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# Multifaceted plant growth promoting potentials of *Pseudomonas aeruginosa* AP isolated from Dandi, Gujarat, India

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**Abstract**
Salt marshes of Dandi are well known for India's Independence Movement, when Mahatma Gandhi undertook the Salt Satyagraha also known as Dandi March in 1930. Bacterial isolate 36 was isolated from Dandi was characterized for its plant growth promoting (PGP) potential. It was examined for P solubilisation (45 µg/ml), ammonia production (9.4 µg/ml) and IAA production (18 µg/ml). Isolate produced siderophore as it showed orange halos on CAS agar. Isolate showed positive HCN production, whereas, negative in starch hydropysis and chitinase production. Isolate was identified by VITEK 2 as *Pseudomonas aeruginosa* AP. PGP efficacy was tested by talcum based formulation on green gram and black gram. Experiment was comprised four treatment T1= Seed (unsterilized-soil), T2 = Seed (sterilized-soil), T3= bio formulated seed (unsterilized-soil) and T4 = bio formulated seed (sterilized-soil) using CRD. T4 is statistical significant compared to other treatments. Thus, *Pseudomonas aeruginosa* AP can be used effectively as PGPR.

**Keywords:** IAA, PGPR, Phosphate solubilization, *Pseudomonas*, Siderophore

## Introduction
Plant root exudates play in key role in proliferating microbial population of particular agricultural niche. It decides the type and density of rhizosphere microbes. *Pseudomonas* a gram negative bacterium is cosmopolitan and used versatile metabolism. *Pseudomonas aeruginosa, P. chlororaphis, P. fluorescens, P. stutzeri, P. pituda* etc. are some well-known nonpathogenic bio control agents showing strong plant growth-promoting activities [12, 18, 26, 27, 32]. Research has already done on root colonization by pseudomonas, factor affecting root colonization, their antagonistic effect and rhizosphere competence [1, 33]. Pseudomonas are very well known for their plant growth promoting activity ranging from P solubilization, siderophore production, IAA production, HCN production and many more.
Due to metabolic versatility, *P. aeruginosa* can adapt itself in both natural and man-made environment [9]. Efficient root colonization in rhizosphere and their acclimatization under adverse soil conditions are options for soil bacteria to survive and compete with other soil microbes [8]. They are ubiquitously present in soil, fresh water, sea water, skin flora, and the rhizosphere [29, 31]. Present study aims to evaluate the efficiency of isolated marine bacterium *P. aeruginosa* for its plant growth promoting traits and it's *in vitro* plant growth promoting ability green gram (*Vigna radiate*, Variety : Vishal) and black gram (*Vigna mungo*, Variety : GU1).

## Materials and Methods
Bacterial isolate 36 isolated previously in our laboratory from saline soil of Dandi, Gujarat (21.3333° N, 72.6333° E) was used in the study. Isolate 36 was evaluated for its following PGPR phenotype.

### Identification of the organism
For identification of bacterial isolate 36, morphologically, colony characteristics and biochemical analysis was performed. Morphological characterization was done using Gram's reaction whereas, for colony characteristics, isolate was grown on nutrient agar. Biochemical characterization was done as per Bergey's Manual of determinative bacteriology. Furthermore, identification was confirmed using VITEK 2 (Bio merieux, USA).

**Qualitative and quantitative estimation of phosphate solubilization**

Bacterial isolate 36 was tested for its phosphate solubilization on Pikovskaya's agar plate. A loopful of culture was placed on pikovaskaya agar (HI Media, India) and incubated for 48 hrs. Size (mm) of the halo around colony was measured. For quantitative estimation, isolate was grown in liquid Pikovskaya's medium as method describe by Pikovskaya [22]. The concentration of the soluble phosphate was estimated by stannous chloride method [15]. From the supernatant after 4 days of incubation.

