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Full Length Research Paper

Isolation, identification andScreening of *Pseudomonas fluorescens* from soil environment of Keffi, Nasarawa state - Nigeria for the production of Bio-fertilizer

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ABSTRACT: Bio-fertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production with no residual harmful effect to the environment.This study on isolation, identification andscreening of Phosphate solubilisationabilityof*Pseudomonas fluorescens* from soil environmentwas carried out from May to September 2018. Soil samples were collected from eight different locations and *Pseudomonas* strainswere isolated and identified using standard microbiological methods.The 16SrRNA gene sequence analysis of the strain showed maximum similarity of 96% with *Pseudomonas fluorescens*reference type straindeposited in RDP GenBank. *Pseudomonas* strains isolated from five different sites showed clear halo zone ranging from 8-18mm. Solubilized Phosphate content produced by *Pseudomonas* ranged from 207.3 mg/l -273.75 mg/l.

Keywords: Bio-fertilizer, *Pseudomonas fluorescens,*Phosphate solubilisation

INTRODUCTION

Phosphorus (P) is oneof the most essential macronutrient elements required by plants for growth and development (Fernandez *et al*., 2007). In agricultural practice, is the most limiting nutrients which deficiencies could led to slow growth and reduced crop production (Venkateswarlu*et al*, 2007).Most agricultural soil contains large amounts of nutrient elements such as phosphorus (P), nitrogen (N) but exist mostly as insoluble mineral complexes as rock phosphate, tricalcium phosphate and di calcium phosphate(Fernandez *et al.,* 2007). Application of chemical fertilizers into the soil to increase soil fertility is not environmentally friendly as it affects native soil microbesand lot of health hazards (Shanmugam*et al*., 2000; Kloepper*et al*., 1991). Residual ill effects of chemical in the soil ranges from degradation of the environment, depletion of top soil nutrients, pollution of ground water, and destruction of soil microbes among others which rendered it unsustainable(Andersona*et al*., 2003; Mehrvarz*et al*., 2008).Incorporation of novel strain bacteria inoculants from any of thesegenera *Pseudomonas*, *Azotobacter*, *Azospirillum* and *Bacillus*into the soil will greatly increase the nutrients content of the soil to improve growth and development of the plants because they are well known as Plant Growth Promoting Rhizobacteria (PGPR) and a powerful agent of bio-fertilizers(Gyaneshwar, 2012).They produces organic acids such as formic, acetic, propionic, lactic, glycolic, fumaric and succinic acidsas while phytohormones as mechanisms of action in the solubilizationofcomplex inorganic compound (di calcium phosphate) in the soil and make soluble phosphate available for plants use(Rashid *et al*., 2004; Ivanova*et al*., 2006).

MATERIALS AND METHODS

Sample collection

Soil sample from eight different locations within keffi, Nasarawa state-Nigeria were collected usinghand shovel at 20mm depth(Appanna *et al*., 1996) and transfer into sterile polyethylene bagbeforetransported to Microbiology laboratory Nasarawa state University, Keffi for further study.

Isolation and subculture of *Pseudomonas* species

Isolation was carried out using a method described by Don Brenner *et al*., (2005). 1.0g of the soil sample was suspended in a test-tube containing 9ml of sterile distilled water. 1ml from the suspended mixture in the initial test-tube was picked and transfer into another test tube

containing 9ml of sterile water. The step was repeated to 10-fold dilutions. 0.5ml of the aliquot dilution was picked from the 6th-dilution tubeusing pipette and spread on prepared King's B agar plate and incubated at 37^{o}C for 48 hrs. Discrete colonies were picked and re-streaked on King's B agar slants for further studies.

