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Isolation and identification of drought resistant bacteria for plant growth promoting properties and their effect on seedling under salt stress

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Abstract

Drought stress affects the water relations of a plant at cellular and whole plant level causing economic losses in agriculture. Inoculation of plants with beneficial micro-organisms promotes plant growth and increases drought tolerance in arid or semiarid areas.

The soil sample were collected from salt rich area for isolation of Phosphatase producing bacteria. The sample were serially diluted and plated on the nutrient agar medium. About 6 different isolates were obtained from the soil sample and it was sub cultured to get pure isolates. The isolates from salt rich area were screened for Phosphatase production in Pikovskaya medium. It is isolated by two methods, streak method and agar well diffusion method. Both the experiments gave a better result for Phosphatase activity. It observed the zone of clearance. Selected bacterial strain was tested for the optimization method.

Optimisation of enzyme production is done using para Nitrophenol Phosphate assay to produce the maximum enzyme activity, in turn the maximum total protein content which was confirmed using Bradford assay. The maximum enzyme activity is seen at temperature of 40^0C, time period of 60 hours and pH of 9 and this is concluded to be the optimised conditions. These conditions are implied to produce phosphatase enzyme in large amount. Also, the ability of phosphatase produced from the potent bacterial strain under optimal conditions to promote growth in maize has also been checked and compared with the common fertilizers available commercially. The results show that the phosphatase enzyme isolated from potent strain in combination with fertilizer produced better growth in terms of weight and length of the shoot and root.

Keywords: Drought stress, Phosphatase, isolation and identification, Optimisation of enzyme.

Introduction

Dessert environment is characterized by high temperature, soil salinity, lack of rainfall and less nutrients. These factors are consider as a major limiting factors of dessert environment and give positive role in adaptation of dessert microorganisms. Province of Gujarat, India covers an area of 4953.7sq km desert named Rann of Kutch. It is located in 22°55" to 24°35" North latitudes and 70°30" to 71°45" East longitudes near the Great Rann of Kutch. In spite of its uniqueness, the diversity of rhizobacteria and its role in adaptation of plants under desert ecosystem is less studied. In arid condition plant growth promotion facilitated by plant growth promoting bacteria (PGPB). Drought resistance PGPB helps direct or indirect way in dessert farming. Many researchers have identified PGPB producing phytohormones such as auxin, cytokinins, gibberellins, and abscisic acid confer the drought resistance to host plant. For that reasons, the search for new PGPB with multiple traits becomes interesting and, they can be used as inoculates for bio-fertilization, phytostimulation and bio-control purposes in desert farming.

Saline soils are known to suppress the growth of plants. Plants grown in saline soil are naturally colonized by microorganisms for their adaptation. In India, chilli (*Capsicum annuum*) is major cultivated plant and its fruits are mainly consumed either fresh or dry. However, chilli is exposed to many biotic (virus, fungi) and abiotic stress, especially salinity, which has a negative effect on chilli growth and yield. The aim of present study was development of dessert farming using drought resistance rhizobacteria. Rhizospheric microorganisms were isolated from rhizospheric soil of Kutch dessert area of Gujarat.

Isolated samples were screened for their drought resistance and PGP properties. Positive isolates were characterized by molecular biological methods. In this way, the aim of this work was to identify and characterize novel bacteria from Kutch desert area of Gujarat exhibiting drought resistance and PGP properties. The plant growth promoting and drought tolerance enhancing ability of those isolates were evaluated on chilli seedlings.

Drought stress affects the water relations of a plant at cellular and whole plant level causing economic losses in agriculture. Inoculation of plants with beneficial micro-organisms promotes plant growth and increases drought tolerance in arid or semiarid areas. Plant growth promoting rhizobacteria (PGPR) are adapted to adverse conditions and protect plants from the deleterious effects of some environmental stresses. Under drought stress bacterial cells accumulate compatible solutes such as amino acids, quaternary amines, and sugars that prevent degenerative processes and improves cell growth under adverse osmotic conditions . The term ‘induced systemic tolerance’ (IST) has been proposed for PGPR-induced physical and chemical changes that result in enhanced tolerance of plants to abiotic stress. Timmusk and Wagner (1999) were the first to show that inoculation with PGPR *Paenibacillus polymyxa* confers drought tolerance in *Arabidopsis thaliana* through the induction of drought responsive gene, ERD15 (early response to dehydration 15). Inoculation of *Azospirillum brasilense* Sp245 in wheat (*Triticum aestivum*) under drought stress resulted in a better water status and an additional ‘elastic adjustment’ leading to better grain yield and mineral quality (Mg, K, and Ca) (Creus *et al*.., 2004). Plants treated with exopolysaccharide (EPS) producing bacteria display increased resistance to water stress. The EPS producing strain *Pseudomonas putida* strain GAP-P45 forms biofilm on the root surface of sunflower seedlings and impart tolerance to plants against drought stress. The inoculated seedlings showed improved soil aggregation and root-adhering soil and higher relative water content (RWC) in the leaves (Sandhya *et al*.., 2009).

