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New primer generated bacterial mapping and biofertilizing potentiality assessment of *Pseudomonas* sp. isolated from cowdung

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Key words: Cowdung, plant growth promoting fluorescent Pseudomonads, MEGA6; species specific primer design, Fast PCR.

Abstract

Modern biofertilizer trend is to use of different plant growth-promoting microorganisms (PGPM), isolated from several sources, including rhizosphere. Here we evaluate the plant growth promoting ability of some fluorescent Pseudomonads, isolated from cow dung. Based on different biochemical tests, we select 7 isolates to observe their effect on *Cicer arietinum* (Bengal gram) seed germination and sprout growth promotion. A new *Pseudomonas* sp Strain TMGR (NCBI Accession Number JX094352) showed best effect with Vigor-index value 4470. 16s rRNA gene sequencing revealed that all isolates have 97-99% similarities with *Pseudomonas aeruginosa*. On the basis of Molecular Evolutionary Genetics Analysis version 6.0 (MEGA6), analysis of 16S rRNA sequences of selected seven isolates with other native *Pseudomonas aeruginosa* strains were available in GeneBank, a phylogenetic tree was constructed to study their relative position. Adopting BioEdit and Fast PCR software programs, we designed *Pseudomonas aeruginosa* specific two primers, 103F and 455R, and their validity was rechecked in wet laboratory. These primers may help in isolation and rapid identification of plant growth promoting *Pseudomonas aeruginosa* strains from different sources in future.

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Introduction

Microbial populations are key component of the soil-plant continuum where they immersed in a framework of symbiotic interactions resulting plant development, growth and other geoactive roles. Microbes supply soluble minerals, bio-stimulants, anti-pathogenic secondary metabolites, while plants help with carbohydrates, amino acids, fatty acids through roots (Kumari *et al*., 2009). For this, bacterial population predominates in rhizosphere but only 2-5% among them help plants growth are known as plant growth-promoting microorganisms (PGPMs) or rhizobacteria (PGPR) (Karnwal, 2009; Rodriguez and Fraga, 1999) and among those strains from genera such as *Pseudomonas, Azospirillum, Azotobacter, Burkholderia, Bacillus, Enterobacter, Rhizobium, Erwinia, Serratia, Alcaligenes, Arthrobacter, Acinetobacter* and *Flavobacterium* are well known (Rana *et al.,* 2014; Rodriguez and Fraga, 1999). These plant growth promoting microorganisms (PGPMs) can stimulate plant growth in two ways. Directly they synthesize different phytohormones, enzymes (like: α-amylase, ACC-deaminase, etc.), fix nitrogen, solubilize as well as mineralize both organic and inorganic phosphates for plants (Shahab *et al*., 2009). In indirect way, they decrease detrimental effects of plant pathogens with the synthesis of hydrogen cyanide, siderophore or different antibiotics (Leong, 1986; Glick, 1995; Jayaprakashvel *et al.,* 2010). For these reasons, PGPMs use as biofertilizer for agricultural improvement has been a focus of researchers in recent years (Adhikari-Rana *et al.,* 2014; Chaiham *et al.,* 2008).

Phosphorus (P) is one of the most essential macronutrient for biological growth (Fernandez *et al.,* 2007). It present at levels of 400-1200 mg/kg of soil and it is estimated that there are almost 40 million tons of phosphatic rock deposits in India (Roychoudhury and Kaushik, 1989; Khan *et al.,* 2009). But the concentration of soluble P is very low, normally at levels of 1 ppm or less. The use of phosphate solubilizing bacteria, as inoculants, simultaneously increase P uptake by the plant and crop yield through the production of organic acids and acid phosphatase (Puente *et al.,* 2004; Rodriguez *et al.,* 2006; Henri *et al.,* 2008). Plant hormones (auxins, gibberellins, ethylene, cytokinins and abscisic acid) are chemical messengers help in organogenesis, tropic responses, cellular responses and gene regulation. There are numerous soil micro floras involved in the synthesis of auxins (native indole-3-acetic acid) or its different derivatives (indole-3-pyruvic acid, indole-3-butyric acid) (Khan *et al.,* 2009). This auxin synthesizing bacteria can be used as a tool for the screening of effective PGPMs-strains.

Pseudomonas is known as 'Giant bacteria' among all bacterial genera (Adhikari-Rana *et al*., 2014). These γ-proteobacterial strains play some important roles in recent times like, plant growth promotion (Rodriguez and Fraga, 1999), bioprecipitation (Renninger *et al.,* 2004), bioremediation (Wasi *et al.,* 2013), anti-pathogenic compounds secretion and so on. Again, among *Pseudomonas* sp., fluorescent Pseudomonades are most important (Ahmadzadeh *et al.,* 2006; Naik *et al.,* 2008; Manjunatha *et al.,* 2012). In India, cow (*Bos indicus*) dung containing Beejamrutha is a good fertilizer (Sreenivasa *et al.,* 2009). Cow dung is a vast source of microbes like *Bacillus, Pseudomonas, Lactobacillus, Azotobacter,* fungi *Aspergillus, Tricoderma,* yeast (Rana *et al.,* 2014; Teo and Teoh, 2011; Punitha *et al.,* 2010; Swain *et al.,* 2007) and till now use as the best biofertilizer in India.

