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Characterization and Plant Growth Promoting Properties of a *Bacillus sp*. Isolated from Maize Roots

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: To isolate plant growth promoting *Bacillus* strains from maize roots and to characterize using molecular methods, the strain with greatest potential for plant growth promotion.
Place and Duration of Study: Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, Awka, between February 2019 and March 2020.
Methodology: The isolation of plant growth promoting rhizobacteria (PGPR) from maize roots was made using Nitrogen Free Bromothymol Blue (NFB) broth. They were screened for Phosphate solubilizing activities on Pikovskaya (PVK) agar. Quantitative determination and solubilization of different types of Phosphates was carried out using Pikovskaya broth. Optimization of factors affecting phosphate such as NaCl concentration, initial pH of the medium, size of inoculum, was done using pvk broth. Evaluation of other plant growth promoting properties were carried out such as IAA, Ammonia, cellulase and HCN production.
Results: Eleven Nitrogen fixing bacteria were isolated using NFB broth based on colour change of the medium from green to blue. Test for phosphate solubilization abilities of the organisms revealed that nine of the isolates solubilized phosphate on PVK agar. Organism coded with IS52 gave the least solubilization index of 1.14 while isolate IS19 gave the highest index of 3.4. Isolate IS19

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yielded the highest amount of 73.5µg/ml P, while isolate IS30 was the weakest solubilizer in PVK broth, yielding 19.4µg/ml P. The best isolate IS19, produced the plant growth hormone Indole Acetic acid at a concentration of 105.4µg/ml. The organism also gave a positive result for ammonia and cellulase production but did not produce Hydrogen cyanide. It was identified as *Bacillus subtilis* using the 16S rRNA gene sequencing.

Conclusion: *Bacillus subtilis* fixed Nitrogen qualitatively and solubilized insoluble phosphates in addition to other plant growth promoting properties, thus *Bacillus subtilis* has potential for plant growth promotion, making it an efficient strain for biofertilizer production

Keywords: PGPR; NFB; phosphate solubilization; PVK.

1. INTRODUCTION

Nitrogen (N) is the main limiting nutrient after carbon, hydrogen and oxygen for the growth and development of plants [1]. Nitrogen is not present in soil parent material despite its abundance in the atmosphere [2]. With the advent of Green Revolution in the second half of the 20th century, our agriculture system relied more on the use of chemical fertilizers [3] so as to meet up with the plant's need.Similarly, Phosphorus (P) is the second limiting nutrient required for plant growth and development involved in important metabolic pathways such as nutrient uptake, biological oxidation, and energy metabolism [4]. The availability of soluble forms of P for plants in the soils is limited because it often forms complexes with iron, aluminum, and calcium cations in the soil [5]. Most soils possess considerable amounts of P, but a large proportion is bound to soil constituents. This often results in deficiency of phosphorus in the soil. Certain approaches have been adopted to circumvent this deficieny. The most common is the application of chemical fertilizers. The large amounts of nitrogen being pumped into the environment from heavily fertilized fields contribute to air pollution and to the entire global warming [6]. The rampant overuse of synthetic agrochemicals for enhancing crop productivity has deteriorated the biological and physicochemical health of the arable soil, leading to a declining trend in agricultural productivity across the globe over the past few decades [3]. The overuse of nitrogenous fertilizers has also resulted in unacceptable levels of water pollution (increasing concentrations of toxic nitrates in drinking water supplies) and the eutrophication of water bodies, leading to algal bloom with consequent death of aquatic lives.

The use of efficient plant growth promoting rhizobacteria (PGPR) as biofertilizers is deliberated as a suitable substitute for minimizing the use of synthetic agrochemicals in crop production [7]. Atmospheric nitrogen (N2)-fixing bacteria inhabit both plant tissues (e.g., nodules, roots) and soil-root rhizosphere interface and can, consequently, supply significant N amounts for plant growth. This is mainly due to the microbially mediated process through a highly sensitive bacterial enzymatic conversion of atmospheric N_2 into ammonia (NH_3), otherwise known as biological nitrogen fixation (BNF). BNF can provide an ecologically acceptable complement or substitute for mineral N fertilizers. Certain PGPR secret organic acids such as gluconic acid, that chelate the cations bound to phosphates [8] releasing the phosphorus and making it available for plant use. PGPR naturally inhabit plant roots and rhizosphere, where they utilize plant-derived nutrients while benefiting the plant through a variety of direct or indirect mechanisms [9]. Members of the genus *Bacillus* constitute an important group of plant growth promoting rhizobacteria (PGPR), which improve growth and yield of crops [10]. The genus represents one of the most abundant and phylogenetically diverse groups of easily cultivable PGPR [11]. *Bacillus* species, as a result of their avid rhizosphere colonization and Plant Growth Promotion characteristics, offer considerable interest for improving crop productivity and yield [12]. These bacteria are favored for commercialization as PGPR owing to their ability to produce heat and desiccation-tolerant endospores. These structures are crucial in maintaining high cell viability and prolonging shelf life in formulations under storage [13]. A number of such PGPR strains are commercially available in the form of formulated products [14].