Drought stress is one of the major agricultural problems reducing crop yield in arid and semiarid regions of the world. Changes in mean global air temperature and precipitation patterns are leading

to longer drought periods and more extremely dry years, and more severe drought conditions will hinder food production in some countries . At present, strategies to increase the ability of plants to tolerate drought stress involve the use of water-saving irrigation, traditional breeding, and genetic engineering of drought-tolerant transgenic plants. Unfortunately, these methods are highly technical and labor-intensive, and thus difficult to apply in practice.

On alternative for growing plants under dry conditions is the use of plant growth promoting rhizobacteria (PGPR). PGPR are a group of bacteria that can be found in the rhizosphere in association with plant root systems, both at the root surface and in endophytic associations, and which can either directly or indirectly facilitate plant growth in optimal, biotic, or abiotic stress conditions. Known mechanisms used by PGPR include nitrogen fixation for plant use, phytohormone production (including auxins, cytokinins, and gibberellins), solubilization of mineral phosphates, and iron sequestration by bacterial siderophores . In addition, PGPR are linked to catabolism of molecules related to stress signaling, such as bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Many PGPR have been shown to alleviate drought stress effects in plants by reducing plant ethylene levels that are usually increased by unfavorable conditions. However, the ability of inoculated bacteria to survive, outcompete with the native microflora, and colonize in the rhizosphere remains to be a critical step for successful application especially in drought-stressed soils since microorganisms not adapted to high water tension will die under these adverse conditions. The drought-tolerant rhizobacteria could thus be advantageous over others to thrive in a new drought environment in the sufficient numbers to deliver beneficial effects on plants.

Drought also induces free radicals affecting antioxidant defenses and Reactive Oxygen Species (ROS) such as superoxide radicals, hydrogen peroxide and hydroxyl radicals resulting in oxidative stress. At high concentrations ROS can cause damage to various levels of organization, like initiate lipid peroxidation, membrane deterioration and degrade proteins, lipids and nucleic acids in plants .Nevertheless, under drought stress the decrease in chlorophyll content was symptom of photooxidation. Decreasing of chlorophyllcontentin Paulownia imperialis, bean andCarthamustinctorius was observed under drought stress. Drought also affects biochemical activities like nitrate reductase (NR), due to lower uptake of nitrate from the soil. It also accentuates the biosynthesis of ethylene, which inhibits plant growth through several mechanisms . Drought as a multidimensional stress, affects at various sub cellular compartment, cell organs and whole plant level . Thus drought negatively affects quantity and quality of growth in plants. Therefore, in order to produce more food, the mitigation of drought stresses is important to achieve the designated goals. Worldwide extensive research is being carried out to develop strategies to cope with drought stress through development of drought tolerant varieties, shifting the crop calendars, resource management practices etc. and most of these technologies are cost-intensive. Recent studies indicate that microorganisms can also help plants to cope with drought stress.

In India, the common method to deal with ‘P’ deficiency is using rock phosphate/ mono-ammonium phosphate/ diammonium phosphate/ superphosphate. This practice in addition to high cost and low efficiency can endanger our environment by irreparable damage to our soil health due to soil sterility, phosphate toxicity and pollution problems . The enhancement in various agronomic yields due to it, has been reported because of the production of growth stimulating phytohormones such as Indole Acetic acid (IAA), Cytokines, Giberrellic Acid (GA), Zeatin, Ethylene and Abscisic Acid (ABA) . Generally, ethylene is an essential metabolite for the normal growth and development of plants. This plant growth hormone is produced endogenously by approximately all plants and is also produced by different biotic and abiotic processes in soils and is important in inducing multifarious physiological changes in plants. Apart from being

a plant growth regulator, ethylene has also been established as a stress hormone. Under stress conditions like those generated by salinity, drought, water logging, heavy metals and pathogenicity, the endogenous level of ethylene is significantly increased which negatively affects the overall plant growth (Munees Ahmad and Mulugeta Kibret, 2014)

