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Effects of Plant Growth Promoting Halotolerant *Pseudomonas aeruginosa* JCM 5962 with Hydrocarbon Degradation Ability, Isolated from Sundarbans Mangrove Area in West Bengal, on *Abelmoschus esculentus* (Okra) Plant Growth

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Abstract

Plant growth promoting rhizobacteria (PGPR) play a key role in sustainable agricultural practices leading to increased crop productivity. Moreover, PGPR with ability to diminish abiotic stresses like salinity and hydrocarbon contamination in soil, can be developed into potent biofertilizers with maximum ecological benefits. Sundarbans mangrove region in West Bengal, a natural reservoir of diverse microbiota is an important source of PGPR adapted to high salinity and other abiotic stresses like hydrocarbon contamination due to oil spillage and water transport systems, rendering the soil unsuitable for farming. In the present study, a potent PGPR has been isolated from rhizospheric soil of Matla riverbed in mangrove areas of Sundarbans, with simultaneous nitrogen fixing, phosphate solubilizing and plant hormone like indole acetic acid (IAA) producing properties as well as high salt tolerance and hydrocarbon bioremediation abilities. The strain has been identified as *Pseudomonas aeruginosa* JCM 5962 (NCBI Accession number MK544832.1) on the basis of 16S rRNA analysis. The isolated *Pseudomonas aeruginosa* strain showed atmospheric nitrogen fixation (3612 ± 2 mg N/ Kg of soil), highest phosphate solubilization index of 3.0 ± 0.06 and 37.14 µg/mL of IAA production. This potent strain also showed salt tolerance upto 7% in culture broth and an uptake of 18.72% of salt. Highest hydrocarbon degradation was shown by this strain in presence of diesel as the sole carbon source. The isolated *Pseudomonas aeruginosa* strain showed overall improvement in growth of *Abelmoschus esculentus* (Okra) plants in pot experiments in different conditions like absence of any abiotic

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stress, presence of 5% salt stress and presence of 1% diesel contaminant. These results indicate that *Pseudomonas aeruginosa* JCM 5962 can be developed as a potent biofertilizer to be used in agricultural lands of Sundarbans mangrove regions and other areas which are plagued by high salinity and increasing hydrocarbons, particularly petroleum contamination.

Introduction

During the past few decades, the world has witnessed the doubling of human population and a concurrent doubling of food production. Plant nutrition plays a key role in the increase in demand for and supply of food. The most limiting nutrients for plant growth are Nitrogen (N) and Phosphorus (P), both of which are found mostly in unavailable forms to be taken up by plant roots.[1] Most of N is present as part of soil organic matter while majority of the soil P is either present as insoluble mineral phosphate or organic phosphate, and hence unavailable for plant uptake. Although inorganic fertilizers supply these two macronutrients in forms acceptable to the plants, tremendous use of these fertilizers along with highly productive and intensive agricultural methods, have led to deterioration of soil quality with less availability of nutrients required for plant growth. Apart from nutrient scarcity, overall growth of plants, food productivity and yield are affected by several biotic and abiotic stresses.[2,3] While the biotic stresses are due to live factors such as plant pests and pathogens, the abiotic stresses include physical conditions like drought, salinity, temperature, heavy metals, and other organic contaminants like hydrocarbons.[4]

For sustainable agriculture, use of plant growth promoting rhizobacteria (PGPR) can enhance soil nutrient availability and alleviate some of the biotic and abiotic stresses hindering plant growth.[5,6,7] PGPRs are free-living bacteria found in the rhizospheric soil, that colonize plant roots and improve plant growth by either direct or indirect methods. The direct mechanisms include atmospheric nitrogen fixation, solubilization of insoluble mineral phosphate, potassium (K), zinc (Zn) etc, production of siderophores[8,9,10] and production of plant growth regulators, particularly, plant hormones like indole acetic acid (IAA).[11] Atmospheric nitrogen (N_2) can be converted into ammonia by certain bacteria (free-living, symbiotic or associative) possessing the nitrogenase enzyme, in a biochemical process called biological nitrogen fixation (BNF).[4] Free-living N_2 fixing bacteria are widely distributed in nature and are estimated to fix 100-290 teragram of N per year in terrestrial ecosystems globally.[12] Some of the free-living N_2 fixing bacteria present in the rhizospheric soil are known to exhibit additional plant growth promoting attributes, which make them suitable as biofertilizers. Another major plant growth promoting trait is solubilization of insoluble mineral phosphate or organic phosphate. After N, the second most important element P is taken up by plant roots in monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) soluble forms. Many PGPRs can convert the unavailable P forms into soluble form by production of organic acids which solubilize the phosphate from inorganic forms by acidification and chelation[13] and/or by production of phosphatases which extract the phosphate from organic sources. Thus, phosphate solubilizing bacteria increase the bioavailability of unavailable P from soil to plants. Plant hormones like IAA is thought to protect plant cells from environmental stresses,[14] promote lateral and adventitious root growth leading to increased uptake of minerals and nutrients.[15] While PGPRs with abovementioned properties can act as biofertilizers, they are also studied for their bioremedial properties like degradation of immobilized soil pollutants such as herbicides, pesticides, solvents, heavy metals and hydrocarbons[16,17] as well as saline stress tolerance.[18]

Sundarbans mangrove region in West Bengal, harbours a rich and diverse microbiota as a natural repository of various beneficial bacteria like PGPR adapted to high salinity. However, a vast majority of the indigenous soil microflora of the Sundarbans, remains unexplored for their potential properties.[19,20,21] In addition to saline stress, soil of this region is also polluted with hydrocarbons, particularly petroleum products, due to regular oil spillage from the water transportation system.[19,22]

One of the most important abiotic stresses in mangrove forests is high salt stress or salinity. Effects of high salinity on the growth, nutrient accumulation and nitrogen fixation of different free-living, symbiotic and other soil bacteria have already been extensively reported. Salinity decreases the uptake and accumulation of essential plant nutrients like N and P due to high osmotic pressure and ion toxicity, leading to decreased plant growth. To relieve salt stress on crop plants, many salt-tolerant crop varieties have been developed using transgenic techniques as well as conventional breeding methods. But, these approaches of producing salt-tolerant crops are labour-intensive, time-consuming and somewhat expensive. An alternative strategy to promote sustainable agricultural practices in saline tracts is to use halotolerant PGPRs.[4] Numerous halotolerant PGPRs have been extensively studied which reduce damages due to salinity in various crops, decrease salt stress, promote nutrient uptake, homeostasis, improve plant health and productivity of crops in saline alkaline soils.[23,24,25,26,27,28]

Oil spillage and other hydrocarbon leakages, result in contamination of soil affecting its physico-chemical properties and fertility adversely. Particularly, petroleum pollution in soil can affect plant nutrient availability by altering soil structure and microbiota composition. Plant growth is greatly affected due to uptake of hydrocarbons by plant roots resulting in different types of phytotoxicity.[29] Bioremediation by both plants and microbes of hydrocarbons have been widely reported. Hydrocarbon degrading bacteria show different rates of degradation of the various components of petroleum products with higher degradation being observed under aerobic conditions[30,31] Recent studies on PGPR based consortia involved in biodegradation of hydrocarbons have shown efficient degradation of hydrocarbons.[32,33,34]

Present investigation involves the collection and screening of rhizospheric soil from Matla riverside near Canning in the Sundarbans mangrove region in order to isolate a potent PGPR with multiple properties such as nitrogen-fixation, phosphate solubilization and IAA production, which will also simultaneously exhibit salt tolerance and hydrocarbon degradation. The aim of this study has been to identify a bacterial strain that could be developed as a potent PGPR, for sustainable agriculture in the nutrient limited, saline and hydrocarbon contaminated tracts of Sundarbans mangrove region as well as other regions with similar soil quality.