In this study, Nitrogen fixing free living bacteria were isolated from the roots of maize crop. Their plant growth promoting traits including phosphate solubilization and production of Indole acetic Acid were evaluated as well as factors affecting phosphate solubilization. Also, the isolate with the greatest plant promotion potential was characterized phenotypically and molecularly based on the 16S rRNA gene sequencing.

2. MATERIALS AND METHODS

2.1 Isolation of PGPR from Maize Roots

The isolation of the PGPR was carried out using a modified method of Al-Humam, [15]. Samples of maize roots were collected from various sites in Nnamdi Azikiwe University, Awka. The maize plants were carefully uprooted and immediately transported to the laboratory. The roots were washed thoroughly to remove adhering soil particles and cut into 1 inch length and thereafter heated in a sterilized Nitrogen Free Bromothymol blue broth (NFB) medium (Sucrose 10 g , K_2HPO_4 0.6 g , $MgSO_4$ 0.20 g , NaCl 0.2 g, K_2SO_4 0.1 g, $CaCO_3$ 2.0 g, distilled water 1000 ml, pH 6.8, 2.0 ml of 0.5% Bromothymol blue solution) at 80^{o}C in a water bath for 60 min. The broth medium containing the root samples were incubated at room temperature for 7 days and growth was observed by colour change of the medium from green to blue as well as turbidity [16]. The isolates were purified by further subculturing unto a solid NFB medium until pure colonies were obtained.

2.2 Test for Phosphate solubilization

The organism's ability to solubilize phosphate was carried out using Pikovskaya PVK [17] medium as modified by Baliah *et al*. [18] (Yeast Extract 0.50 g, Dextrose 10.00 g, Calcium Phosphate 5.00 g, Ammonium Sulphate 0.50 g, Sodium Chloride 2 mg, Potassium Chloride 0.20 g, Magnesium Sulphate 0.10 g, Agar 15.00 g, distilled water 1000 ml). The organisms were inoculated onto PVK medium and incubated for 7 days. Halozone formation showed the ability to solubilize phosphate. Phosphate solubilization index was evaluated according to the ratio of the halozone diameter (colony diameter + halo zone) and the colony diameter [19].

2.3 Quantitative determination of phosphate solubilization

Quantitative phosphate solubilization assay was done using PVK broth, according to the methodology of Nautiyal and Mehta, [20]. The flasks containing 50 ml medium were inoculated with 50 microlitre bacterial culture in triplicates and incubated at room temperature for 5 days in the rotary Shaker. Simultaneously, the uninoculated controls were also kept under similar conditions. The cultures were harvested by centrifugation at 10,000 rpm for 10 min and solubilized phosphate determined using photometric chlorostannous reduced phosphomolybdic acid blue method of Jackson [21].

Fifty microlitre of the sample (supernatant) was transferred to 25 ml volumetric flask using a micropipette. Chloromolybdic acid (5 ml) was added along the sides of the flask and the volume made up to 20 ml using distilled water. Chlorostannous acid (0.5 ml) was added, mixed and the volume was quickly made up to 25 ml using distilled water. Absorbance was read at 600 nm wavelength. The total soluble phosphorus was calculated from the regression equation of standard curve. The pH of culture supernatants was also measured using a pH Meter.

2.4 Evaluation of the Isolates Ability to Solubilize Different Types of Phosphates

The isolates ability to solubilize different types of phosphates was tested in PVK broth medium which was composed as earlier described but only without the addition of agar. The organism was inoculated into 200 ml liquid media containing one at a time, insoluble phosphates Tricalcium phosphate, rock phosphate, $FePO_4$ and aluminium phosphate and incubated for 7 days with shaking. Uninoculated flasks served as controls.

