CHAPTER TWO

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF POTENT PLANT GROWTH PROMOTING RHIZOBACTERIAL (PGPR) STRAINS

2.1. Introduction

Nitrogen (N) is a limiting factor for agricultural crop productivity. Among the macronutrients required by plants, N is required in the greatest quantities for biosynthesis of amino acids, proteins, DNA, RNA, phytohormones and other secondary metabolites. Therefore, N-fertilizers, mainly applied in the form of ammonium (NH_4^+) and nitrate (NO_3^-), play significant role in increasing the productivity of major food crops throughout the world (Chardon et al., 2012; Lassaletta et al., 2014). They have intense effect on the growth and development of plants including growth of roots (Krouk et al., 2010; Lima et al., 2010) and shoots (Hirel et al., 2007; Tschoep et al., 2009). Although the production and application of chemical N-fertilizers have resulted in increased food yield globally and combating world hunger during the past 50 years, their increased use in intensive agriculture has created serious environmental hazards and health issues (Zhang et al., 2012). As plants are unable to use fertilizers efficiently, excess N compounds released from agricultural land create threat to the quality of air, water, and soil. Release of nitrous oxide and other reactive N gases into atmosphere and increase in soil leaching into drainage water, can cause soil acidification causing loss of plant productivity, eutrophication of water bodies leading to death to aquatic life and air pollution (Ottman and Pope, 2000; Diaz and Rosenberg, 2008; Xu et al., 2012; Erisman et al., 2013). Moreover, NO_3^- that accumulates in edible vegetative organs has been considered as source of potential threat to human health. Excessive application of N-fertilizers also impairs soil productivity leading to reduced N use efficiency (NUE) and stagnation of crop yield (Lawlor, 2002; Yousaf et al., 2016). Therefore, there is increasing concern regarding sustainability of technology to provide long-term food security to a growing population. The biggest challenge of 21st century is to continue agricultural productivity in a way that reduces the application of N-fertilizers (Hera, 1995; Xu et al., 2012; Backer et al., 2018).

Researchers all over the world are taking keen interest in achieving better use efficiency of fertilizers with the aid of plant growth promoting rhizobacteria (PGPR) as supplements to fertilizers (Rosier et al., 2018; Xu et al., 2019). PGPR are the soil bacteria inhabiting around/on the root surface that promote plant productivity. They affect plant growth either directly through ability to supply N, phosphorous, potassium and other essential minerals or indirectly through interfering with attacking pathogens (Kloepper

and Schroth, 1981; Olanrewaju et al., 2017). They can be a promising component for integrated solutions to environmental problems because of their capacity to increase seed germination rate, root and shoot growth, chlorophyll content, nutrient uptake, protein and carbohydrate contents, hydraulic activity, tolerance to biotic and abiotic stress, crop yield and delayed ageing or senescence (Mahaffee and Kloepper, 1997; Backer et al., 2018). PGPR play significant role in N cycling and plant utilization of fertilizer N in the plant-soil system (Ames et al., 1983; Adesemoye et al., 2009). Atmospheric nitrogen (N_2) is relatively inert and it does not easily react with other chemicals to form new compounds. N_2 is converted into NH_4^+ by biological nitrogen fixation. Plant N uptake through symbiotic N fixation (Elsheikh and Elzidany, 1997) and non-legume biological fixation/non-associative uptake have been reported (Kennedy et al., 1997; Dobbelaere et al., 2001). The NH_4^+ formed is further oxidized to nitrite (NO_2^-) and NO_3^- by the process of nitrification, which is carried out by nitrifying bacteria, like *Nitrosomonas*, *Nitrosococcus*, *Nitrobacter*, *Nitrococcus* etc. Nitrification is important in agricultural system where fertilizer is often applied as NH_4^+. Conversion of NH_4^+ to NO_3^- increases N absorption as because NO_3^- is more water-soluble than NH_4^+ (Li et al., 2008).

The NO_3^- absorbed by the plant root is assimilated into NH_4^+ via nitrate reduction pathways, whereas NH_4^+ either absorbed directly through root or produced by nitrate reduction is further assimilated by ammonia assimilation pathway involving Glutamine synthetase (GS)/glutamine oxo-glutarate amino transferase (GOGAT). GS catalyses the ATP-dependent condensation of NH_4^+ with glutamate to yield glutamine, whereas GOGAT catalyses the reaction of glutamine with 2-oxoglutarate to yield two molecules of glutamate. The net outcome of the GS/GOGAT cycle is the production of glutamate, which can then be incorporated into other amino acids and nitrogenous compounds (Forde and Lea, 2007; Bernard and Habash, 2009). Plant growth and ultimately, biomass accumulation are critically determined by coordination of N metabolism with carbon metabolism and their optimal functioning (Krapp and Truong, 2005). The reducing equivalents, primarily NADPH produced in photosynthesis are used in the synthesis of C and N assimilates of many different types, particularly carbohydrates and amino acids. These fuel the synthesis of biochemical components and ultimately structure of the whole plant (Krapp and Truong, 2005). Moreover, NO_3^- and other forms of N can also act as signals that regulate the expression of hundreds of genes involved in plant metabolism, physiology, growth and development (Vidal et al., 2010).

Present investigation aims at isolation of PGPR from rhizosphere, their qualitative and quantitative screening for N-fixation and other plant growth promoting (PGP) traits, their identification by using morphological, biochemical methods and finally, selection of potent PGPR for plant application on the basis of principal component analysis (PCA).

2.2. Materials and Methods

2.2.1. Chemical and reagents

All chemicals were from Sigma-Aldrich, USA; Merck, Germany; SRL, India; Promega, USA and were of analytical grade. Microbiological media were purchased from HiMedia Laboratory, India.

2.2.2. Collection of rhizospheric soil

During winters chickpea rhizospheric soil sample was collected from the agricultural field (26°42' 30.88" N longitude and 88°20'62 52.97" E latitude) near University of North Bengal, Siliguri, West Bengal, India. Approximately 10 g of rhizospheric soil sample was collected and kept in sterilized zipped bag. The bags were immediately taken to the laboratory of Department of Biotechnology, University of North Bengal.

2.2.3. Isolation of microorganism from soil

Isolation of rhizospheric bacteria was done by using standard serial dilution and spread plate techniques. 1g soil was suspended in 9 mL of standard 0.85 % saline and vortexed for 10 min to get a uniform suspension. Soil suspension was serial diluted (10^{-1} to 10^{-8}) and 0.1 mL of the serially diluted suspensions were spread plated on nutrient agar and incubated at 37°C for 24 h. The colonies with distinct morphology were observed carefully and isolated. Further the colonies were purified using quadrant streak method on nutrient agar plate and maintained in nutrient agar slant for use. Glycerol stock of the isolated microorganisms was also made and stored at -20°C for further use.

2.2.4. Screening of bacterial isolates for plant growth promoting (PGP) traits

All the isolated strains were tested for their plant growth promoting (PGP) attributes qualitatively and quantitatively, like N-fixation, ammonia production, phosphate solubilisation, zinc solubilisation, synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase.

2.2.4.1. Nitrogen fixation and ammonia production

Bacterial culture grown overnight on nutrient broth (NB) was inoculated into the 100 mL of Asbhy's N-free liquid (ANFL) medium containing (g L^{-1}) mannitol, 15; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.2; $CaCl_2$, 0.2; $FeCl_3$,0.05 and 1 drop of 10% Na_2MoO_4 (w/v) and incubated at 37 ºC for 24 h. Thereafter, the culture was streaked on Asbhy's N-free agar (ANFA) plates (ANFL + 2% w/v agar) and incubated at 37 ºC for 24h. Bacterial strains showing positive growth on the ANFA plates were further checked for quantitative production of ammonia following the method of Goswami et al. (2014). Bacterial culture grown in ANFL medium for 24 h at 37 ºC was centrifuged at 8000 rpm for 10 min at 4 ºC to obtain the clear supernatant. To 0.2 mL of culture supernatant 1 mL Nessler's reagent (Himedia) and 7.3 mL of deionized water were added. The reaction mixture was shaken well and incubated at room temperature (RT) for 15 min. Development of brown to yellow colour is indicative of ammonia production. Ammonia production was estimated spectrophotometrically at 450 nm using the standard curve prepared with 1-10 µg mL^{-1} ammonium sulphate (Fig.2.1).

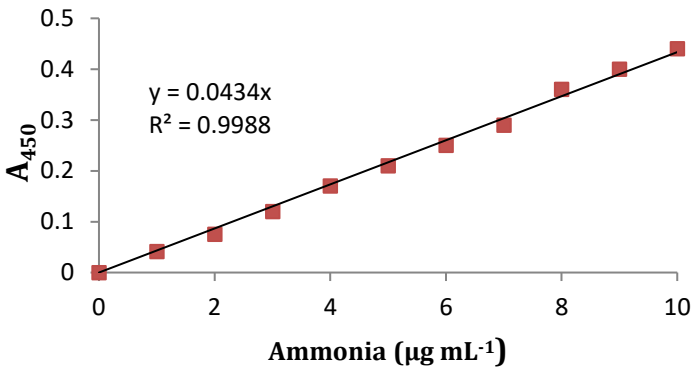


Fig 2.1. Standard curve of Ammonia

2.2.4.2. Screening of free living diazotrophs (Non-symbiotic N fixation)

Screening of free living diazotrophs was done by growing the strains in Jacob's N-free broth (JNFb) medium). Growth capacity of the isolate in N-free medium was detected by the method of Ribeiro and Cardoso (2012). Bacterial isolate was inoculated in screw-cap tube containing 10 mL JNFb liquid medium of the following composition (in g L^{-1}): DL-malic acid, 5; K_2HPO_4, 0.13; $MgSO_4.7H_2O$, 0.25; NaCl, 1.20; $CaCl_2.2H_2O$, 0.25; Na_2SO_4, 2.4; $NaHCO_3$, 0.22; Na_2CO_3, 0.09; K_2SO_4 0.17; agar 1.75; minor element solution 2 mL L^{-1}; Fe-EDTA (1.64%) 4 mL L^{-1}; Vitamin solution 1 mL L^{-1}; pH 7 [Minor element solution consisted of (in g L^{-1}) $CuSO_4.5H_2O$, 0.4; $ZnSO_4.7H_2O$, 0.12; H_2BO_3, 1.4; $Na_2MoO_4.2H_2O$, 1; $MnSO_4.H_2O$, 1.5. Vitamin solution contained (in mg L^{-1}) biotin, 100 g; pyridoxal HCl, 200] and then grown at 37 ºC for 24 h. From there, 1% (v/v) culture was transferred to JNF semi-solid culture medium and incubated at 37 ºC for 7 days. Free living diazotroph was identified by the formation of pellicle in JNF semi-solid media.