Aim and Objectives

Aim

To investigate the use of isolation and characterization of drought resistance bacteria for growth promoting properties and their effect on seedling under salt stress

Objectives

- Collection of rhizosphere soil from salty area
- Isolation of drought resistance bacteria
- Screening of plant growth promotion bacteria using PSB method
- Assay of Plant growth promotion by chemical methods
- Optimization methods
- Production of PGP bacteria
- Application of PGP (pot method)

Materials and Methods

Collection of Soil Samples

Soil samples were collected from rhizosphere region of brinjal and groundnut plants which were taken from farm soils. The samples were stored aseptically in polythene bags under laboratory conditions.

Preparation of Soil Dilution, Inoculation and Incubation

Ten gram of soil was dissolved in 100 ml of sterilized distilled water and shaken for 15 minutes. Subsequent dilution was made following the serial dilution technique of Alexander (1965).

The dilutions from 10-6 to 10-9 were plated into the sterile nutrient agar plates using spread plate method. The sterile saline was used as blank. The plates were incubated at 37ºC for 24 hours. The cultured bacterial colonies were further purified by streak plate method using sterile nutrient agar medium. The oxygenic mono cultures were sub-cultured into sterile nutrient agar slants and nutrient broth for further use.

Isolation of Bacteria from Soil Sample

These soil were taken and subjected to the serial dilution technique using stock soil suspension which contain 1g of soil was dissolved in 10ml of sterilized distilled water and shaken for 15 mins. Subsequent dilution was made following the serial dilution technique of Alexander (1965). The collected sample and used for experiment and sterile saline as a blank and the diluted(10^{-4}) were plated into the sterile nutrient agar plates using spread plate method. The plates were incubated at 37ºC for 24 hours. The cultured bacterial colonies were further purified by streak plate method using sterile nutrient agar medium. The oxygenic mono cultures were subculture into sterile nutrient agar slants and nutrient broth for further use.

Screening for phosphatase activity

The isolated pure bacterial species were screened for the production of extra cellular phosphatase using modified Pikovskaya screening medium.

Modified Pikovskaya Agar medium

Modified medium consists of potassium dihydrogen phosphate and calcium chloride as substrate instead of tricalcium phosphate. This was done to speed up the halo zone formation. The colonies were inoculated in the modified Pikovskaya medium and incubated for 24 hours. The colonies that produced halo zone were considered to produce phosphatase enzyme and the colony which produced a greater zone was given for molecular sequencing and characterization.

The pure cultures were streaked on Sterile PVK plates and the plates were incubated at 37ºC for 2 days. The phosphate solubilization zone was observed around the colony and calculated the solubilization efficiency (SE) (Ramachandran *et al*.., 2002), SE = (Halo zone diameter-Growth diameter of microbe / Growth diameter of microbe) * 100. The positive bacteria which produces maximum alkaline phosphatase enzyme was selected and was used for further experiments.

Sub culturing of selected organism

The positive and better zone forming bacterial species was sub cultured in Nutrient medium agar plates. The pure cultures were retrieved every week and stored at 4°C for further experiment study.

Identification of Bacteria by 16srRNASequencing method

Isolation of bacterial genomic DNA

Procedure

- Nutrient broth was prepared, inoculated and incubated overnight.
- Transferred 1.5 ml of culture to a micro centrifuge tube and centrifuged at 10,000 rpm for 10 minutes.
- The pellet was collected and same procedure was repeated with another 1.5ml of culture containing cells.
- The pellet was re-suspended in 850µl of TE buffer.
- Added 100µl of 10% SDS and 5µl of proteinase-k. Mixed gently and incubated for 1 hour at 37°C.
- Added equal volume of PCI mixture and mixed gently by inverting the tubes until the two phase are completely mixed.
- Centrifuged the mixture at 10,000rpm in a microfuge tube for 10 mins.
- Transferred the upper aqueous layer to a new centrifuge tube and re-extracted with equal volume of PCI mixture.
- Again the mixture was centrifuged at 10,000rpm in a microfuge for 10 minutes.
- The upper aqueous phase was transferred to a new centrifuge tube and added 100 µl of sodium acetate and mixed properly.
- Added 300 µl of isopropanol and mixed gently to precipitate the DNA.
- Mixture was centrifuged at 10,000 rpm for 10 minutes.
- The DNA pellet was washed with 70 % ethanol for 30 seconds and then centrifuged at 5,000 rpm for 5 minutes.
- The pellet was collected and resuspended the DNA pellet in 50µl of TE buffer, run 5µl in a 1% agarose gel.