**Siderophores estimation**

Cells were examined for their siderophore production under iron stressed conditions. Bacterial isolate 36 was grown on Chrome azurol S (CAS) agar plate as method described by Schwyn and Neilands [27]. Plates were incubated at room temperature for 24-48 hrs and observed for their orange color halos near colonies. Isolate was also grown on King's B medium to observe the fluorescence production by cell. Siderophore produced by isolate was also quantified using CAS-shuttle assay [27]. Cells were grown in deferated nutrient broth and incubated at 28 °C. Control comprised inoculated media. 5 ml of sample was withdrawn after 3 days of incubation and centrifuged at 5000rpm for 15 min. 1.5 CAS assay solution was added to 1.5 ml of culture supernatant and was incubated for 20 min. Siderophore binds to dye complex and remove iron from dye. Change in the blue color intensity is recorded at 630 nm and siderophore percentage was calculated formula:

% siderophores = [(Ar – As)/Ar] x 100, where, Ar=absorbance of control (nutrient broth +CAS assay solution), As =absorbance of sample (test culture supernatant + CAS assay solution).

**Indole acetic acid production**

IAA production was estimated using the method described by Brick and co-workers [5]. 1% of 18 hrs grown culture of isolate 36 was inoculated in 50 ml of nutrient broth and nutrient broth supplemented with treptophan (1 mg/ml). Control comprised inoculated nutrient broth. IAA production was examined after 48 hrs of incubation. Sample was collected in aseptic condition and centrifuged at 8000 rpm for 10 min. For quantitative measurement of IAA, each of 1 ml cell-free supernatant was mixed vigorously with 1 ml Salkowsky's reagent (1 ml of 0.5M $FeCl_3$ in 50 ml of 35% $HClO_4$) and two drops of ortho phosphoric acid and assay system was kept at room temperature in dark for 20 min. Developed pink color was measured by optical density using spectrophotometer at 535 nm. The concentration of IAA was extrapolated from standard curve of IAA prepared using 100 µg/ml of standard IAA.

**Ammonia production**

For estimation of ammonia, 1% of 18 hrs grown cells were inoculated into peptone water and incubated for 7 days. Supernatant was collected by centrifugation at 8000 rpm for 10 min. 3 ml of supernatant was mixed with 0.5 ml of Nessler's reagent. Development of brown to yellow color indicates positive for ammonia production [7]. Optical density was measured at 450 nm with help of the spectrophotometer. The concentration of ammonia was extrapolated from standard curve of ammonium.

**Chitinase test and hydrocyanic acid production test**

For chitinase assay, basal medium (medium comprising per liter 0.3 g of MgSO4.7H2O, 3.0 g of (NH4)2SO4, 2.0 g of KH2PO4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 µl of Tween-80, and 0.15 g of Bromocresol purple; pH was adjusted to 4.7 and then autoclaved at 121°C for 15 min) was supplemented with 4.5 g of colloidal chitin. Colloidal chitin was prepared as per method described by Roberts and Selitrennikoff [25]. Cells were streaked on medium and incubated at 28 ± 2 °C and were observed for colored zone formation.

For the qualitative estimation of HCN production, Picrate assay described by Kang and colleague [14] was performed. Briefly, nutrient agar supplemented with 4.4 g/L glycine and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 0.5% picric acid solution was placed between the base and lid of the petri dish. Plates were sealed with par film and incubated at 28 ºC for 48 to 72 hrs. Change in the filter paper color from yellow to orange/brown indicates the production of HCN by isolate.

**Starch hydrolysis**

To evaluate the starch hydrolysis efficiency of the isolate, cells were grown on starch agar (comprised g/L; meat Extract 3.0, peptic digest of animal tissue 5.0, starch soluble 2.0, agar 15.0 g, pH 7.2±0.1 (HI Media). Plates was inoculated with cells and incubated for 24 – 48 hrs at 28 ºC. After incubation, starch agar plate was flooded Grams Iodine to observe colorless zone surrounding the colonies. A clear zone indicates starch hydrolysis whereas, blue or purple zone indicates that starch is not hydrolyzed.

