is crucial for plant nutrition.

The aim of this work is to detect production of siderophore by salt tolerant Azotobacter species and utilize its potentials under salt stress condition for sustainable agriculture.

II. METHODOLOGY

Collection of soil samples

Saline soil samples were collected from various salt-pan areas of Mumbai regions in sterile zipped plastic bags from the rhizosphere region from a depth of 10 -15 cm.

Enrichment and Isolation on Selective Medium

Enrichment of *Azotobacter*, from the saline soil samples collected, was done in modified Ashby's mannitol nitrogen-free broth. It was incubated at 28±2°C for a week. Gram staining was carried of the smear prepared from the enriched broth. Isolation of the culture was done on modified Ashby's mannitol nitrogen-free media. Discrete and well-isolated colonies were observed after 3 days of incubation. Isolated colonies were further transferred on modified Ashby's mannitol Nitrogen-free medium and incubated at 28±2°C for 3–5 days. Pure culture was obtained by repeated subculturing and the isolate was preserved on the same media slant. Morphological characterization and biochemical testing were carried out as described in the Bergey's Manual of Bacteriology and identification was carried out using 16S rRNA sequencing at National Centre for Cell Science, Pune. Identification report was generated using EzBioCloud Database and the confidence in identification is limited by both the availability and the extent of homology shown by the ~1200 bp sequence of the amplified region from the DNA of the isolate with its closest neighbor in the database.

Study of salt-stress tolerance

Ashby's mannitol nitrogen-free broth containing different salt concentrations of NaCl (0, 2, 4, 6, 8 and 10 g% w/v) was inoculated with *Azotobacter* isolate and all the flasks were incubated on a rotary shaker for 5 days at 28 ± 2°C. The growth of the isolate was recorded by measuring the absorbance of the culture broth using a spectrophotometer at 530 nm wavelength.

Screening of Siderophore production

Qualitative detection of siderophore (Plate assay):

Siderophore production was tested qualitatively using Chrome azurol Sulphonate (CAS) agar as described by Schwyn and Neilands. (Kumar V et al; 2017). CAS agar is prepared from four solution which was sterilized separately before mixing. CAS plates is streaked with the isolate and observed for development of orange halo against dark blue background around the colonies after 48-72 h of incubation at 28ºC. A change in color from blue to orange (hydroxamate type siderophore) or purple (catechol type siderophore) will be consider as a positive reaction.

Characterization of Siderophore:

2.4.2.1$FeCl_3$ Test

To 0.5 ml of culture filtrate, 0.5 ml of 2% aqueous $FeCl_3$ solution was added and examined for the appearance of orange or reddish-brown colourwhich was positive indication of siderophore production.

Spectrophotometric assay

Cultured bacterial cells were harvested by centrifuging at 3,000 rpm for 20 minutes. The supernatant was subjected to spectrophotometric analysis to confirm siderophore production. The hydroxamate nature of siderophore was detected by spectrophotometric assay (Jalal et al;1991) where a peak between 420-450 nm on addition of 3ml of freshly prepared 2% aqueous solution of $FeCl_3$ to 1 ml of supernatant indicated presence of Ferrate hydroxamate. Catecholate nature of siderophore was detected by the method of Jalal and Vander Helm using spectrophotometric assay where a peak at 490 nm on addition of 2% aqueous solution of $FeCl_3$ to 1ml of supernatant indicated the presence of siderophores of catecholate nature.

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Tetrazolium Test

For the tetrazolium test two drops of 2 N NaOH was added to a pinch of iodonitrotetrazolium (INT) and 1 ml of culture filtrate was added and observed for the appearance of a deep red colour to determine the presence of hydroxamate siderophores (Sujatha N and Ammani K. 2013).

Arnow's method for the estimation of Catechol-type Siderophores

The Arnow's method (Arnow 1937) is based on the fact that catechol, when combined with nitrous acid (HNO_2), gives off a yellow color. Arnow's assay is performed by combining the following in order, mixing between each step:

1) 1 ml culture supernatant/ uninoculated medium
2) 1 ml 0.5 M HCl
3) Nitrite-molybdate reagent (10 g sodium nitrite + 10 g sodium molybdate dissolved in 100 ml D/W)
4) 1 M NaOH (4.0 g NaOH dissolved in D/W to make a final volume of 100 ml)

After all components have been added, incubate at room temperature for approximately 5 minutes to allow the colour to fully develop. Again, supernatant of cultures grown under high iron conditions as well as uninoculated media with reagents is used as controls. Once developed, the absorbance of the solution is measured at 500 nm, using the uninoculated modified Fiss minimal medium with no added iron and components 2-4 added as a blank. The control assay is colourless, and a positive reaction is indicated by a pink to deep red colour being produced, depending on intensity (based on amount of catechol present) (Arnow 1936).