Materials and Methods

Screening and isolation of plant growth promoting bacteria (PGPB) showing simultaneous nitrogen-fixation and phosphate solubilization properties

Rhizospheric soil samples were collected aseptically in sterile polypropylene containers from Matla riverbed near Canning (22.3104° N, 88.6579° E) in Sundarbans, South 24 Parganas, West Bengal, India and transported to the laboratory for further microbiological analysis. The pH, temperature and soil type were determined during soil sample collection at the sampling site. 1g of dry soil was suspended in 10 mL sterile water and then 10-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) were made in sterile 0.85% saline solution. Plant growth promoting bacteria exhibiting simultaneous nitrogen-fixation and phosphate solubilization were isolated by plating the 10-fold serial dilutions of the soil sample on N_2-free Burk's media amended with 0.2% insoluble tricalcium phosphate or TCP (Sucrose 20.0 gL^{-1}, magnesium sulfate $MgSO_4.7H_2O$ 0.2 gL^{-1}, dipotassium hydrogen phosphate K_2HPO_4 0.8 gL^{-1}, potassium dihydrogen phosphate KH_2PO_4 0.2 gL^{-1}, calcium sulfate $CaSO_4$ 0.13 gL^{-1}, ferric chloride $FeCl_3$ 0.00145 gL^{-1}, sodium molybdate $Na_2 MoO_4.2H_2O$ 0.000253 gL^{-1}, agar 15.0 gL^{-1}, tricalcium phosphate $Ca_3(PO_4)_2$ 2.0 gL^{-1}] by spread-plate technique. The inoculated agar plates were incubated at 37°C for 5-7 days. Based on colony morphology and characteristics, bacterial colonies were selected and purified by repeated subculturing using streak-plate method in the same medium.

Determination of phosphate solubilization index (PSI)

Phosphate solubilization ability of the bacterial isolates obtained was determined by spot inoculating the isolates in Pikovskaya agar medium[35] (Yeast Extract 0.5 gL^{-1}, dextrose 10.0 gL^{-1}, tricalcium phosphate $Ca_3(PO_4)_2$ 5.0 gL^{-1}, ammonium sulfate $(NH_4)_2SO_4$ 0.5 gL^{-1}, potassium chloride KCl 0.2 gL^{-1}, magnesium sulfate $MgSO_4.7H_2O$ 0.1 gL^{-1}, manganese sulfate $MnSO_4$ 0.0001 gL^{-1}, ferrous sulfate $FeSO_4.7H_2O$ 0.0001 gL^{-1}, agar 15.0 gL^{-1}) and incubating at 37°C for 3-4 days. Phosphate

solubilization was indicated by the clear halo zones obtained around the bacterial colonies and was measured as phosphate solubilization index using formula of Edi *et al.*[36]

Phosphate Solubilizing Index (PSI) = (CD + ZD) / CD;

where CD= Colony Diameter and ZD= Zone Diameter both measured in cm.

Most potent PGPR isolate was selected on the basis of PSI calculated and this strain was chosen for further studies.

Morphological and Biochemical Characterization Of PGPBS3 Isolate

Colony morphology of PGPBS3 isolate was observed on both Nutrient agar and N_2-free Burk's agar media containing 0.2% $Ca_3(PO_4)_2$. Gram staining of the bacterial cells was done using Crystal Violet solution, Gram's Iodine and Safranin according to standard protocol. Bacterial cells were observed under scanning electron microscope (SEM) to determine the cell shape and size. For this, a fresh culture of the bacterial isolate was taken, the cells were washed with phosphate buffered saline, pH 7.4 and fixed with 0.5% glutaraldehyde. The sample was dehydrated after ethanol wash, dried overnight, coated with platinum, re-dried and observed using a high-resolution SEM (Zeiss EVO 18). To characterize the PGPBS3 isolate, various biochemical tests like IMViC, carbohydrate and amino acid utilisation, different enzyme assays, motility test, tests for growth in different selective media and antibiotics sensitivity tests were performed.

Molecular Identification of PGPBS3 Strain

Genomic DNA of the strain was isolated using HiPurA Bacterial Genomic DNA purification Kit (Himedia) according to the manufacturer's protocol. Identification of the isolate was done by polymerase chain reaction (PCR) amplification of the 16S rRNA gene sequence in an ABI Veriti 96 well thermal cycler using two universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was performed in a total reaction volume of 25 µL with 2.5 µL buffer containing $MgCl_2$, 2 µL template DNA, 0.5 µL each of the forward and reverse primers, 0.5 µL dNTP mix, 0.25 µL Taq DNA polymerase and 18.75 µL nuclease free water under the following reaction conditions: initial denaturation at 94°C for 5 mins followed by 30 cycles of 94°C for 40 s, 54°C for 40 s, 72°C for 95 s and final extension at 72°C for 8 mins. The amplified product was purified using HiPurA PCR clean-up system kit (Himedia) and sequenced by an automated DNA sequencer (Applied Biosystems 3500XL). The sequence obtained was analysed and identified using BLAST search and was compared against bacterial 16S rRNA sequence database available on NCBI. The sequences were aligned by using Clustal W followed by construction of neighbour joining phylogenetic tree, using MEGA 7.

Estimation of Plant Growth Promoting Properties of PGPBS3 Isolate

Qualitative Estimation of Phosphate Solubilization

Phosphate solubilization and simultaneous acid production was qualitatively assessed by spot inoculating the bacterial isolate on Pikovskaya agar medium containing a pH indicator bromothymol blue and incubating at 37°C for 3-4 days.

Quantitative Estimation of Phosphate Solubilization of PGPBS3

Phosphate solubilization by the bacterial isolate was assayed for 6 days post inoculation. Briefly, 25 mL of Pikovskaya broth was inoculated with 0.1 mL of the bacterial culture and incubated at 37°C for 6 days. 1.5 mL of aliquots were withdrawn from the culture on 1st, 2nd, 3rd, 4th, 5th and 6th days post inoculation and centrifuged for 10 mins at 10,000 rpm at room temperature to obtain the supernatants. Estimation of phosphate and determination of pH of the supernatants were done on each day. Phosphate was estimated by adding 4 mL of Phosphate reagent containing 2.5% ammonium molybdate, 6N H_2SO_4 and 10% ascorbic acid to 1 mL of the supernatant of each day and measuring the absorbance at 829 nm in a Shimadzu UV-1900 UV-Visible Spectrophotometer according to Clesceri and group.[37] Phosphate concentrations were calculated from a standard curve set up with different concentrations of sodium dihydrogen phosphate solution using the Phosphate reagent in a similar manner.

