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Evaluation of Plant Growth Promoting Potential and Nitrogen Fixing Efficiency of Halotolerant *Azospirillum* sp. Isolated from Coastal Paddy Fields

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Salinity is a major abiotic constraint affecting rice quality and productivity. Traditional methods and synthetic nitrogen fertilizers pose risks to the bio-economy and environment. Thus, the usage of halotolerant diazotrophic bioinoculants can be a sustainable approach for improving nitrogen nutrition and ameliorating saline stress in rice plants. On account of that, three *Azospirillum* sp. (AZS1, AZS2 & AZS3) were isolated from the rhizospheric soil of the coastal rice field in Odisha, India. Among them, AZS1 showed significantly high salt (8%) tolerance, various plant growth-promoting attributes (IAA, GA, HCN, ammonia production and inorganic P-solubilization), and extracellular enzymatic (amylase and cellulase) activities. Additionally, the isolate also showed elevated levels of superoxide dismutase (SOD) and proline production under increasing NaCl stress. This potential diazotroph (AZS1) was confirmed as *Azospirillum* sp. (OP503586) by 16S rRNA gene sequence. Further, the detection of the nifH gene and determination of the increasing percentage of nitrogen in culture media indicated the nitrogen-fixing ability of this isolate. In general, this study suggests the potential to utilize this indigenous halotolerant *Azospirillum* sp. as a potential bioinoculant to mitigate the salt stress in coastal agro-ecosystem. Nonetheless, a field study is highly essential in this regard prior to its practical application.

(***Key words:*** *Bioinoculants, Diazotrophs, Halotolerant, Plant growth-promoting attributes, Salinity)*

Rice (*Oryza sativa* L.), a global dietary staple, plays a significant role in food security by providing a relatively inexpensive and accessible source of sustenance. In an agriculture-dominated country like India, considering the expansion of rapid population, decline of cultivated land, global warming and other cultural changes, vertical growth through productivity enhancement and crop protection is the need of the hour to ensure food security (Banik *et al.,* 2016). On the other hand, salinization of arable land, especially in the coastal areas is one of the most detrimental abiotic factors coupled with physiological and metabolic abnormalities limiting both the vegetative and reproductive stages of growing rice plants (Daliakopoulos *et al.,* 2016). Additionally, the coastal regions are at risk of increasing salinization of land due to frequent natural calamities like storms, cyclones, tidal surges etc. All of these factors contribute to increased soil salinity, waterlogging, submergence, and siltation, resulting in maximum paddy crop loss. Plant breeders and biotechnologists are consistently facing difficulty in developing improved salt-tolerant rice varieties through natural selection or genetic manipulation due to several factors that include genetic complexity, environmental interactions, trade-offs with other traits, etc. (Qadir *et al.,* 2014). Similarly, incorporating excessive inorganic compounds like gypsum, limestone and inorganic fertilizers with high salt index can neither be sustainable for the environment nor economically feasible for small-scale farmers. In this context, using halotolerant diazotrophic microbial inoculants can be a more promising and sustainable approach to mitigate the negative impacts of salinity stress on plant growth and productivity. Among diazotrophs, *Azospirillum* sp., a gram-negative, non-fermentative, microaerophilic bacteria that belongs to the family *Azospirillaceae* is one of the most efficient nitrogen-fixing microflora inhabiting the rhizospheric region of rice. Apart from nitrogen fixation, Bashan

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and de-Bashan (2010) postulated the "theory of multiple mechanisms" in which *Azospirillum* operates a collective or synchronous pattern of impacts in host plants like synthesize and supplement a wide array of physiologically active phytohormones including auxins, cytokinin, gibberellins, ethylene, salicylic acid, siderophore and extracellular enzyme production, hydrolyse organic and inorganic phosphorus, control phytopathogens and alleviate abiotic stressors such as salinity and drought by inducing induced systemic tolerance (IST). Thus, it is imperative to explore, isolate, and characterize more efficient indigenous strains of *Azospirillum* considering their compatibility with the crop and local niche to ensure successful inoculation with long-term consistency and highly competitive to thrive in extreme conditions. In light of the above, the present study was designed to explore native halotolerant *Azospirillum* sp. isolated from the rhizospheric soil of the coastal rice field in Odisha and evaluate their plant growth promoting attributes, stress adaptability, antioxidant and extracellular enzymatic activities and nitrogen fixing potential. Future research on the formulation of effective biofertilizers for such challenging soils will benefit from the findings of this study.

MATERIALS AND METHODS

Sampling sites and physicochemical analysis of soil sample

Four rhizospheric soil samples were collected aseptically from the two coastal rice fields of Puri and Paradeep in Odisha, India [GPS geoplaner: Location: Satapada, (Village - Siandi), 19°34′49.4″ N, 85°17′40.3″ E, Elevation: 21m amsl, Fig. 1; Location: Paradeep, (Village - Pradeepgarh), 20°18′11.9″ N, 86°35′56.6″ E; Elevation: 12m amsl, Fig. 2.] and taken to the Department of Microbiology, Odisha University of Agriculture and Technology, Bhubaneswar, Odisha, India. Both the sites are located very close to the Bay of Bengal (around 3km) which has a significant impact on the weather and the soil properties. Soil samples were subjected to analyses for different physico-chemical parameters such as pH, electrical conductivity and soil texture which were measured following standard procedures (Dane and Topp, 2020).