Csaky's assay for the detection of hydroxamate siderophores

The Csaky's assay is quantitative for hydroxamic acids, and is thus used to detect and quantify hydroxamate siderophores such as the desferrioxamines. It was developed by Csaky (1948). In the procedure, equal volumes (e.g. 1 ml) of culture supernatant and acid (H2SO4; 6 M) are mixed and autoclaved at 121°C for 30 min. Upon cooling, 1.0 ml of an acid-iodine solution (sulfanilic acid [1% w:v] prepared in acetic acid [30% vol:vol in water] + 0.5 ml iodine [1.3% wt:vol prepared in acetic acid 30% [vol/vol]) is added. The mixture is then incubated for 5 min at room temperature, after which excess iodine is removed by adding a 1 ml solution of tri-sodium arsenite [Na_3AsO_2 (2% w:v); prepared in water]. Subsequently, a 1 ml solution of α- naphthylamine [0.3% w:v] prepared in acetic acid [30% v:v] is added. The color of the solution at this point would change from orange to red. The final volume can be raised to 10 ml by adding distilled water, to bring the solution to a measurable range, if the red color is too intense. Absorbance is measured at 526 nm after 30 min at room temperature. Purified hydroxylamine hydrochloride (red color in solution) wasused as a standard.

Effect of salt stress on siderophore production

The isolate was grown in iron deficient modified Ashby's mannitol broth at different salt concentration i.e. 2, 4, 6, 8 %. Siderophore production at these different salt concentration was estimated using the Csaky's assay described above.

Bioassay of Siderophore

A bioassay using culture supernatant was carried out to check the ability of siderophore produced by the isolate to promote growth of other bacterial culture under iron restricted medium.

III. LITERATURE REVIEW

Salinity is one of the harshestabioticfactors limiting the growth and productivity of crop plants and the area of land affected by it is increasingdue to several reasons both natural and human activities. Poor irrigation practices can lead to secondary salinization, which affects about 20% of irrigated land globally (Glick et al., 2007). Soil salts exist as ions released from minerals during weathering, added through irrigation or fertilizers, or migrated upward from shallow groundwater. When rainfall is insufficient to flush these ions from the soil, they accumulate, resulting in soil salinity (Blaylock et al., 1994).According to a report of Global Map of Salt-affected Soils (GSASmap) under the soil-portal of FAO, a record from 118 countries covering 85% of global land area, it shows that more than 424 million hectares of topsoil (0-30 cm) and 833 million hectares of subsoil (30-100 cm) are salt-affected: 85% of salt-affected top soils are saline, 10% are sodic and 5% are saline-sodic and 62% of salt-affected subsoils are saline, 24% are sodic and 14% are

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saline-sodic. A wide range of adaptations and mitigation strategies are required to cope with such impacts. Cost effective and sustainable strategies need to be developed to manage soil salinity. Microorganisms could play a significant role in this respect, if we deedwith their ability of tolerance to saline conditions, synthesis of compatible solutes, production of plant growth promoting hormones, bio-control potential, bio-stimulant property and their interaction with crop plants.

Azotobacter spp. are most specifically known for their nitrogen fixing ability but they also have the ability to produce different growth hormones (IAA, Gibberellins, etc.), solubilizes phosphate, siderophores and hydrogen cyanide. (Narula et al. 1981, Neito & Frankenberger 1989, Tindale 2000). Azotobacter also produces Poly β- hydroxybutyrate granules which is a reserve food material used under adverse condition as a source of carbon and energy. PHB Granules enhances the survival of bacteria under hyperosmotic stress. (Obruca et al; 2017). The organism is known to produce cyst which also helps in surviving under stress condition. (Sivapriya S.L; 2017).