In this paper, an attempt has been made to isolate and characterize some fluorescent Pseudomonads from both fresh as well as stored cow dung and studying their biofertilizing ability. This study also able to design and validate species specific primers and use them as molecular probes for PCR based detection of important *Pseudomonas aeruginosa* bacteria and that new set of primer have been designed in order to obtain a complete sequence and facilitate the sequencing.

Materials and methods

Cow dung samples and bacteria isolation

Both fresh cow dung (FCD) and stored cow dung (SCD) samples were collected from Rangamati, Paschim Medinipur, West Bengal, India (Latitude- 22°25'00" to 22°57'00" north, Longitude- 87°11' east, Altitude - 23 meters from mean sea level). Every time pH, electro-conductivity (EC), percentage of organic carbon (% OC), available nitrogen (N_2), available potassium (K_2O) and available phosphorus (P_2O_5) of each sample was measured (Rana *et al.,* 2013). 10 gm either FCD or SCD samples were dumped in 100 ml freshly prepared fluorescent Pseudomonades specific King's B (KB) (Jayaprakashvel *et al.,* 2010; King *et al.,* 1954) broth in 250 ml Earlenmair flasks and incubated at room temperature (Hall *et al.,* 1996; Lemamceau, 1992). After 24 hrs 1ml of screened KB broth of both FCD and SCD were serially diluted up to 10^{-9} times and pour-plated (100 µl) with respective agar media. After 24 hr single colonies were isolated by random sampling, purify them and stored in 40% glycerol solution at -80°C prior to characterization (Rana *et al.,* 2012).

Screening of phosphate solubilizing bacteria

All isolates were spotted on modified Pikovskaya's plates containing aluminum phosphate [$AlPO_4$; Solubility product (Ksp) = 6.3×10^{-19} mol^5dm^{-15}], calcium phosphate [$Ca_3(PO_4)_2$; Ksp = 2.0×10^{-29} mol^5dm^{-15}], iron(III) phosphate [$FePO_4$; Ksp = 1.3×10^{-22} mol^5dm^{-15}], magnesium phosphate [$Mg_3(PO_4)_2$; Ksp = 1.0×10^{-25} mol^5dm^{-15}] and zinc phosphate [$Zn_3(PO_4)_2$; Ksp = 9.0×10^{-33} mol^5dm^{-15}] and incubated at 30±2 °C for 7days (Mahdi *et al.,* 2011). The halo-zone formation around the bacterial colonies was considered to have the phosphate solubilizing activity. Isolates were streaked thrice on same media to confirm the activity. The solubilization efficiency (E) was determined by measuring the halo-diameter (HD in cm.) and the colony diameter (CD in cm.), to obtain the relation of: E = (HD/CD) × 100 (Ramamoorthy *et al.,* 2002).

Spectrophotometric assay of phosphate solubilization and IAA production

All isolates were grown separately in 10 ml modified Pikovskaya's broth having calcium phosphate [$Ca_3(PO_4)_2$] and Luria-Bertani broth. After 14 days, all broths were centrifuged separately at 7000 for 20 min. Amount of phosphate released was measured spectrophotometrically (UV-VIS spectrophotometer; ELICO-INDIA, Model no. 159) by Mo-Blue method from 1ml Pikovskaya's broth supernatant of different isolates (Watanabe and Olsen, 1965) and the precipitated calcium phosphates [$Ca_3(PO_4)_2$] were taken to Mastersizer-2000 (Malvern, UK, E Ver. 5.2, Sl. No. MAL1017204) for the particle size analysis with respect to calcium phosphate used. Similarly, *in vitro* IAA biosynthesis was determined spectropohotometrically from LB-broth by the method of Salkowski (Reddy *et al.,* 2008; Shahab *et al.,* 2009).

In vitro response in gram seed germination and seedling growth

200 ml of different bacterial inoculums, containing 3×10^8 cfu/ml, were separately centrifuged at 7000 rpm for 20 min (Cooling Centrifuge, REMI C-24 BL, India) and supernatant were discarded. Bacterial pallets were washed three times with sterile distilled water and finally suspended with carboxymethylcellulose solution (1mg CMC in 100 ml sterile distilled water). Surface-sterilized seeds of *Cicer arietinum* were then kept separately on water agar media in culture tubes keeping a non-treated sterilized gram seed as control. *In vitro* plant growth-promoting activity by selected microbes, were assessed after 7 days and the seedling Vigour-Index (VI) values were calculated (Muhammad and Amusa., 2003; Ramamoorthy *et al.,* 2002; Glick *et al.,* 1997).