2.2.4.3. Solubilisation of inorganic phosphate (Pi)

Phosphate solubilisation property of bacterial isolates was determined by the method of Katznelson and Bose (1959). Bacterial culture was grown on NB for overnight at 37 ºC and then 0.1 mL of the culture was transferred to 100 mL of Pikovskaya broth (PKB) containing (g L^{-1}) glucose, 10; dicalcium (DCP) or tricalcium phosphate (TCP), 5; $(NH_4)_2SO_4$ 0.5; NaCl, 0.2; $MgSO_4$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4.H_2O$, 0.0001; $FeSO_4.7H_2O$ 0.0001, pH 7 and then grown at 37 ºC for 48 h. The culture obtained was point inoculated on Pikovskaya agar (PKA=PKB + Agar) plate containing DCP or TCP as insoluble Pi source and incubated at 37 ºC for around 7 days and then observed for appearance of clear zone around the colonies. Phosphate solubilisation index (PSI) was calculated (Vazquez et al., 2000) from the following equation:

$$PSI = \frac{\text{Diameter of phosphate solubilization zone (mm)}}{\text{Growth diameter of spot inoculant (mm)}} \quad \text{(Eq 1)}$$

The quantitative phosphate liberation capacity of the isolates was evaluated using the Vanadomolybdophosphoric acid method of Barton (1948). For this, isolate showing significant PSI was grown in 100 mL of PKB containing DCP, for 48 h at 37 ºC. The

culture broth was then centrifuged at 10,000 rpm for 10 min at 4°C to obtain the cell free extract (CFE). To 1 mL of CFE 0.5mL of Vanado-molybdate reagent [2.5 g ammonium molybdate $(NH_4)_6Mo_7O_{24}.4H_2O$) dissolved in 30 mL dH_2O (Solution A) and 0.125 g ammonium metavanadate (NH_4VO_3) dissolved in 30 mL dH_2O by heating and 33 mL of concentrated HCl added after cooling (solution-B), and finally, 30 mL of solution-A mixed with 63 mL of solution-B and the final volume of the mixture made 100 mL with dH_2O] was added. The resulting reaction mixture was incubated for 10 min and thereafter, absorbance was recorded at 400 nm using spectrophotometer. The amount of solubilised phosphate was estimated using standard curve of KH_2PO_4 (10-100 µg mL^{-1}) as reference (Fig 2.2).

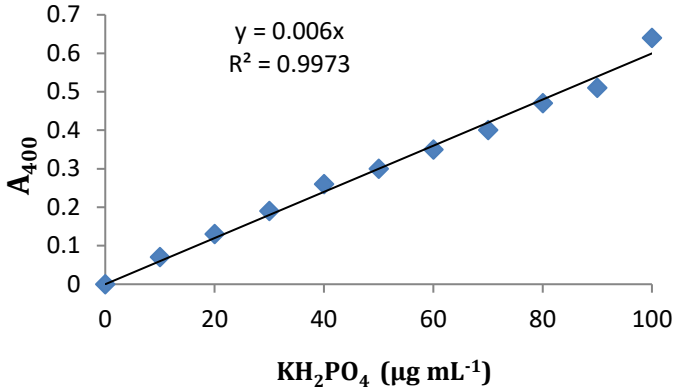


Fig 2.2. Standard curve of KH_2PO_4

2.2.4.4. Solubilisation of organic phosphate (OP)

Screening of bacterial isolate for organic phosphate (sodium phytate) solubilisation capacity was based on the production of phytase that hydrolyze phytate to release inorganic phosphate (Pi). Isolated organisms were grown in phytate screening media (PSM) containing (g L^{-1}): glucose, 20; KCl, 0.5; $CaCl_2$, 2; NH_4NO_3, 5; $MgSO_4$, 0.5; $MnSO_4$, 0.01; $FeSO_4$, 0.01; sodium phytate, 2; and agar, 15 (pH 7) at 37 ºC for 2-3 days. Formation of clear zone around the colony indicates phytase production. Phytase positive strains were analysed for phytase activity as described by (Pal Roy et al., 2016). Bacterial strain was grown in phytase production media (PPM) (g L^{-1}): glucose, 5.0; yeast extract, 3.0; $MgSO_4$, 0.5; KCl, 0.5 g; $CaCl_2$,0.1; sodium phytate,1.0 (pH 6) for 72 h at 37 ºC. Culture aliquot was withdrawn at 24 h intervals for 72 h and centrifuged at 10,000 rpm for 10 min and the supernatant was used as crude enzyme extract (CEE). 2 mL of reaction mixture containing 100 mM buffer [citrate buffer for pH 4.5 and 5.5, phosphate buffer for pH 6.5 and 7.5], 1 mM sodium phytate, 200 µl of CEE was incubated at 37 ºC for 30 min followed by addition 10 % (v/v) tri-chloroacetic acid (TCA) to stop the reaction. Then Pi reagent (ammonium molybdate, 0.5 %; concentrated H_2SO_4, 0.5 N; and ascorbic acid 2 %) was added to the reaction mixture and kept for 10 min at 37 ºC followed by the monitoring absorbance at 610 nm. The amount of Pi released from phytate was

determined by using Pi standard curve (Fig. 2.3). One unit (U) of phytase activity represents 1 µmol of Pi released per min under standard assay conditions.

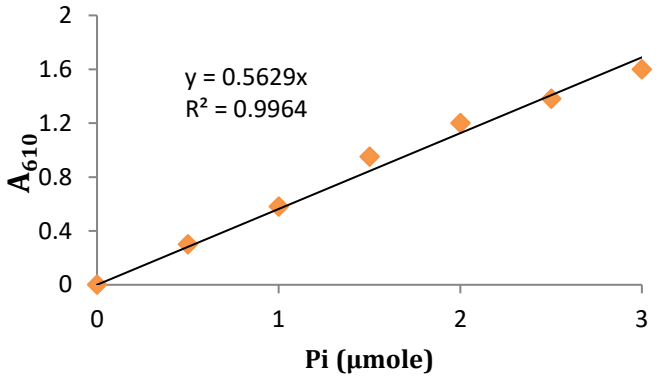


Fig.2.3. Standard curve of inorganic phosphate (Pi)

2.2.4.5. Solubilisation of Zinc

The ability of the bacterial cultures to solubilise zinc was done by the method of Dinesh et al. (2018). Bacterial isolate grown on NB for 24 h was spot inoculated on zinc solubilizing medium (ZSM) containing (g L^{-1}) glucose 10, $Zn_3(PO_4)_2$ [Zinc phosphate (ZP)] or $ZnCO_3$ [Zinc carbonate (ZC)],10; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0.2; KCl, 0.2; Agar 15, pH 7 and incubated at 37 ºC for 5 days. Zinc solubilisation index (ZSI) was calculated applying the following equation:

$$ZSI = \frac{\text{Diameter of Zinc solubilisation zone (mm)}}{\text{Growth diameter of spot inoculants (mm)}} \quad \text{(Eq 2)}$$

2.2.4.6. Production of Indole Acetic Acid (IAA)

The production of IAA was determined by following the method of Patten and Glick (2002). Bacterial culture was grown in 100 mL of Luria-Bertani (LB) broth with (0.2 mg mL^{-1}) or without tryptophan at 37 °C under shaking at 120 rpm for 2 days. Then the culture was centrifuged at 10,000 rpm for 10 min and 1 mL of CFE was mixed with 2 mL of Salkowski's reagent (2 % 0.5 $FeCl_3$ in 35 % Perchloric acid solution) and incubated for 30 min at RT (25 ºC). Development of pink colour indicated IAA production and its optical density was recorded at 530 nm. Concentration of IAA was estimated using standard curve prepared with 1-20 µg mL^{-1} IAA (Fig 2.4).

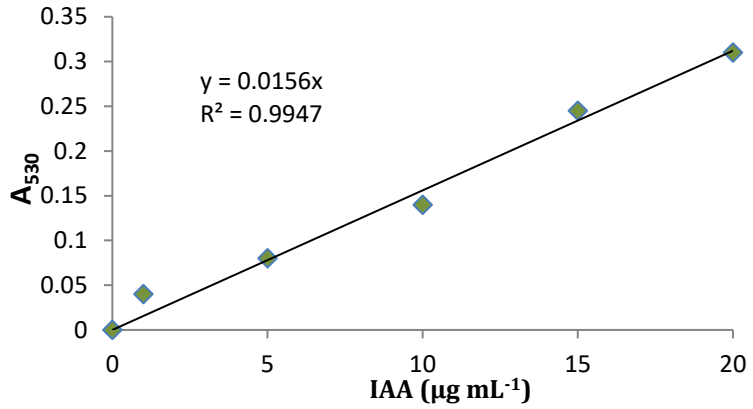


Fig 2.4. Standard curve of IAA

2.2.4.7. Screening for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase

Qualitative screening for ACC deaminase production by the isolates was performed by the method of Kumar et al. (2012). Bacterial isolate was grown on LB medium at 37 ^{o}C and mid log phase cells were harvested by centrifugation at 6000 rpm for 10 min. The bacterial pellet was washed twice with sterile saline and resuspended in 0.5 mL of 0.85% saline. The resuspended cells were spot inoculated on minimal media plates containing ACC as sole N-source and incubated at 37 ^{o}C for 3 days. Medium with ammonium sulphate as N-source served as control.

