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INDOLE ACETIC ACID PRODUCTION PROPERTY AND MOLECULAR IDENTIFICATION OF PHOSPHATE SOLUBILIZING BACTERIA FROM RHIZOSPHERE SOIL USING 16SrRNA GENE SEQUENCING

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Abstract
Phosphorus is critical to the growth and yield of plants, because it stimulates growth, initiates nodule formation as well as influences the efficiency of rhizobium-legume symbiosis. Deficiency of phosphorus in plants is manifested in terms of stunted growth, reduced yield and delayed maturity. Application of organic or inorganic chemical fertilizers or combinations of both in order to increase fertility, over a period of time builds up total and available phosphorus in the soil. However, this high level of total phosphorus added to the soil is rapidly fixed as insoluble forms soon after application and becomes unavailable to the plants regardless of the fertilizer brand or chemical composition. Excessive application of phosphorus to soil increases phosphorus fixation and the long-term effect is the gradual loss of phosphorus from such soil into underground waters, leading to contamination. A large number of microorganisms present in the rhizosphere are known to solubilize and make available the insoluble phosphorus in the available form to the plants. This study presents insights into the screening and morphological properties of some phosphate solubilizing microorganisms, which are organisms that can solubilize these fixed forms of phosphorus and make it available to plants. A total of 35 Phosphate Solubilizing Microbial colonies were isolated on Pikovskaya agar medium, containing insoluble tricalcium phosphate (TCP) from agricultural soil. Among these, 3 isolates with strong halo zone formation were studied. The 3 isolates, AFS-A, AFS-B, and AFS-M were subjected to molecular identification, based on the 16SrRNA gene amplification and sequencing technique. The molecular identification showed isolate AFS-A as Enterobacter hormaecheii with a percentage identity of 96.57 (accession number CP017180.1). AFS-B was identified as Enterobacter cloacae, with a percentage identity of 96.57 (accession number CP026850.1). AFS-M was identified as Clostridium beijerinckii, with 97.88 percentage identity (accession no.MK522142.1). Studies on their PSI and PSE showed that E. cloacae solubilized TCP more with a PSI of 3.22 ± 0.1 and a PSE of 2.39.0 ± 1.0. E. hormaecheii had a PSI of 1.36 ± 0.0 and a PSE of 149.3 ± 1.0. C. beijerinckii had a PSI of 0.25 ± 0.0 and PSE of 146 ± 1.0. All isolates lowered the pH of PVK broth. Highest IAA produced was recorded by E. cloacae (44.0 ± 0.0). This was followed by C. beijerinckii (19.1 ± 0.0) and E. hormaecheii (12.3 ± 0.2). This serves as a foundation for future exploration of the bio-prospective potential of rhizosphere bacteria in this unique ecological niche. This could lead to the eventual replacement of chemical fertilizers in agricultural practice by increasing the bioavailability of phosphorus already present in soils. This will lend support to food security and equally protect the environment as a natural practice and health of various life forms.

Key Words: *Phosphate Solubilizing Microorganisms, Accession number, Pikovskaya, C. beijerinckii, E. hormaecheii; E, cloacae*

Introduction

Phosphorus is a chemical element found on earth in numerous compound forms like phosphate ion (PO_4), located in water, soil and sediments. It plays an important role in virtually all major metabolic processes in plants. It is a component of biological molecules such as DNA, RNA, ATP and Phospholipids. On a macro level, it affects root development, stalk and stem strength, crop maturity and nitrogen fixation in legumes (Khan *et al*., 2020). phosphorus availability for plants stimulates early plant growth and hastens maturity. Since the concentration of available P in soil is lower than what is found in healthy plant tissues, it is common agricultural practice to apply mineral Phosphorus fertilizers in the form of readily available monocalcium phosphate or monopotassium phosphate (Krystal *et al.*, 2023). Although phosphorus is abundant in soil in both inorganic and organic forms, it is a major limiting factor for plant growth as it exists in unavailable forms for root uptake. Adequate inorganic phosphorus occurs in soil, mostly in insoluble mineral complexes. Some of them appearing after frequent application of chemical fertilizers. These insoluble precipitated forms cannot be absorbed by plants (Rengel and Marschner, 2019).