Estimation of Total Nitrogen in Soil After Treatment with the Bacterial Isolate

An alternative experiment based on estimation of total nitrogen of soil by Kjeldahl method was designed to determine the nitrogen-fixation ability

of the PGPBS3 isolate in soil. To 500 g sterile soil, PGPBS3 culture broth was added (~10^{11} cells) and incubated at 37°C for 10 days. A control sterile soil sample (500 g) without any inoculum was also incubated at 37°C for 10 days. After incubation, the total nitrogen of the control and test soil samples were estimated by Kjeldahl method. Briefly, the soil samples were digested with concentrated sulfuric acid (H_2SO_4) and potassium sulfate : copper sulfate in the ratio of 10:1 (catalyst) in presence of salicylic acid and sodium thiosulfate. This converted all the inorganic and organic nitrogen present in the soil to ammonium ion which was then estimated by titration with standard sulfuric acid and the total nitrogen was calculated according to the following conversion factor and formula :

1 mL of 0.1 N standard sulphuric acid = 0.0014 g of nitrogen

Total Nitrogen in mg / Kg = (t-b) x 14 x N x 1000/10

where, t= Titre value of sample, b= Titre value of blank and N= Normality of sulphuric acid

Indole Acetic Acid (IAA) Production by the Bacterial Isolate

PGPBS3 isolate was inoculated in Burk broth with 0.2% TCP and incubated for 5-7 days at 37°C. The cells were then centrifuged for 10 mins at 10000 rpm at room temperature to obtain the supernatant. IAA production was determined by adding 2 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% $HClO_4$) to 1 mL of the PGPBS3 culture supernatant and incubating for 90 mins in dark. Observation of pink colour confirmed production of IAA and its absorbance was measured at 530 nm in a spectrophotometer (Shimadzu UV-1900 UV-Visible Spectrophotometer). IAA concentration was determined from a standard curve of pure IAA prepared by recording the absorbance at 530 nm of increasing concentrations of IAA

Study of Salt Tolerance by PGPBS3 Isolate

Salt tolerance of the PGPBS3 bacterial isolate was assessed by inoculating the strain in 5 mL of Nutrient broth enriched with increasing concentrations of NaCl (1% to 15% w/v) and incubating at 37°C in a shaker-incubator for 5 days. Control sets were also prepared which contained Nutrient broth and increasing concentrations of NaCl but no inoculum. The resultant turbidity at different salt percentages was measured by recording the absorbance at 600 nm. The highest concentration of salt at which significant growth was observed, was used in another experiment, where 5 mL of Nutrient broth containing 7% NaCl was inoculated with PGPBS3, incubated at 37°C in a shaker-incubator for 5 days. After 5 days, the bacterial cells were centrifuged at 10000 rpm for 10 mins and the supernatant was collected. The electrical conductivity (EC) of the supernatant was measured and compared to the control (supernatant of uninoculated Nutrient broth with 7% NaCl) to estimate the salt uptake by the bacterial strain. Salt uptake percentage was calculated by the following formula:

$$\% \text{ of salt uptake} = [100 \times (EC_{control} - EC_{test}) / EC_{control}] \%$$

Bioremediation of Hydrocarbon Contaminants by PGPBS3 Strain

Hydrocarbon Degradation by PGPBS3 Strain

Hydrocarbon degradation ability of PGPBS3 strain was studied by inoculating the pure culture in Bushnell-Haas medium (minimal medium composed of magnesium sulfate $MgSO_4.7H_2O$ 0.2 gL^{-1}, potassium dihydrogen phosphate KH_2PO_4 1.0 gL^{-1}, dipotassium hydrogen phosphate K_2HPO_4 1.0 gL^{-1}, ammonium nitrate NH_4NO_3 1.0 gL^{-1}, ferric chloride $FeCl_3$ 0.05 gL^{-1}) containing 1% of different hydrocarbons like petrol, diesel, naphthalene, camphor and hexane respectively. The cultures were incubated at 37°C for 7 days in shaking condition and subsequent appearance of turbidity was measured spectrophotometrically at 600 nm.

GC-MS Analysis of Diesel Degradation by PGPBS3

Biodegradation of diesel by PGPBS3 bacterial isolate was estimated by the following protocol. To 50 mL of Bushnell-Hass media in two separate sets of Erlenmeyer flasks (each set consisted of three flasks), 1% of diesel (v/v) was added as the sole carbon source. 1 mL of PGPBS3 culture was added to the set of test flasks. The uninoculated flasks constituted the control set. Both the test and control sets were incubated at 37°C for 6 days in a shaker incubator. After 6 days, the test and control broths were mixed with 40 mL of petroleum ether in separating funnels respectively and

shaken vigorously to get single emulsified layer. Subsequently, acetone was added to each flask and shaken gently to break the emulsification, resulting in two distinct layers. The top layer containing petroleum ether mixed with diesel was passed through anhydrous sodium sulphate to remove moisture. Petroleum ether and acetone were evaporated by heating in a water bath. The oil (diesel) obtained by this treatment from both test and control sets were further analysed by GC-MS (Trace-GC-Ultra, Thermo Fisher Scientific, USA). 1 µL sample (test and control) was injected in the polar column TR WAXMS. Software XCALIBUR and MS library NIST (version 2014) was used for monitoring the process. Mass spectrometer (MS) POLARSQ model was used.

Determination of Plant Growth Promoting Activity of PGPBS3 Bacterial Strain on *Abelmoschus esculentus* (Okra) Plant

Preparation of Bacterial Inoculum For Plant Studies

PGPBS3 isolate was used for studying plant growth promoting activity in two plant experiments. For this, PGPBS3 culture was grown in 100 mL of Luria Bertani broth in a shaker incubator (150 rpm) at 37°C for 24 h. The overnight culture was then serially diluted (10^{-1} to 10^{-7}) and 0.1 mL of each dilution was spread plated onto Luria Bertani agar plates, incubated at 37°C for 24 h and the CFU/ mL was evaluated. The final concentration of PGPBS3 isolate was maintained at $4x10^8$ cells/mL for inoculation.

Bioassay of PGPBS3 Isolate in *Abelmoschus esculentus* (okra) plant

The effect of PGPBS3 isolate in plant growth promotion was tested by performing experiments on *Abelmoschus esculentus* (okra). Seeds of *A. esculentus* were planted in pots containing sterile soil. Sterile soil was prepared by collecting soil from agricultural fields and then sterilizing the soil at 121°C and 15 lbs pressure.