Screening of various salt-tolerant strains of Azotobacter has revealed that some strains are able to colonize the rhizosphere successfully and promote plant growth in saline soils. (Van Oosten et al; 2018.) Inoculation of maize plants with Azotobacter has been reported to improve growth in control and saline stress conditions (Rojas-Tapias et al; 2012). Two salt tolerant strains were also reported to alleviate saline stress by improving sodium exclusion and potassium uptake. (Rojas-Tapias et al; 2012)

Azotobacter salinestris belongs to family *Pseudomonadaceae* and genus *Azotobacter*. *Azotobacter salinestris* is gram negative coccobacilli (oval with pointed end), older cells are round, pleomorphic and are often found in pairs and chains of six to eight cells. The organism is known to form cyst. Cells are motile by means of peritrichous flagella. Cells from old cultures are non-motile. Physiological characteristics include growth at room temperature (30^{o}C to 36^{o}C) and a neutral to slightly alkaline pH requirement.

Most strains, except type strain, produce a capsule and synthesize poly β-hydroxybutyrate. Strains are urease positive, amylase positive, and catalase positive. Carbon sources include fructose, galactose, glucose, sucrose, mannitol, melibiose, 0.25% sodium benzoate, and starch. A brown black to tan-brown pigment, allomelanin (a catechol melanin), is produced by most strains when grown at high aeration under N-fixing conditions.

The organism has been isolated from saline soil of different countries like Western Cananda (William J. Page and Shailaja Shivprasad; 1991), Bangladesh (Akhter et al; 2012), Argentinea (Rubio et al; 2013), Egypt (Omer AM et al; 2016), Allahabad, India (Hafeez M. et al; 2018), West Java, Indonesia (Reginawanti Hindersah et al; 2019) and Eastern Kenya (Priscillah Wanjira et al ; 2022).William J. Page, 1991 had showed the dependency of *Azotobacter salinestris* on Na+ ion for growth. He incubated the cells in Burk's medium for 16 hours with different Na+ ions concentration. In this experiment he observed an inverse relation between the lag phase and the Na+ ion concentration i.e. that there was an increased lag phase with decreased Na+ ion.The organism had shown tolerance to NaCl upto 8% (Chennappa G. et al; 2016)

Its tolerance to high salt concentration makes this organism as a sustainable approach under saline condition (Omer A.M. et al; 2016). Pot experiment carried by Omer A. M. et al too evaluate the effect of *Azotobacter salinestris* on the morphological and biochemical characteristics of sorghum gave significant result on the parameter studied. Siderophore production have also been demonstrated in soil-plant systems (Buyer et al. 1993; Dimkpa C. 2016). The application of siderophore-producing microbes or siderophore-containing microbial preparations for improving plant growth and enhancing metal remediation of polluted environments.

IV. RESULT AND DISCUSSION

4.1 Sampling, isolation and Identification

The isolate was isolated from saline soil of Mumbai region (Wadala salt pan area) by serial dilution spread method. The isolate gave big, mucoid, light brown colonies on Ashby's Mannitol N-free media plates. (Fig 1). The isolate was Gram negative. It was positive for nitrate reduction, starch hydrolysis, urease test and oxidase test and negative for catalase, citrate and H_2S production similar to the features matching with *Azotobacter* species as per the Bergey's manual of determinative bacteriology.

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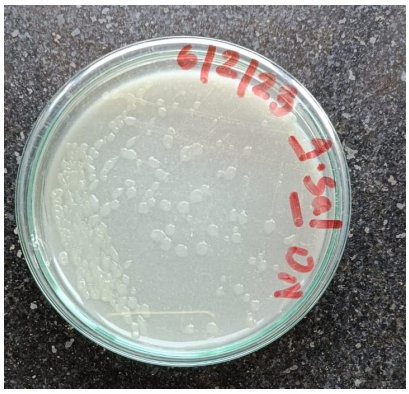

Fig 1 – Colonies of *Azotobacter salinestris* strain isolated from saline soil of Mumbai region.