2.2.5. Identification of the bacterial isolates

2.2.5.1. Morphological and biochemical characterization

For morphological characterization of the bacterial isolates gram staining, growth pattern, motility test, spore formation test were done. Growth pattern of the bacteria was monitored in nutrient agar media for identification of their aerobic/anaerobic nature; motility test was performed in the sulphide indole motility agar medium; spore formation capability was checked by endospore staining using malachite green staining; and gram staining was performed to classify the microorganisms in specific genera. The biochemical characterisation studies included catalase test, Voges-Proskauer test, methyl red test, urease test, nitrate reduction test, oxidase test, citrate utilization, indole test, starch hydrolysis test, casein hydrolysis, gelatine liquefaction test, lipid hydrolysis, growth at 45 ^{o}C, growth in 7 % NaCl, fermentation of sugars (glucose, fructose, mannitol, lactose, sucrose, maltose, starch, xylose, sorbitol, mannose) (Smibert and Kreig, 1994).

2.2.5.2. Molecular identification of the isolates

2.2.5.2.1. Isolation of genomic DNA from bacterial isolates

Genomic DNA was isolated by Murmur's method (Murmur 1961). Bacterial culture was inoculated in LB broth and incubated for overnight at 37 ^{o}C and then centrifuged at 8,000 rpm for 10 min at 4 ^{o}C. The cell pellet was washed with 0.1M EDTA: 0.15 M NaCl

solution (1:1) followed by centrifugation at 10,000 rpm for 5 min. Cell pellet was resuspended in 2-3 mL of 0.1 M EDTA: 0.15 M NaCl solution and was stored at -20 °C for 4 h. Frozen cells were thawed at 55 ^{o}C in water bath till dissolution and incubated with 50 µg mL^{-1} solution of lysozyme (prepared in 0.1 M Tris-HCl pH 8) at 37 °C for 30 min. To the cell lysate, SDS was added and incubated at 55 °C for 15 min. The resulting mixture was then treated with proteinase K (4 µg mL^{-1}) at 55°C for 30 min. Genomic DNA was purified from the lysate by sequential extraction with equal volume of Tris-saturated phenol (pH 8), Tris-saturated phenol:chloroform (1:1) and chloroform (Sambrook et al., 1989). DNA was precipitated from the aqueous phase by adding double volume of absolute ethanol followed by centrifugation at 10,000 rpm for 10 min at 4°C. The DNA pellet was washed with 75 % ethanol, air dried and dissolved in TE buffer [10 mM Tris HCl and 1 mM EDTA (pH 8.0)].

2.2.5.2.2. Agarose gel electrophoresis of DNA

1% agarose in 1X Tris-acetate EDTA (TAE) buffer (1 litre of 50X solution contains 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA) was melted, cooled to 50-60 °C and then supplemented with 5µg mL^{-1} ethidium bromide (EtBr). The melted agarose was then poured in a casting tray fitted with a teflon comb forming wells. DNA sample was mixed with 1X DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in distilled water) and loaded onto the wells. Electrophoresis was performed in a horizontal electrophoresis tank using 1X TAE buffer at 50 V. DNA band was visualized on a UV-Transilluminator.

2.2.5.2.3. PCR amplification of 16S rRNA

PCR amplification of 16S rRNA gene was done by using bacterial genomic DNA as template. The reaction mixture in total volume of 25 µl containing 12.5 µl ultrapure water, 5µl 5X Go Tag Green buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 2µl $MgCl_2$ (2mM), 1µl dNTPs (10 mM), 1 µl 27F forward primer (10 µM, 5'-AGAGTTTGATCCTG GCTCAG-3'), 1 µl 1492R reverse primer (10 µM, 5'-TACGGTTACCTTGTTACGACT T-3'), 2 µl genomic DNA (50 ng) and 0.50 µl Taq polymerase enzyme (5 U μl^{-1}, Promega, USA). PCR was performed with initial denaturation at 94 ^{o}C for 5 min followed by 30 cycles of denaturation at 94 ^{o}C for 30 sec, annealing at 58 ^{o}C for 30 sec and extension at 72 ^{o}C for 1 min and then a final extension at 72 ^{o}C for 7 min. The PCR product was resolved on 1% agarose-TAE gel along with 500 bp DNA ladder. The bright band of around 1500 bp was cut from the gel, and then extracted and purified using gel extraction kit (QIAGEN, India). The purified PCR product was cloned in pGEM-T easy vector (Promega, USA) using the manufacturer's instruction. The ligation mix consisted of 5 µl of 2X ligation buffer, 1 µl pGEM-T vector, 2 µl PCR product and T4 DNA ligase, which after overnight incubation at 10^{o}C was transformed into competent *Escherichia coli* JM109. The transformants were selected in LB-medium containing ampicillin (50 µg mL^{-1}), X-gal (80 µg mL^{-1}) and IPTG (0.5 mM). The recombinant plasmid was isolated from the transformed cell by the Alkaline lysis method (Birnboim and Doly, 1979). The

nucleotide sequence of the 16S rRNA gene was determined by dideoxy chain termination methods using vector based T7 and SP6 primers.

2.2.5.2.4. Phylogenetic analysis

The phylogenetic relationship of the bacterial isolates were determined by comparing their 16S rRNA sequences, with closely related neighbour sequences retrieved from the GenBank database of the National Centre for Biotechnology Information (NCBI), via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1990). Phylogenetic analysis was performed by using the software package MEGA 4 (Tamura et al., 2007) after obtaining multiple alignments of the data available from public database by Clustal W (Thompson et al., 1994). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 replicates (Russo and Selvatti, 2018).

2.2.6. Statistical analysis

All the experiments were performed in triplicate. The data represented as mean along with standard error and their significance was further checked by 1 and 2 way analysis of variance (ANOVA) using SPSS version 16 (Saha and Ghosh, 2014). Homogeneity of mean was further analysed by Duncan's multiple range test (DMRT) at a level of significance $p< 0.05$ (Glen, 2017). Selection of the potent PGPR strains were done using Principal component analysis (Jolliffe and Cadima, 2016) of the qualitative plant growth promoting (PGP) traits of those isolates which showed at least 4 PGP traits, using SPSS version 16.

2.3. Result

2.3.1. Isolation of rhizosphere bacteria and qualitative screening of PGP traits

Analysis of the rhizosphere soil sample showed approximately10^8 cfu g^{-1} of soil on nutrient agar plate. Initially 82 bacterial isolates were screened based on morphologically distinct characteristics, of which 27 isolates showing the ability to fix atmospheric N were further tested for other PGP traits, like IAA production, inorganic phosphate solubilisation, organic phosphate solubilisation, Zn solubilisation, ACC deaminase production. Among the N fixing isolates, 12 produced IAA (44 %), 22 solubilised DCP (81 %), 6 solubilised TCP (22 %), 16 produced phytase (59 %), 14 and 16 isolates showed zone of solubilisation in media with ZP (52 %) and ZC (59 %), respectively, 10 isolates showed positive for ACC deaminase (37 %). The distribution of PGP traits between the bacterial isolates has been shown Table 2.1. The strain RS3, showing the positive result for almost all PGP traits (Table 2.1).

The PGP traits shared by the bacterial isolates are represented by venn-diagrams (Fig 2.5a & b, Fig 2.6a & b, Fig 2.7a & b). From the results, it is apparent that three isolates shared N_2 fixation, IAA production and ACC deaminase production (Fig 2.5a) traits; seven isolates were common in sharing the traits of N_2 fixation and Pi (DCP) solubilisation; two

were common in sharing the traits of N_2 fixation, Pi (DCP) and Pi (TCP) solubilisation; two isolates were common in sharing the traits of N_2 fixation, DCP, TCP and organic

Table 2.1.
Screening of PGPR for plant growth promotion attributes of Nitrogen fixation, Phosphate solubilisation, Zinc solubilisation, IAA production, ACC deaminase. ('+' = positive for PGP traits and '–' = negative for PGP traits)

PGPR	N_2 fixation	Phosphate solubilization			Zinc solubilization		IAA production	ACC deaminase
		Inorganic		Organic				
		DCP	TCP		$ZnCO_3$	$ZnPO_4$		
RS2	+	-	-	+	+	+	-	+
RS3	+	+	+	+	+	+	+	+
RS5	+	+	-	+	-	+	-	-
RS6	+	+	-	+	+	-	-	+
RS7	+	+	+	-	-	-	+	-
RS9	+	+	-	-	+	+	+	-
RS10	+	+	-	-	-	+	+	+
RS11	+	+	-	+	-	-	+	-
RS14	+	-	-	+	+	+	-	-
RS16	+	+	-	-	-	+	-	+
RS20	+	+	+	+	-	-	-	-
RS23	+	+	-	+	+	-	+	-
RS26	+	+	-	+	+	+	+	+
RS31	+	+	-	-	-	+	+	-
RS39	+	+	-	+	+	-	-	-
RS46	+	+	-	-	+	-	+	-
RS48	+	-	+	-	+	-	+	-
RS49	+	+	-	+	+	-	+	-
RS51	+	+	-	-	-	+	+	-
RS53	+	+		+	+	-	-	+
RS59	+	+	+	-	-	+	-	+
RS60	+	+	-	+	-	-	-	+
RS61	+	-	-	+	+	+	-	-
RS65	+	-	+	-	+	-	-	+
RS71	+	+	-	+	-	+	-	-
RS74	+	+	-	-	+	+	-	-
RS77	+	+	-	+	+	-	-	-

phosphate (OP) solubilisation; eleven bacteria shared N_2 fixation, DCP and OP solubilisation properties; two isolates shared the traits of N_2 fixation and Pi (TCP) solubilisation; three isolates were common in sharing N_2 fixation and OP solubilisation attributes (Fig 2.5b); seven isolates shared the traits of N_2 fixation, zinc carbonate (ZC) and zinc phosphate (ZP) solubilisation (Fig 2.6a); one isolate showed ZC, ZP, DCP, TCP and OP solubilisation properties (Fig 2.6b); one isolate showed the traits of IAA, OP, TCP and DCP solubilisation (Fig 2.7a); four isolates were common in sharing ZC solubilisation and IAA production; one isolate showed ZC and ZP solubilisation, IAA production and ACC deaminase production; and two isolates shared ZC solubilisation,

IAA production and ZP solubilisation traits (Fig 2.7b). Isolates showing the positive result in the qualitative screening were further evaluated quantitatively for their PGP trait.