The following experimental set-up was done for determining the plant growth promoting effect of PGPBS3 isolate on A. *esculentus* growth.

Table 1: Experimental set-up for *A. esculentus* plant growth promotion by PGPBS3 isolate:

Experimental Sets	PGPBS3 inoculum	5% NaCl (w/w)	1% Diesel (w/w)
Set 1 (Control)	–	–	–
Set 2	Inoculum added	–	–
Set 3	–	5% NaCl added	–
Set 4	Inoculum added	5% NaCl added	–
Set 5	–	–	1% Diesel added
Set 6	Inoculum added	–	1% Diesel added

In all the six experimental sets, *A. esculentus* seeds were planted in potted sterile soil. PGPBS3 bacterial inoculum ($4x10^8$ cells/ mL) was added to Sets 2, 4 and 6 respectively. Growth of the saplings of the control (Set 1) and test sets (Sets 2, 3, 4, 5 and 6) were recorded till 40 days by measuring the height of plants and length of leaves at regular intervals of 10 days. Readings for at least 5 saplings were observed for each set.

Statistical Analysis

All experiments were done in triplicate and subsequent standard errors were calculated according to the formula $SE = \sigma / \sqrt{n}$ where SE is the standard error, σ is the sample standard deviation and n is the sample size.[38] Calculations of standard deviation and standard error were done using MS-Excel.

Results and Discussion

Isolation and Screening of Potent Plant Growth Promoting Bacteria from Rhizospheric Soil of Sundarbans Mangrove Region

The pH and temperature of the soil sample collected were initially determined at the site of the collection. The pH was found to be between 7.0-8.0 (neutral to alkaline) and temperature recorded was 31°C. The soil type was assessed to be silt-loam to clayey which corroborated with earlier reports.[39,40] Serial

dilutions of rhizospheric soil from Matla riverbed near Canning, Sundarbans mangrove region, West Bengal, were inoculated in N_2-free Burk's agar medium containing insoluble tricalcium phosphate (TCP) i.e., $Ca_3(PO_4)_2$, in order to screen for free-living nitrogen fixing bacteria with simultaneous phosphate solubilization property. Fig.1.a shows some of the bacterial isolates obtained in Burk's agar (N_2-free) medium amended with 0.2% TCP. Seven such morphologically different isolates were obtained and named PGPBS1-7 (PGPBS - plant growth promoting bacterial strains). These bacterial isolates were then inoculated in Pikovskaya's agar medium separately to test for their phosphate solubilization ability. The phosphate solubilization index (PSI) was calculated for all the isolates according to the formula given in the methods section. Isolate PGPBS3 showed the highest value of PSI of 3.0 ± 0.06 (Table.2). Halo zone of clearance for the PGPBS3 isolate in Pikovskaya's agar plate is shown in Fig. 1.b. Plant growth promoting attributes of PGPBS3 isolate are summarized in Table.2. Plant growth promoting bacteria can act as good biofertilizers because of their ability for solubilizing insoluble phosphate, nitrogen fixation and production of some plant growth regulators[18] like the plant hormone indole acetic acid. In this study, PGPBS3 showed highest IAA production (37.14 µg/mL) in the culture supernatant (Table.2). Since PGPBS3 strain showed the capability of fixing atmospheric nitrogen (growth in N_2-free medium), phosphate solubilization and IAA production, this isolate was chosen as a potent plant growth promoting bacterial isolate. Among the 7 isolates, PGPBS3 was chosen for all further studies on the basis of maximum value of PSI and highest production of IAA obtained. PGPBS3 isolate was then purified by repeated subculturing and maintained in Burk's agar media with 0.2% TCP.

Table 2: Plant growth promoting properties of PGPBS3 isolate

Plant growth promoting properties studied	Observations
Atmospheric N_2 fixation	+ (Growth in Burk's agar medium)
Phosphate solubilization	+ (Growth in Pikovskaya's agar medium)
Phosphate solubilization index (PSI)	3.0 ± 0.06
Indole acetic acid production	37.14 µg/mL

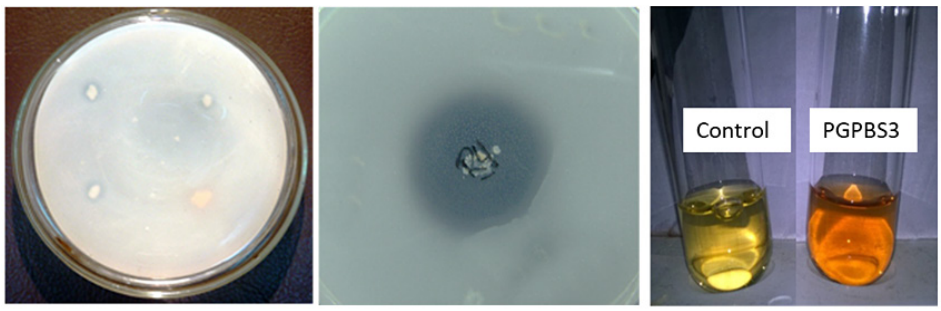
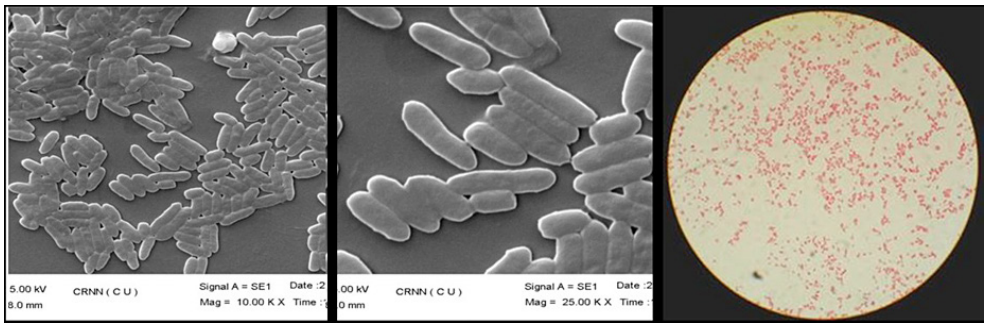


Fig.1: Isolation and characterization of PGPBS3 bacterial isolate. (a) Some bacterial isolates screened on N_2-free Burk's agar medium amended with 0.2% TCP; (b) Halo zone of phosphate solubilization of PGPBS3 on Pikovskaya's agar; (c) IAA production by PGPBS3 isolate.



a b c

Fig. 2: Scanning electron micrographs of PGPBS3 isolate under (a) 10,000x magnification and (b) 25,000x magnification; (c) Gram stain of PGPBS3 isolate (100x magnification)