Isolation of *Azosprillum* sp.

Isolation of *Azospirillum* spp. from rhizospheric soil was carried out according to Usha and Kanimozhi (2011). Ten grams of rhizospheric soil was subjected to the serial dilution method and semi-solid nitrogen-free malate medium containing L-malic acid 5 g, K_2HPO_4 0.5 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.02 g, trace element solution 2 mL, bromothymol blue (0.5% dissolved in KOH) 2 mL, Fe EDTA (1.64% solution), and yeast extract 0.02 g L^{-1} was used for enrichment. The tubes were incubated at 28°C for 7 d. Isolates that showed subsurface pellicle growth after 7 d were selected. Subsequently, the optimum incubation period was determined from the growth curve pattern of the isolates.

Morphological and biochemical characterization of the bacterial isolates

Pure colonies of the bacterial isolates were characterized according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). All the selected bacterial isolates were subjected to Gram's staining, colony morphology, biochemical (indole, methyl red, Voges-Proskauer, citrate utilization, nitrate reduction, H_2S, oxidase, catalase, TSI, motility) and carbohydrate (lactose, xylose, fructose, dextrose, sucrose, glycerol, galactose, trehalose, salicin, raffinose, rhamnose) utilization tests and examined after 48 h incubation using standard procedures (Bhavna *et al.,* 2019; Krishnan *et al.,* 2016).

Abiotic stress tolerance of the isolates

NaCl and KCl tolerance of the isolates

Aliquots of 100 µL of 5 d old grown pure bacterial isolates were inoculated in 20 mL sterilized Okon's broth (Malic acid 5 g, KOH 4 g, K_2HPO_4 0.50 g, $FeSO_4.7H_2O$ 0.05 g, $MnSO_4.7H_2O$ 0.01g, $MgSO_4.7H_2O$ 0.10 g, NaCl 0.02 g, $CaCl_2$ 0.01 g, Na_2MoO_4 0.002 g, Bromothymol blue; 0.5% in 95% methanol, 2.00 mL, Agar 1.8 g 100 mL^{-1}, NH_4Cl 1 g and distilled water 1L) amended with varied (0, 2, 4, 6, 8 and 10%) concentrations of NaCl and incubated at 28°C for 5 d in a shaker (120 rpm). The same procedures were followed to assess KCl tolerance (Akhter *et al.,* 2011). The optical density of the sample was noted at 600 nm using UV-VIS Spectrophotometer (UV 2550 Shimadzu, Koyoto, Japan) after 5 d. Controls (Uninoculated different salt concentration amended

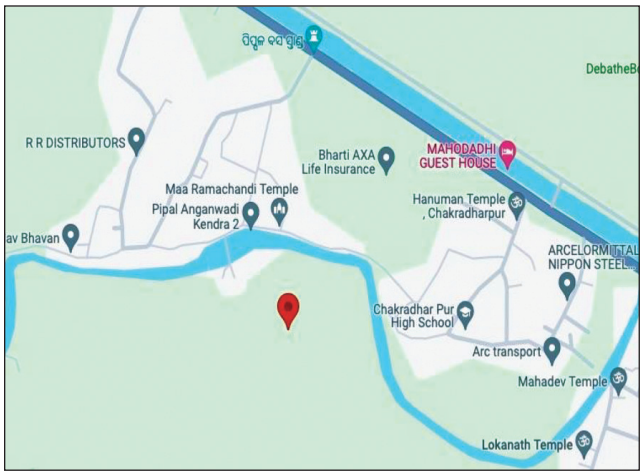

Fig. 1. *Sample site location of Satapada*

Fig. 2. *Sample site location of Paradeep*

Okon's broth) was also maintained for assay, and all the experiments were done in triplicates to minimize the error. Isolates survived up to 8% NaCl (OD> 0.5) stress was only considered for further experiments.

pH tolerance of the isolate

Aliquots of 100 µL of culture of the isolate was inoculated into 20 mL of Okon's broth adjusted with different pH (5, 6, 7, 8, 9, and 10) and incubated at 28°C for 5 d. After the incubation period the optical density of the sample was measured at 600 nm using UV-VIS Spectrophotometer (UV 2550 Shimadzu, Koyoto, Japan). Control (uninoculated different pH amended Okon's broth) was set, and all the experiments were done in triplicates to minimize the error.