Only 0.1% of total phosphorus exists in a soluble form for plant uptake (Billah *et al*., 2019), because of its fixation into an unavailable form. The term, "phosphorus fixation", is used to describe reactions that remove available phosphate from the soil solution into the soil solid phase (Liu *et al*., 2023). This can occur via either of these two types of reactions: (a) phosphate sorption on the surface of soil or (b) phosphate precipitation by free Al^{3+} and Fe^{3+} in the soil solution (Rahul *et al.,* 2023). It is for this reason that soil phosphorus levels are supplemented on most agricultural soils by adding chemical phosphate fertilizers, which not only represent a major cost of agricultural production, but also impose adverse environmental impacts on overall soil health and degradation of terrestrial, freshwater and marine resources (Lobo *et al.,* 2019)

Increased levels of phosphorus were identified as a main factor for eutrophication of surface waters that may lead to algal blooms (Zhang *et al.*, 2023). The repeated and careless application of chemical phosphorus fertilizers leads to loss of soil fertility (Chen *et al.*, 2020), by disturbing microbial diversity and consequently reducing crop yield. The long-term effect of different sources of phosphate fertilizers on microbial activities includes inhibition of substrate induced respiration by fungal and bacterial activities and microbial biomass carbon (Shahab *et al.*, 2018). The efficacy of applied chemical phosphorus fertilizers in chemical form rarely exceeds 30% due to its fixation, either in the form of iron/aluminum phosphate in acidic soils (Krystal *et al*., 2023) or as calcium phosphate in neutral to alkaline soils (Tang *et al.,* 2023). Since phosphorus supplies are not easily replenished in comparison to nitrogen, it is important to better utilize Phosphorus reserves in the soil and reclaim chemically bound Phosphorus (Liu *et al.*, 2023)

Phosphate solubilizing bacteria (PSB) in the plant rhizosphere play a significant role in releasing P from its insoluble complexes to a form that is more readily usable by plants. The inorganic forms of P can be solubilized by microorganisms that

secrete low molecular weight organic acids to dissolve phosphate-complexed minerals (Billah *et al.*, 2019) and chelate cations that partner with P ions (PO_4^{3+}) to release P directly into the surrounding soil solution system (Chen *et al*., 2020). With the current public interest in promoting more sustainable agricultural practices, using PSB, either in conjunction with or as a replacement for expensive and environmentally damaging fertilizers, would be advantageous to the agricultural industry (Barea *et al*., 2015).

This study represents an effort to isolate bacteria and identify PSB strains from soils sampled from the rhizosphere of three (3) different crops (*Vernonia amygdalima*, *Ocimum gratissimum* and *Moringa oleifera.)*. We isolated some PSB, with efficient phosphate solubilization and plant growth promoting capabilities.

Materials and Methods

Experimental Conditions

The experiments were carried out at the Faculty of Agriculture farm, University of Benin, Benin City, Edo state, located on Latitude 6º 20′.32″N and Longitude 5º 36′0.53″E and 239.16 meters (784.65 feet) above sea level. Benin City has a tropical or savannah climate with a yearly temperature of 28.78ºC (83.8°F), which is -0.68% lower than Nigeria's average. Relative humidity is 81.51%.

Soil Sample Collection

Rhizosphere soil samples were randomly collected from the rhizosphere of three (3) economically important plants at the Faculty of Agriculture Research farm, University of Benin Ugbowo main campus. The plants were Vernonia amygdalima (Bitter leaf), Ocimum gratissimum (Scent leaf) and Moringa oleifera. The soil samples were collected from a depth of 0-30cm, packaged in sterile disposable bags and transported to the Soil Science Research Laboratory, Faculty of Agriculture and stored at -4°C for further analysis (Mahantesh and Patil, 2022).

Isolation and Purification of phosphate solubilizing Bacteria from Rhizosphere soil samples

A 10g sample from each soil was suspended in 90ml of sterilized water in 250 Erlenmeyer flasks to make 1:10 dilution. These were agitated on a rotary shaker at 125rpm for 30mins to break clogs. A ten-fold serial dilution was made to obtain an appropriate dilution factor. From dilutions 10^{-4}, 0.1ml suspension was transferred to Petri dish containing Pikovskaya's medium which consists of glucose (10g), $Ca_3(PO_4)^2$ (5g), (NH4)S04 (0.5g), Yeast extract (0.5g), $MgSO_4$7H20 (0.1g), $MnSO_4$(0.002g), $FeSO_4$(0.002g), NaCl (0.2g), Agar (15g), Distilled water (1000ml), pH (7.0) (Pikovskaya, 1948) Incubation was done at 30°C for 7days. Colonies of bacteria were counted on the 5th to the 7th day. Isolates showing discreet halo zones around colonies were assumed to be phosphate solubilizers. These were selected, purified and preserved on agar slants at -4°C for further use.

Identification and Characterization of Bacteria Isolates

All selected isolates were examined for morphological (colony, morphology, cell shape and colony type) and microscopic (gram staining) characteristics with reference to (Krieg and Holt, 2022; Kucey, 2018).