The identification of the isolate was done as *Azotobacter salinestris* strain based on 16S rRNA sequencing. The sequence is shown in Fig 2. Based on the extent of homology shown by the ~1200 bp sequence of the amplified region (99.41% similarity) from the DNA of the isolate with its closest neighbor in the database, the isolate was identified as *Azotobacter salinestris.*

Sequence Text (in FASTA format font: courier new 10):

>B_OCT_22_029

CCAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGCAGCGGGACCT
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ACTCGCGCTAATACCGCATACGTCCTACGGGAGAAAGTGGGGGACCTTCGGGCCTCACGCTAACAGATGAGCCTA
GGTCGGATTAGCTGGTTGGTGGGGTAACGGCCCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGT
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCC
TGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGCCGGGAGGAAGGGCTGTAG
GCGAATACCCTGCAGTCTTGACGTTACCGGCAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATAC
GAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGGTAAGTTGGATGTGAAAGC
CCCGGGCTCAACCTGGGAACTGCATCCAAAACTGCCTGGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGT
AGCGGTGAAAGCGTAGATATAAGGAAGGACCACCAGTGGGGAAGGGGACCCCCTGGACTGATCTGACACTGAGGT
GCGAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGC
TCCTTGAGAGCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA
AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGG
CCTTGACATCCTGCGAACTGAGTAGAGATACCCGGGTGCCTTCGGGAACGCAGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCGATTCG
GTCGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTA
CGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGCGGAGCTAATCCCAGAA
AACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAA
TGTCGCGGTGAATACGTTCCCGGGCCTTGT

Fig 2 – 16s rRNA sequence of *Azotobacter salinestris* strain identification using the EzBioCloud database

4.2 Salt stress tolerance study

The isolate identified as *Azotobacter salinestris* was subjected to salt tolerance studies. Growth and survival of the isolate was observed up to 8% NaCl but the growth of culture was significantly reduced at this concentration. At 10% NaCl concentration there was no growth of the isolate (Table 1)

Table 1: Growth analysis of *Azotobacter salinestris* isolateat different (0% to 8%) NaCl concentration

Salt Concentration (%)	0	2	4	6	8	10
Azotobacter isolate	++++	++	++	+	+	-
Key: + → Growth, - → No growth						

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4.3 Screening of Siderophore

4.3.1 Qualitative test for Siderophore

The detection of siderophores was confirmed by spot inoculating *Azotobacter salinestris* isolate on the CAS agar plate. Yellow colour colonies with clearance around it was observed (Fig. 3) indicating the production of siderophore.

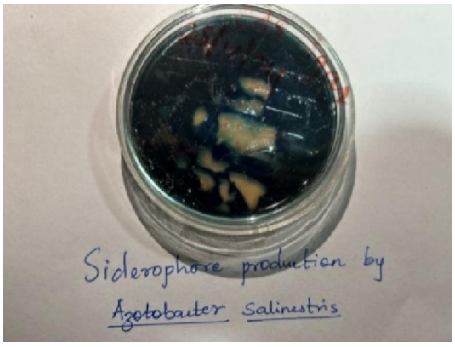

Fig 3. Yellow coloured colonies of *Azotobacter salinestris* isolate with clearance around it on CAS agar plate.

4.3.2 Characterization of type of Siderophore

Azotobacter salinestris isolateproducing siderophore was characterized for type of siderophore produced (Table 2). The isolateshowed maximum peak between 420 and 450 nm in the $FeCl_3$ test (Fig 4) and deep red color in the tetrazolium test which indicates hydroxamate type of siderophore.Further confirmation was done by Csaky's assay. (Fig 5)

Table 2: Characterization of Siderophore produced by *Azotobacter salinestris* isolate

Isolate	FeCl3 Test		Catechol (Arnow's Test)	Hydroxamate (tetrazolium test)	Hydroxamate (Cskay's Assay)
	Peak at 495 (Catechol)	Peak at 425 (Hydroxamate)			
Azotobacter salinestris isolate	-	+	-	+	+
Key: + -- Positive result , - Negative result					

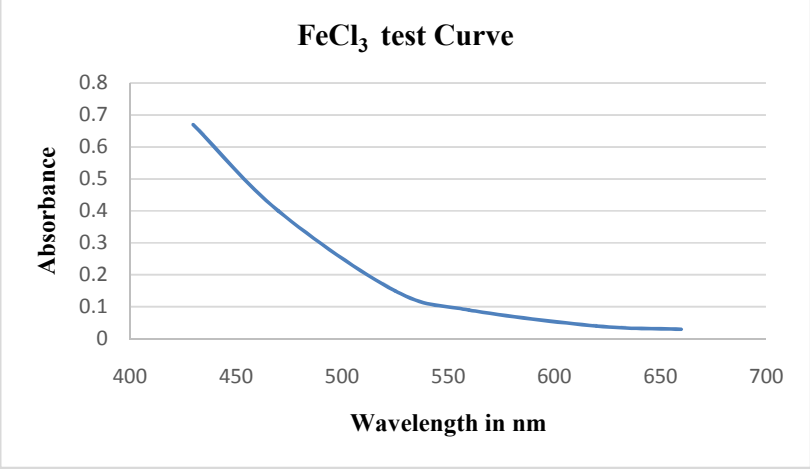

Fig 4. Characterization of Siderophore by $FeCl_3$ test showing absorbance maxima at 425 nm