Agarose Gel Electrophoresis

- 1X TAE was prepared by diluting appropriate amount of 50X TAE buffer .
- 0.5 g of agarose was weighed and it was added to 50 ml of 1X TAE. This gives 1% agarose gel.
- It was boiled till agarose dissolves completely and a clear solution results.
- The combs were placed in an electrophoretic set such that it is approximately 2 cm away form the cathode.
- 10 µl ethidium bromide was added per 100 ml of agarose gel once the temperature of the agarose solution reaches 50 C.
- The agarose gel was poured in the central part of the tank and was observed that air bubbles are not generated.
- The gel was kept undisturbed at room temperature for the agarose to solidify.
- 1X TAE buffer was poured into the gel tank till the buffer level stands at 0.5 to 0.8 cm above the gel surface.
- The combs were lifted gently, it was ensured that wells remain intact.
- The power cord was connected to the electrophoretic power supply and the samples were loaded in the wells in the desired order.
- The voltage was set at 50 V and the power supply was switched ON.

Amplification of 16S ribosomal RNA fragment

The isolated genomic DNA was used as a template in PCR amplification of 16S rRNA. The Universal primers used for amplification of 16S rRNA are 16S-RS-F (forward) 5' CAGGCCTAACACATGCAAGTC-3' and 16S-RS-R (reverse) 5'-GGGCGGWGTGTACAAGGC-3'. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM TrisHCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM MgCl2, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward and reverse primers and template DNA. The amplification reactions was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) that was programmed as follows: initial denaturation at 95ºC for 5 mins; 35 cycles of denaturation of 95ºC for 30 sec, annealing at 60ºC for 40 sec, extension at 72ºC for 60 sec and final extension at 72ºC for 7 mins. The PCR products were stored at −4ºC for further use. The amplified products were quantified in 1.2% agarose gel electrophoresis. PCR product (5 μL) and 1 μL loading dye was loaded along with 5 µL of 1 kb ladder into the separate wells. The gel was run at 100 volts for 1 hour and the amplified products were visualized under UV transilluminator.

Phylogenetic analysis

The Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequences obtained from both forward and reverse primers were edited and contigs assembled using DNA Dragon - DNA Sequence Contig Assembler (Sequentix version, 1.6.0. – Digital DNA Processor, Germany). The online program BLAST (NCBI-2012) was used in identifying the related sequences with known taxonomic information available at the databank of National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA). A phylogenetic tree was constructed with the MAFFT version 7 (Katoh et al., 2019), which involved sequence alignment and neighbor-joining method. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Preparation of Inoculum

For further production of enzyme and other parameters, the inoculum was prepared using Luria Bertani (LB) broth (Zolta´nPra´Gai, 2001). The pure culture was inoculate into sterile broth and was incubate at 37°C in a rotary shaker for overnight. The overnight culture was used as an inoculum for the mass production of enzyme.

Phosphatase Production Medium

The enzyme production was carried out by Shake Flask Fermentation using Pikovskaya production media. 500 ml of sterile production broth wasprepared and 5% inoculums were transferred aseptically in to the production medium. The inoculated medium was incubated at 37°C for 3 days and placed in a shaker at 120 rpm rotation for better aeration and growth of the organism.

Plate assay (Qualitative analysis)

The plate assay was performed using Pikovskaya agar medium. After solidification of the agar plates, around 10 mm diameter of well was cut out aseptically with the help of a cork borer. The well was filled with the culture filtrate (test) and other was filled with non-culture filtrate (control) and incubated at 37°C for 3 days in humid chamber. The observation was made to see the phosphate solubilizing zone around the well.