Identification of *Pseudomonas* species

Cultural and morphologicalcharacteristics of *Pseudomonas* species isolated on King's B agar were carried out by gram's staining method described bySulbara`n*et al*., 2007. Biochemical test such as methyl red, Vogesproskauer, indole, catalase, oxidase, urease, citrate utilization, motility, starch hydrolysis, gelatin hydrolysis, carbohydrate utilization and cyst formation were carried according to the method described in Bergey's Manual of Determinative Bacteriology 9th edition(Singh *et al*., 2007; Maki *et al.,* 2011; Breed *et al.,* (2011).Amplification of the 16SrRNA gene from the extracted genome of the bacteria strain was performed using the universal primer pairs 27F (5′-AGAGTTTGATCATGGCTCAG-3′) as forward primer and 1492R (5′-GGTTACCTTGTTACGACTT-3′) as the reverse primer (Lane, 1991; Turner *et al*., 1999; Ahmed *et al.*, 2007). Full length sequencing of the amplified 16SrRNA gene product (amplicon)of the *Pseudomonas* strain was send to Epoch Life science (USA) for purification and Sanger's sequencing as outlined in QIAquick purification protocol (Qiagen, Crawley, UK)(Glyselinck*et al*., 2013).

The DNA extraction procedures

500µl overnight culture broth of the bacteria strain was spun at 1000 rpm for 5min. The pellet was re-suspended in 300µl lysis buffer and 2µl RNase A and later Vortex vigorously for 30-60sec. 8µl proteinase K was added to the mixture by pipetting and then incubated at 60^{0}c for 10min and later cools down for 5mins. Thereafter, 300µl binding buffer was added and vortex briefly before the tube was place on an ice for 5mins and later centrifuge for 5mins at 10,000 rpm. 40-50 µl elution buffer was added into the centre of the column then incubated at room temperature for 1min before it was centrifuge at 10,000g for 2mins and the DNA pellet was stored at 4^{0}C or -20^{0}C as outline in Jena Bioscience extraction protocol(Nishiguchi*et al*., 2002; Vural, Ozgun, 2011).

PCR Amplification of 16SrRNA gene of the bacteria species isolated

The 16SrRNA extracted genome DNA of the bacteria strain was amplified using Polymerase chain reaction (PCR) amplification processes by mixing a set of universal primers, 27F (5′-AGAGTTTGATCATGGCTCAG-3′) as forward primer and 1492R (5′-

GGTTACCTTGTTACGACTT-3′) as the reverse primer (Lane, 1991; Turner *et al*., 1999; Ahmed *et al.*, 2007).A typical PCR reaction mixture was prepared by adding 2 µl of the template DNA with the 5X HOT FIREPol Blend Master mix (Solis Biodyne) containing 1.5 µl of forward and 1.5 µl reverse primers (BIOMERS, Germany), 5µl of 10X PCR buffer (Solis Biodyne), 1.5µl $MgCl_2$, 2.0µl mM of deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 1.5 µl Taq DNA polymerase enzyme (Solis Biodyne), and 5µl of nuclease free water alongside 5µl cells pellet of the extracted bacteria DNA and the entire reaction mixture was make up to 25µl by total concentration. The PCR reaction mixture tubes wereplaced on an Eppendorf vapo protect thermo cycler machine (Nexus series) to run 30 cycles programme under a given PCR conditionsfor an initial denaturation at 94°C in 2minutes (30 cycles), follow by denaturation at 94°C for 45 seconds, annealing at 61°C for 1 min, extension at 72°C for 1 minute 30 seconds (30 cycles)and finally extension at 72°C for 5 minutes (1cycle) and hold at 4°C. The amplified 16SrRNA gene product wererun on a 1.5% agarose gel electrophoresisafter staining with ethidium bromide standard solution (aliquot fraction of the PCR product and one DNAladder were loaded per comb or gel well) for 1 hour 30 minutes at 80V. After electrophoresis, distinct DNA bands well separated of the bacteria species were compare with molecular ladder of the standard DNA of the gene size or molecular marker of 1500bp (Solis Biodyne)(Devereux, Wilkinson, 2004; Silva *et al*., 2013).

Sequencing of the amplified 16SrRNA gene

Fraction of the amplified genome of the bacteria strains were send (Epoch Life science (USA) for purificationaccording to QIAquick purification protocol described by (Glyselinck*et al*., 2013).The purified product was send to Epoch Life science (USA) for sequencing (Sanger sequencer machine) and analysis using ABI 3100 software module (version 5.2) (Giovannoni*et al.,*1990).Thus,the resulted sequences obtained were compared with the published nucleotide sequences of close related bacteria species type strain deposited in the GenBank National Centre for Biotechnology Information (NCBI) database and Ribosomal Database Project via BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Nikunjkumar, 2012; Tamura *et al*., 2011).