Molecular Identification by 16SrDNA Sequence analysis

Genomic DNA Extraction

50-100mg (wet weight) bacterial cells (PBS) that have been resuspended in up to 200µl of isotonic buffer was added to a ZR Bashing™ Lysis tube. 750µl Lysis Solution was added to the tube. A bead fitted with 2ml tube holder was secured and processed at maximum speed for 5min. The ZR BasingBead™ Lysis Tube in a microcentrifuge was centrifuged at 10,000 x g for 1min. 400µl of supernatant was transferred to a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7,000 x g for 1min. The base of the Zymo-Spin™ Spin filter was snapped off prior to use. 1,200µl of Bacterial DNA Binding Buffer was added to the filtrate in the collection Tube from step 4. 800 ul of the mixture from step 5 was transferred to a Zymo-Spin™ IIC Column in a Collection Tube and centrifuged at 10,000 x g for 1 minute. The flow was discarded from the Collection Tube and step 6 was repeated. 200ul of DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in new Collection tube and centrifuged at 10,000 x g for 1min. 500µl bacterial DNA Wash buffer was added to the Zymo-Spin™ IIC column and centrifuged at 10,000 x g for 1min. The Zymo-Spin™ IIC Column was transferred to a clean 1.5ml microcentrifuge tube and 100µl (35ul minimum) DNA Elution Buffer was added directly to the column matrix. Centrifuge at 10,000 x g for 30 seconds to elute DNA.

Polymerase Chain Reaction (PCR) amplification of 16S rDNA and Sequencing.

2 µl of the extracted DNA was used as a template for PCR amplification. The 25µl PCR reaction generally contained 0.4 µl 10mM dNTP, 2.5µl 10x PCR buffer, 2.5µl 25mM $Mgcl_2$, 0.2µl (5"-AGAGTTTGATCCTGGCTCAG-3') and 1µl (10mM) of reverse primer 1492r(5'-GGTTACCTTGTTACGACTT-3'). The PCR program included a denaturation step of 5min at 95°C, followed by 30cycles of 95°C for 30s, 50°C for 45s, 72°C for 1hr, 30min and a final extension step of 10min at 72°C. PCR products were Sanger-sequenced using the forward primer 27F at the International Institute of Tropical Agriculture Ibadan. The sequences were edited using Bioedit (http://www.mbio.ncsu.edu/Bioedit/). A blast search on NCBI gene Bank Database (WWW. Ncbi.nlm.nih.gov/) was used to identify the isolates.

Phylogenetic Analysis

Sequence data were multiple aligned using clustal W and compared with available sequences of bacterial linage from the NCBI database. A phylogenetic tree was constructed by using Neighbor-forming method from distance matrices on MEG A4 program (Tamura *et al.*, 2013). The evolutionary history was inferred using the Neighbor-joining method (Saiton and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein, 1985). The evolutionary distance was computed using the Maximum composite likelihood method (Tamura *et al.*,2013) and were given in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

Analysis of Phosphate Solubilizing Activity

Qualitative Estimation of Phosphate Solubilization

Sterilized pikovskaya agar plates were spot inoculated with 20µI of pure culture of each isolate, allowed to solidify and incubated at 30°C for 7days. Formation of

a clear zone around the spot was visually observed and measured. Their phosphate solubilization index (PSI) were calculated according to Edi-Premono *et al*. (2000). Based on their solubilization index along with abundance, isolates were selected and used for quantitative analysis of phosphate solubilization efficiency in PVK broth.

$$SI = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony Diameter}}$$

Quantitative Estimation of Phosphate Solubilization

500mg of $Ca_3(PO_4)_2$ was aseptically added to Pikovskaya broth then dispensed into 250ml flasks. All flasks were autoclaved and inoculated with 100ml test isolate culture suspension. The flasks were incubated at room temperature on a shaker water bath at 120rpm for 12 days. 5ml sample were withdrawn periodically on day 4, 8 and 12. The sample was analyzed for phosphate solubilization. The samples were centrifuged at 15,000rpm for 30mins. Available phosphorus in the culture supernatant was analyzed for phosphorus content using the phosphor-molybdate method. Uninoculated medium was used as control (Alam *et al*., 2022).

Quantitative Measurement of pH of the Media

Initial pH and change in pH were also recorded on same interval of 4th, 8th and 12th day by digital pH meter (OAKTON, pH700). The experiment was conducted in triplicates and values were expressed as their mean (Alam *et al*., 2022).

Results

Total Viable Bacterial Count (log_{10}cfu/ml) on PVK Agar at 37°C for 5-7 Days

There was a significant difference in the total viable count (log10 CFU/ml) for all the four isolates. *E. cloacae* had significantly more cells, ranging from 1.57± 1.0 on the day 5 to 2.98 ± 0.2 on the day 7.