16S rRNA gene sequencing

Chromosomal DNA was extracted by procedure of Wilson (1987) with some modification. 500 mg of wet-bacterial cells were resuspended in 100µl TE-buffer (10mM Tris-HCl, 1mM EDTA, pH 8·0). Cells were lysed by 10% SDS and proteinaseK (20 mg/ml; HIMEDIA). After isolated DNA was further purified by chloroform/isoamyl alcohol (24:1, v/v), phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and RNaseA (10mg/ml; HIMEDIA). DNA concentration and purity of each DNA sample were

determined by measuring the optical density at A_{260} and A_{260}/A_{280} ratio, respectively (Murray and Thompson, 1980).

The 16S rRNA gene was amplified using PCR with *Taq*-polymerase (Merck, Bioscience) (Saiki *et al.,* 1988) and the universal primer pair of 27F (5'-AGAGTTTGATCCTGGTCAGAACGCT-3') and 1459R (5'TACGGCTACCTTGTTACGACTTCACCCC-3'), described by Weisburg *et al.* (1991). PCR amplifications were performed in 50 µl reaction mixture containing buffer, 1.5 mM $MgCl_2$, 0.5 nmol each deoxynucleoside triphosphate, 10 pmol each primer, 2.5 U *Taq*-polymerase and the template DNA, by using the DNA thermal cycler (Mastercycler personal, Eppendrof AG-22331, Germany) with the following temperature profile: an initial denaturation at 94°C for 2min; 30 cycles of denaturation (1min at 94°C), annealing (1min at 55°C) and extension (1min at 72°C); and a final extension at 72°C for 10min (Scarpellini *et al.,* 2004). The amplicons were purified and sequenced directly using a *Taq* DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems). Sequence reaction mixtures were electrophoresed and analyzed with an Applied Biosystems model 377A DNA sequencer.

Sequence alignment, Phylogenetic tree construction

Sequences analyses of selected strains were performed using BLAST search tool available on the NCBI homepage (http://www.ncbi.nlm.nih.gov). A Maximum Likelihood (ML) tree was constructed with the 16S rRNA sequences of closely related members of isolated strains as retrieved from the NCBI GenBank (http://www.ncbi.nlm.nih.gov) (Kwon *et al.,* 2005). Sequence data were aligned with ClustalW 1.6 and evolutionary tree construction was done by using Molecular Evolutionary Genetics Analysis 6.0 (MEGA6) software (Tamura *et al.,* 2013). A bootstrap analysis of 1000 replicates was performed to estimate the confidence of tree topologies (Adhikari-Rana *et al.,* 2014).

Design and assess of PCR 16S rRNA Pseudomonas aeruginosa Species-Specific Primer

All 16S rRNA sequences of our selected isolates and some other sequences of *Pseudomonas aeruginosa* were taken from NCBI. Multiple alignments of those sequences were performed with ClustalW multiple program in BioEdit software to get consensus sequence. 103F (5'-AATACCGCATACGTCCTGAGG-3') and 455R (5'-CCCTTCGTATTACCGCGGCT-3'), two *Pseudomonas aeruginosa* specific primers, were designed with the Fast PCR program. Validity of those new primers was rechecked both in *in silico* way by using OligoCalc software (Kibbe, 2007) and in wet laboratory with some *Pseudomonas aeruginosa* strains and some other species like *Bacillus subtilis subsp*. strain TGNS and *Enterobacter* sp Strain TSNG. PCR amplified (see Table S4 for PCR conditions) products were electrophoresed in 1% agarose gel. DNA bands of the expected size of 372bp for all *Pseudomonas aeruginosa* species specific primers extracted from the gel and further sequenced for validation of those species specific primers (Chakraborty, 2014).

Results

Evaluation of phosphate solubilizing ability and IAA production ability

Total 55 bacterial strains were isolated by three time repetition of isolation processes and random sampling. Among 30 isolates of FCD, 29 showed phosphate solubilizing activity on calcium phosphate [$Ca_3(PO_4)_2$], magnesium phosphate [$Mg_3(PO_4)_2$] and zinc phosphate [$Zn_3(PO_4)_2$] containing Pikovskaya's plates (Figure 1) and 9 produced cherry red colored ring upon addition of Kovac's reagent with LB-liquid media. But among 25 isolates of SCD (isolates are denoted by superscript 'S' in strain mark), 21 had phosphate solubilizing activity and 13 could synthesize IAA. Spectrophotometric results showed, only 2 isolates were most efficient phosphate solubilizers and 2 had high IAA production ability among 30 of FCD, while among 25 of SCD, 8 were most efficient phosphate solubilizers and 6 had high IAA production ability (Table 1). Based on these tests results seven isolated were separated to carry out the whole investigation. Different phosphate solubilization efficiency in plate assay and

spectrophotometric assay of all selected isolates are shown in Table 1. No isolates can solubilize aluminum phosphate [$AlPO_4$] and ferric phosphate [$FePO_4$].