Phosphate solubilizing activity of *Pseudomonas* species isolated

The phosphate solubilizing activities of the *Pseudomonas* species isolated were determined both qualitatively and quantitatively by agar plate screening method and broth method,

respectively. **2.7Screening for Phosphate solubilisation by *Pseudomonas* species isolated on Pikovskaya's agar**

*Pseudomonas*isolates were tested on a Pikovskaya's agar and incubated at 37^0C for 3-5days by a method described by SubbaRao 1999, Naikar 2008 and Sangeeta*et al.,* 2011. After the 3rd day, colonies showing halo zones around them (clear halo zone) were considered positive for Phosphate-solubilisation and were measured as solubilisation index (SI) for each of the bacterial species.

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

Quantitative estimation of Phosphate solubilized by *Pseudomonas* species isolated on Pikovskaya'sbroth

Alltheeight*Pseudomonas* species were further inoculated each on a 250 ml flask of Pikovskaya'sbroth (without agar) for 9 days.Molybdo-vanadate methodin conjunction with UV-Spectrophotometer at 470 nm as described by Subba Rao *et al*., 1982 was employed. (Solution A: 25g $NH_4MoO_4.2H_20$ was dissolved in 400mls sterile water. Solution B: 1.25g ammonium vanadate dissolved in 300ml boiling water on a water bath, allow coolingbefore mixed with 250ml concentrated $HN0_3$solution). Barton's reagent was prepared by mixing A and B and make up the volume to 1000ml. A greenish colour was form as outline by molybdo-vanadate James (1995).2ml of the clear filtrate was mixed with 5ml Barton's reagent and the volume was made up to 10ml with sterile water. After 10minutes, the mixture solution turned yellowish and the intensity of the colour were measured at 470nm wavelength. 0.1mg/mlstandard solution of Potassium di hydrogen phosphate (KH_2PO_4)was prepared and the absorbance measured were used to constructa standard calibration curve by plotting absorbance values against varying concentrations of the standard solution (mg/ml). Straight line graph is formed and the amount of solubilized phosphate produced (unknown sample concentration) in the experiments was calculated using the regression equationof the straight line graphAOAC (2016).The amount of soluble phosphorous (P) released in the broth culture by each *Pseudomonas* species wascalculated.

Statistical analysis

Data obtained were analysed using R Console software (Version 3.2.2). Pearson's Chi-square test was used to compare the proportion of activity and ability of bacterial isolates in

solubilisation of complex phosphate in relation to locations where soil samples was collected. P-values < 0.05 were considered statistically significant.

RESULTS

Isolation and Identification of *Pseudomonas* species

Cultural, morphology and biochemical characteristics of the *Pseudomonas* species isolated is given in Table 3.1

Percentage occurrence of the bacteria species isolated from different locations in keffi

Percentage occurrence of the *Pseudomonas* species isolated from different locations in keffi showed that Angwanlambu (AL) and GRA had the highest occurrence of *Pseudomonas* isolates with 100% while High court, Kofarhausa, Old barrack, BCG, pyanko, and Angwanmada had 50% occurrence as given in Table 3.2

16SrRNA Sequenced gene of the *Pseudomonas* strain

16SrRNA gene sequence of selected *Pseudomonas* strain with hyper activity is given in Figure 3.1. The sequences obtained was compared with 16SrRNA gene sequences of reference type strain available in the RDP Genbank database (http: 11rdp (me.msu.edu/).

Rate of solubilisation activity of *Pseudomonas* strains on Pikovskaya's agar plates

Figure 3.2 shows the rate of solubilisation activity of *Pseudomonas* strains were the highest halo zone was observed in *Pseudomonas* species isolated from Angwanlambu (AL) with 18mm followed by Angwanmada (AM) with 15mm, GRA with 14mm, High court (HC) with 11mm and the least was observed from isolate from BCG with 8mm.