This was followed by *C. beijerinckii* with a mean viable count of 7.2± 0.0 on day 5 to 1.96 ± 0.0 on day7. *E. hormaecheii* grew exponentially from 9.6 ± 0.1 on day 5 to a stationary phase with a mean viable bacterial count of 1.84 ± 1.0 on day 7.

Table 1: Total Viable Bacterial Count (log_{10}cfu/ml) ON PVK Agar At 37°C For 5-7 Days

Isolate	Microbial count Day 5 (log10cfu/ml)	Microbial count Day 6 (log_{10} cfu/ml)	Microbial count Day 7 (log_{10} cfu/ml)
E. hormaecheii	9.6 ± 0.1[c]	1.84 ±1.0[c]	1.84 ±1.0[c]
E. cloacae	1.57± 1.0[d]	2.64 ±1.0[d]	2.98 ± 0.2[d]
C. beijerinckii	7.2± 0.0[a]	1.52 ±1.0[a]	1.96 ±0.1[a]

Values are mean ± standard deviations of triplicate counts

Qualitative estimation of phosphate solubilization index (PSI) of PSB isolates after 7 days of incubation

This table shows that there was a significant difference in the solubilization index of the isolates after incubation for 7days. Maximum PSI was observed by *E. cloacae* with a solubilization index of 3.22 ± 0.1. This was followed by *E. hormaecheii,* with a moderately strong PSI of 1.36 ± 0.0. *C. beijerinckii* had a weak PSI of 0.25 ± 0.0 after incubation for 7days.

Table 2: Qualitative estimation of phosphate solubilization index (PSI) of PSB isolates after 7 days of incubation

Plant source	Inferred PSB	Rate of Solubilisation	Solubilisation Index
Mangnifera indica	*E. hormaecheii*	Moderate	1.36 ± 0.0c
Vernonia amygdalima	*E. cloacae*	Strongly	3.22 ± 0.1a
Cocos nicifera	*C. beijerinckii*	Weak	0.25 ± 0.0d

Means followed by different alphabets are different at 5% level of significance

Effect of Cowpea varieties, Biofertilizer and Inorganic fertilizer on Plant height, No of Leaves, No. of Branches, Stem girth and Leaf area

Effect of the treatments was significant for plant height. Plant height varied from 17.67cm to 29.56cm at 60DAS. Treatment $C_2B_2I_1$ gave the tallest plants (29.56cm). This was followed by treatment $C_1B_3I_1$ (26.56). Plant height was, however, significantly reduced by treatment $C_2B_1I_2$ (17.67).

Maximum number of Leaves was obtained from treatment $C_1B_3I_1$ (23.23). This was followed by treatment $C_2B_2I_1$ (22.67). Treatment $C_2B_1I_2$ (15.67) gave the smallest number of leaves.

Maximum number of branches was obtained by treatment $C_2B_2I_1$ (6.90). This was followed by treatment $C_1B_3I_1$ (6.89). Treatment $C_2B_1I_2$ (4.56) gave the lowest number of branches.

Stem girth was significantly affected by the treatments. Increase in stem girth ranged from 2.99 to 3.39. Treatment $C_2B_2I_2$ gave the widest stem girth of 3.39. This was followed by treatment $C_1B_3I_1$ (3.32).

Effect of the treatments was significant on leaf area. Maximum leaf area was obtained by treatment $C_2B_2I_1$ (8.54). This was followed by treatment $C_3B_4I_1$ (8.01). Treatment $C_1B_2I_2$ gave the lowest in leaf area (3.88).

pH and quantitative estimation of phosphate solubilization efficiency (PSE) of PSB isolates from Day 4 to Day 12.

Phosphate solubilization efficiency of the isolates was confirmed by quantitative analysis of available phosphorus in PVK liquid medium. All the isolated microorganisms solubilized TCP, though they varied in their ability and the growth period.

The phosphate solubilization efficiency of the isolates varied from 4.6 +_ 0.1µg/ml to 239.0 ± 1.0 µg/ml from day 4 to day 12. Among the 3 isolates, *E. cloacae* showed the highest phosphate solubilization efficiency on day 12 (2.39.0 + 1.0 µg/ml). pH varied from an initial value of 6.2 ± 0.1 for *E. hormaecheii* to 4.7 ± 0.1 for *E. cloaca.* The progressive decrease in pH was the same for all the isolates within the 12-day incubation period.

Table 3: pH and quantitative estimation of phosphate solubilization efficiency (PSE) of PSB isolates from Day 4 to Day 12.