Particle size analysis data also revealed a good size decrement for solubilize calcium phosphate granules (Figure 2). Every time particle size analyzer analyzed the particle size range between 0.100 µm to 1000.000 µm. Where 15.55% obscuration was found for control and 14.41% obscuration was found for treated particle. Residual weight decreased from 0.318% to 0.242%, mean surface weight decreased from 9.571 to 1.221 µm and mean volume weight decrease from 34.726 to 25.560 µm during the solubilization.

Table 1. Selected isolates identification, phosphate solubilization, indole-3-acetic acid production and plant growth promotion ability.

Isolates	*Strain name*	*GeneBank Accession Number*	*SE of $Zn_3(PO_4)_2$*	*SE of $Mg_3(PO_4)_2$*	*SE of $Ca_3(PO_4)_2$*	*Amount of phosphate released (in Kg/Hector)*	*IAA (ppm)*	*VI*
Control	No strain used	-	000.00	000.00	000.00	0.000	0.000	885
E_{57}	*Pseudomonas sp* Strain GRTM	KC169988	280.00	200.00	140.00	1.152	5.048	4290
B_{11}	*Pseudomonas sp* Strain GATS	KC169987	185.71	255.60	160.67	0.972	0.048	4410
P_{64}	*Pseudomonas sp* Strain GNST	KC169994	175.00	111.10	100.00	1.026	5.128	3710
P_{76}	*Pseudomonas sp* Strain TMGR	JX094352	225.00	210.00	160.00	1.250	7.932	4470
P^S_{46}	*Pseudomonas sp* Strain GTRS	KC169990	144.44	162.50	150.00	1.056	5.326	2830
P^S_{71}	*Pseudomonas sp* Strain TSSG	KC169991	171.43	142.90	150.00	1.008	0.765	1680
P^S_{111}	*Pseudomonas sp* Strain GTNS	KC169989	216.67	122.20	115.38	0.954	0.946	3680
Z^S_{16}	*Pseudomonas sp* Strain TSGR	KC169995	166.67	108.30	111.11	0.936	0.726	3930

Supplementary materials

Table S1. Fertilizing-parameters measurement values of FCD and SCD.

Sample collection place and type	*pH*	*EC*	*% of OC (Kg/ Hect.)*	*Avl. N_2 (Kg/ Hect.)*	*Avl. P (Kg/ Hect.)*	*Avl. K (Kg/ Hect.)*
Panskura, FCD	7.2	2.70	3.68	3542	>500	400.0
Rangamati, FCD	7.6	2.40	3.48	3480	>500	300.0
Delua, FCD	7.4	2.20	3.40	3422	>500	280.0
Average, FCD	7.4	2.43	3.52	3481	>500	326.6
Panskura, SCD	7.9	0.21	3.38	3388	350.0	350.0
Rangamati, SCD	7.7	0.23	3.33	3222	320.0	250.0
Delua, SCD	7.8	0.25	3.30	2980	300.0	220.0
Average, SCD	7.8	0.23	3.34	3197	323.3	273.3

Table S2. Maximum Likelihood fits of 24 different nucleotide substitution models.

Strain	Accession No	Country
Pseudomonas sp. GRTM	KC169988	India; Cowdung
Pseudomonas aeruginosa	AB108690	Japan
Pseudomonas aeruginosa P60	KF670598	Korea
Pseudomonas aeruginosa D1	KF113578	China
Pseudomonas sp. GATS	KC169987	India; Cowdung
Pseudomonas aeruginosa R14	DQ095879	India
Pseudomonas aeruginosa NT1	JQ229776	India
Pseudomonas aeruginosa 57	FJ972538	South-Korea
Pseudomonas aeruginosa K2	FJ972528	South-Korea
Pseudomonas sp. Mexd38	JX436405	France
Pseudomonas fluorescens CIAH-Pf-196	HM484975	India
Pseudomonas sp. CGR-E2	HM171373	India
Pseudomonas sp. GNST	KC169994	India; Cowdung
Pseudomonas aeruginosa 21R	GU263805	China
Pseudomonas aeruginosa NO6	FJ972534	South-Korea
Pseudomonas aeruginosa strain SXYC6	JN999830	China
Pseudomonas aeruginosa strain P-9	HQ224529	India
Pseudomonas sp. TMGR	JX094352	India; Cowdung