Table 3: Biochemical Characterization of PGPBS3 isolate

Biochemical characteristics	**PGPBS3 bacterial isolate**
Colony morphology[a]	Circular, medium sized, smooth, opaque colonies which turn greenish in colour after 5-7 days
Gram character	Gram negative, rod-shaped cells
Motility	+
Oxidase	+
Catalase	+
Urease	–
Protease	+
Nitrate reductase (NO^{3-} to NO^{2-})	+
Optimum pH and temperature for growth	pH 9.0; 37°C
Indole production	–
Voges-Proskauer test	–
Methyl Red test	–
Citrate utilization	+
Gelatin hydrolysis	+
Starch hydrolysis	+
Utilization of:	
Glucose	+
Mannitol	+
Sucrose	+
L-Valine	+
L-Serine	+
L-Histidine	+
Growth in MacConkey Agar	+ (non-lactose fermenter)

+ indicates positive test and - indicates negative test

[a] colony morphology observed in Nutrient agar medium

Morphological and Biochemical Characterization of PGPBS3 Isolate

PGPBS3 isolate is found to be Gram-negative bacilli (Fig.2.c) with rod-shaped cells in clusters observed in the SEM micrographs (Fig.2.a and b). Biochemical tests showed PGPBS3 strain to be a motile, oxidase, catalase and nitrate reductase positive, urease negative bacteria. Results of the biochemical

characterization are shown in Table.3. Morphology and preliminary biochemical tests done according to Bergey's manual indicated that the isolate belongs to the *Pseudomonas* genus.

Molecular identification of PGPBS3

Molecular identification of isolate PGPBS3 was done by PCR amplification of the 16S rRNA gene sequence. The amplified product, which was approximately 1440 bp in size, was sequenced and sequence alignment using BLAST-N in the NCBI Nucleotide database was done. PGPBS3 was identified as *Pseudomonas aeruginosa* strain JCM 5962 (NCBI Accession number MK544832.1) and found to have 95.76% sequence similarity with *Pseudomonas aeruginosa* strain BBK-3 16S ribosomal RNA gene, partial sequence (KC237280.1). The phylogenetic tree of PGPBS3 isolate, as shown in Fig.3, was constructed using the neighbor joining method.

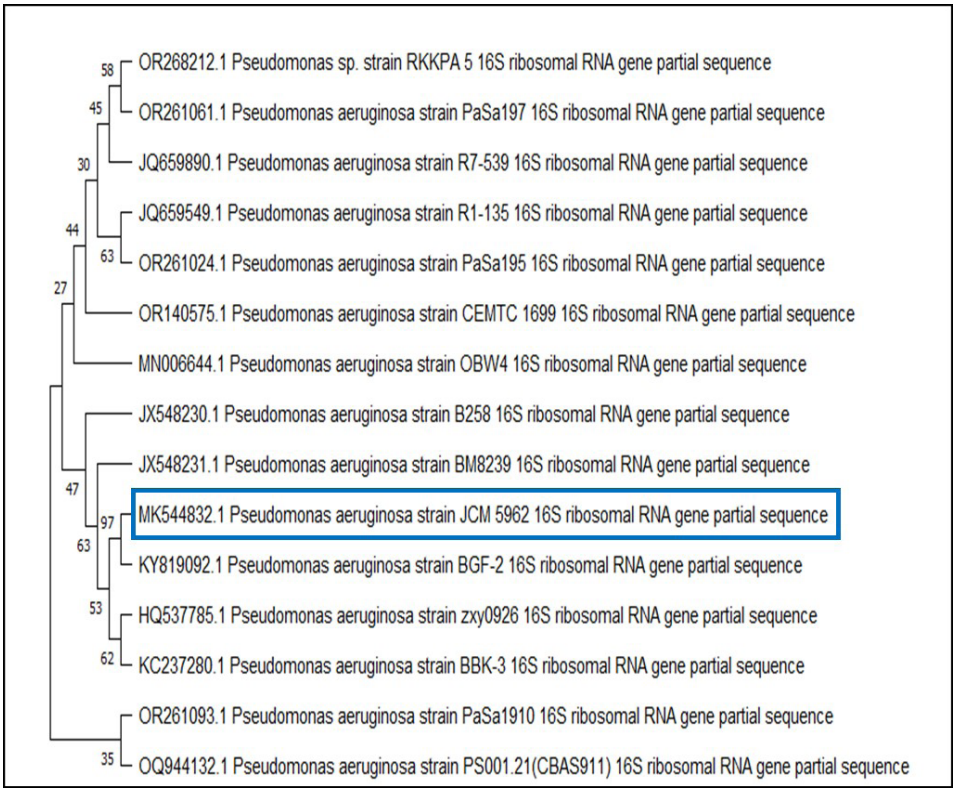


Fig. 3: Phylogenetic tree of PGPBS3 isolate (*Pseudomonas aeruginosa* strain JCM 5962) constructed by neighbor-joining method, obtained from 16S rRNA gene sequence analysis.

Elucidation of the PGPR Traits of *P. aeruginosa* JCM 5962 (PGPBS3) Strain

Phosphate Solubilization

To determine the extent of phosphate solubilization by *P. aeruginosa* JCM 5962 (PGPBS3) strain in Pikovskaya's broth culture, soluble phosphate of the culture supernatant was measured each day upto 6 days post inoculation. For *P. aeruginosa* JCM 5962, the soluble phosphate concentration increased from 6.02±0.9 µg/mL at 1 day post inoculation to 34.48±1.7 µg/mL at 6 days post-inoculation (Fig.4.a). Simultaneously, the pH of the supernatant was also measured each day and found to reduce from pH 7.0 at 1 day post inoculation to pH 5.14 at 6 days post-inoculation (Fig.4.b). This observation was in agreement with the production of yellow zone around colonies of the isolate in Pikovskaya's agar containing bromothymol blue pH indicator (Fig.4.c). Bromothymol blue pH indicator is yellow in acidic, green in neutral and blue in alkaline conditions. Thus,

the yellow zone obtained (shown in Fig.4.c) indicated a lowering of the pH during phosphate solubilization, while the rest of the plate remained green due to neutral pH. One of the important mechanisms by which PGPR solubilize insoluble mineral phosphate is by releasing organic acids into the soil, reducing the surrounding pH and helping in breaking down the insoluble phosphate. Organic acids produced might act as chelators of divalent cations like Ca^{2+}, releasing the phosphate in soluble form from the insoluble calcium phosphate and thus making it available for uptake by plants from the soil.[41,42] So, it can be inferred that the pH drop observed is due to organic acid production by the bacterial isolate.