The cultures were centrifuged at 5000 rpm for 15 minutes and solubilized phosphate determined using photometric chlorostannous reduced phosphomolybdic acid blue method [21].

2.5 Effect of Sodium Chloride (NaCl) Concentration on Solubilization of P by the Isolates

This was carried out according to the method of Ramakrishnan *et al*. [22]. The PVK was prepared with different concentrations of NaCl as follows; 0.5%, 1.0%, 1.5%, 2.0% and 2.5% and inoculated with a loopful of each of the isolates. The incubation was done for 7 days under shake-flask fermentation condition. It was thereafter assayed for P concentration.

2.6 Effect of the Initial pH of the Medium on the P Solubilizing Ability of the Isolates

This was done using PVK broth media as described by Fengling *et al.,* [23]. The pH of the

medium was adjusted with 0.1M NaOH and 0.1M HCl to the values 3, 5, 7 and 9. The broth was inoculated with a loopful of the selected isolates, incubated for seven days under the shake-flask fermentation condition. The final pH of each medium was taken after seven days. It was thereafter assayed for phosphate concentration.

2.7 Effect of Inoculum Size of the Isolates on the Phosphate Solubilization

This was done using a modified method of Vassilev *et al*., [24]. The size of the inoculum was determined by inoculating several colonies of organisms into 10 ml nutrient broth and incubating for 48 hrs. After 48 hours, a 9- fold serial dilution was carried out using peptone water. One mililitre of the culture was plated out from the dilutions 10^{-3} -10^{-7}, on a nutrient agar and colonies counted after 48 hrs incubation. five milliliters of the broth culture from the dilutions 10^{-3} -10^{-7} was used to inoculate flasks containing 25 ml pvk broth and incubated for 5 days with shaking in a rotary shaker at 150 rpm. The effect of inoculum concentration on solubilization of phosphates was determined by assaying the amount of soluble phosphate from the various dilutions.

2.8 Evaluation of other Plant Growth promoting Properties of the potential isolate

2.8.1 Test for ammonia production

The isolate was tested for the production of ammonia in peptone water. Freshly grown culture was inoculated into 10 ml peptone water in each tube and incubated for 48 h at 30°C. Nessler's reagent (0.5 ml) was then added to the tube. Development of brown to yellow colour showed a positive test for ammonia production [25].

2.8.2 Cell wall degrading enzyme production

Colonies was screened for cellulase activity by plating on Carboxymethyl (CMC) agar 1.88 g, sodium citrate 0.5 g, K_2HPO_4 7.0 g, KH_2PO_4 2.0 g, $(NH_4)_2SO_4$ 1.0 g, $MgS0_4.7H_20$ 0.1 g, Agar 10 g, Congo red 0.20 g, pH 7.0, Distilled water 1000 ml. The agar plate was prepared and spot inoculated with test organism and incubated at 30°C for 5 days. Development of halo zone around the colony was considered as positive for cellulase production. To visualize the hydrolysis zone, the plates were flooded with an aqueous solution of 1% (w/v) Congo red for 15 min and discolored with 1 M NaCl for 15 min [26].

2.8.3 Hydrogen Cyanide production

Screening of bacterial isolate for hydrogen cyanide (HCN) production was done as per methodology described by Castric [27]. Bacterial culture was streaked on nutrient agar medium containing 4.4 g per liter of glycine. A Whatman filter paper No.1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of the plate. Plates were sealed with parafilm and incubated at 30°C for 4 days. Development of light brown to dark brown color indicated HCN production.

2.8.4 Detection of Indole acetic acid

2.8.4.1 Culture growth conditions

Fifty mililiters of Nutrient broth (NB) containing 0.1% DL tryptophan was inoculated with 500 microlitre of 24h old bacterial culture and incubated with shaking at 180 rpm for 48h. The bacterial culture was centrifuged at 6,000 rpm for 10 min. Estimation of indole-3-acetic acid (IAA) in the supernatants was done using colorimetric assay [28].

2.8.4.2 Colorimetric estimation

One millilitre of supernatant was mixed with 4 ml Salkowski reagent and absorbance of the resultant pink colour was read after 30 min at 535 nm in UV/Visible Spectrophotometer. Appearance of pink colour in test tubes indicated IAA production as described by Gordon and Weber [29].