Pseudomonas aeruginosa R7-734	JQ659920	Singapore
Pseudomonas aeruginosa IFS	JQ041638	Pakistan
Pseudomonas aeruginosa CB3	KJ854401	India
Pseudomonas sp. DGD4	KJ700306	Viet Nam
Pseudomonas sp. GTRS	KC169990	India; Cowdung
Pseudomonas aeruginosa strain LES4	HQ123431	India
Pseudomonas aeruginosa strain LPT5	HQ123430	India
Pseudomonas sp. TSSG	KC169991	India; Cowdung
Pseudomonas sp. G4(2013)	KC502895	China
Pseudomonas aeruginosa NO3	FJ972531	South-Korea
Pseudomonas aeruginosa NO2	FJ972530	South-Korea
Pseudomonas aeruginosa K2	FJ972528	South-Korea
Pseudomonas sp. GTNS	KC169989	India; Cowdung
Pseudomonas aeruginosa SK9	KC790300	China
Pseudomonas fluorescens FPVC18	JQ660571	India
Pseudomonas fluorescens BCPBMS-1	HQ907732	India
Pseudomonas aeruginosa 63-2A	HM104655	China
Pseudomonas sp. TSGR	KC169995	India; Cowdung
Pseudomonas aeruginosa LCD12	FJ194519	China
Pseudomonas sp. AMO3	GQ483505	Iran
Pseudomonas aeruginosa ELA-6.20	FJ195006	South Africa
Pseudomonas sp. XP-M2	EU410508	China

Response to seed germination and plant growth

It was found that, among 7 isolates P_{76} can influence the plant growth with highest Vigour Index value than others selected bacterial isolates (Figure 3). In all cases, two seeds were germinated (germination energy 100% without any percent disease index) and sprouts gave highest average growth. Mean Vigour Index values of sprouts after a week influenced by different selected bacteria are shown in Table 1.

Table S3. Maximum Likelihood Fits of 24 Different Nucleotide Substitution Models. JC+G is thus best fitted model with low BIC value for phylogenetic study.

Model	#Param	BIC	AICc	lnL	Invariant
JC+G	78	2709.214119	2076.842693	-960.1708687	n/a
K2+I	79	2710.182823	2069.710514	-955.5983464	0.885190574
JC	77	2711.527451	2087.257071	-966.3844092	n/a
JC+G+I	79	2713.570552	2073.098244	-957.2922113	0.555670186
K2+G	79	2714.216217	2073.743908	-957.6150436	n/a
K2	78	2716.497939	2084.126513	-963.8127787	n/a
HKY+G	82	2719.010032	2054.236058	-944.8413287	n/a
T92+G	80	2720.465117	2071.892089	-955.6826192	n/a
HKY	81	2721.203823	2064.530241	-950.9950984	n/a
JC+I	78	2721.608212	2089.236786	-966.3679153	0.004175226
T92	79	2722.702807	2082.230498	-961.8583384	n/a
K2+G+I	80	2724.298284	2075.725257	-957.599203	0.004279285
HKY+G+I	83	2728.988945	2056.114742	-944.7739105	0.018263528
TN93+G	83	2729.010478	2056.136276	-944.7846771	n/a
T92+G+I	81	2730.517613	2073.84403	-955.6519933	0.008276755
HKY+I	82	2731.290624	2066.51665	-950.9816244	0.003400662
TN93	82	2731.312282	2066.538308	-950.9924536	n/a
T92+I	80	2732.441233	2083.868206	-961.6706775	0.046485641
TN93+G+I	84	2738.969537	2057.99527	-944.7073322	0.02113064
TN93+I	83	2741.321268	2068.447066	-950.9400723	0.01320771
GTR+G	86	2758.59706	2061.423157	-944.4073457	n/a
GTR	85	2758.667373	2069.593206	-949.4993761	n/a
GTR+I	86	2759.894192	2062.720289	-945.0559115	0.682206725
GTR+G+I	87	2765.843948	2060.570472	-942.9739151	0.335888841

Table S4. PCR for 16SrRNA Species-Specific Primers for *P. aeruginosa*.

Primer Name	*Primer Pair (Forward and Reverse 5'-3')*	*Size of PCR Product (bp)*	*Annealing Temperature (O°c)*	*Specificity*
1F3_1_103-123 1R3_1_455-474	AATACCGCATACGTCCTGAGG CCCTTCGTATTACCGCGGCT	372	55	*P. aeruginosa*

PCR conditions were as follows: initial denaturation for 2 min at 94°C,followed by 40 cycles of denaturation for 45s at 94 °C, annealing temperature for 45s as indicated for *P. aeruginosa* species, and elongation for 1 min at 72 °C, followed by a final 10 min elongation at 72 °C and finally keep amplicon at 4 °C for indifinite time.

Identification of selected isolates

The amplified 16S rRNA products of all selected isolates were sequenced and all nucleotide sequences of RNA fragments were compared to the sequences available in NCBI GenBank. Sequence analysis of these isolates was also performed using BLAST (Blastn) search tool (http://www.ncbi.nlm.nih.gov) available on the NCBI homepage. All isolated strains exhibited 97 to 99% sequence similarity with *Pseudomonas aeruginosa*.