Amount of solubilized phosphate produced by *Pseudomonas* strains on the broth cultures

Figure 3.5shows theamount of solubilized phosphate by*Pseudomonas fluorescens*were the highest amount was observed in *Pseudomonas fluorescens* isolated from Angwan-lambu (AL) with 273.75 mg/ml followed by Angwan-mada (AM) with 267.30 mg/ml, followed by GRA with 265.25 mg/ml and High court (HC) with 263.00 mg/ml respectively while low

amount was observed in *Pseudomonas* isolates from BCG with 261.00 mg/ml, followed by Pyanko with 241.75 mg/ml, followed by Old-Barrack (OB) with 218.25 mg/ml and the least amount was observed in isolate from Kofar Hausa (KH) with 207.30 mg/ml respectively.

Table 3.1 Cultural morphology and biochemical characteristics of the bacterial species

Cultural	Morphology		Biochemical characteristics											Inference
	G	S	Ca	O	I	MR	VP	M	CUT	SH	UH	GH	CF	
G/Y, GL on KB Agar	**-**	**rod**	**+**	**+**	**-**	**-**	**-**	**+**	**+**	**+**	**-**	**-**	**-**	***Pseudomonas sp.***

G = Gram reaction, S = shape, Ca = catalase, O = Oxidase, I = indole, MR = Methyl red, VP = Voges-proskauer, M = Motility, CUT = Citrate utilization test, SH = Starch hydrolysis, UH = Urease hydrolysis, GH = Gelatin hydrolysis, CF = Cyst formation, -negative, +positive, G/Y = Green/Yellowish, GL = Glistening

Table 3.2Percentage occurrence of the bacteria species from different locations in keffi

Locations	No. Sample	% *Pseudomonas* isolates
AL	**2**	**2 (100)**
HC	**2**	**1 (50)**
KH	**2**	**1 (50)**
GRA	**2**	**2 (100)**
OB	**2**	**1 (50)**
BCG	**2**	**1 (50)**
PYANKO	**2**	**1 (50)**
AM	**2**	**1 (50)**

AL = Angwanlambu, KH = Kofarhausa, Pyanko, HC = High court, OB = Old barrack, AM = Angwanmada, % = Percentage

CGGAAGGAACACATAGAGTATGATCCTGGCTCAGAAATCTCAAAACGGGAACCC
CAAAAGTTTTTTTTCCACACACTTAGGGGGCCGGGAAAACCCCCCGGGGTTTTTT
TTTTAAACTTTTCCGGGGCCGGGGACCAACCCCCAGGTGTTTTTTTTTGCACTTTC
TGGGGGCGGGGAACAACCTCATTGTGTTTTTTTTTTTTTTTTCTTGGGAGGGGGG
AAACACCAAATTTTTTTTTTTTTTTGTTCTTCCGGCGCCAAACAAAAACAAAAAAT
ATTTTTTTTTTTTGGCCTTTATTCCCAGGGAAGAAAAAAAAAAACATTGTTTGTCT
TTTATTTAAAAGGCCCCACAAAAAAACCAACCGGTGTTTTTTTTTCTTGAAATCC
GGGAGAGGTGCGAAAAACCTTTGTTTTTTGTGTGCGAAACGCGGGCAGGGGTCC
TAATATTTGTTTTCTCCCGTTCCTCCCACTCGCTGCAGCCAAAATAGGATACATTC
CCATTAGAAGATACCTGTTGTTCGCCCGGAAGAAGGGATTGTCGAAGGGATCAA
GAAAACGTGGCGCCCCGGACGGCAAAGAATAACTTGTACTCCTAGAGAAAGGAC
GTCACGAGGTGCTTTGGC