2.9 Identification of Isolates

Bacterial isolate was identified using the Bergey's Manual of Determinative Bacteriology [30] based on physiological and biochemical characteristics. This was followed by 16S rRNA gene sequencing of the bacterium with the highest PGPR activity.

2.10 Moleular Identification

Extraction of DNA was done using CTAB method, the 16S rRNA gene was amplified by PCR using universal primer for bacteria: 16S forward primer (27F TCCTCCGCTTATTGATATGS) and reverse

primer (1525R GGAAGTAAAAGTCGTAACAAGG). The amplified 16S rRNA gene PCR products (gene fragment of 1000 bp length) from this isolate, after purification by 2 M Sodium acetate wash techniques was directly sequenced on the Gene Sequencer 3130XL genetic analyzer from Applied Biosystems. The 16S rRNA gene fragment (1500 bp length) sequenced in both direction to obtain gene sequence in the form of A, C, T and G was then blasted on http://ncbi.nlm.nih.gov to assess the DNA similarities.

2.11 Statistical Analysis

All experiments were performed in 3 replicates and the results were subjected to statistical analysis using analysis of Variance (ANOVA) using Origin Pro.

3. RESULTS AND DISCUSSION

Eleven free living nitrogen fixing bacteria (NFB) were isolated from maize plant roots (*zea mays*), collected from different farms at the Nnamdi Azikiwe University, Awka. Several Nitrogen Fixing Rhizobacteria have been previously isolated from maize plants. Nitrogen free bromothymol blue (NFB) broth and agar media were used for isolating the organisms. Before inoculating the root into the media, the media colour was green owing to the presence of the indicator. However, after 7 days incubation, the colour of the media was changed from green to blue (Fig. 1). The control media without the root sample remained green in colour. It was assumed that NFB accumulated excreted ammonia into the media and pH of the media was increased. Hence, the increase in the pH of the media resulted in the colour change from green to blue since bromothymol gives a blue colour at alkaline pH. Similar findings were made by Gothwal *et al*., [32]; Yu and Ullrich, [32]; Latt *et al*., [33]; Harca *et al*., [34]. All the isolates were able to fix atmospheric nitrogen and it was on this basis that they were selected.

In general, the degree of phosphate (P) solubilization on the PVK agar varied amongst the isolates. Isolate IS19 was found to be superior (Fig. 2) to all the other organisms with a solubilization index of 3.4, while IS52 had the lowest solubilization index value of 1.47 (Table 1). Several phosphate solubilizing rhizospheric bacteria have been previously isolated [35,36,37].

The five selected isolates solubilized different types of insoluble Phosphate in the liquid Pikoskaya (PVK) medium (Fig. 3). The organisms presented the same efficiency in solubilization of P in similar to that obtained on the PVK agar, in other words, the result of the quantitative assessment corresponds with that of qualitative evaluation. This contradicts what was reported by Yongbin *et al.,* [38]. Phosphate solubilizing ability is an important parameter while assessing PGPR. There is no metal-P complex that can act as the general selection factor for phosphate solubilizing bacteria because of the variability of soils in pH and some chemical properties [39]. Hence, it is imperative to measure the P solubilization ability using different types of P complexes. Insoluble Tricalcium P, Rock P, AlPO4, and FePO4 were elected for this evaluation. All strains solubilized $Ca_3(PO_4)_2$ to a greater extent than the other insoluble P. Similar results were reported during quantitative determination of the P solubilization ability [40]. Tricalcium phosphate was considerably more soluble than iron/aluminum phosphate. One-way Anova showing variations in $Ca_3(PO4)_2$ solubilization in liquid broth is as shown in Table 2.

Several factors are known to influence the ability of rhizospheric bacteria to solubilize insoluble organic and inorganic phosphates [8]. Such factors include; temperature, pH, salinity etc. PGPR inocula must therefore be able to tolerate a wide range of environmental factors. In the test for the effect of NaCl on Tricalcium Phosphate solubilization, the amounts of soluble phosphates were more at low concentrations of the salt. However, NaCl concentration of between 1-1.5%, generally enhanced the performance of the organism (Fig. 4). Srinivasan *et al.,* [41] and Thant *et al.* [42] made similar observations. Increasing concentration of NaCl in PVK medium, progressively inhibited the ability of the isolate to solubilize the different insoluble phosphates tested. One-way Anova showing variations in effect of NaCl concentration on Tricalcium P solubilization is as shown in Table 3