***In vivo* PGP efficacy**

Plant growth promoting efficacy was tested by seed germination assay. Green gram (*Vigna radiate*, Variety : vishal) and black gram (*Vigna mungo*, Variety : GU1) were procured from Mega Seed, Pulses and Castor Research Unit, Navsari Agricultural University, Navsari and used in experiment. To access the *In vivo* PGP efficiency of isolate, a talcum based formulations. 20% of 18 hrs grown culture was mixed with sterilized talcum powder mixture (1.5% CaCO3 and 1.0% CMC) under aseptic conditions. This formulation was used for seed factorization of seeds. Seeds were surface sterilized before application of talcum based bio formulation using 0.3% mercury chloride [24]. After sterilization, seeds were mixed with talcum based bio formulation under aseptic condition and incubated for 6 hrs to coat seed with bacterial isolate. Here, CMC allows bacterial adhesion to seed and talcum works as carrier material. Seed germination assay was performed in plate as well as in pot culture. In plate assay, seed treated with above bio formulated bacteria and untreated seeds (control) were incubated for 7 days in sterile petriplates (15 cm diameter size). For pot experiment four treatment T1=Control seed (No treatment, unsterilized soil), T2 = (No treatment, sterilized soil), T3= bio formulated seed (unsterilized soil) and T4 = bio formulated seed (sterilized soil) was used. Sterilized soil was used to minimize the effect of other PGPR present in soil on seed germination and plant growth promotion. Now, bacterial coated seeds (10 seeds in each pot) were sowed in pot (15 cm diameter, 9 cm height) and plant growth promoting parameter i.e., number of leaves, shoot length and root length were measured after 14$^{th}$ day of incubation. Soil used for experiment was analyzed for its physical and chemical properties. Soil used in pot experiment

was analyzed for pH, EC, N, P, K, Exchangeable sodium% and cation exchange capacity using standard methods.

**Statistical analysis**
Plant growth promoting efficacy of bacterial isolate 36 was alanyzed using CRD with four repetitions. For seed germination in plate assay, data were analyzed after 7$^{th}$ days while in pot experiment, data were analyzed after 14$^{th}$ days of incubation.

## Results

**Identification**
Bacterial isolate 36 was isolated from the water sample collected from marine water collated from Dandi. Total 36 bacteria were isolated from the marine water. Isolate 36 was screened for its PGPR activities. Morphological characterization using Gram's reaction showed that isolate was Gram negative short rod. Colony characteristics suggested that isolate produced greenish blue color colony typical characteristic of *P. aeruginosa*. Biochemical analysis revealed that oxidase, Citrate utilization, nitrate reduction, gelatinase, manitol were positive whereas, indol, MR, VP, $H_2S$ production, uric acid utilization, glucose, lactose, sucrose, maltose, xylose and urease negative. Identification was confirmed using VITEK 2 and isolate was named as *Pseudomonas aeruginosa* AP. PGPR traits were identified by following tests.

**Phosphate solubilising ability**
To evaluate the ability of P solubilisation, isolate was grown on pikovaskaya agar plate. Isolate showed a halo around the colonies indicates P solubilisation. It shows 1.4 cm zone and solubilise the phosphate. For estimation of quantitative P solubilisation, cells were grown in Pikovskaya's broth. It solubilizes 45 µg/ml of tri-calcium phosphate present in media.

**Siderophore estimation**
Qualitative assay for siderophore production was performed on CAS agar. Isolate showed 1.2 cm in diameter orange haloes near colony indicates siderophore production (Figure 1b). When grown on King's B medium, it produced yellow green pigment and fluorescent under UV light (Figure 1a). It produced 84.6% siderophore after 3 days of incubation as estimated by CAS shuttle assay. Thus isolates has ability to solubilize iron and provide to plant.

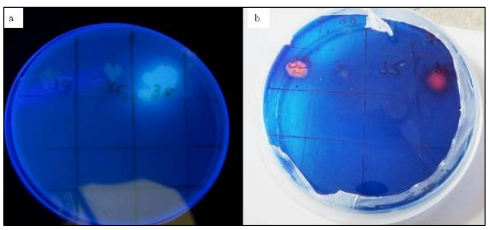

**Fig 1:** Fluorescence under UV light (a) and orange halo near colony on CAS agar by *P. aeruginosa* AP

**IAA production**
Induction of indole acetic acid production was measured after 48 hrs of incubation. Cells were grown in LB broth and LB broth supplemented with treptophan (1 mg/ml) to observe the effect of treptophan on IAA production. Cells grown in treptophan supplemented media produced higher IAA (31 µg/ml) compared to cells grown only in LB broth (18 µg/ml). Thus, data suggests that addition of tryptophan trigger IAA production.