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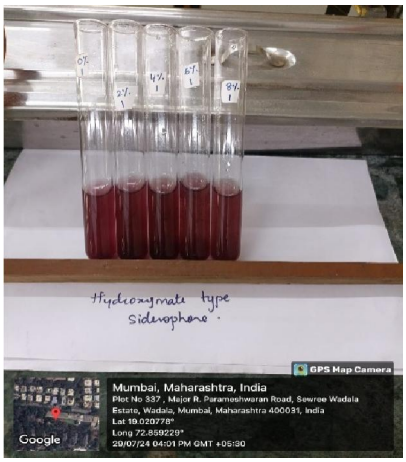

Fig 5. Deep red colour indicating hydroxamate type siderophore by Cskay's assay.

4.3.3 Quantitative method

The isolate was inoculated in 50 ml Modified Ashby's Mannitol Broth for 24 hours, broth was centrifuged,supernatant was collected and used to detect siderophore by Csaky's method.

Csaky's methodwas utilized to quantify the hydroxamate-type siderophore. The siderophore production by the *Azotobacter salinestris* isolate is found to increase with the growth (Fig. 6).

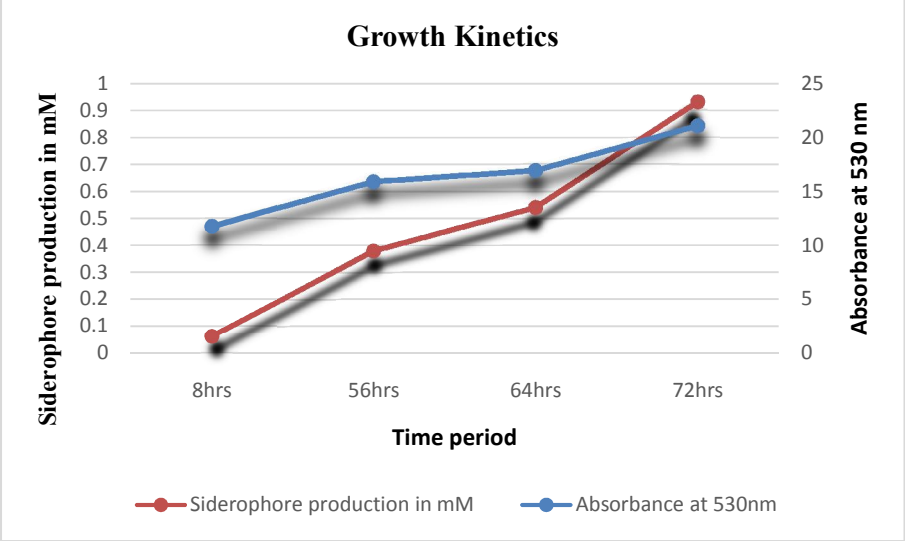

Fig 6: Growth Kinetics of Siderophore production by *Azotobacter salinestris* isolate

The results of Siderophore production were found positive for the tested salt concentration at different incubation period. The highest concentration of siderophore (9.3 x 10^{-1}mM) was produced by *Azotobactersalinestris* in control after 72 hrs whereas with 4% NaCl concentration the maximum concentration (8.6 x 10^{-1}mM)was observed after 72 hrs of incubation period at 28 ± 2 °C. (Fig 7). Further increase in salt stress resulted in decrease in siderophore production.