Extracellular enzymatic activities of the isolate AZS1

In-vitro extracellular enzymatic activity, such as amylase, gelatinase, protease and cellulose of the isolate (AZS1), were evaluated by following standard protocols described earlier (Tirry *et al.,* 2021). The enzyme amylase was detected by spot inoculating the bacterial culture on soluble starch yeast extract medium plates containing 1% soluble starch and incubated at 28°C for 5 d. The plates were flooded with iodine solution for five minutes and the appearance of a clear zone formed around the colonies was checked. Similarly, gelatin liquefaction was tested by spot inoculating them on nitrogen free Jensen's agar containing 1% gelatin and incubating them at 28°C for 5 d. The plates were flooded with 15% acidic mercuric chloride solution. The development of a clear zone surrounding the colonies was considered positive. Further, bacterial isolate was spot inoculated in skim milk agar medium and incubated at 28°C for 5 d to evaluate the protease activity. The appearance of a clear zone around the colony indicates proteolytic activity. The bacterial isolate was screened for cellulose degradation by spot-inoculating the bacterial culture on Carboxy methyl cellulose (CMC) agar. The plate was flooded with 0.1% Congo red for 10 min, followed by the addition of 1N HCl for 5 min andthen 1N NaOH for another 5 min. Observation of a clear zone around the spot against red background is considered positive test for cellulase production.

Assessment for plant growth promoting (PGP) traits of the isolate AZS1

In - vitro PGP properties, namely, phytohormone (IAA and GA), ammonia, hydrogen cyanide (HCN), siderophore production, and P-solubilization potential of the isolate were evaluated by following standard techniques as described earlier (Narayanan *et al.,* 2022). In brief, Salkowski's (2% of 0.5M $FeCl_3$ in 35% $HClO_4$) method was used to detect (Indole acetic acid) IAA production and absorbance was measured at 530 nm. Gibberellic acid (GA) production was estimated by using DNPH (2,4-dinitrophenyl hydrazine) and ethyl acetate. The colour intensity of the solution was determined by taking absorbance at 430 nm. IAA and GA production was estimated using the standard curve prepared with a known concentration of IAA and GA (10-200 µg mL^{-1}), respectively. Ammonia production by the isolates

was evaluated by adding Nessler's reagent (5 mL) to 2 d old culture. HCN production was detected by the change of colour of dry sterilized filter paper (Whatman no.1) soaked in alkaline picrate solution (2% sodium carbonate prepared in 0.5% picric acid) inserted in the culture flask without touching the wall and medium. Siderophore production and phosphate solubilization were assessed using the spot inoculation technique on chrome azurol S agar (CAS) plates and Pikovskaya's agar plates, respectively. Positive results were indicated by a colour change in the former and the presence of a halo zone in the latter.

Proline production by the isolate (AZS1) under NaCl stress

The proline content of bacterial isolate under NaCl stress was evaluated according to Khoma *et al.* (2021). Briefly, the bacterial isolate was cultured in M9 medium with varied (2, 4, 6, 8 and 10%) concentrations of NaCl and incubated at 28°C for 48 h. Then, 3% aqueous sulfosalicylic acid was added to the supernatant and centrifuged at 13,000 rpm for 10 min. Further, supernatant and distilled water (1:1) were combined with 1 mL of glacial acetic acid and 1 mL of ninhydrin and incubated at 90°C for 1 h. The suspension was cooled in an ice bath before adding 2 mL of toluene and vortexed for 2 min (red colour development). The upper phase was collected, and the absorbance was measured at 520 nm.

Effect of NaCl on superoxide dismutase production

In-vitro antioxidant enzyme, superoxide dismutase (SOD; EC 1.15.1.1) of bacterial isolate was evaluated as described earlier (Chakraborty *et al.,* 2015). In brief, 0.1 mL cell-free extracts of the isolate grown in varied (2, 4, 6, 8 and 10%) concentration of NaCl was added to the reaction mixture comprised of 13.5 mM methionine, 75 µM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), and 50 mM sodium carbonate. Then, 0.1 mL riboflavin (2 mM) was added to initiate the reaction under illuminating (two 15 W fluorescent lamps) chamber for 15 min, and the absorbance was measured at 560 nm. The SOD activity was expressed as unitmg protein^{-1} min^{-1}.

Quantitative estimation of carbon and nitrogen

A loopful of the selected isolate was inoculated into 5 mL sterilized broth. After 0, 7 and 14 d of incubation, 2 mL broth was taken and immediately filtered through a membrane filter (0.3 µm) to obtain cell-free extract in sterilized vials and refrigerated at 4°C and used for estimation of CHNS% in the CHNS analyser.

Assay for nitrogen fixation under NaCl stress

Micro-Kjeldahl analysis was used to assess the competence of nitrogen fixing ability of the isolate as described by Richard *et al.* (2018) by inoculating the isolates in Okon's medium containing 0, 2, 4 and 6% NaCl and incubating at 28°C for 5 d. Triplicate samples were maintained for each isolate. Nitrogen fixing efficiency of the AZS1 was expressed as mg Ng^{-1} substrate.

Amplification of NifH gene

PCR amplification of the nifH gene was carried out using universal diazotrophic primer pairs Pol-F and Pol-R according to Lin *et al.* (2016). The thermal profile is as follows: initial denaturation for 45 s at 95°C; denaturation for 45 s at 60°C; annealing for 30 s at 60°C; primary extension for 30 s at 72°C; back to step 1 for another 39 cycles. Final extension for 10 min at 72°C was followed.