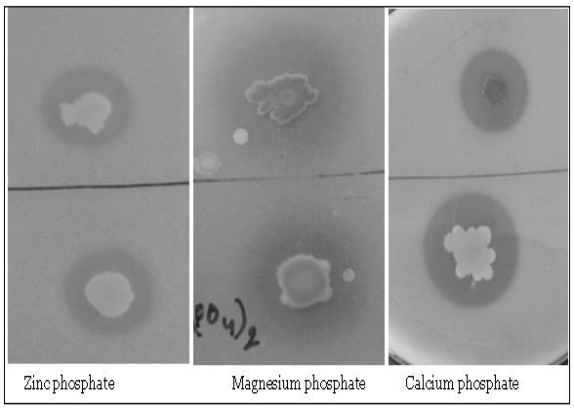

Fig. 1. Zinc phosphate, magnesium phosphate and calcium phosphate solubilization by *Pseudomonas* sp. Strain GRTM (top) *Pseudomonas* sp. Strain TMGR (bottom), respectively.

Phylogenetic Affiliation of 16S rDNA of Pseudomonas sp

Forty 16S rRNA sequences, including all isolated Pseudomonas strains from cowdung, were taken initially from NCBI database. According to MEGA6, JC+G model (Jukes-Cantor + discrete Gamma distribution) was found as Best-fit Model with lowest BIC score (2709.214119) (Table S3). Multiple alignment file was used to create phylogram using JC+G model based on bootstrap analysis of 1000 replicates and estimate the confidence of tree topologies. In this study, all the isolated strains from stored cow dung (Strains: TSGR, GTNS, TSSG and GTRS) were grouped into same top-cluster where strains isolated from fresh cow dung (Strains: GATS, GNST, TMGR and GRTM) got different positions. The Phylogenetic tree (Figure 4) revealed that all the native strain fit in to an evolutionary cluster comprising members of *Pseudomonas aeruginosa* and other *Pseudomonas* sp. grouped into separate cluster according to their relatedness.

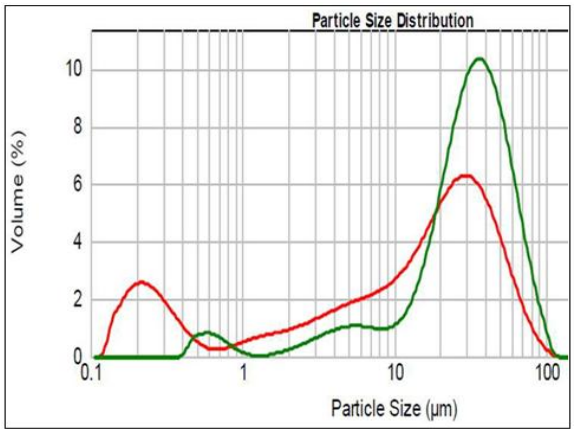

Fig. 2. Particle size decrement after solubilization by *Pseudomonas* sp Strain TMGR.

Validation of species specific primer

This study was done to differentiate *Pseudomonas aeruginosa* strains from other closely related fluorescent Pseudomonads. This was done by applying the assay to purified genomic DNA template from isolates of thirty known isolated native *Pseudomonas aeruginosa* strains form different origin and fifteen non *Pseudomonas aeruginosa*. Result from agarose gel was able to correctly confirm all the *Pseudomonas aeruginosa* isolates tested whereas there were no amplification products observed for *Bacillus subtilis* subsp. GRTM and *Enterobacter* sp. TSNG strains controls were similarly analyzed (Figure 5 and S1).

Fig. 3. *Cicer arietinum* seed germination and sprout growth promotion by (a) E_{57} [*Pseudomonas sp* Strain GRTM] and (b) P_{76} [*Pseudomonas sp* Strain TMGR] on water agar media. *Pseudomonas sp* Strain TMGR can influence smatter among all selected isolates.

Discussion

Both fresh and stored cow dung have huge source of *Pseudomonas* sp. All fertilizing parameters of fresh cow dung are so high than stored cow dung (Table S1) that fresh cow dung may be toxic for plant and thus should not be use as fertilizer. These fertilizing parameters must be governed by those strains. *Pseudomonas* isolates isolated from fresh cow dung are active phosphate solubilizers and phytohormone suppliers than those from stored one.