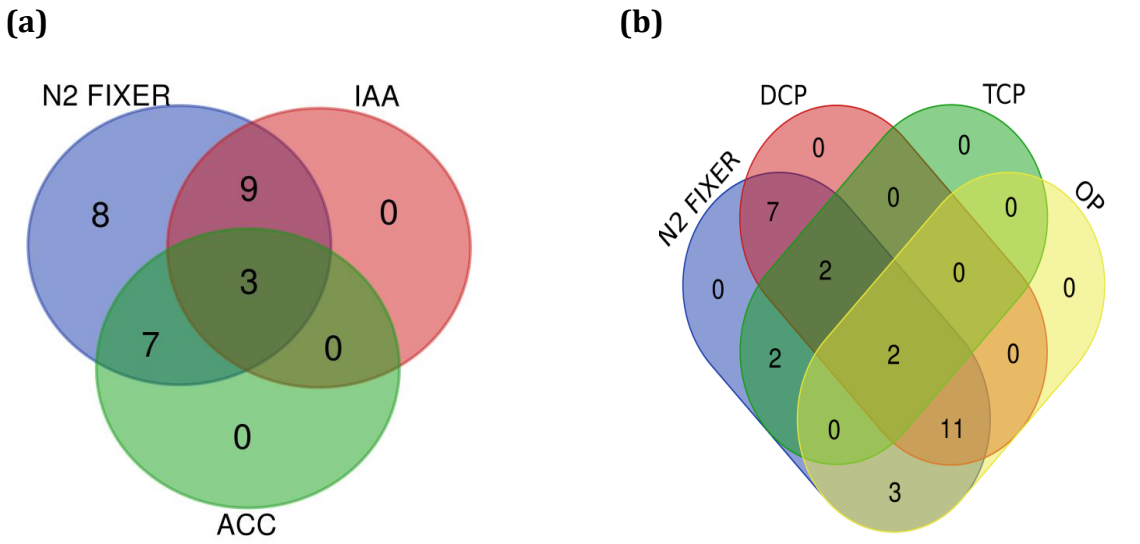


Fig 2.5. Distribution of PGP traits among PGPR isolates as shown by venn-diagram (a) N_2-fixation, IAA production and ACC deaminase production, (b) N_2-fixation, Dicalcium phosphate (DCP), Tricalcium phosphate (TCP) and Organic phosphate (OP) solubilisation.

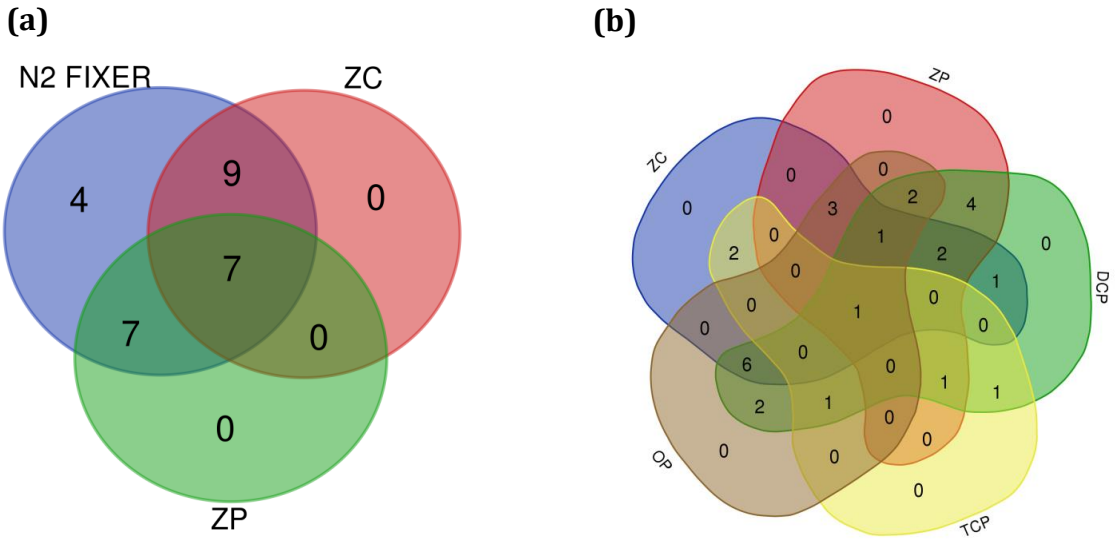


Fig 2.6. Distribution of PGP traits among PGPR isolates as shown by venn-diagram (a) N_2-fixation, Zinc carbonate (ZC) and Zinc phosphate (ZP) solubilisation (b) Zinc carbonate (ZC) and Zinc phosphate (ZP), Di-calcium phosphate (DCP), Tri-calcium phosphate (TCP) and Organic phosphate (OP) solubilisation.

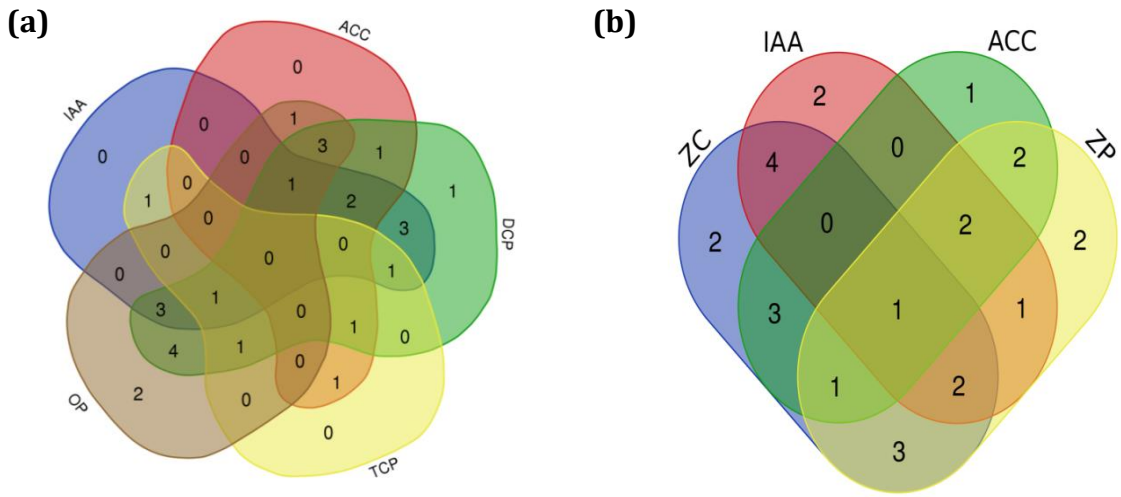


Fig 2.7. Distribution of PGP traits among PGPR isolates as shown by venn-diagram (a) Indole acetic acid (IAA) production, ACC deaminase production, Di calcium phosphate (DCP), Tri calcium phosphate (TCP) and Organic phosphate (OP) solubilisation (b)Indole acetic acid (IAA) production, ACC deaminase production, Zinc carbonate (ZC) and Zinc phosphate (ZP) solubilisation among the isolates.

2.3.2. Morphological and biochemical identification of the isolated PGPR

Twenty seven isolated potent plant growth promoting rhizobacteria bacteria (PGPR) were identified based on their colony morphology in nutrient agar plates, gram nature and biochemical reaction up to genus level. The biochemical test results of all the 27 PGPR isolates are depicted in the Table 2.2 and from the result it can be found that **one** of the isolates belongs to the genus *Cedecea* (RS3), **two** bacteria affiliated to the genus *Streptococcus* (RS9, RS51), **seven** to the genus *Bacillus* (RS2, RS5, RS16, RS23, RS39, RS53, RS60), **five** to the genus *Pseudomonas* (RS6, RS7, RS1, RS46, RS49), **two** of the isolates are *Klebseilla* sp. (RS26, RS71), **four** of the isolates are *Acinetobacter* sp. (RS31, RS59, RS65 & RS77), **three** of the isolates belongs to the genus *Azotobacter* (RS10, RS14 & RS19) and **three** of the isolates belongs to the genus *Burkholderia* (RS48, RS61 and RS74).

2.3.3. Quantitative screening of PGP traits

2.3.3.1. N_2-fixation and ammonia production

The first criterion in screening of PGPR in this study was to isolate bacterial strains with the ability to fix atmospheric N_2. Among the 82 rhizospheric isolates 27 selected isolates were able to grow in N-free Asbhy's agar media indicating their ability to fix N_2 to ammonia. Among the 27 isolates three strains RS3, RS26 and RS23 were found to form pellicle in the JNFb semi-solid N-free medium which further confirmed them as free living N_2-fixers. All these 27 selected isolates were further screened for quantitative

Table 2.2
Morphological and biochemical characteristics of the Plant growth promoting rhizobacterial isolates

Sl. No	Isolates	Morphological and Biochemical characteristics	Identity of the bacteria
1	RS2	Gram positive, rod shaped, colony diameter 3.2 mm, white, convex and irregular margin colony, spore formation(+ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (G), glycerol (G), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG)	*Bacillus* sp.
2	RS 3	Gram negative, rod shaped, colony diameter 1.2 mm, yellow, small and smooth, slimy colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (AG), glycerol (AG), maltose (+ve), glucose (AG), xylose (AG), arabinose (AG), cellobiose (AG), sucrose (G), fructose (G)	*Cedecea* sp.
3	RS5	Gram positive, rod shaped, colony diameter 3.3 mm, creamy white, convex colony, spore formation (+ve), motility (+ve), growth under aerobic condition (+ve), Starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalse (+ve), oxidase (-ve), lipase (+ve), citrate utilization (-ve), Voges- Proskauer test (-ve), methyl red (+ve), indole (-ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (G), glucose (AG), xylose (AG), arabinose (A), cellobiose (AG), fructose (A), sorbitol (A)	*Bacillus* sp.
4	RS6	Gram negative, rod shaped, colony diameter 1.2 mm, creamy yellow, small and rough colony, spore formation (-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (-ve), glycerol (AG), maltose (-ve), glucose (G), xylose (A), arabinose (A), cellobiose (AG)	*Pseudomonas* sp.