Figure 3.3 16SrRNA gene sequences of *Pseudomonas fluorescens* species

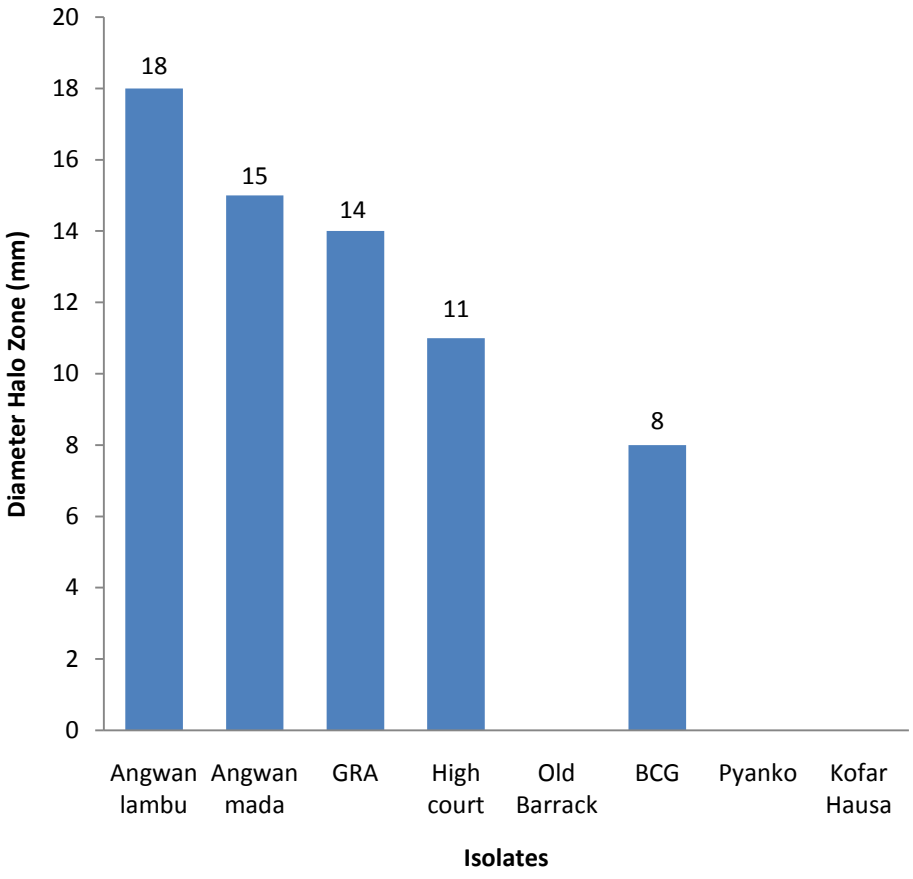

Figure 3.4 Rate of Phosphate solubilisation activity of *Pseudomonasfluorescens*on Pikovskaya's agar plate