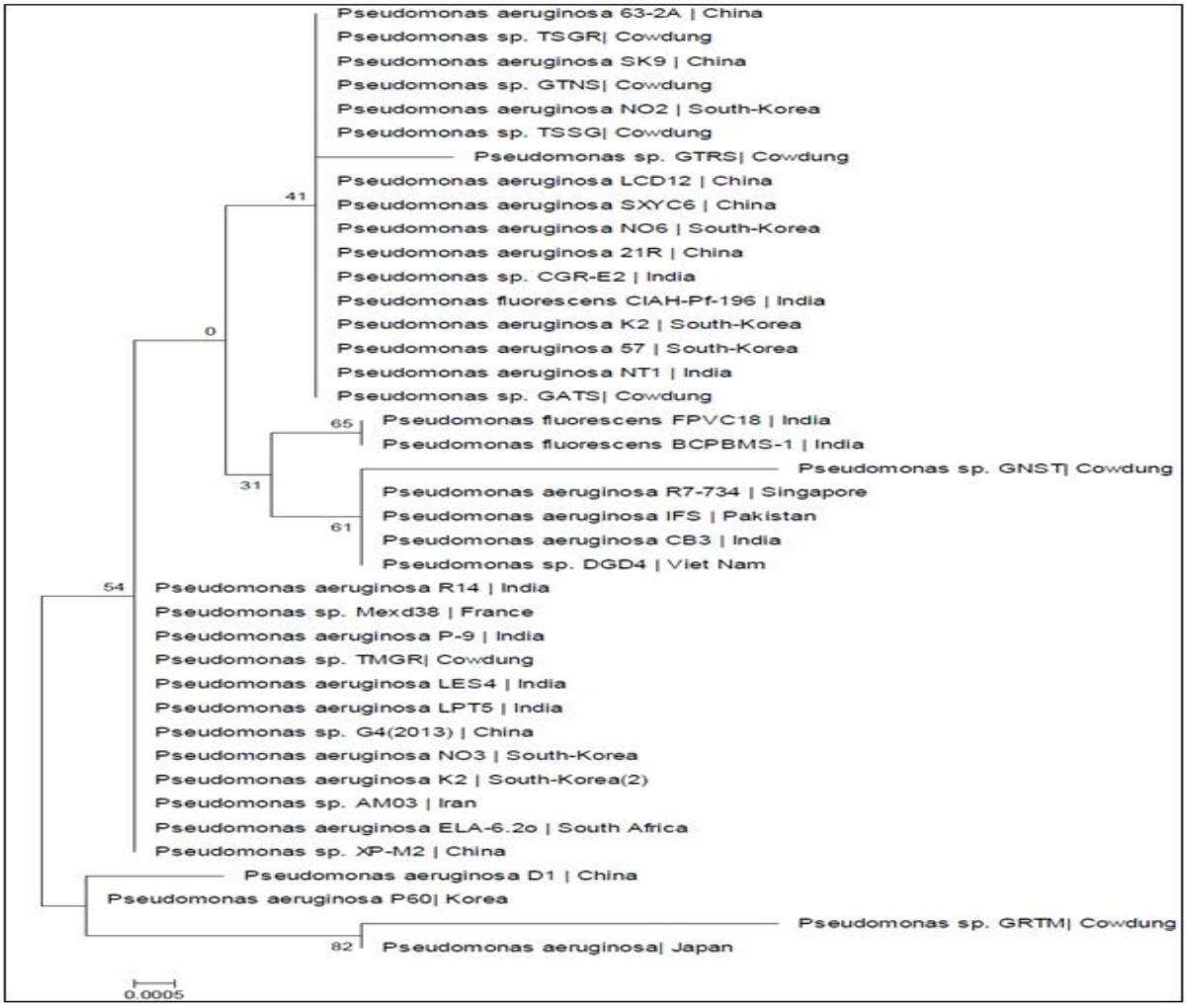

Fig. 4. Maximum Likelihood relationship among different fluorescent Pseudomonads. The number at each branch points is the percentage supported by 1000 bootstrap trees. The bar represents 0.0005% sequence divergence. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 0.0005% significance level.

The solubility product constant, **K_{sp},** is the equilibrium constant for a sparingly soluble solid substance dissolving in an aqueous solution. It represents the level at which a solute dissolves in solution. Higher is the K_{sp} value of a solid substance, higher is the solubility and vice versa. So that, the solubility sequence of studied phosphate salts is $Zn_3(PO_4)_2$> $Ca_3(PO_4)_2$> $Mg_3(PO_4)_2$> $FePO_4$> $AlPO_4$. Our selected *Pseudomonas* sp.s can readily solubilize highly insoluble phosphates viz.; $Zn_3(PO_4)_2$, $Ca_3(PO_4)_2$ and $Mg_3(PO_4)_2$ but cannot solubilize $FePO_4$ and $AlPO_4$. In soil, $Ca_3(PO_4)_2$ present in highest amount among $Zn_3(PO_4)_2$, $Ca_3(PO_4)_2$ and $Mg_3(PO_4)_2$. Again, Ca^{2+} is known as the macro-nutrient while Zn^{2+} and Mg^{2+} are considered as the micro-nutrient for plant growth and nutrition. For these reasons, in this study, we did quantitative estimation of phosphate solubilization by taking calcium phosphate as reference phosphate salts. P_{76} (*Pseudomonas* sp Strain TMGR), an isolate from fresh cow dung, as best biofertilizer strain while E_{57} (Pseudomonas sp Strain GRTM), getting second position in this race, is also a fresh cow dung resident (Figure 1 and Table 1). They are different from other and showed molecular difference in evolution as well as diversity during phylogenetic analysis.