Evolutionary analysis of the isolate

The genomic DNA of AZS1 was isolated by DNA extraction kit (Qiagen, USA) and then sent to Eurofins Genomics India Pvt. Ltd. Bangalore, India for 16S rDNA sequencing. Amplification and sequencing of the 16S rRNA genes were carried out using Sanger sequencing method taking two universal primers, specifically 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTTACGACTT-3'). The consensus sequence of 16S rDNA was analysed by Bio-Edit software v7.2 and then submitted to NCBI (http://blast.ncbi.nlm.nih.gov/blast.cgi) to obtain accession number. Alignment of the nucleotide sequence of 16S rRNA of the isolate was done using MEGA software 10.0.2 (http://www.megasoftware.net) and a phylogenetic tree was constructed following the Neighbor-Joining (p-distance model) method. The evolutionary tree topologies (nodes bar 0.02 and 0.05 substitutions per site for 16S rDNA) values were

evaluated through bootstrap (500 replicates) analysis (Tamura *et al.,* 2013).

RESULTS AND DISCUSSION

Physicochemical characteristics of soil and isolation of the *Azospirillum* sp.

Two coastal rice fields in Odisha, India were selected because the sampling sites were very close to the sea, fluctuating weather conditions and repeated intrusion of estuarine water. Physico-chemical parameters of the soil samples are depicted in Table 1. The pH of the soil sample was ranged from 6.1 to 6.4, indicating the slightly acidic nature. Electrical conductivity (measured in 1:2 soil: water suspension) values observed at these locations were 5.64 dSm^{-1} at Satapada and 5.57 dSm^{-1} at Paradeep, respectively indicating the saline nature of these rhizospheric soil samples (Zhang *et al.,* 2015). Textures of the soil samples were found to be loamy sandy, and the sand percentage of the soils was 21.94% for Paradeep and 23.67% for Satapada, respectively.

A total of three bacterial isolates were obtained from the soil samples using Okon's medium and maintained in the laboratory for morphological, biochemical, and physiological characterization. Growth of *Azospirillum* was detected by the formation of thin white layer of pellicle at the sub-surface of the media which is presented in Fig. 3. Narayan *et al.* (2018) also reported white sub-surface pellicle growth of the *Azospirillum*

Table 1. *Soil sampling sites, variety of rice cultivated and soil physicochemical properties. Values are mean ± se of three replications and different letters indicate significant differences at p <0.05 by one-way ANOVA analysis*

Soil sampling sites	Latitude & longitude	Rice variety cultivated in sampling sites	pH	Sand (%)	EC_2 ($dS\ m^{-1}$)	Textural class
Satapada	19°34′49.4″ N 85°17′40.3″ E	Luna Suvarna (Salinity resistant)	6.1±0.25a	5.34±0.3c	5.87±0.1c	Loamy sand
Paradeep	20°18′11.9″ N 86°35′56.6″ E	Lunishree (Salinity resistant)	6.2±0.2d	5.27±0.5d	5.19±0.3e	Loamy sand

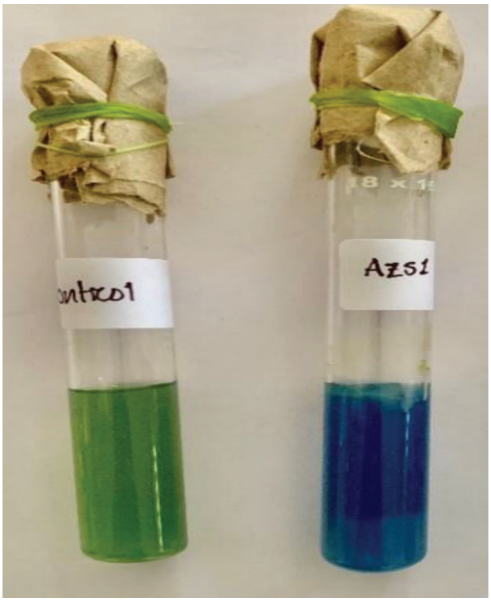

Fig. 3. *Subsurface pellicle growth*

isolates when grown in NFb semi-solid medium after 7 d of incubation. All the isolates were gram-negative, curved rods, and motile (Nawadkar *et al.,* 2015). Based on the morphological and biochemical characteristics, three *Azospirillum* (AZS1, AZS2, and AZS3) isolates were identified and maintained at the laboratory for further studies. The morphological and biochemical characteristics are described in Tables 2, 3 and 4.

Characterization of bacterial isolates for abiotic stress tolerance

The *Azospirillum* isolates were first screened for salt tolerance (NaCl and KCl) as the identification of an indigenous halotolerant isolate was the main objective of this study. Among three *Azospirillum* isolates, the AZS1 strain showed up to 8% NaCl and 8% KCl tolerance, which is significantly higher than AZS2 and AZS3 (Figs. 4 and 5). Therefore, only AZS1 was

Table 2. *Morphological characteristicsof the bacterial isolates.*

Isolate No.	Shape	Colour	Colony	Margin	Elevation	Texture	Gram reaction	Bacteria under SEM
AZS1	Regular	Pale yellow	Round	Smooth	Flat	Mucoid	-ve	Curved rod
AZS2	Irregular	clear	Round	Curled	Flat	Viscid	-ve	Vibrio
AZS3	Irregular	clear	round	Entire	Flat	Mucoid	-ve	Curved rod