**Ammonia production**
Isolate 36 has ability to utilize peptone and convert it into ammonia. On addition of Nessler's reagent to supernatant, it developed a brown color. It produced 9.4 µg/ml of ammonia after 7 days of incubation.

**Chitinase test and hydrocyanic acid production test**
To evaluate the chitniase production, cells were grown on media containing chitinase as sole carbon source. Growth was not observed even after 72 hrs of incubation indicated absence of chitinase production. Isolate showed positive for HCN production. Picric acid dipped yellow color filter paper started to turn brown after 24 hrs of incubation. After 48 hrs of incubation, it was completely converted yellow to brown.

**Starch hydrolysis**
On starch agar, on addition of lugol's iodine no color change was observed and no clearance zone near colony was found. Thus, isolate showed negative for starch hydrolysis.

**Seed germination**
Result of seed germination in plate assay showed higher root length and shoot length in treated seeds while numbers of leaves are in same in all the treated seeds (Figure 2). However the size of leaves was bigger compared to control in both Vishal and GU1 seeds. In pot culture number of leaves, soot length and root length were higher in T4 in both Vishal and GU1 variety. Data are statistically significant as shown in Table 1. In Vishal variety, higher numbers of leaves were in T4 treatment followed by T3 and T2 and least was in T1. In similar pattern root length and shoot length was also higher in T4 treated seeds followed by T3, T2 and least in T1. In GU1 variety, number leaves were higher in T4 followed by T3 and T2. Root length and shoot length of GU1 were also higher in seed treated with T4, followed by T3 and T2. Control comprised least growth in all parameters.

**Soil analysis**
Soil used in pot experiment was analyzed for physic-chemical properties (Table 2). It has pH 6.75 and EC 0.055 ds/m. It contains 196.38 kg/H nitrogen, 52.253 kg/H phosphate and 164.82 kg/H of potassium present in the soil sample. Exchangeable sodium percentage was 1.51% and cation exchange capacity was 22.95 mg/100g.

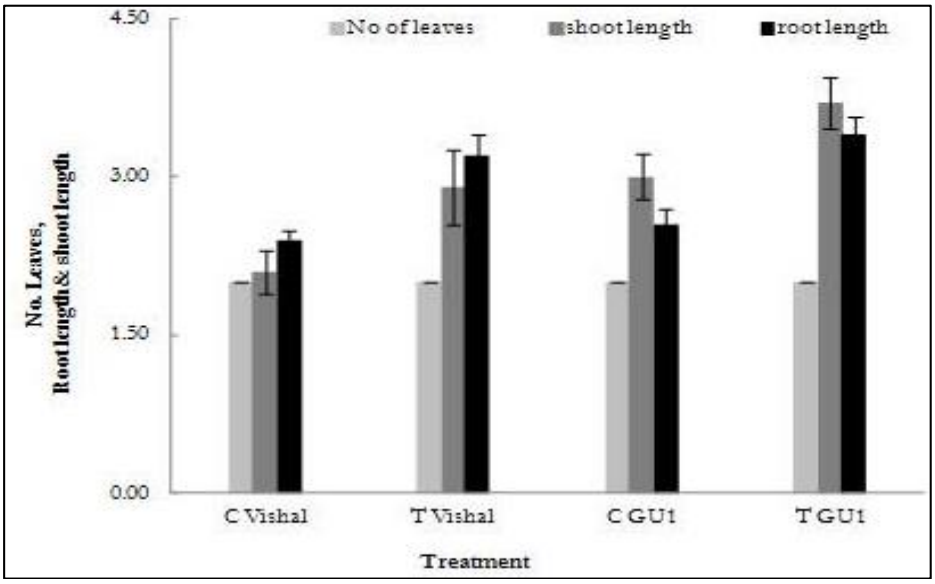


**Fig 2:** Effect of *P. aeruginosa* AP on *Vigna radiate and V. munga* seed germination in plate assay (C=Control; T=Test; error bar indicates ± standard deviation)