PSB	$Ca_3(PO_4)_2$					
	Day 4		Day 8		Day 12	
	pH	p(µg/ml)	pH	p(µg/ml)	pH	p(µg/ml)
E.hormaecheii	6.5±0.1c	5.2±0.1a	5.8±0.1b	158.0±1.0b	5.2±0.1b	149.3±1.0b
E. cloacae	5.6±0.1a	4.8±0.1a	5.1±0.1a	284.0±1.0d	4.7±0.1a	239.0±1.0a
C. beijerinckii	5.8±0.1b	5.3±0.1a	5.2±0.1a	164.0±1.0c	5.0±0.1b	146±1.0c

Means followed by different alphabets are different at 5% level of significance

Indole-3-Acetic Acid (IAA) production efficiency of PSB isolates

All the PSB isolates produced IAA. The amount of IAA produced by the isolates ranged from 12.3 ± 0.2 to 44.0 + 0.0 µg/ml. E. cloacae produced the maximum amount of IAA (44.0 ± 0.0 µg/ml). This was followed by E. beijerinckii with a total IAA production of 19.1 ± 0.0 µg/ml. The minimum amount of IAA was produced by E. hormaecheii (12.3 ± 0.2 µg/ml).

Table 4: Indole-3-Acetic Acid (IAA) production efficiency of PSB isolates

PSB	IAA (µg/ml)
E. hormaecheii	12.3 ± 0.2d
E. cloacae	44.0 ± 0.0a
C. beijerinckii	19.1 ± 0.0c

Means followed by different alphabets are different at 5% level of significance

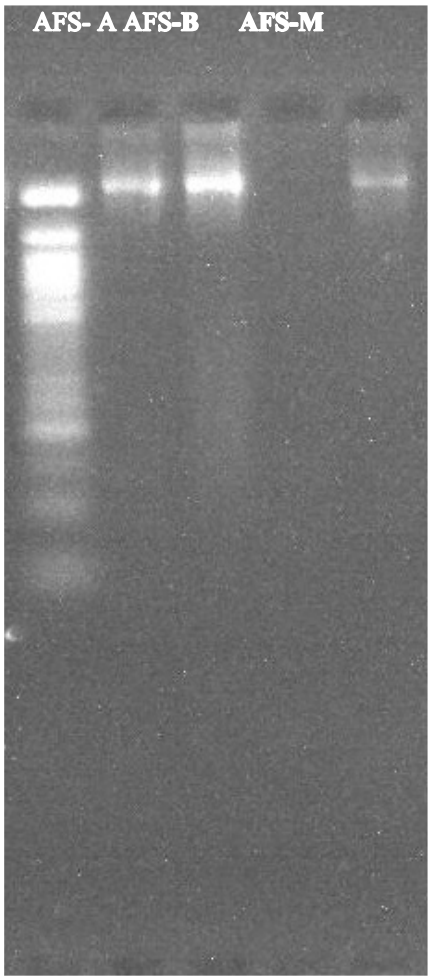

Plate 1: Gel picture showing the presence of 16Sr RNA region of isolates

Table 5: Molecular Characterization of PSB Isolates

Sample code	Source	Identity	Query cover (%)	Percent Identity	Accession number
AFS- A	Rhizosphere	*Enterobacter hormaechei*	82	96.57	CP017180.1
AFS - B	Rhizosphere	*Enterobacter cloacae*	82	96.57	CP026850.1
AFS- C	Rhizosphere	*Enterobacter asburiae*	64	86.96	MK104512.1
AFS - D	Rhizosphere	*Clostridium beijerinckii*	61	95.68	LC020497.2
AFS - F	Rhizosphere	*Clostridium beijerinckii*	90	97.88	MK522142.1

Sample code	Source	Query cover (%)	Percent identity	Accession number	Identity
AFS-A	Rhizosphere	82	96.57	CP017180.1	*E. hormaecheii*
AFS-B	Rhizosphere	82	96.57	CP026850.1	*E. cloacae*
AFS-M	Rhizosphere	90	97.88	MK522142.1	*C. beijerinckii*

The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor 1969).

The tree with the highest log likelihood (-2610.8450) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths, measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 619 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.05 software package (Tamura *et al*., 2013).