"-ve" = Gram's negative. SEM = Scanning Electron Microscopy

Table 3. *Biochemical characteristics of the bacterial isolates*

Biochemical tests	AZS1	AZS2	AZS3
IAA production	+ve	+ve	+ve
Methyl red	+ve	+ve	+ve
Voges-Proskauer	-ve	-ve	-ve
Citrate utilization	+ve	+ve	+ve
Mannitol motility	+ve	+ve	+ve
Catalase	+ve	+ve	+ve
Oxidase	-ve	+ve	-ve
Triple sugar iron	-ve	-ve	-ve
H_2S production	+ve	+ve	+ve
Nitrate reduction	+ve	+ve	+ve
ONPG	+ve	+ve	-ve

"+": Positive; "-": Negative

Table 4. . *Sugar utilization pattern of the bacterial isolates*

Sugar utilization tests	AZS1	AZS2	AZS3
Lactose	+ve	+ve	+ve
Xylose	+ve	-ve	+ve
Fructose	+ve	+ve	+ve
Dextrose	+ve	+ve	-ve
Sucrose	+ve	-ve	+ve
Glycerol	-ve	-ve	-ve
Galactose	+ve	+ve	-ve
Trehalose	+ve	+ve	+ve
Salicin	+ve	+ve	-ve
Raffinose	-ve	-ve	+ve
Rhamnose	-ve	-ve	-ve

"+": Positive; "-": Negative

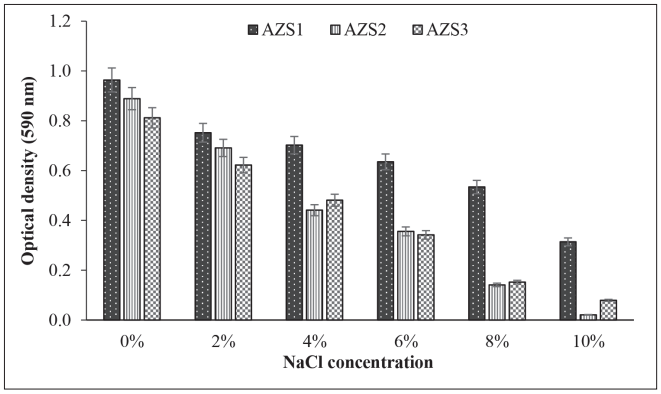

Fig. 4. *NaCl tolerance of the bacterial isolates (Vertical bars represent Standard Error of the Mean values)*

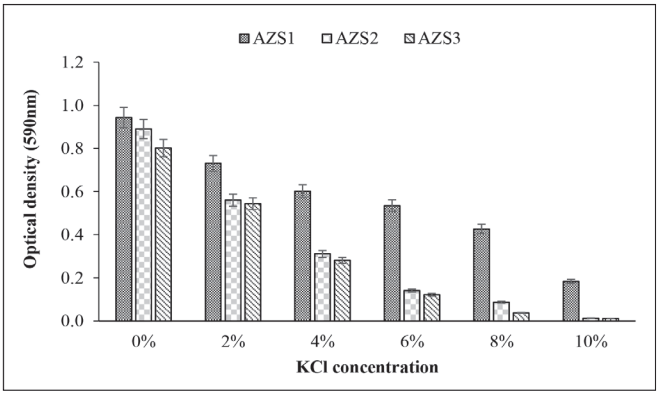

Fig. 5. *KCl tolerance of the bacterial isolates. (Vertical bars represent Standard Error of the Mean values)*

selected for their potential salt tolerance and subjected to further experiments. Similar findings were obtained by Zarea *et al.* (2012) who reported a salt-tolerant *Azospirillum* sp. from Khuzestan Province, an arid area in southwest Iran, showed NaCl tolerance up to 6% NaCl stress. Studies conducted by Zaied *et al.* (2009) showed that the mutant strains of *Azospirillum* sp. could withstand NaCl up to 50%. Halotolerant bacteria have a flexible mode of either adaptation (by cell envelope) or osmo-adaptation (production of potassium ions, glycine betaine, proline, trehalose and ectoines) strategies to protect intracellular integrity and maintain the exact cell volume and under salinity stress (Nina *et al.,* 2018). The optimum pH for the growth of the AZS1 was recorded at pH 7, however, this isolate was also able to grow at pH 9, indicating their alkali tolerance nature. At pH 5 no growth was seen (Fig. 6).