Optimization studies - Effect of Various Parameters for phosphatase production

Time:

250 ml of sterile production medium was prepared and 5% inoculum was added aseptically. The inoculated medium was incubated at 37°C

with shaking around 150 rpm. The culture was periodically drowned at 12 hrs intervals up to 108 hrs. After incubation the culture filtrate was examined for the total protein content and phosphatase activity. And the absorbance were noted at 400nm in uv-visible spectrophotometer. Followed by Bradford assay had been done and the values were noted at 595nm spectrophotometer.

pH:

250 ml of sterile production medium was prepared in different conical flasks and each flask was adjusted to different pH such as 5, 6, 7, 8, 9, 10 using 0.1N NaOH and 0.1N HCl. After sterilization flasks were inoculated with 5% inoculum. The flasks were incubated at 37 ºC at shaker around 10000 rpm for 20 minutes. The protein estimation and enzyme activity were estimated.46The O.D values were noted at 400nm. Followed by Bradford assay had been done and the values were noted at 595nm spectrophotometer.

Temperature:

250 ml of sterile production medium was prepared in different conical flask and inoculated with 5% inoculum. Each flask was incubated at different temperatures such as 25ºC, 30ºC, 35ºC, 40ºC, and 45ºC for 48 hours. The protein estimation and enzyme activity were estimated. The O.D values were noted at 400nm. Followed by Bradford assay had been done and the values were noted at 595nm spectrophotometer.

Chemical Assay (Quantitative analysis)

Alkaline phosphatase activity was measured spectrophotometrically by monitoring the release of p-nitrophenol from p-nitrophenyl phosphate (pNPP) at 400 nm. A typical reaction mixture contained 100 µl of enzyme diluted in 2ml of 200 mMTris buffer (pH 8.5), 100 µl of 5mM CaCl2, 100 µl of 500 µmolpNPP and the final volume was made up to 3 ml using distilled water. Blank was prepared by mixing 2 ml of 200 mMTris buffer (pH 8.5) and 1ml distilled water.

The reaction was performed at 37 C for 30 min and stopped by addition of 500 µl of 4 M sodium hydroxide. One unit of phosphatase is the amount, which hydrolyses 1 µmol of substrate per min. The standard curve obtained by absorbance of p-nitrophenol (0 - 500 µmol) at 400 nm was used for quantification of enzyme activity.

Assessment of phosphate solubilizing bacteria and its effect on growth of shoot and root in *Vignaradiata* (green gram)

Pot Trial

Group I: Control
Group II: Fertilizer
Group III: Enzyme
Group IV: Enzyme + Fertilizer

Procedure:

A pot experiment for phosphate utilization in Green Gram was made in surface sterilized pots (Plastic pots of about 6 X 10 inches). 8 kg soil was prepared and pH was adjusted to pH 8.

The soil was divided into eight equal parts and sterilized. After sterilization, 1kg of soil was filled in eight pots each, labelled as Control, Fertilizer, Phosphatase enzyme, Phosphatase Enzyme + Fertilizer.

10g seeds were soaked overnight and 10 seeds were sown in each pot. A thin layer of soil was sprinkled on the green gram. The pots were kept and 75% of moisture was maintained by pouring 15ml of source solution directly on seeded area. Growth was observed every day.

Data collection and analysis

The whole plant was pulled out from the experimental pot and used for experimental observation. Before placing the roots in the bucket, a sieve was placed at the bottom of bucket. Then by gradual movement of the roots in water, the roots were separated from the soil. Roots were further cleaned in gently running tap water and peat masses were removed with

forceps. Any broken root portion collected in the sieve was carefully washed out. The root portion was separated from shoot. The length of the shoot was measured from the base of the stem to the growing point of the youngest leaf. The length of root was measured from the growing point of root to the longest available lateral root apex. For fresh weight of shoot and root the portion was blotted dry and the weight was recorded before the materials could get desiccated. The number of primary and secondary branching was also counted by eye estimation.

Results

Isolation of microorganisms

After 24 hrs incubation in nutrient agar medium, few colonies were isolated based on the morphology, colour, shape, etc. Totally six colonies were isolated (Fig. 1).