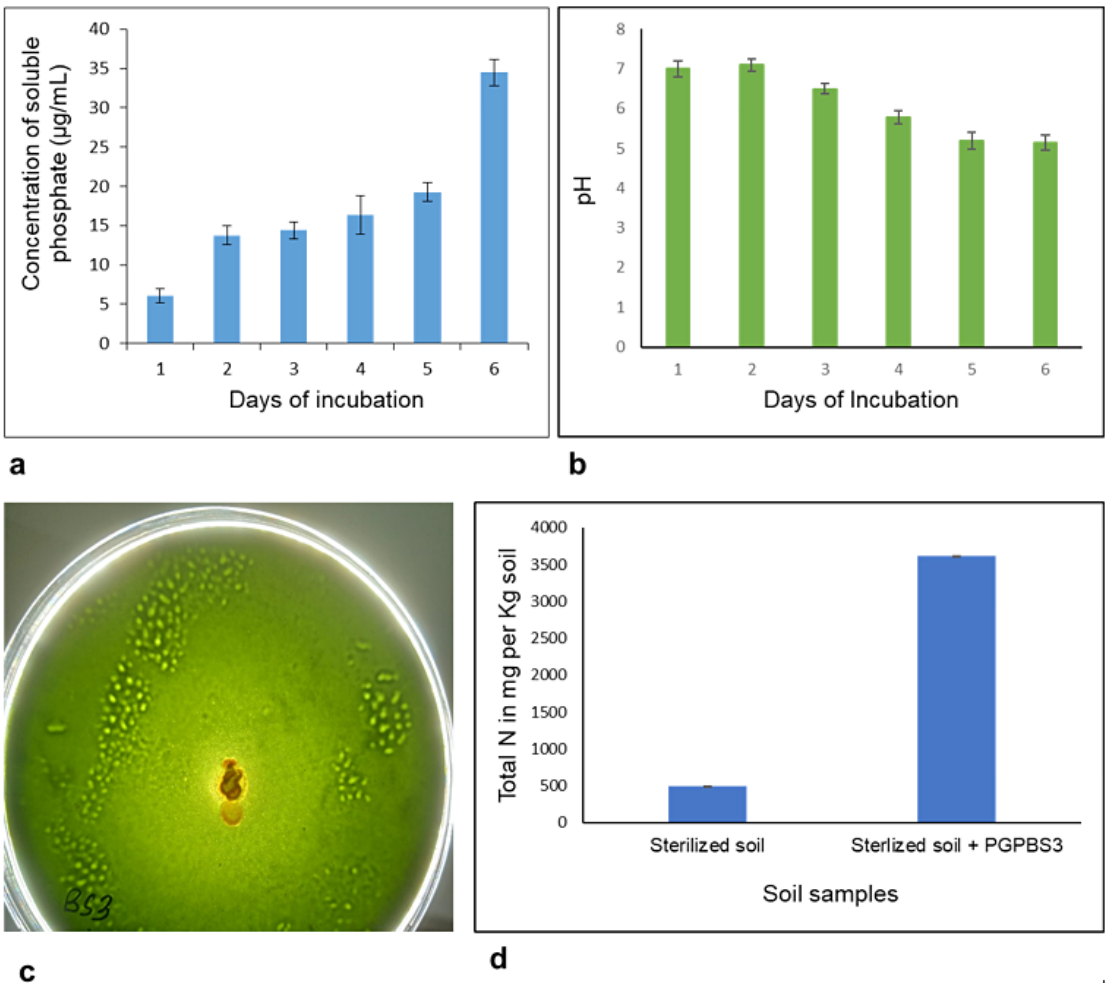


Fig. 4: Estimation of phosphate solubilization and nitrogen-fixation by *P. aeruginosa* JCM 5962. (a) soluble phosphate concentration in µg/mL produced in broth culture upto 6 days post inoculation due to inorganic phosphate solubilization; (b) simultaneous decrease in pH upto 6 days post inoculation; (c) organic acid production on Pikovskaya's agar medium containing bromophenol blue indicator and (d) total nitrogen estimation of sterile soil with and without *P. aeruginosa* JCM 5962 inoculum.

Atmospheric Nitrogen-Fixation

Fig.4.d shows the nitrogen-fixation of PGPBS3 bacterial strain estimated by a modified total nitrogen method. This modified total nitrogen (N) estimation experiment was designed to detect atmospheric nitrogen-fixation by the PGPBS3 isolate, since, any detectable nitrogenase activity could not be obtained using the conventional acetylene reduction assay

(ARA). The total N content of sterilized soil was estimated and compared to the total N of sterilized soil inoculated with *P. aeruginosa* JCM 5962. A large increase in the total nitrogen (mg/ Kg of soil) was observed between the sterilized soil without any inoculum and the *P. aeruginosa* JCM 5962 inoculated sterilized soil (Fig.4.d). 500 g of soil used in the control and the test experiments were sterilized and then total N was measured to eliminate any contribution towards the increase in nitrogen by the soil's natural microbiota. The increased value of total N obtained for sterilized soil with *P. aeruginosa* JCM 5962 inoculum (3612 ± 2 mg N/ Kg of soil) indicated fixation of atmospheric nitrogen by this isolate. Total N of only sterile soil (without *P. aeruginosa* JCM 5962 inoculum) was 490 ± 2.88 mg N/ Kg of soil (Fig.4.d). Additionally, *P. aeruginosa* JCM 5962 could be cultured repeatedly in nitrogen free Burk's media, proving that this isolate was capable of fixing atmospheric nitrogen.

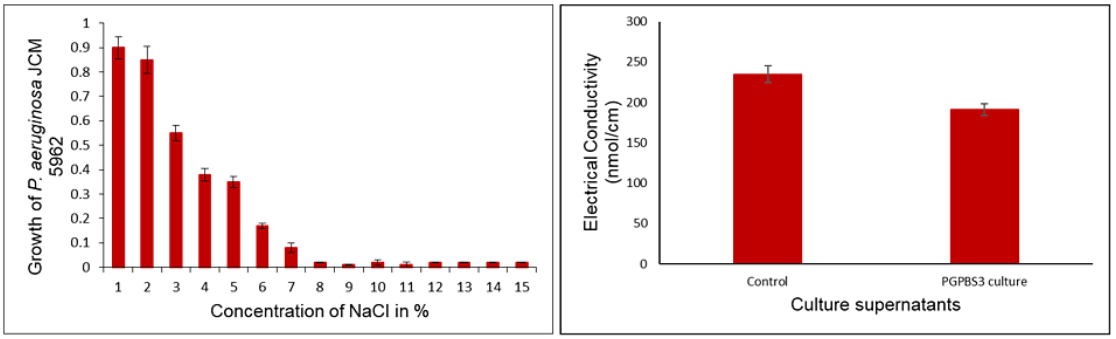


Fig. 5: Effect of salinity on growth of *P. aeruginosa* JCM 5962. (a) Growth of the strain in presence of increasing concentration of NaCl (%) and (b) estimation of electrical conductivity in culture supernatant of *P. aeruginosa* JCM 5962.