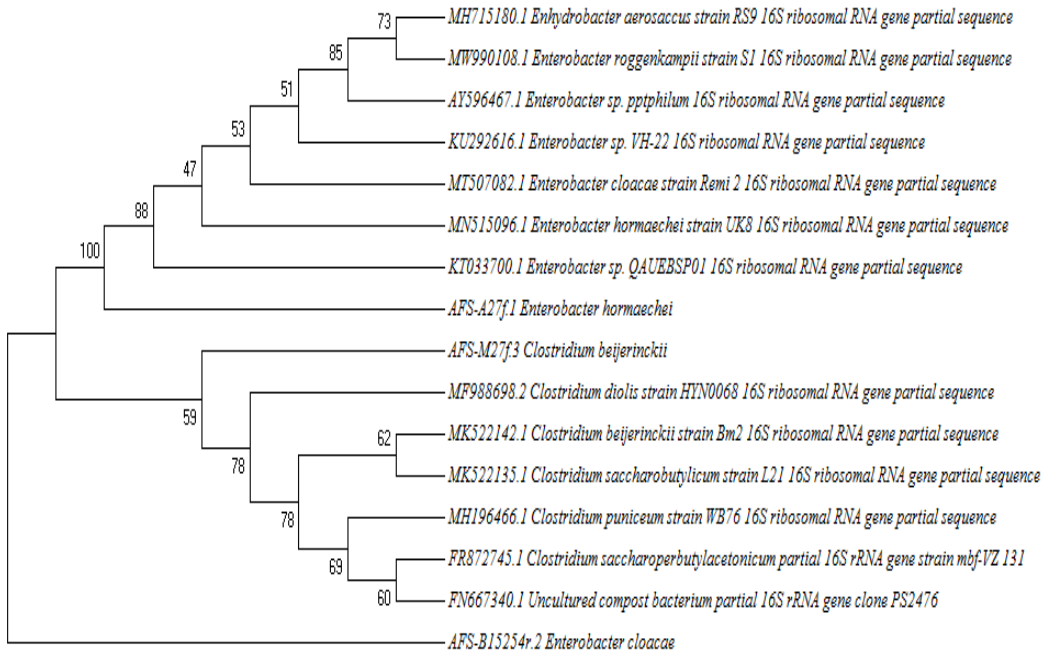

Fig 1: Molecular Phylogenetic analysis by Maximum Likelihood method

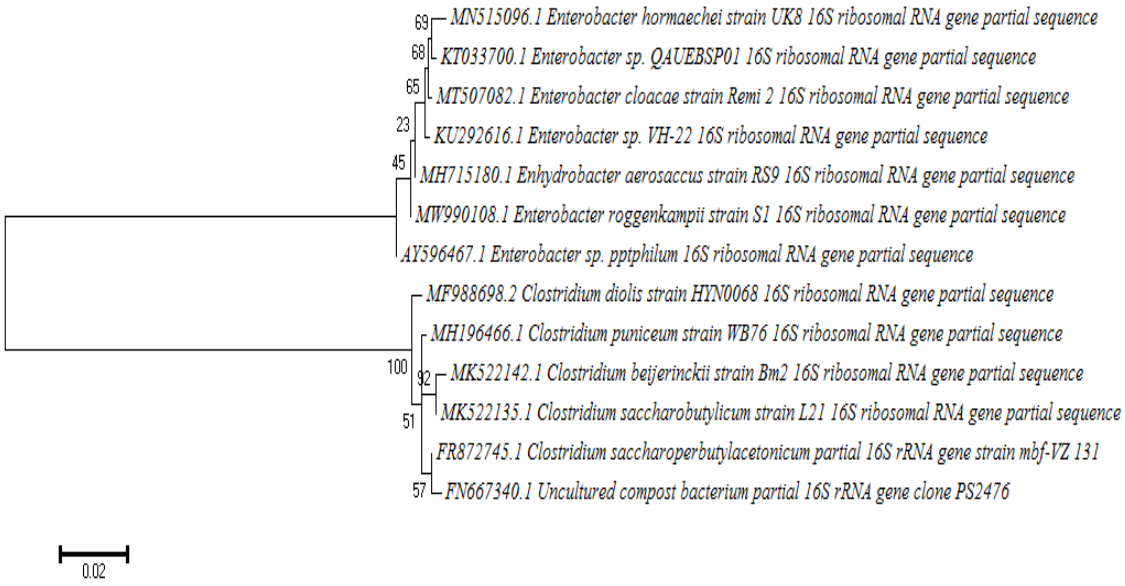

Fig 2: Molecular Phylogenetic analysis by Maximum Likelihood method

Discussion

Phosphorus is one of the major plant nutrients, lack of which limits plant growth. Most agricultural soils contain large reserves of total Phosphorus, commonly in the range of 200-5000mgPka-1, with an average of 600mgPka-1. These phosphorus reserves keep accumulating due to regular application of chemical fertilizer (*Fernandez et al.,* 2018). Phosphate Solubilizing Microorganisms brings about mobilization of insoluble phosphates in the soil and increase plant growth under conditions of poor phosphorus availability. These beneficial bacteria enhance plant growth by improving soil nutrient status, secreting plant growth regulators and suppressing soil borne pathogens (*Gulati et al*., 2020).