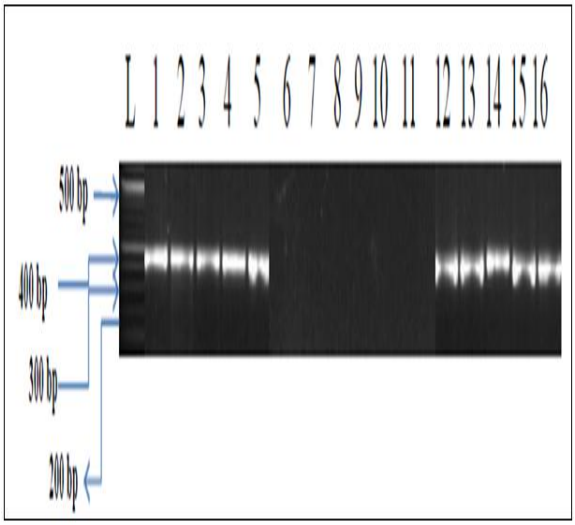

Fig. 5. Agarose gel electrophoresis of PCR amplified *Pseudomonas aeruginosa* species specific 372 bp 16S rDNA amplicons. Lanes are named with respective isolates; L: 100 bp DNA Ladder, 1: *Pseudomonas aeruginosa* GRTM; 2: *Pseudomonas aeruginosa* GATS; 3: *Pseudomonas aeruginosa* GNST; 4: *Pseudomonas aeruginosa* TMGR; 5: *Pseudomonas aeruginosa* GTRS; 6: *Bacillus subtilis* subsp. TGNS; 7: *Enterobacter* sp. TSNG; 8: *Alcaligenes faecalis*; 9: Control (without any DNA); 10: *Alcaligenes faecalis*; 11: *Enterobacter* sp.; 12: *Pseudomonas aeruginosa* CIFP7; 13: *Pseudomonas aeruginosa* TSGR; 14: *Pseudomonas aeruginosa* TSSG; 15: *Pseudomonas aeruginosa* GTNS; 16: *Pseudomonas aeruginosa* CIFP4.

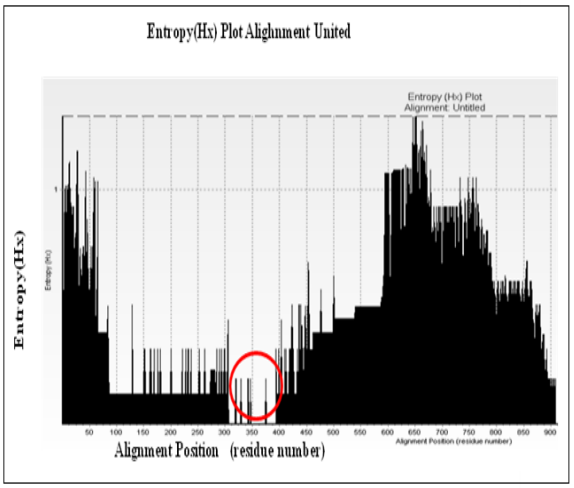

Fig. S1. Conserved Region to design Species specific (372bp) Primer Entropy (Hx) Plot Alignment.

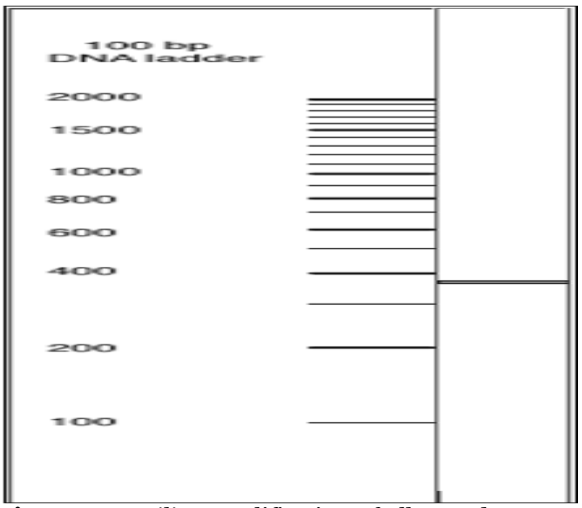

Fig. S2. *In Silico* amplification of all *Pseudomonas aeruginosa* native strain showed a specific band size at 372 bp by using OligoCalc Software.

103F and 455R are two *Pseudomonas aeruginosa* specific primers which are checked both *in silico* and wet laboratory analysis (Figure S2 and Figure 5, respectively). This primer set can amplify 16S rRNA

region of *Pseudomonas aeruginosa* and give a sharp band at 372bp region with satisfied low entropy value (Figure S1). These primers can be used for rapid and specific identification of *Pseudomonas aeruginosa* isolated from any type of cattle dung and most importantly for bacterial mapping. These isolates have high biofertilizing and anti-pathogenic activity (HCN, Siderophore, citric acid and salicylic acid production ability were found during biochemical characterization of selected strains). So that cow dung is the best and cheapest biofertilizer from ancient age and modern microbiologist should be started biofertilizer strains from it immediately.

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