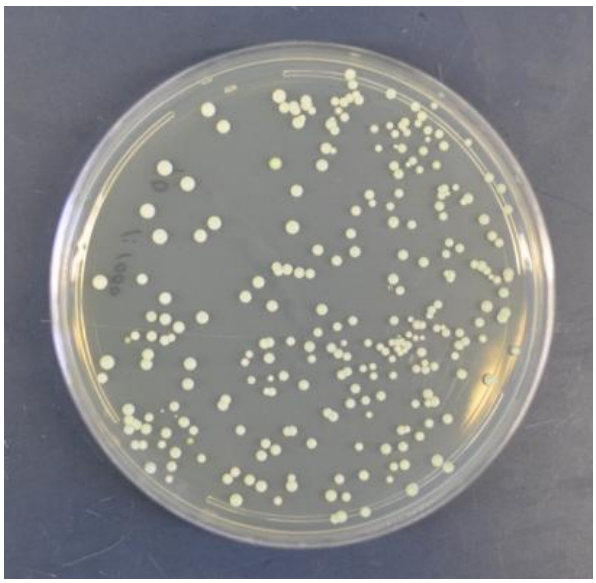

Fig. 1. Spread plate

These colonies were again sub-cultured in the nutrient agar medium for 24hrs for obtaining pure colonies (Fig. 2). These colonies were used for the screening for phosphatase activity.

Fig. 2. Pure cultures of isolated colonies

Screening for phosphatase activity

In this study, the modified Pikovskaya medium is used to screen the production of phosphatase enzyme. In correlation to our screening medium, Salcedo *et* al (2014) used Pikovskaya medium for screening the phosphatase enzyme.

The six colonies were transferred into the modified Pikovskaya's agar medium to screen the phosphatase enzyme. The plates were incubated at room temperature for 24hrs. After the incubation period, it was found that the isolates 2, 4, and 5 showed halo zone. The colonies that produced the halo zone concluded the production of phosphatase enzyme. This step was again repeated to find the potent strain. The colony which showed the maximum zone of inhibition was found to be isolate 4 (Fig. 3).

Fig. 3. Screening of isolated colonies for phosphatase activity

Optimization Studies

Time

The bacterial culture was withdrawn and checked for enzyme activity at every 12 hrs (0hr, 12hr, 24hr, 36hr, 48hr, 60hr, 72hr) and the enzyme activity was determined. The pNPP assay was performed on all the supernatants of the culture and absorbance was measured at 400nm.

Table 1. Phosphatase production on different incubation time period

S.No	Time of culture withdrawal (hr)	Absorbance
1	0	0
2	12	0.646
3	24	0.657
4	36	0.722
5	48	1.143
6	60	0.973
7	72	0.875

Based on these values we inferred that, 48 hrs of incubation showed the maximum enzyme activity (Table 1) and this was concluded to be the optimized condition. The same assay when performed on *E. coli* by Qureshi *et* (2010) showed the maximum enzyme activity at 24hrs incubation.

5.3.2 Effect of pH

Next to the temperature, the pH is the important parameter which determines the growth of the organism and phosphatase production. Usually most of the bacteria require medium pH for its growth. The requirement of the pH for its better growth is also based on the environmental pH where they grow.

Table 2. Influence of pH on phosphatase production

S.No	pH	Absorbance
1.	4	0.336
2.	5	0.472
3.	6	0.486
4.	7	0.525
5.	8	0.547
6.	9	0.480

In the present study, results showed that the higher production have been recorded at optimum pH range from 8 (Table 3), whereas Atekan*et al*(2014) isolated PSB from sugarcane and the optimization results were high at pH 6. Somashekar*et al*., (2010) optimized the extracellular ALP production from *Bacillus sp*and the results were almost similar to this study which was found to be pH 8.8.

Temperature

The environmental parameters are showing great influence in the growth of the organism and the production of enzyme. One of the main parameter is the temperature. To optimize the optimum temperature for better yield, production was made in various temperatures (25°C, 30°C, 35°C, 40°C, 45°C). The temperature requirement of the organism is based on the nature of environment where they grow. The same chemical assay was performed to check the absorbance.

Table 3. Effect of temperature on phosphatase production

S.No	Temperature (ºC)	Absorbance
1.	25	0.419
3.	30	0.902
4.	35	0.771
5.	40	0.665
6.	45	0.453

It was found that the isolate has shown higher phosphatase activity at 30°C (Table 2). Similar results were obtained by Karpagam and Nagalakshmi (2014) and their optimized temperature was found to be 35° to 40°C. Sanjotha and Sudheer (2016), in their study, reported that temperature of 42°C showed maximum enzyme activity.