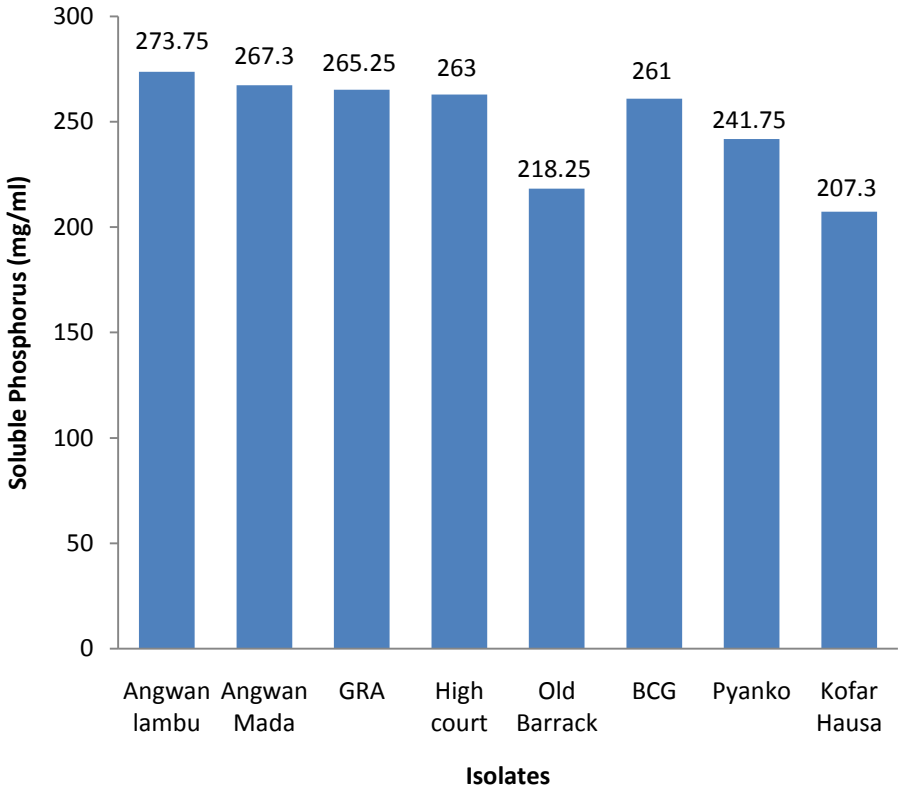

Figure 3.5. Amount of solubilized phosphate produced by *Pseudomonas fluorescens* in Pikovskaya's broth

DISCUSSION

Eight *Pseudomona*s species were isolatedand their cultural features was observed and identified by various biochemical characteristics and 16SrRNA sequencing analysis. Microscopic examination of the strainsshowed thatthey are gram negative, rod shaped and motile in nature. The 16SrRNA sequenced analysis of strain with hyper phosphate solubilisationactivity showed maximum similarity of 96% with *Pseudomonas fluorenscesof* the reference type strain. Therate of solubilisation activity was measured and quantified qualitatively and quantitatively. Solubilisation activity of *Pseudomonas* strains with the highest halo zone was observed in *Pseudomonas* species isolated from Angwanlambu (AL) with 18mm *fluorescens*followed by Angwanmada (AM) with 15mm, GRA with 14mm, High court (HC) with 11mm and the least was observed from isolate from BCG with 8mm. The halo zone formed is due to the production of organic acid or secretion of phosphatase enzyme by *Pseudomonas fluorescens* on the Pikovskaya's agar. These findings agreed with

Fernandez *et al*. (2007) and Kumar *et al*. (2012) that *Pseudomonasfluorescens* is capable of forming a halo zone on Pikovskaya`s agar plate due to production of organic acid.

Similarly, *Pseudomonas* isolatewith the highest amount of solubilized phosphate was observed in *Pseudomonas* isolated from Angwan-lambu (AL) followed by Angwan-mada (AM), GRA and High-court (HC) on Pikovskaya's culture broth while strains isolated from BCG, Pyanko, Old-Barrack (OB) and Kofar Hausa (KH) produces the lowest amount of solubilized phosphate of 261.00 mg/ml, 241.75 mg/ml, 218.25 mg/ml and 207.30 mg/ml respectively as presented in Figure 3.5 above. Solubilisation occurred due to the production of organic acids or secretion of phosphatase enzyme by the *Pseudomonas* species in the medium and this is the main principal mechanism of phosphate solubilisation(Kumar *et al*., 2001).

Already it has been established in the literature that Phosphate solubilising Bacteria (PSB) solubilizes insoluble phosphate in the soil by secreting organic acid. Statistically results of solubilized phosphate in relation to the different locations showed significant difference ($P<0.05$)

(χ^2 =17.159, df = 7, P = 0.0164). These findings are in agreement with research worked postulated by Rodriguez *et al*., (2006) and Collavino*et al*., (2010) that phosphate solubilizing *Pseudomonas* (PSP) has the potential in solubilizing insoluble phosphate compound on broth medium and make phosphorus available in soluble. Although not all the amount of phosphorus produced on the broth are calculated, some fractions have already been used up by thespecies in carrying out its biochemical activities.

Thus, the *Pseudomonas fluorescens* used for this study might have used the same mechanism to solubilize the inorganic phosphate incorporated on the broth (Kumar *et al.,* 2001).

Conclusion

In this study a potential phosphate solubilizing bacteria species was isolated from the rhizospheric soil of AngwanlambuKeffi, Nasarawa state-Nigeria. The efficiency of the strain resulted in formation of a halo zone.This *Pseudomonas* strain isolated could be a potential candidate for bio-fertilizer production.

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