Bioremediation of Abiotic Stresses by *P. aeruginosa* JCM 5962

Effect of Salinity on *P. aeruginosa* JCM 5962

Salinity of soil reduces the uptake and accumulation of N and P in plants leading to poor growth of plants. Plant growth promoting bacteria are known to reduce stress due to salinity by imparting salt tolerance to plants. Since *P. aeruginosa* JCM 5962 was isolated from the soil of riverbed of Matla flowing through the Sundarbans mangrove area (region of high salinity), this isolate was tested for its salt tolerance property. The isolated strain was grown in Luria Bertani medium containing 1% to 15% of NaCl and it was observed that *P. aeruginosa* JCM 5962 could tolerate upto 7% NaCl concentration. There was no growth of the strain in higher percentages of salt concentration (Fig.4.a). Electrical conductivity (EC) measurement of the bioinoculated culture supernatant containing 7% salt was 191 nmol/cm and EC value of the control supernatant (uninoculated media with 7% NaCl) was 235 nmol/cm. Thus, there is a decrease in the EC value of the inoculated broth supernatant from the control, indicating uptake of salt by the bacterial isolate, as shown in Fig.4.b. The percentage of salt uptake by the isolate under the experimental conditions was 18.72% (calculated according to the formula given). Recently, Pallavi *et al.* reported several halotolerant bacterial species with potent plant growth promoting properties from the Sundarbans mangrove area which can have potential use as biocontrol agents.[43] The PGPR isolate obtained in this study, however, shows promising bioremedial properties (discussed in the next section) along with high tolerance for salt stress.

Hydrocarbon degradation by *P. aeruginosa* JCM 5962

One of the major abiotic stresses to the Sundarban mangrove soil is polyaromatic hydrocarbon (PAH) contamination due to the oil spillage from waterways transportation system plying through the rivers of this region.[22] So, *P. aeruginosa* JCM 5962 was also evaluated for its potential to degrade hydrocarbons like diesel, petrol etc. *P. aeruginosa* JCM 5962 showed considerable growth in presence of hydrocarbons like petrol, diesel, naphthalene,

camphor and hexane in minimal media (Fig.6.a). Diesel degradation by *P. aeruginosa* JCM 5962 was the maximum as highest turbidity was obtained in presence of diesel after 7 days (Fig.6.a). This strain also showed moderate degradation of naphthalene, hexane and petrol (Fig.6.a). GC-MS analysis of *P. aeruginosa* JCM 5962 treated diesel showed a decrease in the area of major peaks of the control sample (untreated diesel), indicating breakdown of the main compounds, while appearance of few new peaks represented the degradation products or metabolites (Fig.6.b and c). The control sample consisted of peaks mostly for PAH (Fig.6.b) whereas the treated diesel sample showed very few peaks for PAH and more peaks for straight chain alkanes, alkenes and alkynes (Fig.6.b). Recently, Bakaeva and coworkers have also shown that several *Pseudomonas* strains with plant growth promoting properties are capable of degrading hydrocarbons as well.[44] The findings of the present investigation, thus corroborates well with other studies done.

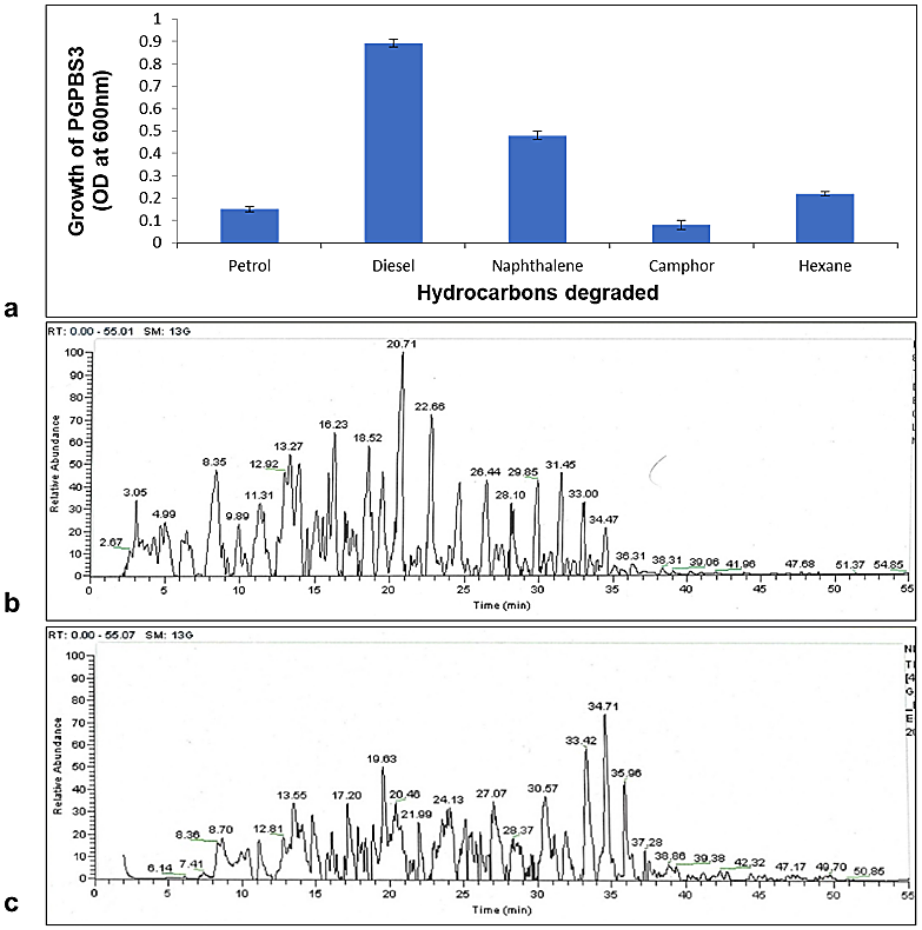


Fig. 6: Biodegradation of hydrocarbons by *P. aeruginosa* JCM 5962 strain. (a) Degradation of different types of hydrocarbons by the bacterial strain *P. aeruginosa* JCM 5962. (b) GC-MS analysis of untreated diesel (control) sample and (c) GC-MS analysis of *P. aeruginosa* JCM 5962 treated diesel sample.

Determination of Plant Growth Promoting Potential of *P. aeruginosa* JCM 5962 on *Abelmoschus esculentus* (Okra)