Plate assay

A petri dish with the Pikvoskaya's agar medium was taken and two wells were created. One was considered to be the control and other was taken as the test well. The supernatant of the organism which showed the maximum zone of inhibition was introduced into the test well. The control well was left empty. After 24hrs incubation a clear zone was seen around the test well (Fig. 4). This proves that the extracellular phosphatase enzyme was secreted by utilizing the Pikvoskaya's agar.

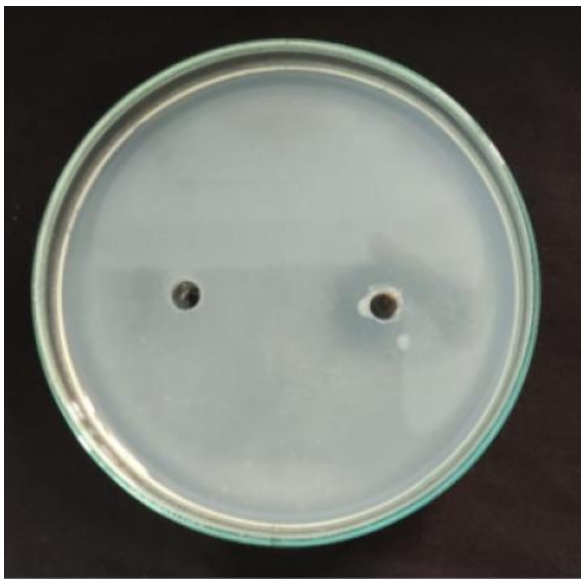

Fig. 4. Plate assay

Assessment of phosphatase solubilizing bacteria on growth of green gram

The efficiency of phosphatase produced from the potent bacterial strain under optimal conditions to promote maize growth has also been tested and compared with commercially available fertilizers (Fig. 5). The results show that using the phosphatase enzyme isolated from a potent strain in combination with fertilizer resulted in better growth in terms of shoot and root weight and length (Fig. 6).

Day 1

Control

Fertilizer

Enzyme

Enzyme + Fertilizer

Day 5

Control

Fertilizer

Enzyme

Enzyme + Fertilizer

day 10

Control

Fertilizer

Enzyme

Enzyme + Fertilizer

Day 15

Control

Fertilizer

Enzyme

Enzyme + Fertilizer

Fig. 5. Assessment of phosphatase solubilizing bacteria on growth of Green gram (Pot trial).

Fig. 6. Assessment of length and weight of shoot and root growth

DNA Amplification

Genomic DNA was amplified and 16sr RNA sequencing was done.

16SrRNA gene sequencing (~652bp)

ACTCAAGCCTGCCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCTGACTTA
ACAGACCGCCTGCGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTAC
CGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCTTCTGCGAGTAACGTCAATCACCAAGGTTA
TTAACCTTATTGCCTTCCTCCTCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACG
CGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAG
TCTGGACCGTGTCTCAGTTCCAGTGTGGCTGGTCATCCTCTCAGACCAGCTAGGGATCGTCGCC
TAGGTGAGCCATTACCCCACCTACTAGCTAATCCCATCTGGGCACATCCGATGGCAAGAGGCC
CGAAGGTCCCCCTCTTTGGTCTTGCGACATTATGCGGTATTAGCTACCGTTTCCAGTAGTTATC
CCCCTCCATCGGGCAGTTTCCCAGACATTACTCACCCGTCCGCCACTCGTCACCCGAGAGCAA
GCTCTCTGTGCTACCGTTCGACTTGCATGTGTTAGGCCTGCCGCCAGCGTTCAATCTGAGCCAG
GATCAAACTCTAAAT