In the present study, the rhizosphere regions of some plants were selected for isolation of phosphate solubilizing bacteria. This habitat was selected for isolation because of the greater possibility of occurrence of phosphate solubilizing bacteria. *Panhwar et al*. (2019), found considerably higher numbers of PSB population in the rhizosphere in comparison with non-rhizospheric or bulk soil. The population level varied in different rhizosphere soils. This is supported by the findings of Kucey (2018) who reported that PSB isolates have been found in almost all soils tested although the number varies with soil, climatic and cropping history. This large variation in the distribution of PSB in different soils may be due to the differences in organic carbon content of the soil. (Yadav and Singh, 2022).

In this study, 8 bacterial isolates produced a halo zone around colonies in Pikovskaya agar medium. These were selected and purified on the same medium. This is in line with the findings of Gaind (2020), who reported that PSB strains were isolated using the Pikovskaya medium based on the formation of halo zone around these microorganisms. These organisms grew exponentially in PVK agar at 37^{o}C for 5-7days (Table 1). Sanjotha and Sudheer (2020), also isolated microbial colonies from soil of Karwar Costal Region, which showed clear zones around the microbial growth and was considered as phosphate solubilization. Three isolates (AFS-A, AFS-B, AND AFS-M) were selected from the 8 bacterial isolates on the bases of their phosphate solubilization index (Table 2), Phosphate Solubilization Efficiency and pH in liquid medium (Table 3).

The clear or halo zone was formed due to the solubilization of insoluble phosphates by several mechanisms including the production of Organic acids like gluconic, ketogluconic, oxalic and succinic acid, (*Vazquezet et al.,* 2022), Polysaccharides (*Goenadi et al.*, 2020) and Phosphatase enzymes, mainly acid phosphates (*Rodriguez et al.,* 2022). Several reports have mentioned the production of organic acids by *S. marcescens* (Chen *et al*., 2020), Burkholderia gladioli (Stephen and Jisha, 2022), *Enterobacter* spp (Wan *et al.*, 2020) and *Pseudomonas* spp (Rasul *et al*., 2020). In this study, *Enterobacter cloacae* had the highest PSI (3.22 $\pm$ 0-1) while *Clostridium beijerinckii* (0.25 $\pm$ 0.0) had the least phosphate solubilization in solid PVK medium. This is in line with the findings of Zhao *et al*. (2018) who stated that Screening of PSB clearly indicated that there was wide variation in the PSB strains in solubilization zone formation (PSI), pH change, and P solubilization in

the liquid medium. These characters were used to determine the efficient strains of PSB. Phosphate solubilization efficiency was highest on the 8th day. This is in line with recent studies done by *Zhao et al*. (2018) and Kumar *et al*. (2021), who found that the content of soluble Phosphate does not change over time because PSBs can degrade TCP by producing organic acids to promote the content of soluble P in culture medium, thus the content of soluble P does not decrease over time. With increase in culture time and nutrient consumption, the insoluble P in the culture is converted to a form that plants can absorb. (Patel *et al.*, 2021). Therefore, the Phosphate solubilization activity declines after peaking.

pH of the medium turned acidic, indicating that production of organic acids by PSB, can facilitate the solubilization of phosphate. (Rasul *et al*., 2021; Singh *et al*., 2021). Maximum decline in pH was recorded with *Enterobacter cloacoa*, from 5.6 $\pm$ 0.1 to 3.7 + 0.1 at the end of the 12day incubation period. *C. beijerinckii* had the least decline in pH from 5.8 $\pm$ 0.1 to 5.0 $\pm$ 0.1 from day 4 to day 12. A fall in pH accompanied phosphate solubilization but due to the production of organic acids no correlation could be established between acidic pH and quantity of Phosphate liberated (Pichersky and Lewinsohn, 2020). Medium pH tended to decrease in all cases of growth; however, no constant relationship was found between amounts of P released and the drop in pH. The lack of relationship between pH drop and P solubilized may be due to the liming effect of rock Phosphate and the production of other metabolites by the microbes (Kucey, 2018)

In addition to Phosphate solubilization, PSB also produced other secondary metabolites like indole-3-Acetic acid (IAA). (Table 4). Several evidence have demonstrated plant growth promotion by PSB through the production of IAA (Yadav *et al*., 2022; Shahab *et al*., 2018). The result on IAA production from the PSBs tested here, support the observations of Barea *et al.*, (2015), who reported the production of PGPS like IAA, gibberelins, cytokinins or their combinations from 50 phosphate solubilizing bacteria. Several workers like Singh *et al*. (2021), Shahab *et al.* (2018) and Kuklinsky-Sobral *et al*. (2016) reported on the production of IAA by PSBs.