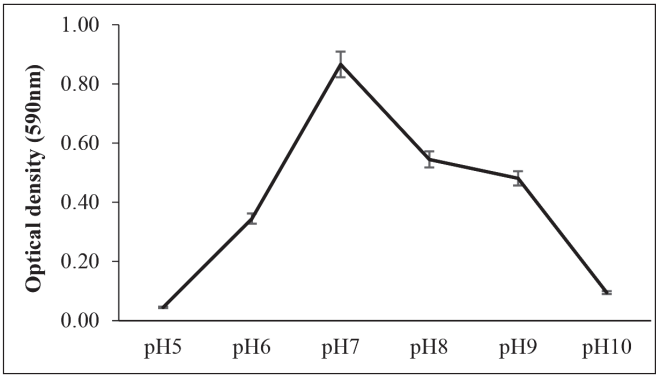

Fig. 6. *pH tolerance of isolate AZS1(Vertical bars represent standard error of the mean values)*

In-vitro PGP activities of the isolate

The various PGPR traits, such as the production of IAA, ammonia, HCN and siderophore and phosphate solubilisation were evaluated for AZS1 (Table 5). It showed indole production (36.52 µg mL^{-1}) when supplemented with 3 mg of tryptophan. IAA is always proven to be the most effective phytohormone which regulates cell division and acts as a growth regulator in plants during saline conditions. None of the isolates showed siderophore producing ability. Gibberellic acid (GA) is also a very important plant growth promoting phytohormone produced by AZS1 (38.35 µg mL^{-1}). However, neither any growth nor halo zone in the Pikovskaya medium was observed for the AZS1. Based on earlier reports *Azospirillum* sp. demonstrates an

Table 5. *Extracellular enzymes and plant growth promoting activities of AZS1*

Extracellular enzymes	AZS1
Amylase (Starch)	+ve
Gelatinase (Gelatin)	-ve
Proteinase (Casein)	-ve
Cellulase (Cellulose)	+ve
PGP traits	
IAA production	+ve (36.52 µg mL^{-1})
Ammonia production	+ve
Siderophore production	-ve
Phosphate solubilization	-ve
HCN production	+ve
Gibberellic acid production	+ve (38.35 µg mL^{-1})

"+": Positive; "-": Negative

inability to metabolise disaccharides, such as sucrose, when provided as the exclusive carbon source in Pikovskaya medium (Haiyambo and Chimwamurombe, 2018). AZS1 also showed the ammonia producing potential, which is another crucial characteristic, for plant development. HCN production by AZS1 is a significant characteristic as it indicates the antifungal potential. Based on the screening of plant growth promoting (PGP) traits, AZS1 were positive for four traits which can promote growth and enhance production for rice plants either independently or synergistically.

Production of extracellular enzymes

The potential of the production of various extracellular enzymes by the AZS1 isolate was assessed and depicted in Table 5. It showed positive results for starch and cellulase hydrolysis test, while no hydrolysis was observed in the gelatinase and casein hydrolysis test. Similar results were observed where the *Azospirillum brasiliense* isolate was positive for starch and cellulase hydrolysis in the experiment conducted by El-Katatny (2010).

Proline production by the isolate under NaCl stress

The proline content of AZS1 under NaCl was measured by the method of Bates *et al.* (1973) as depicted in Fig. 7. Highest proline production was observed under 8% NaCl (0.16 mmol mg^{-1} protein) while the lowest was observed at 0% NaCl (0.02 mmol mg^{-1} protein) under different salt concentration ($p<0.05$). However, at 10% NaCl proline concentration is slightly decreased (0.07 mmol mg^{-1} protein). This may be due to the reduction of cell population under higher salt stress. Similarly, García *et al.* (2017) reported that *A. vinelandii* strains Az19 and Az63 produced proline in higher amounts in drought stress conditions. Accumulation of proline is considered a metabolic parameter of salinity stress and is suggested to play an important role by decreasing the cell osmotic potential and providing osmotic stabilization and protection of cell membranes from the damaging effects of salt (Nabti *et al.,* 2015).

Effect of NaCl on superoxide dismutase (SOD) production

Isolate AZS1 produced a significant amount of SOD ($p<0.05$) with increasing concentration of NaCl (Fig. 8). Highest superoxide dismutase was recorded at 8% NaCl (1.18 mg $protein^{-1}$ min^{-1}), whereas the lowest activity was observed at 2% NaCl (0.12 mg $protein^{-1}$ min^{-1}). However, at 10% NaCl production of SOD was slightly lower (0.75 mg $protein^{-1}$ min^{-1}) because of the reduction in population size of the bacteria. Similar findings were reported by Molina *et al.* (2021) where *Azospirillum brasiliense* (strain Az39) showed increasing SOD activity under oxidative stress. Antioxidant enzymes have been reported for a long time because of their role in scavenging reactive oxygen species (ROS) and reducing oxidative stress in plants (Kazemeini *et al.,* 2016) and bacteria (Yasmeen *et al.,* 2020) under elevated salinity stress.

Carbon, nitrogen and carbon/nitrogen (C/N) ratio

The percentage of carbon, nitrogen and carbon/nitrogen (C/N) ratio in the isolates was measured with CHNS analyser and the data is presented in Fig. 9. Since the isolates were grown in nitrogen-free media; the nitrogen concentration at 0 d was noted to be negligible with low concentration of carbon. On the 7th day, all the isolates showed a stable increase in the concentration of nitrogen. On the 14th day, there was the highest increase in the concentration of nitrogen. The C/N ratio was highest at 0 d and lowest at 14 d. The result indicates the production of nitrogen by the isolate and utilisation of carbon.