Phylogenetic tree

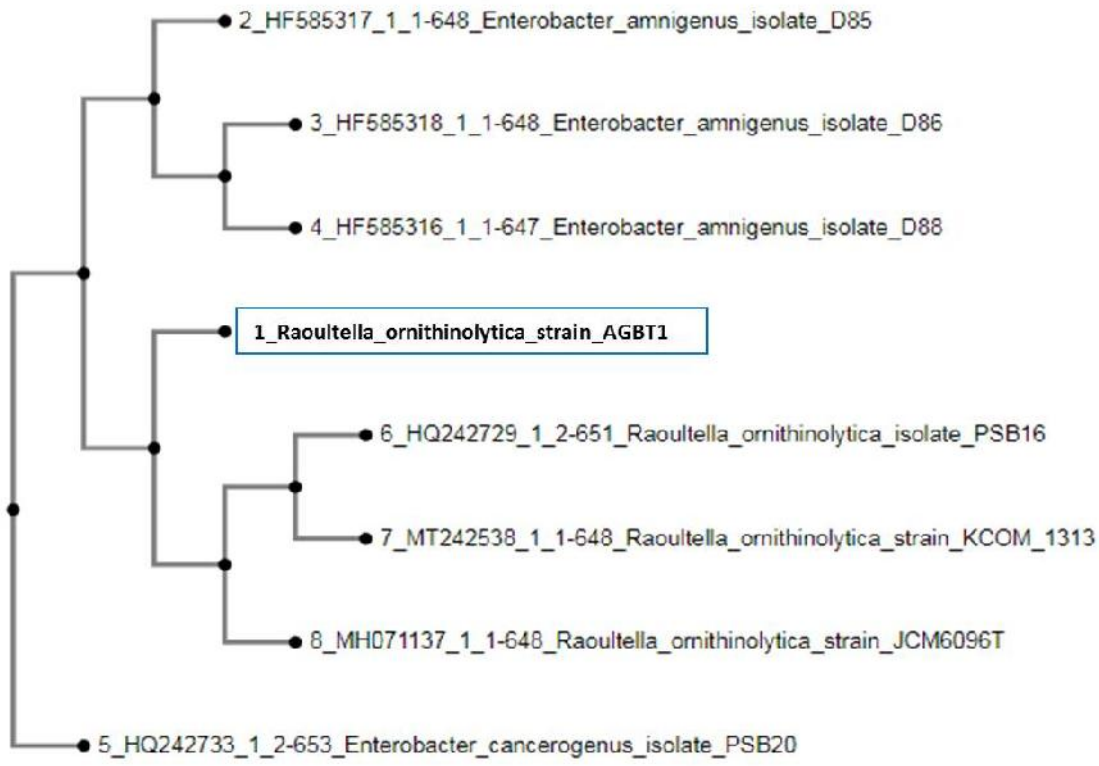

Fig. 7 Phylogenetic tree generated from the 16S rRNA gene sequence data using neighbor-joining tree building algorithm. The colored name indicates the sequence generated from the present study.

Discussion

Plant are constantly exposed to abiotic stresses such as salinity, drought, temperature etc…. lead to poor plant growth; (Sandhya et al….2010). Management of abiotic stress gaining importance through tolerant strain from strened ecosystem can enhance growth and development of plants. The most efficient stress tolerant strain were selected for plant growth promoting studies. Whereas, in Phosphate solubilization only six isolates showed Phosphate solubilization in tricalcium Phosphate amended Pikovskaya medium.

The selected strain were identified by sequencing of 16rRNA gene to ascertain their taxonomic positions. However, the presence of Alcaligenes in salty soil may be due their diverse metabolisms and high tolerance to a variety of physical conditions (Nakano *et al.*, 2013). They are previous report pertaining to the plant growth capabilities of Alcaligenes species. The work carried out by Sayyed *et al*. (2010) showed siderophore producing *Alcaligenes faecalis* BCCM 2374 enhanced seed germination (8.75%), root length (9.35%),shoot length (16%) and chlorophyll content (8.0%) in Arachis hypogaea over control treatment under pot culture conditions. Naseem and Bano (2014) have also shown the ability of exopolysaccharide producing *Alcaligenes faecalis* increased the root length of maize up to 50% in no stress and 42% in drought stress compared to control.

Conclusion

Biofertilizers can be the best alternatives to chemical fertilizers which drastically affect the land quality. This study has revealed that bacterial strains *Raoultella ornithinolytica*, isolated from agricultural fields oil have the ability to produce extra-cellular alkaline phosphatase which can be used as biofertilizers**.** The strains that produced the phosphatase enzyme are screened using plate assay and chemical assay.

This work indicated the potential of drought resistant bacteria for green gram growth promotion under salinity stress. Besides this, efficient strains reported in this study could be formulated to alleviate salt stress in green gram plants.

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