**Table 1:** Effect of talcum based bio formulation of *P. aeruginosa* AP on seed germination of *Vigna radiate* and *Vigna mungo* under pot condition

| Variety | *Vigna radiate* (Variety Vishal) | | | *Vigna mungo* (Variety GU1) | | |
|---|---|---|---|---|---|---|
| **Treatment** | **No of leaves** | **Shoot length** | **Root length** | **No of leaves** | **Shoot length** | **Root length** |
| T1 | 1.95 | 4.68 | 4.30 | 2.03 | 7.93 | 4.80 |
| T2 | 2.08 | 5.75 | 5.00 | 2.15 | 8.53 | 5.13 |
| T3 | 2.13 | 6.10 | 5.30 | 2.13 | 9.68 | 5.78 |
| T4 | 2.35 | 7.95 | 7.90 | 2.50 | 10.15 | 6.35 |
| S.Em | 0.04 | 0.09 | 0.06 | 0.05 | 0.08 | 0.05 |
| CD @ 5% | 0.14 | 0.27 | 0.20 | 0.17 | 0.23 | 0.17 |
| CV% | 4.19 | 2.86 | 2.30 | 5.00 | 1.66 | 1.99 |

T1=Control seed (No treatment, unsterilized soil), T2 = (No treatment, sterilized soil), T3= bio formulated seed (unsterilized soil) and T4 = bio formulated seed (sterilized soil)

**Table 2:** Physico-chemical properties of soil used for *in vitro* study

| Sr. No | Physico-Chemical parameter | Amount present |
|---|---|---|
| 1 | pH | 6.75 |
| 2 | EC | 0.055ds/m |
| 3 | Exchangeable Na% | 1.15% |
| 4 | Cation exchange capacity | 22.95 mg/100g |
| 5 | Nitrogen | 196.38 kg/H |
| 6 | Phosphorus | 52.253 kg/H |
| 7 | Potassium | 164.82 kg/H |

## Discussion

Application of haphazard use of chemicals fertilizers and pesticide leads to incredible harm to ecosystems and all biota. Alternative to these chemical applications, biofertilizer are one of the best solution to combat the hazard. These microbes can be used as both plant growth promoting bacteria and as biocontrol agent for suppression of plant pathogenic microbes. Most commonly known plant growth promoting bacteria are *Bacillus, Rhizobia, Pseudomonas, Azotobacter* etc. However, the genus *Pseudomonas* is one the best characterized as PGPR and as biocontrol agent [30]. Being a PGPR, it has ability to solubilize phosphate, nitrogen fixation, iron chelation by siderophores and phytohormone production whereas, being a biocontrol agent it has ability to produce HCN and secondary metabolites to suppress the growth of plant pathogenic microbes [10, 11, 13, 23, 28]. Hence, due to its multifaceted capability makes it appropriate as biofertilizer and biocontrol agent in agriculture field.

In present study isolated 36 bacterium identified as *Pseudomonas aeruginosa* AP have multifaceted plant growth promoting traits. Phosphorus is the most important key element in the nutrition of plants after nitrogen. It requires for photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration and thus plays a pivotal role in plants. Mostly soil are rich in phosphorus, however it is not utilized by plant as generally these phosphate is either in its insoluble form or immobilized in soil [20]. Here, PGPR play a role by solubilizing phosphate via organic acid production [6]. *Pseudomonas aeruginosa* AP has ability to produce organic acid and able to solubilize phosphate and make it available for plant absorption.

Here isolate is also able to produce iron scavenging molecule Siderophore. Siderophore chelate the iron and make it unavailable for the other plant pathogenic microbes and thus indirectly works as bio control agent. Siderophore from *Pseudomonas* is well studied and characterized [4, 16, 19]. Many PGPR have ability to produce plant hormone IAA and is helpful in root initiation, cell division and cell enlargement [21]. In present study *Pseudomonas* produced IAA and tryptophan addition stimulate more production of IAA. Asghar and co-worker reported L -tryptophan- dependent auxin production and observed that it increase the grain yield and the number of branches [2, 3]. In present study isolated *Pseudomonas* increase the number of leaves, root length and shoot length in both the tested seed of *Vigna radiate* and *Vigna munga.* Thus it can be used as plant growth promoter for both these plants.

*Pseudomonas* aeruginosa AP isolated from the Dandi, saline soil is able to solubilize phosphate and produce siderophore. It has ability to increase plant growth by IAA production and addition of tryptophan increase the IAA production. It showed also showed PGP activity on seed germination indicates it efficacy as biofertilizer. However, field trial is needed to check its efficacy in field and its survival in environmental condition.

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