5	RS 7	Gram negative, rod shaped, colony diameter 1.4 mm, yellow, small and smooth colony, spore formation (-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (+ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), indole (-ve), nitrate reduction (+ve), acid and gas production from starch (G), lactose (-ve), glycerol (AG), maltose (+ve), glucose (G), xylose (A), arabinose (A), cellobiose (AG), sorbitol (G)	*Pseudomonas* sp.
6	RS 9	Gram negative, rod shaped, colony diameter 3 mm, watery to translucent, mucoid, conical and entire margin colony, growth period 3-5 days, spore formation (-ve), motility (-ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from glucose (AG), arabinose (AG), tryptophan (+ve), mannose (AG), mannitol (AG)	*Rhizobium* sp.
7	RS 10	Gram negative, rod shaped, colony diameter 1.1 mm, white, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalse (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (+ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (AG), cellobiose (AG), sucrose (G), fructose (AG)	*Azotobacter* sp.
8	RS 11	Gram negative, rod shaped, colony diameter 1mm, yellow, small and smooth colony, spore formation(-ve), motility (-ve), growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (+ve), catalse (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), indole (-ve), nitrate reductase (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (+ve), glucose (G), xylose (AG), arabinose (AG), cellobiose (AG), sorbitol (G), Fructose (G)	*Pseudomonas* sp.

9	RS 14	Gram negative, rod shaped, colony diameter 1.2 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (+ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (+ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (AG), cellobiose (AG), sucrose (G), fructose (AG)	*Azotobacter* sp.
10	RS16	Gram positive, rod shaped, colony diameter 2.8 mm, creamy white, convex and irregular margin colony, spore formation (+ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalse (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (AG)	*Bacillus* sp.
11	RS20	Gram negative, rod shaped, colony diameter 2.8 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (+ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), Methyl red (+ve), Indole (+ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (G), cellobiose (AG),sucrose (G), fructose (AG)	*Azotobacter* sp
12	RS23	Gram positive, rod shaped, colony diameter 2.8 mm, creamy white, convex and irregular margin colony, spore formation (+ve), motility (+ve), Growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), Indole (-ve) nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (AG)	*Bacillus* sp.

13	RS26	Gram negative, rod shaped, colony diameter 1.2 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (+ve), methyl red (-ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (AG), cellobiose (AG), sucrose (G), Fructose (AG)	*Klebseilla* sp.
14	RS31	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (-ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (NF), lactose (NF), glycerol (NF), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G), sucrose (NF), Fructose (NF)	*Acinetobacter* sp.
15	RS39	Gram positive, rod shaped, colony diameter 3.4 mm, creamy white, convex and entire margin colony, spore formation(-ve), motility (+ve), Growth under aerobic condition (+ve), starch hydrolysis(+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (G), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (G)	*Bacillus* sp.
16	RS46	Gram negative, rod shaped, colony diameter 1.2 mm, creamy yellow, small and rough colony, spore formation (-ve), motility (+ve), Growth under aerobic condition (+ve), starch hydrolysis(-ve),casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), indole (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose(-ve), glycerol (AG), maltose (-ve), glucose (G), xylose (A), arabinose (A),cellobiose (AG), sorbitol (G)	*Pseudomonas* sp.

17	RS48	Gram negative, cocco bacilli shaped, colony diameter 1.1 mm, red, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (NF), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (NF), cellobiose (AG), sucrose (G), fructose (AG)	*Burkholderia* sp.
18	RS49	Gram negative, rod shaped, colony diameter 1.2 mm, creamy yellow, small and rough colony, spore formation(-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (-ve),casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges- Proskauer test (-ve), methyl red (+ve), indole (-ve), nitrate reductase (-ve), acid and gas production from starch (G), lactose(-ve), glycerol (AG), maltose (-ve), glucose (G), xylose (A), arabinose (A), cellobiose (AG),	*Pseudomonas* sp.
19	RS51	Gram negative, rod shaped, colony diameter 3.2 mm, watery to translucent, mucoid, flat and entire margin colony, growth period 3-5 days, spore formation (-ve), motility (-ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from glucose (AG), arabinose (AG), tryptophan (-ve), mannose (AG), mannitol (AG)	*Rhizobium* sp.
20	RS53	Gram positive, rod shaped, colony diameter 2.8 mm, creamy white, convex and entire margin colony, spore formation (-ve), motility (+ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (+ve), methyl red (-ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (AG), glucose (AG), xylose (A), arabinose (A), cellobiose (AG), sorbitol (AG)	*Bacillus* sp.

21	RS59	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), Motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (-ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (NF), lactose (NF), glycerol (AG), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G) ,sucrose (NF), fructose (NF)	*Acinetobacter* sp.
22	RS60	Gram positive, rod shaped, colony diameter 3.3 mm, creamy white, convex colony, spore formation (+ve), motility (-ve), growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (-ve), Voges-Proskauer test (-ve), methyl red (+ve), nitrate reduction (-ve), acid and gas production from starch (AG), lactose (AG), glycerol (AG), mannose (G), glucose (AG), xylose (AG), arabinose (A), cellobiose (AG), fructose (A), sorbitol (AG)	*Bacillus* sp.
23	RS61	Gram negative, cocco bacilli shaped, colony diameter 1.1 mm, red, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (+ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (NF), glycerol (AG), maltose (AG), glucose (AG), xylose (G), arabinose (NF), cellobiose (AG), sucrose (G), fructose (AG)	*Burkholderia* sp.
24	RS65	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalse (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), Acid and gas production from starch (NF), lactose (NF), glycerol (AG), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G), sucrose (NF), fructose (NF)	*Acinetobacter* sp.

25	RS71	Gram negative, rod shaped, colony diameter 1.1 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, Growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (-ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (+ve), methyl red (-ve), indole (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (G), glycerol (AG), maltose (G), glucose (AG), xylose (AG), arabinose (AG), cellobiose (G), sucrose (G), fructose (G)	*Klebseilla* sp.
26	RS74	Gram negative, cocco bacilli shaped, colony diameter 1.1 mm, red, small and smooth, spherical colony, spore formation (-ve), motility (+ve), non-encapsulated, growth under aerobic condition (+ve), starch hydrolysis (+ve), casein hydrolysis (+ve), gelatin hydrolysis (+ve), lysine decarboxylase (+ve), urease (+ve), catalase (+ve), oxidase (-ve), lipase (+ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (+ve), indole (-ve), H_2S production (+ve), nitrate reduction (-ve), acid and gas production from starch (G), lactose (NF), glycerol (AG), maltose (AG), glucose (AG), xylose (AG), arabinose (NF), cellobiose (AG), sucrose (G), fructose (NF)	*Burkholderia* sp.
27	RS77	Gram negative, cocco bacili shaped, colony diameter 1.6 mm, yellow, small and smooth, spherical colony, spore formation (-ve), motility (-ve), encapsulated, growth under aerobic condition (+ve), starch hydrolysis (-ve), casein hydrolysis (+ve), gelatin hydrolysis (-ve), lysine decarboxylase (+ve), urease (-ve), catalase (+ve), oxidase (-ve), lipase (-ve), citrate utilization (+ve), Voges-Proskauer test (-ve), methyl red (-ve), H_2S production (-ve), nitrate reduction (-ve), acid and gas production from starch (NF), lactose (NF), glycerol (AG), maltose (G), glucose (AG), xylose (NF), arabinose (NF), cellobiose (G), sucrose (NF), fructose (NF)	*Acinetobacter* sp.

A= Only acid produced, AG= Acid and gas produced, G= Only gas produced, NF= Not found,
+ve = present , -ve = absent

production of ammonia in N-free Ashby's broth. The result in Fig 2.8 shows that the isolates RS3, RS23, RS26 and RS51 produced more than 6 μg mL^{-1} of ammonia with significantly higher production by RS3 (9.52 μg mL^{-1}) and RS26 (10.13 μg mL^{-1}).

2.3.3.2. Phosphates solubilisation

Among the nitrogen fixers, twenty-two were found to solubilise DCP, six solubilised TCP, and sixteen produced phytase. The phosphate solubilisation index (PSI) of DCP solubilising bacteria are shown in Fig 2.9a & b. It can be seen that isolates RS3, RS7, RS10, RS26, RS46 and RS49 displayed PSI $\geq$ 2. The PSI of RS3, RS23 and RS26 positively correlated with their ability to solubilise DCP.