The study on the effect of initial pH on the phosphate solubilizing ability of the isolate revealed that increasing initial pH of the medium reduced the ability of isolate to solubilize phosphate. This is as shown in Table 4. Higher yields of soluble phosphates were obtained at neutral initial pH. This implies that phosphates will be more readily soluble by phosphate solubilizing bacteria in a soil whose pH is within

the neutral range, than in both acidic and alkaline soil. The solubilization of the different insoluble phosphates by the isolates was characterized by a drop in pH of the medium. The principal mechanisms for microbial phosphate solubilization are the production of organic acids and acid phosphatases. [8,42] Production of organic acids by phosphate solubilizing bacteria lowers the pH of the surrounding [5]. Similarly, Collavino *et al*., [44] showed that acidification of the broth medium coincided with P solubilization. The isolate might have used same mechanism to solubilize the Phosphate which ultimately caused a decline in the pH of culture filtrate.

Increased phosphate solubilization rate was observed with an increase in inoculum concentrations of the organism. This is in line with the reports made by; Vassilev *et al.*, [45] The optimum inoculum concentration for the organisms was 2.43 x 10^9cfu/ml. This is as shown in Fig. 5. However, data showed that inoculation with higher amounts of bacterial inoculum resulted in lower soluble P concentrations, with 8.2% decrease compared to the optimal yield. This was probably provoked by the higher amounts of *Bacillus subtilis* biomass in broth medium leading to nutrient depletion.

Table 1. Solubilization Index (Sol. Index) on PVK Agar (72 hrs)

Isolate	Diameter halozone (mm)	Diameter colony (mm)	Sol. Index
IS19	17.00	5.0	3.40
IS28	10.30	4.2	2.45
IS30	9.00	4.3	2.09
IS33	10.00	6.0	1.67
IS35	10.50	3.9	2.69
IS36	0.00	0.0	0.0
IS39	0.00	0.0	0.0
IS43	9.35	7.1	1.32
IS47	9.45	8.1	1.16
IS52	9.54	8.3	1.14
IS53	1.17	10.86	9.3

Table 2. One-way Anova showing variations in $Ca_3(PO4)_2$ solubilization in liquid broth

Isolate	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
IS16	3	1	79.6	1.50997	0.87178
IS19	3	1	72.83333	1.60728	0.92796
IS28	3	1	42.86667	1.62891	0.94045
IS30	3	1	19.46667	1.50111	0.86667
IS33	3	1	28.66667	3.05505	1.76383
IS35	3	1	52.7	2.52389	1.45717
Control	3	1	0.33333	0.57735	0.33333

At the 0.05 level, the population means are significantly different.

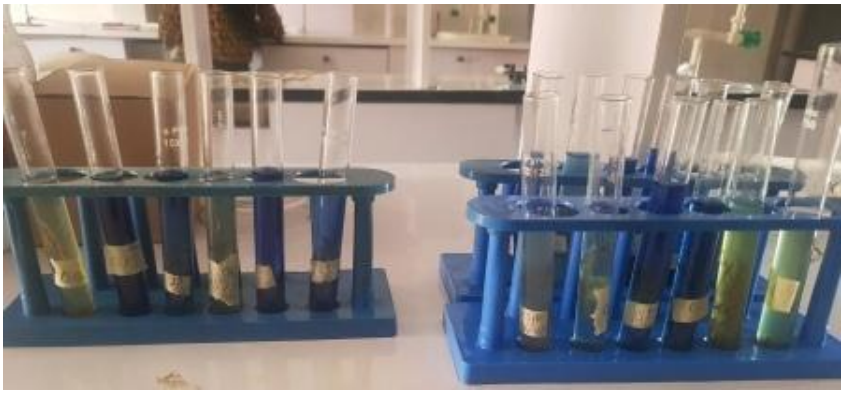

Fig. 1. Nitrogen fixation in NFB broth

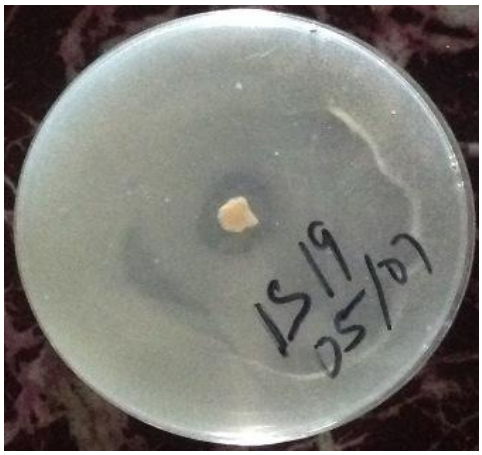

Fig. 2. Solubilization of insoluble phosphates on PVK Agar medium by isolate ISI9