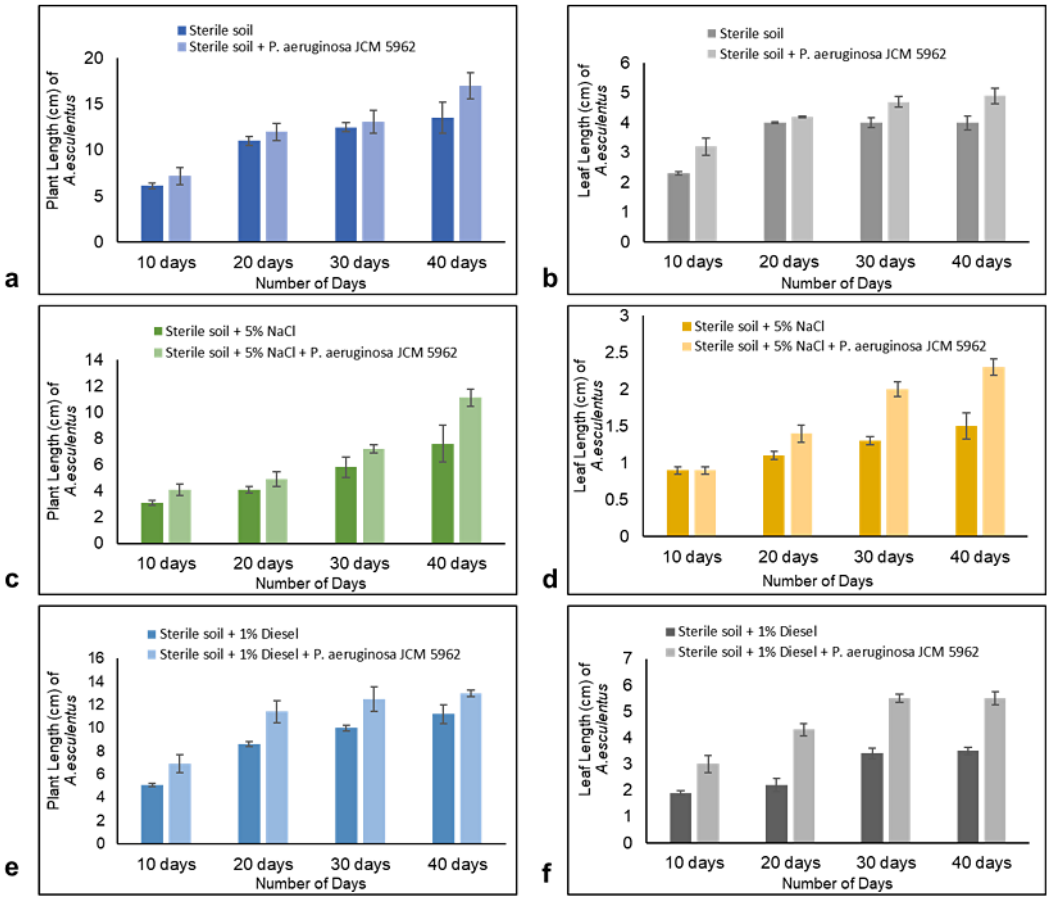


Fig 7: Study of plant growth promoting effect of *P. aeruginosa* JCM 5962 on *Abelmoschus esculentus* (Okra) plant. (a) Plant length and (b) leaf length of control set without inoculum (Set 1) and test set with *P. aeruginosa* JCM 5962 inoculum (Set 2); (c) plant length and (d) leaf length of saline soil without bioinoculum (Set 3) and with bioinoculum (Set 4); (e) plant length and (f) leaf length of diesel contaminated soil without bioinoculum (Set 5) and with bioinoculum (Set 6).

Plant growth promotion of PGPBS3 isolate was demonstrated through a series of experiments on *Abelmoschus esculentus* (Okra) plant. *A.esculentus* is a vegetable that is cultivated regularly in rice-based cropping system in the Sundarbans mangrove saline soil as the alternative crop in between two seasons of rice cultivation and hence was chosen for this study.[45,46] Before attempting direct field trials of *P. aeruginosa* JCM 5962 on plant growth promotion in the Sundarbans mangrove soil, potted experiments were done to evaluate the potency of this isolate as a future biofertilizer. In the potted experiments, the soil used was sterilized so that the contribution, if any, from the natural microbiota of that soil, would be eliminated. Details of the experimental setup is given in Table.1 in the methods section. In the control set (Set 1), no inoculum was added and *A.esculentus* seeds were planted. In Set 2, the effect of addition of *P. aeruginosa* JCM 5962 isolate on the growth of *A.esculentus* seeds was observed. Both the plant length and the leaf length increased significantly after 40 days when the bioinoculum

was added to the sterilized soil (Set 2) compared to the uninoculated control soil Set 1 (Fig.7.a and b). In Sets 3 and 4, the effects of addition of the bioinoculum was evaluated under saline conditions. Fig.7.c and d shows the increase in both plant length and leaf length of *A.esculentus* plants observed after 40 days, on addition of the PGPBS3 isolate to the soil. Introduction of 5% NaCl in the soil decreased the overall growth of *A.esculentus* whereas addition of *P. aeruginosa* JCM 5962 inoculum to the saline sterile soil increased the overall growth of the plants (Fig.7.c and d). In Sets 5 and 6, 1% diesel was added to the potted sterile soil to mimic the hydrocarbon contaminated soil condition. In presence of the bioinoculum, the plant length and leaf length of A.*esculentus* increased significantly after 40 days (Fig.7.e and f). In general, the application of *P. aeruginosa* JCM 5962 bioinoculum significantly increased both plant length and leaf length of *A.esculentus* plants under normal, saline and diesel contaminated conditions respectively. Thus, this study indicates that *P. aeruginosa* JCM 5962 with the ability to fix atmospheric nitrogen, solubilize insoluble phosphate and produce indole acetic acid, can be used as a potent biofertilizer in saline and hydrocarbon contaminated soils for nutrient management for crops.

Conclusion

In the present study, a potent plant growth promoting *Pseudomonas aeruginosa* JCM 5962 bacterial strain with bioremedial and salt tolerance properties have been isolated from the rhizospheric soil of Matla riverbed in Sundarbans mangrove area. In the last decade, a considerable amount of research has been dedicated to the isolation and characterization of either PGPRs with halotolerance or PGPRs involved in bioremediation of soil contaminants. The application of PGPR in petroleum hydrocarbon degradation is a relatively new field of research which is still largely unexplored.[47] Although, previously, different species of the genus *Pseudomonas* have been reported for their plant growth promoting traits,[4] halotolerance[20,43] and hydrocarbon degradation abilities,[48] the present investigation is relevant and significant in this context because the *Pseudomonas* isolate obtained here shows all of these properties simultaneously. *P.aeruginosa* JCM 5962 strain isolated in this work, can fix atmospheric nitrogen and solubilize insoluble mineral phosphate simultaneously with concomitant IAA production (37.14 µg/mL), high halotolerance (upto 7%) and significant bioremediation of hydrocarbon (particularly diesel) contaminants in soil. Plant-based assay with *Pseudomonas aeruginosa* JCM 5962 bioinoculum has also shown significant improvement in the overall growth of *A.esculentus* (Okra) plants in three types of soil conditions, namely, absence of any abiotic stress (normal condition), presence of high salinity (5% salt stress) and presence of 1% diesel contamination. The increase observed in plant length and leaf length of the bioinoculum treated plants compared to the control plants clearly indicated that *Pseudomonas aeruginosa* JCM 5962 can be developed as a potent biofertilizer for its plant growth promoting characteristics as well as alleviation of abiotic stresses like high salinity and high hydrocarbon pollution, thereby aiding sustainable agriculture in the harsh soil environments of Sundarban mangrove regions.

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Conflict of Interest

There is no conflict of interest and the research work is not submitted to any other journal.

Data Availability

Data will be made available on request.

Authors Contribution

Biswajit Saha – Conceptualization, Experiment, Validation, Data analysis, Resources,

Dr. Aparna Sen – Supervision, Conceptualization, Data analysis, Writing-original draft, review and editing, Resources

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