Nitrogen fixing capacity of the isolates

Isolate AZS1 showed the potential to fix nitrogen. AZS1 fixed 13.25 mg N g^{-1} without any NaCl concentration (Table 6). However, increasing salt concentration did not significantly affect nitrogen fixation (AZS1 at 2% NaCl fixed 12.45 mg N g^{-1}, at 4% NaCl 12.1 mg N g^{-1}, at 6% NaCl fixed 11.65 mg N g^{-1} (Table 6). Sulaiman *et al.* (2019) also reported similar findings where the range of nitrogen fixing ability of *Azospirillum* sp. was between 3.3-9.3 mg N g^{-1}. According to Hossain *et al.* (2014), *Azospirillum* sp. had a nitrogen fixing efficiency that ranged from 10.03 to 13.11 mg N g^{-1}. The findings indicated that inoculation of AZS1 can increase the bioavailability of nitrogen in the soil to compensate for N deficiency under salinity stress.

nifH gene amplification

The genomic DNA obtained from the isolate AZS1 was of good quality without any shearing or protein contaminations. The concentration of the DNA was measured (581.4 ng μl^{-1}) through Nanodrop UV

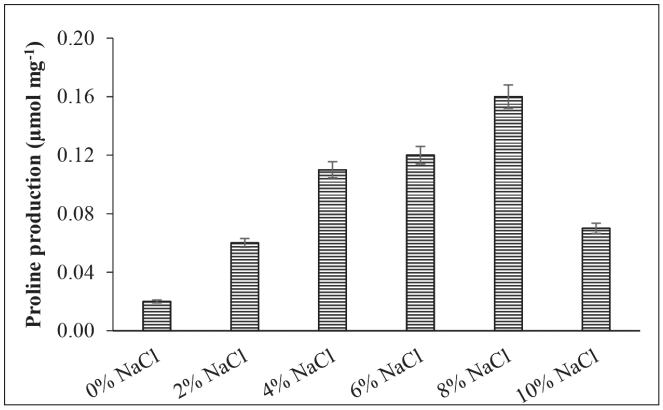

Fig. 7. *Proline production by isolate AZS1 under NaCl stress (Vertical bars represent standard error of the mean values)*

Table 6. *Nitrogen fixing efficiency of the AZS1 under NaCl stress*

NaCl concentration	mg N g^{-1} substrate
0% NaCl	13.25
2% NaCl	12.45
4% NaCl	12.1
6% NaCl	11.65

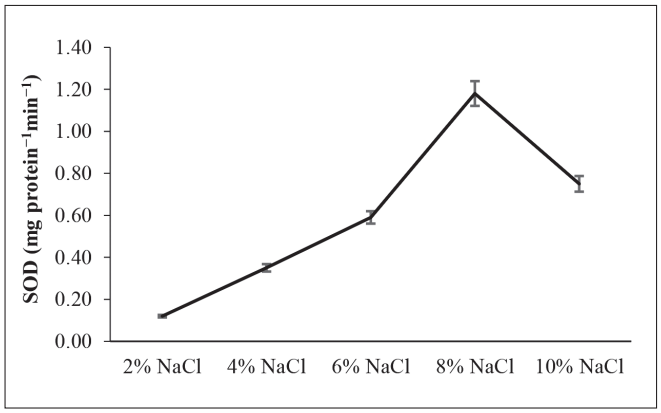

Fig. 8. *Superoxide dismutase(SOD) production of AZS1 under NaCl stress*

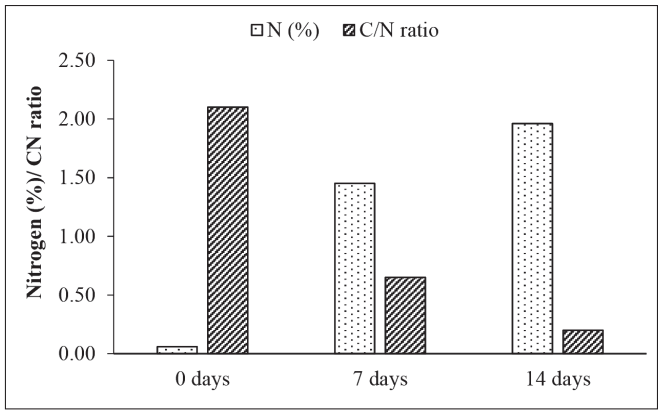

Fig. 9. *Nitrogen (N) and carbon/nitrogen (C/N) ratio changes of AZS1*

Spectrophotometer (Thermo Fisher Scientific, USA). After genomic DNA purification, the presence of *nifH* gene was checked using Pol F and Pol R primers and genomic DNA as template. For the validation of the PCR result, *Azotobacter chrococccum* (MTCC NO 3853) and *E. coli* were taken as positive and negative control respectively. The result of the PCR followed by the gel run is presented in Fig.10. As it is evident from the figure both the positive control and the tested organism (AZS1) showed similar positive amplicon size of 360-400 bp. Visualization of the amplified product clearly indicated the presence of the *nifH* gene. *nifH* gene which encodes for the dinitrogenase reductase is evolutionarily conserved and is the most widely established molecular marker for the study of diazotrophs in natural microbial communities (Gaby and Buckley, 2012). The rates of nitrogen fixation have been associated with *nifH* abundance and thus knowledge of diazotroph community structure and dynamics is required to understand the ecological constraints on nitrogen fixation in *Azospriilum*. Earlier the expression of the *A. brasilense* structural nitrogenase *nifH* gene has been analyzed on the plant roots by means of a *nifH-gusA* (*E. coli*) fusion (Oda and Jos, 2000). However, many factors appear to affect the association between *Azospirillum* and the plant. Therefore monitoring the expression of putative *Azospirillum*-plant interaction genes may lead to a better understanding towards engineering and exploitation of more efficient plant growth-promoting *Azospirillum* strains.