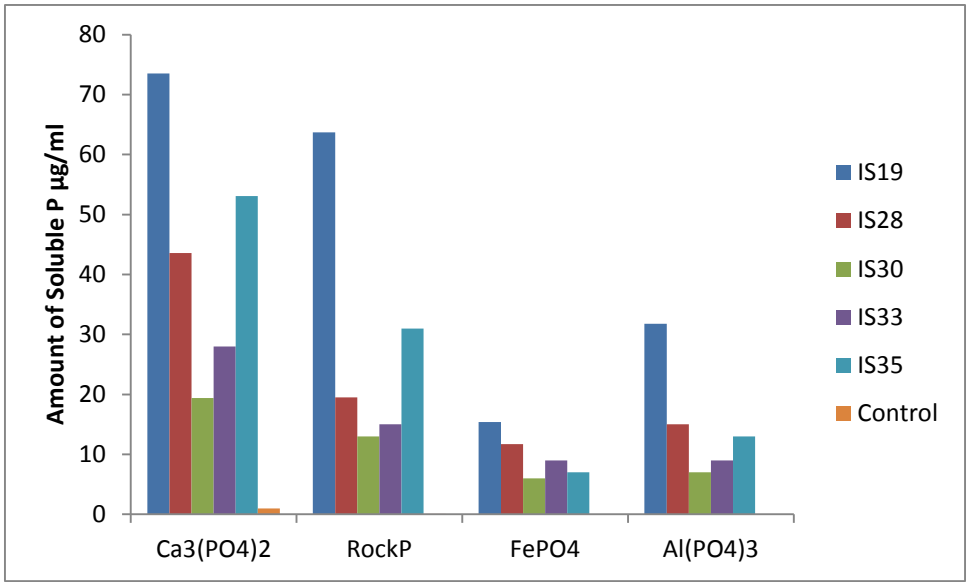

Fig. 3. Solubilization of different types of Insoluble Phosphates in PVK broth

Table 3. One-way Anova showing variations in effect of NaCl concentration on P solubilization

NaCl conc (%)	N Analysis	N Missing	Mean	Standard Deviation	SE of Mean
0.5	3	1	46.33333	3.78594	2.18581
1	3	1	75	7	4.04145
1.5	3	1	69.33333	1.52753	0.88192
2	3	1	21	2	1.1547
2.5	3	1	15.66667	2.08167	1.20185

At the 0.05 level, the population means are significantly different

Table 4. Effects of Initial pH on Solubilization of insoluble phosphates

	Amount of soluble P (µg/ml)			
Initial pH	$Ca_3(PO_4)_2$	Rock P	$FePO_4$	$AlPO_4$
3	9 (1.4)	8 (2.2)	7 (2.6)	7 (2.3)
5	54 (3.3)	34(4.1)	23(4.7)	30(4.4)
7	73 (4.8)	54(5.4)	39(6.0)	54(5.8)
9	17 (7.8)	18(8.1)	8(8.6)	14(8.3)

NB: Final pH are shown in parentheses

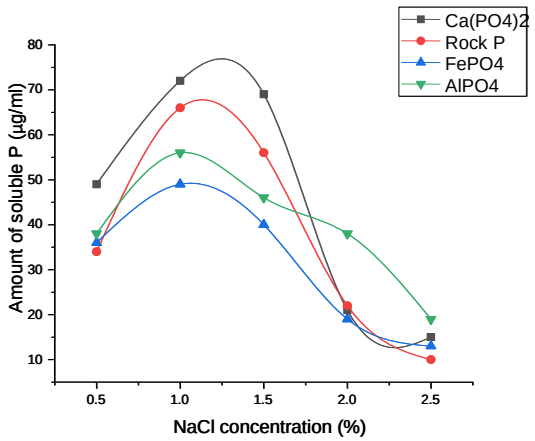

Figure 4: Effect of NaCl concentration on Phosphate solubilization by *B subtilis*