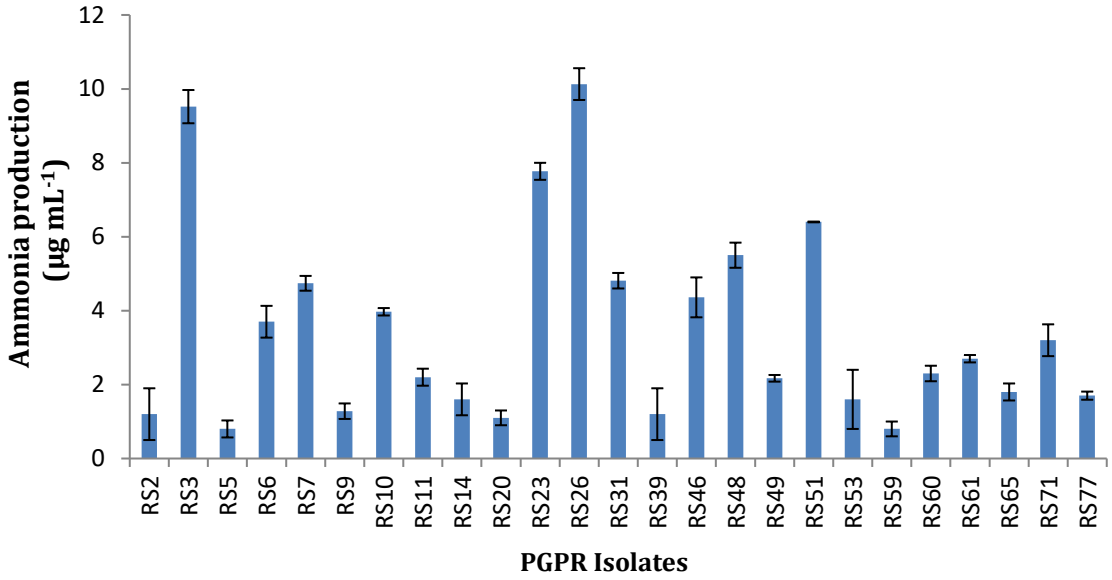


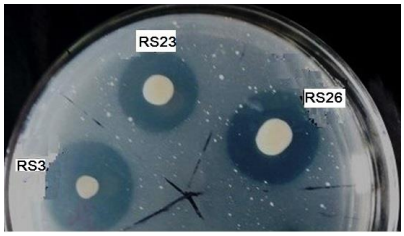
Fig 2.8. Production of ammonia by rhizosphere isolates in N-free Asbhy's broth media. Data were represented as triplicate of mean±standard deviation.

The liberation of Pi by RS3, RS23, RS26 and RS49 were recorded to be 49, 40, 26 and 30 µg mL^{-1}, respectively (Fig 2.9c). The release of Pi was found to be associated with decline in the medium pH from neutral to 3, 4, 5.5 and 5 in case of RS3, RS23, RS26 and RS49, respectively. The organic phosphate (phytate) solubilisation attribute was determined by inoculating and incubating the isolates on PSM plates containing sodium phytate. Sixteen bacteria were found to utilize phytate phosphorous. Further their ability to produce the enzyme phytase was also checked and it was found that almost all the sixteen bacteria produced phytase with significantly higher activity of RS3, RS23, RS26 and RS49 in the pH range 5.5 to 6.5 (Fig 2.10).

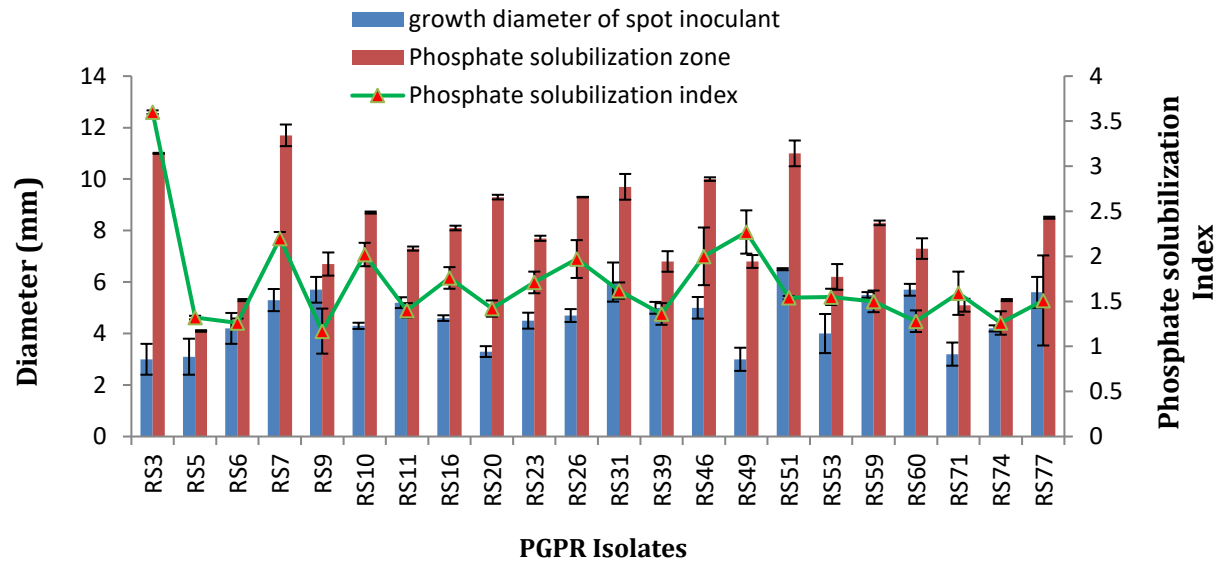
2.3.3.3. Zinc solubilisation

The property of zinc solubilisation of the selected isolates was determined by cultivating them on agar media containing either $ZnCO_3$ or $Zn_3(PO_4)_2$. In total sixteen isolates were found to solubilise $ZnCO_3$, whereas fourteen isolates were recorded to solubilise $Zn_3(PO_4)_2$ (Fig 2.11a). PGPR isolates RS2, RS3, RS6, RS9, RS14, RS23, RS26, RS39, RS46, RS48, RS49, RS53, RS60, RS61, RS71, RS77 were found to solubilise insoluble $ZnCO_3$ whereas, RS2, RS3,RS5, RS9, RS10, RS14, RS16, RS26, RS31, RS51, RS59, RS61, RS71 and RS74 solubilised $Zn_3(PO_4)_2$. The result in the Fig 2.11b shows the zinc solubilisation index (ZSI) of the $ZnCO_3$ solubilising isolates. The isolates RS3, RS9 and RS26 showed ZSI of 2.34, 2.06 and 2.07, respectively.

(a)



(b)



(c)

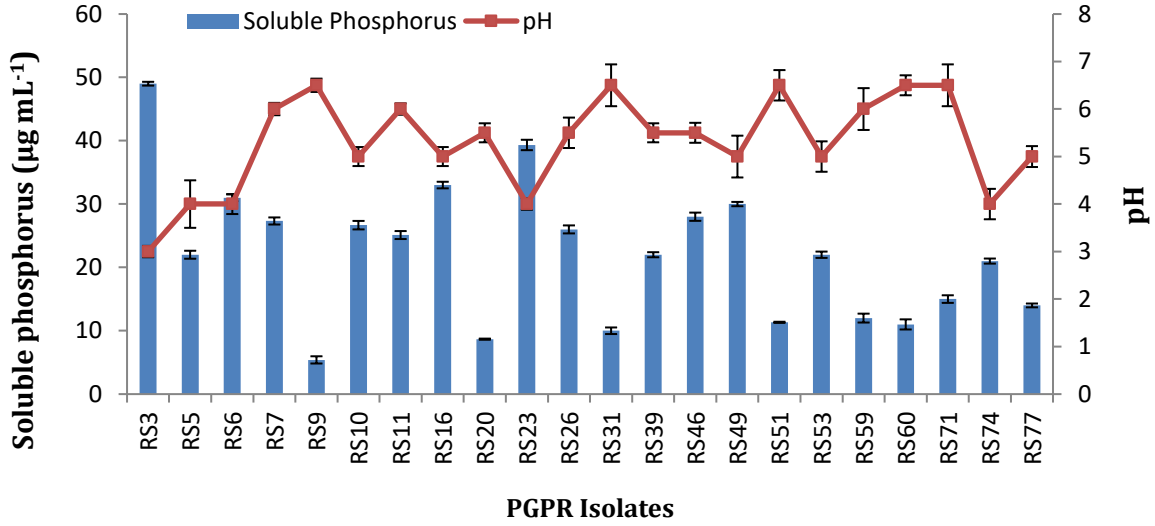


Fig 2.9. (a) Phosphate solubilisation zone of di calcium phosphate (DCP) formed by the PGPR isolates RS3, RS23 and RS26 (b) Phosphate solubilization index (PSI) formed by PGPR isolates on Pikoskaya's agar medium, Diameter (mm) on Y axis refers to the colony growth and diameter of phosphate solubilisation zone and (c) Quantitative estimation of inorganic phosphate liberated during solubilisation of dicalcium phosphate by selected phosphate solubilizers. Data are triplicates of mean ± SD.

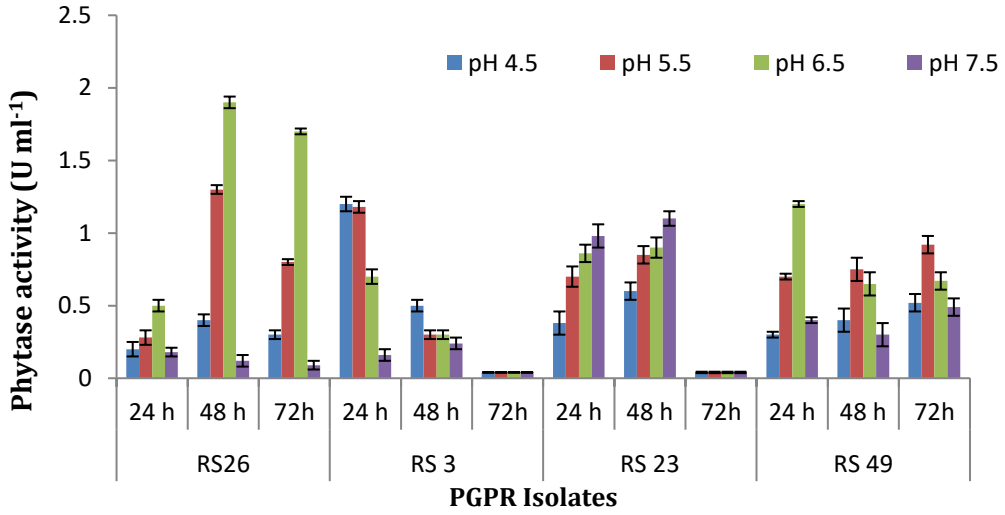


Fig 2.10. Phytase activity of the isolates RS3, RS23, RS26 and RS49 at different pH and different time interval