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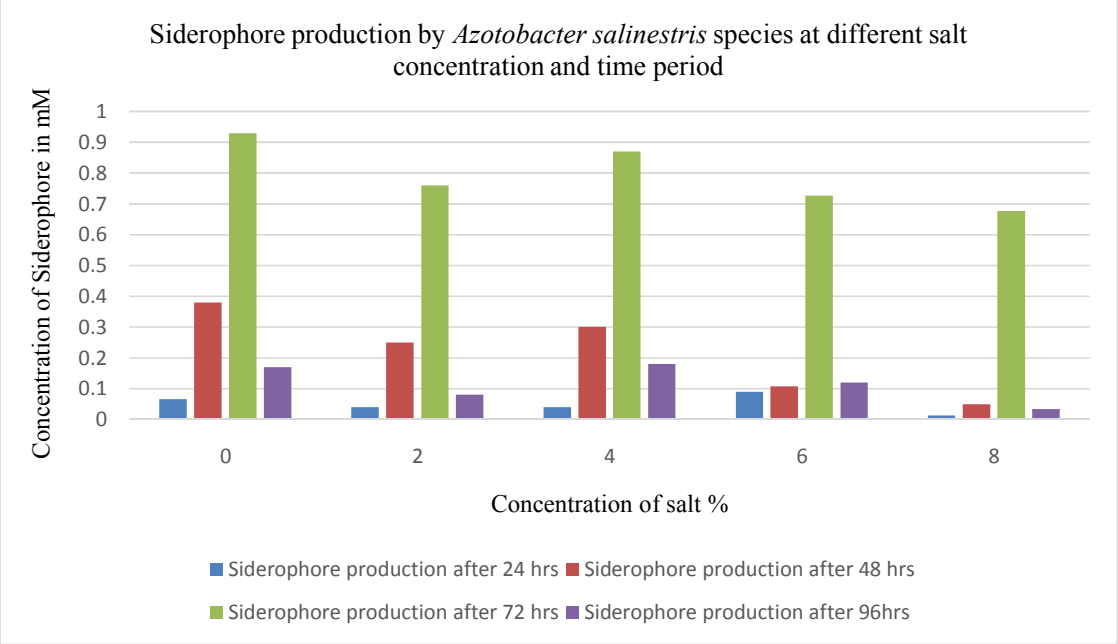

Fig 7. Effect of salt on Siderophore production

4.3.4 Bioassay of siderophore

A zone of exhibition was observed around the bacterial culture tested for growth promotion provided by a siderophore in iron-restricted conditions. (Table 3 and Fig 8). The siderophore from *Azotobacter salinestris* was shown to stimulate growth of Klebsiella and Pseudomonas but not *E.coli, Proteus, Salmonella or Staphylococcus* under iron limited conditions.

Table 3: Zone of exhibition in test organisms

Bacterial Culture tested	**Diameter of Zone of Exhibition in mm**
Escherichia coli	zero
Klebsiella pneumoniae	22
Pseudomonas aeruginosa	25
Proteus mirabilis	zero
Salmonella typhi	zero
Salmonella paratyphi B	zero
Staphylococcus aureus	zero
Bacillus subtilis	zero

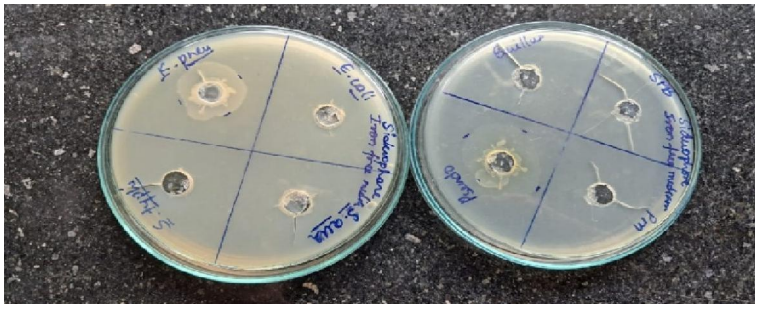

Fig 8. Zone of exhibition around the bacterial culture tested on iron restricted agar plate

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V. CONCLUSION

*Azotobacter salinestris*isolate exhibits a notable ability to produce hydroxamate-type siderophores, under increasing salt concentrations. The findings indicate that the production of siderophores increases with higher salinity, peaking at a 4% salt concentration and further decreases. This suggests that *Azotobacter salinestris* can adapt to saline environments and efficiently compete for iron, even under iron-limited conditions, which is critical for plant growth in saline soils. Such traits can be exploit for sustainable agricultural practices, especially in areas affected by salinity, by enhancing soil fertility and plant health through the inoculation of this bacterium as biofertilizer. Therefore, the use of *Azotobacter salinestris* could be a promising biotechnological strategy to improve crop productivity in saline-affected soils.

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