Fig. 4. Effect of NaCl concentration on Phosphate solubilization by *B. subtilis*

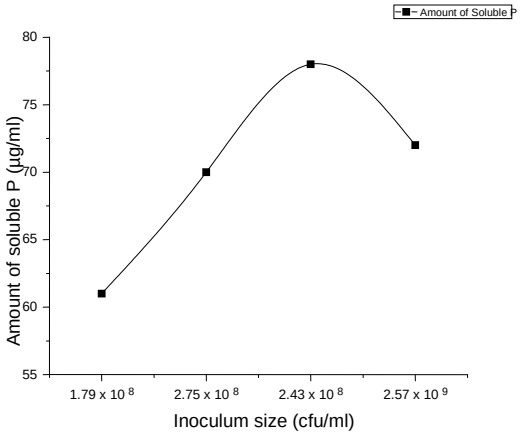

Fig. 5. Effect of Inoculum size on P solubilization by IS19

Table 5. Plant Growth Promoting Properties of the Isolates

Isolate	IAA(µg/ml)	NH_3	HCN	Cellulase
IS19	105.4	+	-	+

Plant Growth Promoting Rhizobacteria are known to employ series of mechanisms in promoting plant growth. These include the production of phytohormones such as Indole Acetic Acid, secretion of cell wall degrading enzymes such as cellulase, production of various compounds such as Hydrogen Cyanide, Ammonia etc. *In vitro* screening for characteristics commonly associated with plant growth promotion revealed that the strain was able to produce IAA with concentration of 105.4µg/ml (Table 3). PGPR may induce significant changes in the architecture of root system through the production of phytohormones (e.g. IAA), which enhance lateral root branching and formation of root hairs [46]. Cellulolytic microorganisms play a major role in the synthesis of cellulases crucial for the breakdown of complex polysaccharides into simple assimilable sugars. The strain also produced cellulase enzyme (Fig. 6). Many cellulose producing *Bacillus subtilis* strains have been previously isolated from soil [47,48].

Table 6. Characterization and identification the Isolate IS19

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
+	**Rod**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**-**	**-**	**-**	**+**	***Bacilllus***	**LC535007.1**	**96.65**	***Bacillus subtilis***

Key - 1: Gram Reaction, 2: Shape, 3: Catalase, 4: Starch Hydrolysis, 5: Citrate, 6: Spore staining, 7: Glucose, 8: Lactose, 9: Fructose 10: Ribose, 11: Maltose, 12: Mannitol, 13: Methyl Red, 14: Indole test, 15: Urease test, 16: Motility test, 17: suspected isolate, 18: Accession, 19: Identity (%), 20: Closely related Taxa