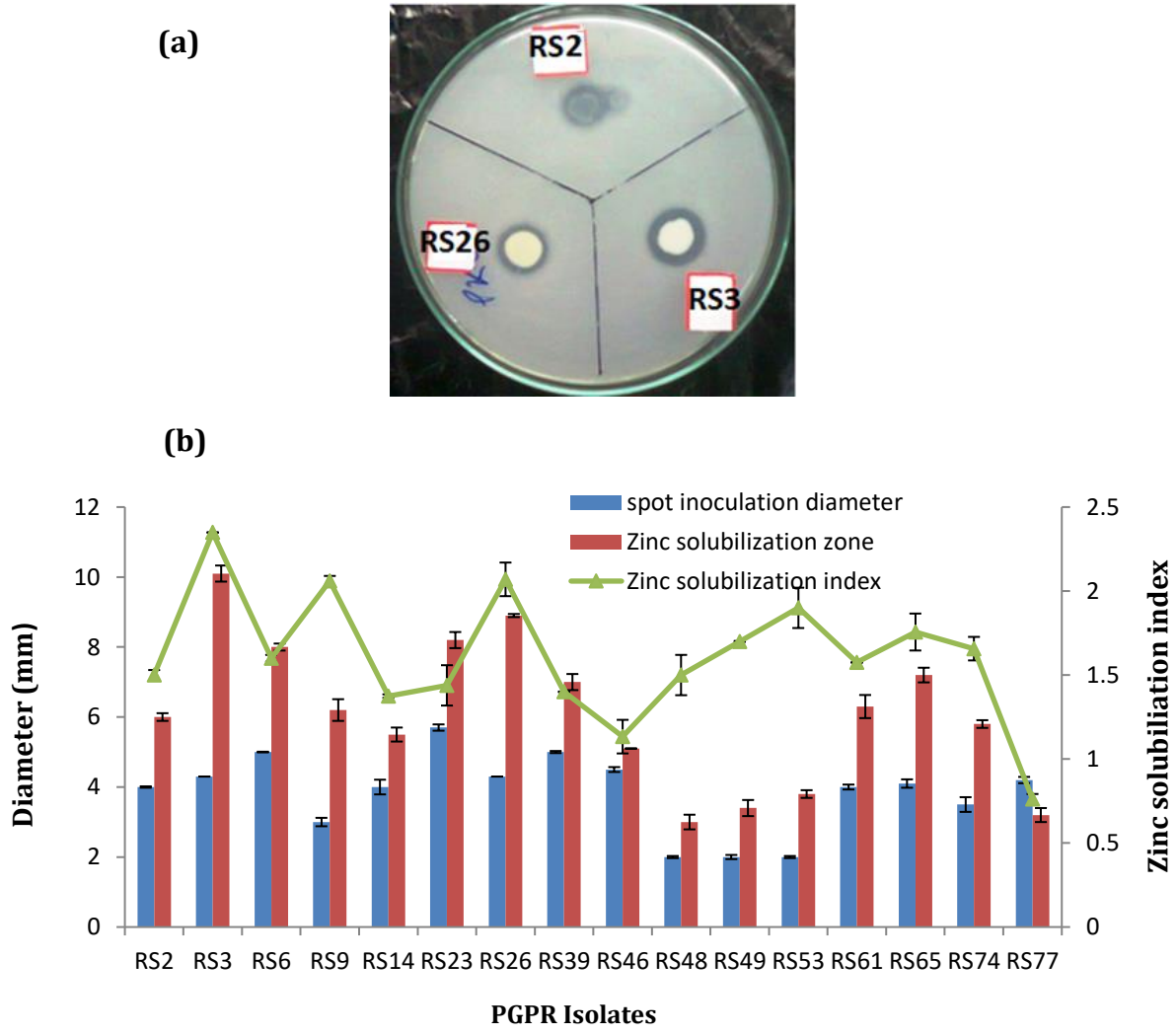


Fig 2.11. Zn solubilisation (a) Solubilisation of insoluble zinc carbonate by RS2, RS3 and RS26 with formation of halo around the colonies (b) Zinc solubilisation index (ZSI) of the PGPR isolates in the zinc carbonate containing media. Diameter (mm) on Y axis refers to the colony growth and diameter of zinc solubilisation zone.

2.3.3.4. Production of IAA

Production of indole acetic acid (IAA) by the PGPR isolates was monitored by growing them in LB broth in presence or absence of tryptophan. The result in Fig 2.12 represents the production of IAA by the isolates. Bacterial strains RS3 and RS26 produced significantly greater quantity of IAA and their respective production levels were 5.06 and 7.13 µg mL^{-1} in absence of tryptophan and 10.13 and 14.51 µg mL^{-1} in presence of tryptophan.

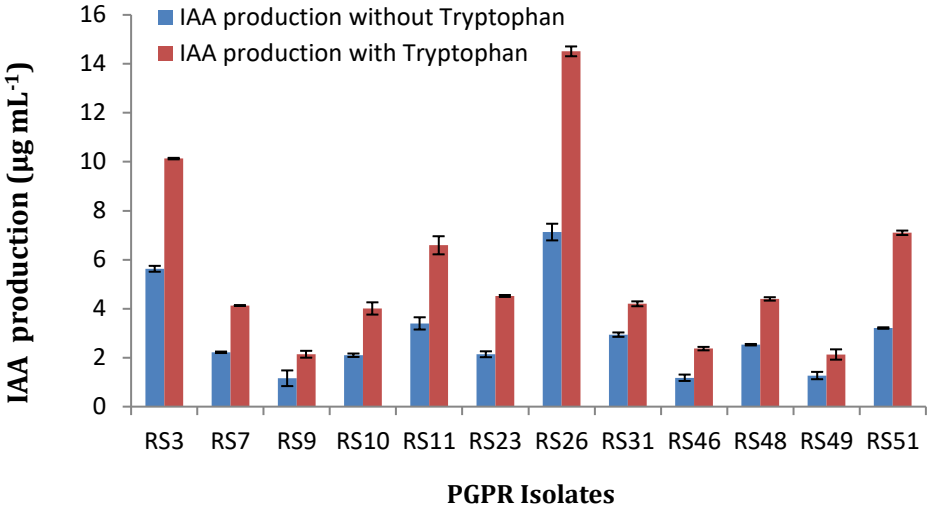


Fig 2.12. Production of Indole acetic acid (IAA) by the PGPR isolates in presence or absence of tryptophan.

2.3.3.5. ACC deaminase synthesis

About 40% of the screened isolate i.e 11 isolates showed positive for ACC deaminase. They can able to grow in minimal media plates containing ACC as sole N-source. RS2, RS3, RS6, RS10, RS16, RS26, RS53, RS59, RS60 and RS65 were ACC deaminase positive.

2.3.3.6. Selection of PGPR strain for application in plant growth

For pot experiment, PGPR isolates (those showing at least 4 PGP traits) were selected based on their presence of maximum PGP attributes as well as by principal component analysis (PCA) using the statistical tool, SPSS (**Fig 2.13**). Combined approach for selection of factors on the basis of Eigen values, scree plot and variance explained criterion of 60%, considered two principal components (PCs), PC1 and PC2 which explain 32.7 and 30.1 % variability, respectively. The major contributory factors to PC1 were ammonia production (0.857), IAA production without tryptophan (0.878), IAA production with tryptophan (0.874), phosphate solubilisation index (0.82), phosphate solubilisation (0.633), zinc solubilisation index (0.642). The microbial strains RS3 and RS26 contributed to PC1 with a correlation coefficient of 0.607 and 0.602, respectively.

The second principal component PC2 represented ACC deaminase production (0.875), phytase (0.76) and organic phosphate solubilisation (0.846). The results of principal component loading plot indicate that microbial strain RS3 and RS26 have significant positive contribution to most of the PGP attributes of PC1 as reflected by the formation of tight cluster by them in the loading plane. PCA thus suggested RS3 and RS26 as potent PGPR and these two strains with more than four PGP attributes were selected for pot trial experiments on mustard (*Brassica campestris* L.) plant.

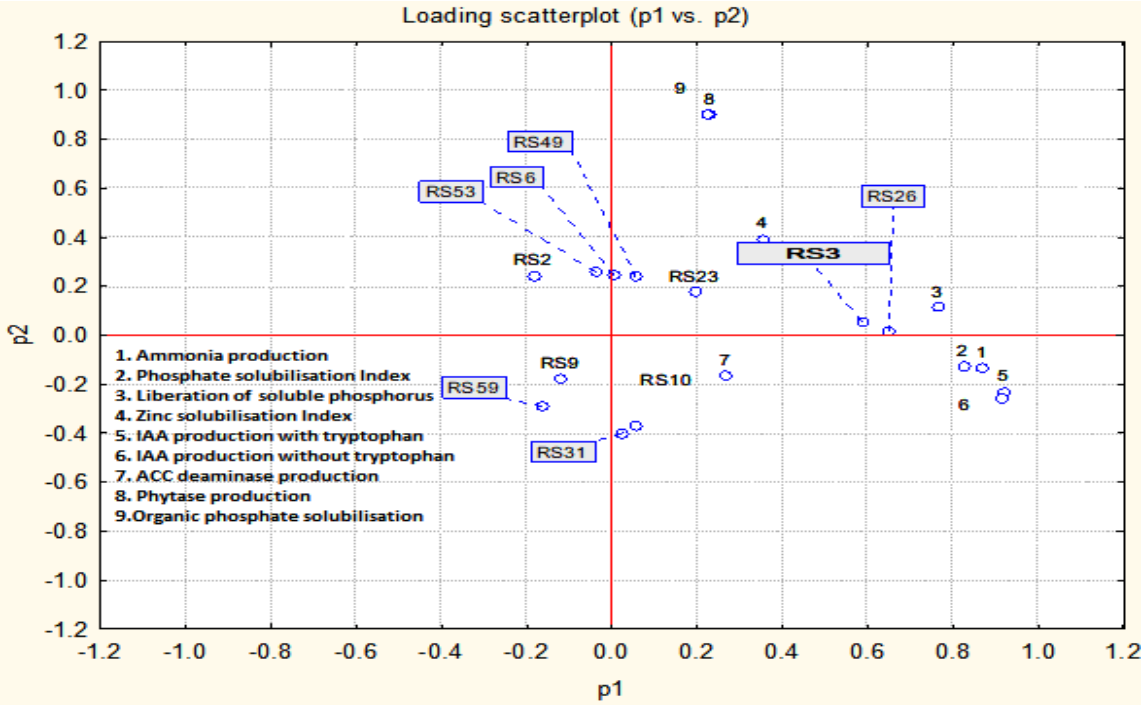


Fig 2.13. Principal component analysis of the plant growth promoting traits (PGP) (presented in Arabic numerical) along with the PGPR isolates, showing the association of isolates with PGP traits.