Molecular identification and phylogenetic analysis of the potential isolates

The 16S rRNA gene sequence analysis was done to confirm the AZS1 strain to be *Azospirillum* and to determine its taxonomic positions. Isolate was

further subjected to phylogenetic analysis using the neighbourhood joining method, with 100 bootstrap sampling. The results revealed that the AZS1 strain belongs to the genus *Azospirillum* of the family *Azospirillaceae* and showed 98.5% similarity with the 16 S rRNA gene sequence of *Azospirillum* sp. (Fig.11). The accession number of the isolate provided by the NCBI GenBank is OP503586.

Increasing salinization is becoming a serious threat to agriculture and global food security. Exploitation of potential halotolerant diazotrophs would be a better sustainable strategy to ameliorate salt stress in host plants and enhance crop productivity. In this study, out of three rhizospheric halotolerant *Azospirillum* isolates, AZS1 showed significantly better salt-tolerant properties. Moreover, the production of phytohormones (IAA and GA), ammonia and HCN by the isolate would be useful for the improvement of growth and productivity of rice under salinity stress. Production of antioxidant enzymes by the isolate can cope with free radicals and ROS and suppress damaging effects on plants under salt stress. The result of the study also suggested that the isolate is a potent nitrogen fixer. Therefore, the utilization of this organism as a bioinoculant with multi-faceted attributes is expected to have a substantial positive impact on the coastal agro-ecosystem. However, proper field trials and management practices are essential to optimize the use of these microbial inoculants.

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CONFLICTS OF INTEREST

The authors do not have any conflict of interest to declare.

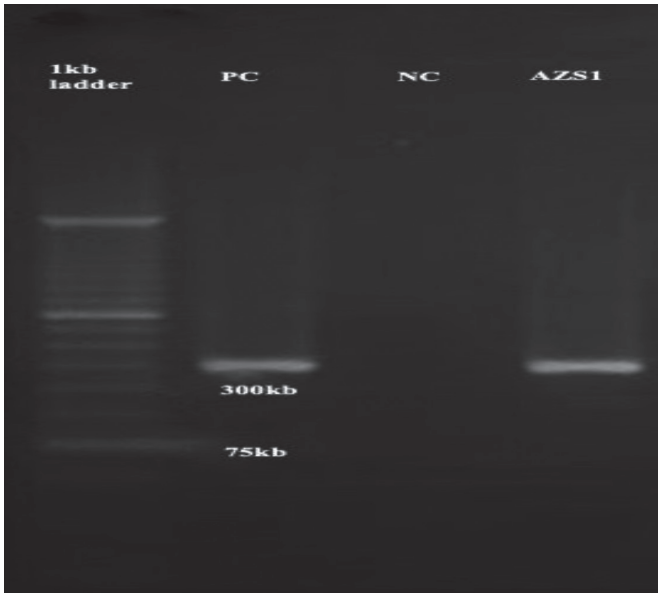

Fig. 10. *PCR gel showing amplification of nifH gene*

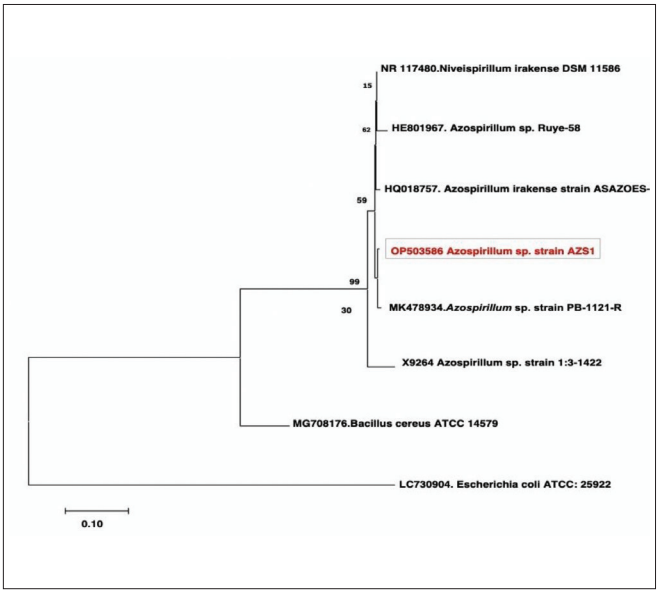

Fig. 11. *Phylogenetic analysis of AZS1*

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