Enterobacter cloacoa produced the maximum quantity of IAA (44.0 $\pm$ 0.0), while *Clostridium beijerinckii* produced the least amount (19.1 $\pm$ 0.0). The amount and range of IAA produced in general in this study by the PSB isolates were comparable to those reported by several authors about different PSB strains (Liu *et al*., 2023; Guilherme *et al.,* 2020; Khalid *et al*., 2017) and also supported the view that auxin production is common among rhizosphere bacteria. (Ismail *et al*., 2017; Maria-micaela *et al*., 2020). The large variation in the amount of IAA produced by different strains has been attributed to the variability in the metabolism of the different strains of PSBs (Pichersky and Lewinsohn, 2020). Auxins produced by PSBs can influence several plant growth components including nutrients, thus increasing plant growth (Thiamann, 1972). Thus, it is possible that the strains tested here can also provide additional growth promotional activity apart from releasing P into the rhizosphere.

Production of different organic acids by PSBs has been reported earlier, which

are in line with the results of present investigation but it did not reveal whether there was any interaction between organic acids and IAA production. Further studies are necessary to illustrate any plausible interaction between production of organic acids and plant growth promoting substances.

The assessment of the bacterial 16SrRNA gene sequence has emerged as a preferred genetic technique as it can better identify weakly described, rarely isolated or phenotypically aberrant strains. (Panhwar *et al*., 2019; Barea *et al*., 2015). In this study, molecular phylogenetic approach based on 16SrRNA sequences to identify pure potential isolates was used. According to the sequence of the 16SrRNA gene, 2 isolates belong to the *Enterobacter* sp. and one, to the *Clostridium* sp. were identified. Sequences from the 3 isolates were almost 97-98% similar to other 16SrRNA sequences from the NCBI database, as shown in Table 5. Bacterial isolate AFS-A were identified as *Enterobacter hormaecheii* (96.57% sequence homology), AFS -B, was identified as *Enterobacter cloacoa,* (96.57% sequence homology) while isolate AFS-M was identified as *Clostridium beijerinckii* (97.88% sequence homology). This is in line with the findings of Ismail *et al*., (2017), who isolated 7 *Enterobacter* strains including *E. hormaecheii* from the roots of the Quinoa plant. Maria-Micaela *et al.*, (2020) Reported that *Enterobacter* sp was one of the most efficient strains isolated from the rhizosphere with the potential to enhance tomato plant performance. In addition, Liu *et al*. (2023), also noted that *E. cloacae* species are an extremely diverse group of bacteria that are associated with plants, soils and humans. Guilherme *et al.* (2020) reported that the genus *Clostridium* which is composed of a large spectrum of gram positive mesophilic and anaerobic species acts in various environments, providing agro ecological benefits in plant growth promotion and participation in industrial processes and replacing in both cases, chemicals, harmful to the environment. The sequences of the 16S rDNA of the 3 isolates were submitted to NCBI database and the resulting accession numbers were assigned those strains (Table 5)

Figure 1. shows the molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor, 1969). The tree with the highest log likelihood (-2610.8450) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 16 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 619 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

Figure 2. This is just the relationship among all percentage similarities. It shows the molecular Phylogenetic analysis by Maximum Likelihood method. The

evolutionary history was inferred by using the Maximum Likelihood method based on the Jukes-Cantor model (Jukes and Cantor 1969). The tree with the highest log likelihood (-1708.5626) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 708 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

Conclusion

Based on the nucleotide sequences obtained, the neighbor joining tree constructed showed that these organisms had strong relationships with other members of their genus as shown in figure 1. *E. homaecheii* and *C. beijerincki* clustered very closely with other species that have previously been reported by other researchers. The identification of highly efficient phosphate solubilizing bacteria, could lead to eventual replacement of chemical fertilizers in agricultural practice by increasing the bioavailability of phosphorus already present in soils. This will lend support to food security and equally protect the environment as a natural practice and health of various life forms.

More studies are required to identify and understand the significance and mechanism underlying the formation of soluble phosphate by PSB and its benefits as bio-inoculants. Based on the results of the indole acetic acid production, the currently isolated bacterial strains, especially *E. cloacoa* and *E. hormaecheii* could be useful for bio-fertilizer individually or as a consortium for plant growth promotion, stress tolerance, rhizosphere engineering, as well as an alternative approach to chemical fertilizers. Further studies are needed to develop certain formulations for the largescale commercial application in various crop field uses.

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