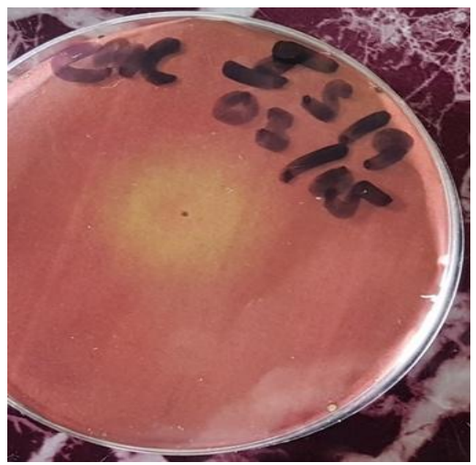

Fig. 6. Cellulase production by *B. subtilis*

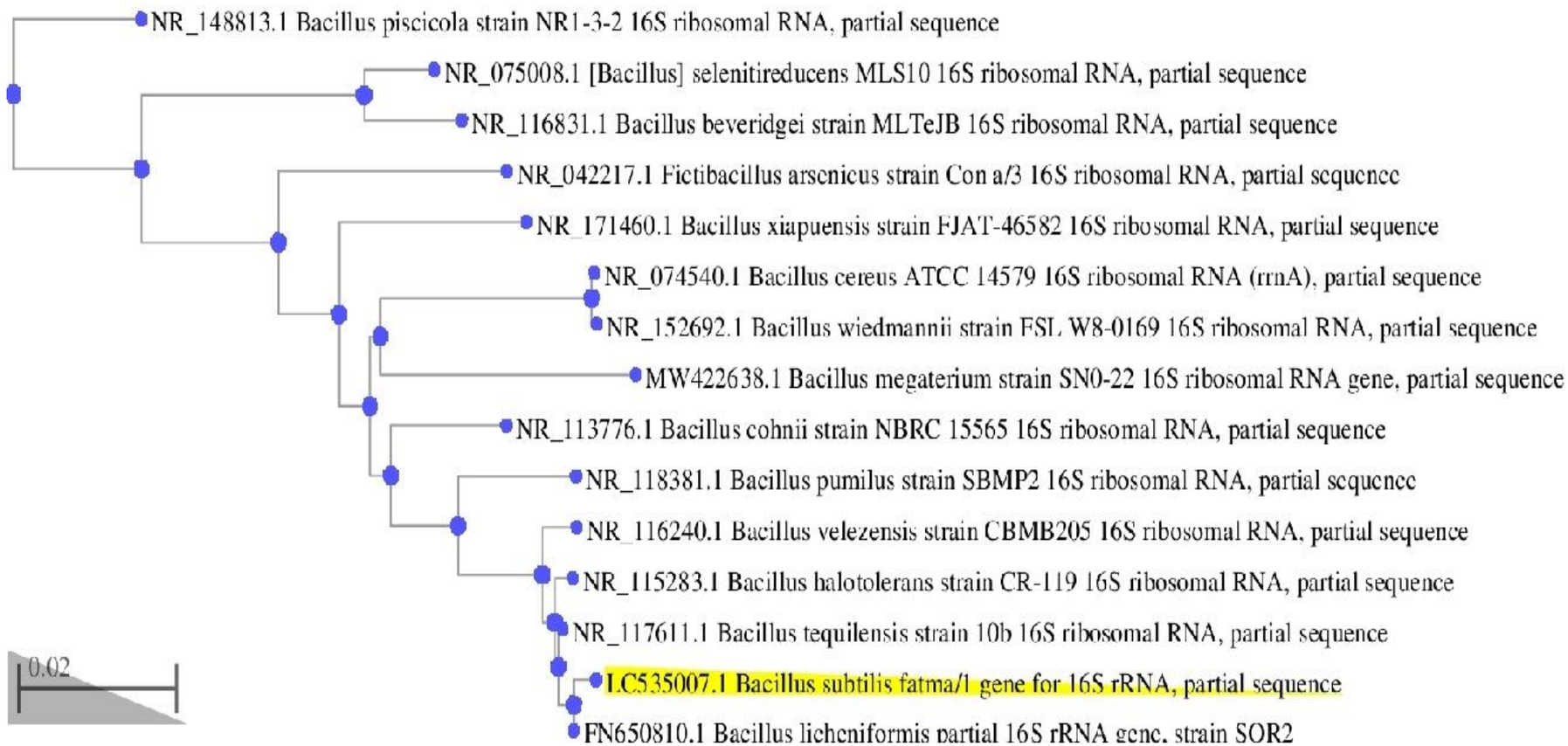

Fig. 7. Phylogenetic tree based on 16S rRNA sequence showing the position of the strain with other closely related strains. These 16S rRNA sequences of related strains were downloaded from NCBI GenBank database

Ammonia production can help satisfy the nitrogen demand of the host plant and in excess reduces the colonization of plants by pathogens. Ammonia production by PGPR is one of the essential traits linked to plant growth promotion. In general, ammonia produced by PGPR has been shown to supply nitrogen to their host plants and thereby promote root and shoot elongation and their biomass [49]. The tested organism gave a positive result for ammonia production (Table 5). The biochemical and phenotypic characterization of the isolate IS19 is as shown in Table 6. Based on the 16S rRNA gene sequences, the PGPR isolate IS19, in this study, had 96.65%. similarity with the *Bacillus subtilis*. The phylogenetic tree based on 16S rRNA sequence showing the position of the strain with other closely related strains is as shown in Fig. 7.

4. CONCLUSION

The PGPR isolates obtained from maize roots fixed atmospheric Nitrogen and solubilized phosphates. The organism with the best potential was characterized and identified as *Bacillus subtilis* using the 16S rRNA gene sequencing. It produced other plant growth promoting properties such as IAA, Ammonia and cellulase enzyme. Our findings indicated that *B.subtilis* can be an efficient strain for microbial inoculants used in agriculture

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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