2.3.3.7. Molecular identification of the isolated potent PGPR

Strain RS3 was further identified using phylogenetic analysis based on 16S rRNA gene sequence comparisons which showed that the isolate RS3 (GenBank accession number **KX101223**) belong to the branch encompassing members of genus *Cedecea* and was most closely related to *Cedecea davisae* with 99% 16S rDNA sequence similarity and hence identified as *Cedecea davisae* RS3 (Fig 2.14).

Another potent strain RS26 was identified by 16S rDNA sequence analysis followed by phylogenetic tree construction (Fig 2.15). The result showed that the strain RS 26 (Gene bank Accession number **MH 819506.1**) is belonging to the genera *Klebseilla* and showed 99% sequence similarity with *Klebsiella pneumoniae* ATCC 13884. Hence, identified as *Klebsiella pneumoniae* RS26.

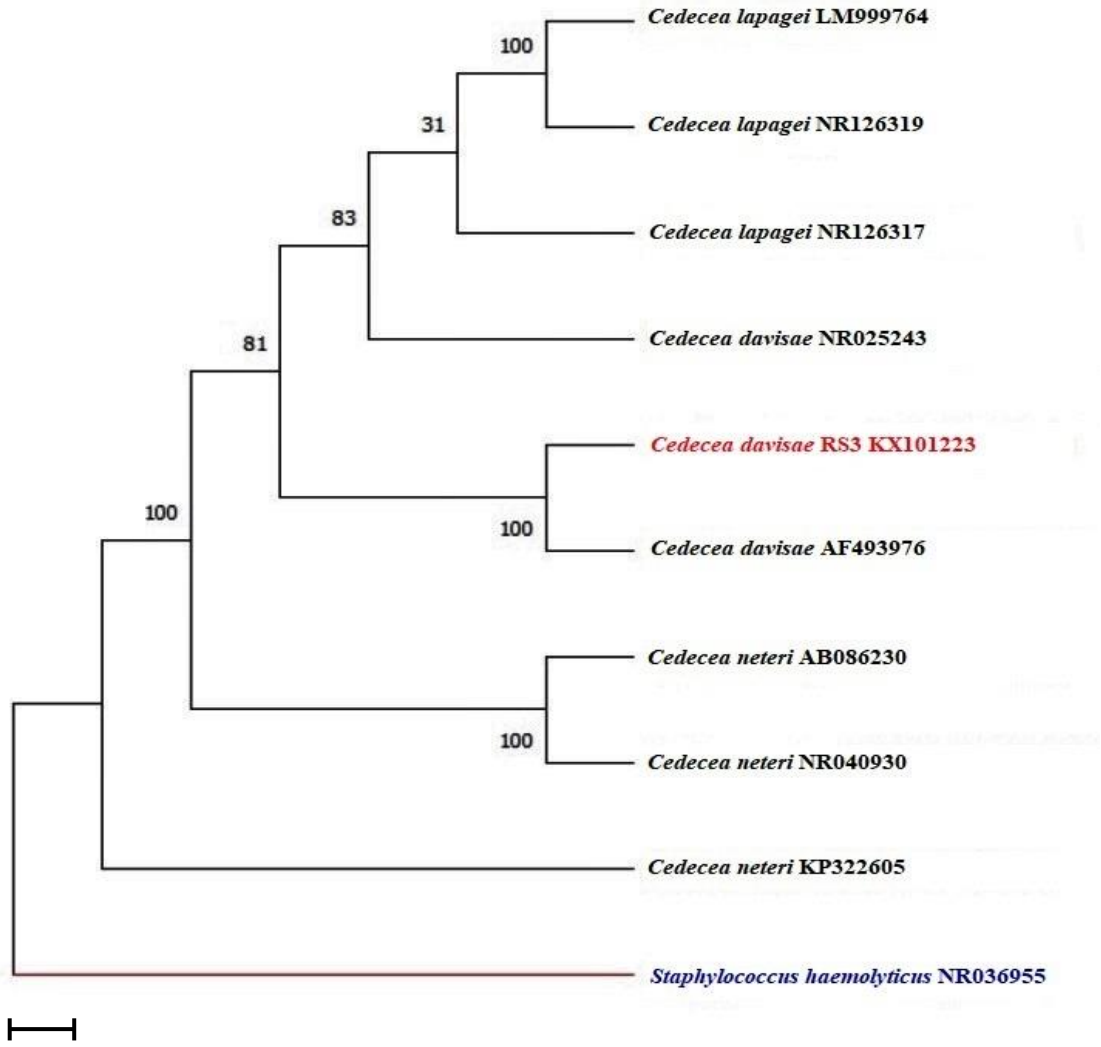


Fig 2.14. Phylogenetic tree created using 16S rRNA gene sequences representing the position of PGPR strain RS3 with the other *Cedecea* species. Bar reflects 1 nucleotide substitution per base. Numbers at nodes shows bootstrap values. At extreme right of the strain names NCBI accession numbers are given in paranthesis.

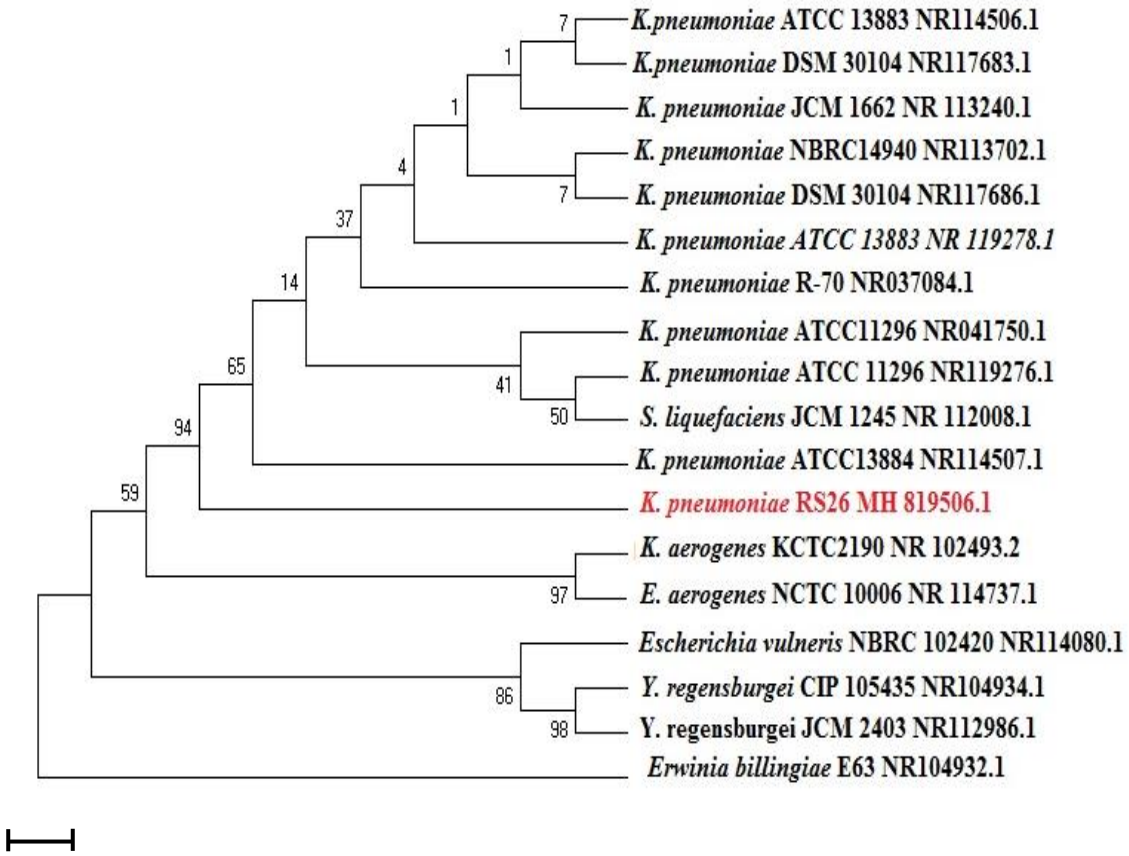


Fig 2.15. Phylogenetic tree created using 16S rRNA gene sequences representing the position of PGPR strain RS26 with the other *Klebsiella* species. Bar reflects 1 nucleotide substitution per base. Numbers at nodes shows bootstrap values. At extreme right of strain names NCBI accession numbers are given.

2.4. Discussion

During past the demand for legumes, cereal crops, and other agriculturally important plants has been met through the increased application of N-fertilizers to the agricultural fields. However, the extensive and indiscriminate use combined with the inefficient uptake of N-fertilizers by the plants is associated with several health and environmental issues. In this context, PGPR having N_2 fixing ability can be a suitable alternative to N-fertilizers or they can be used in combination with lower dose of chemical based N-fertilizer to combat the harmful effects (Dobbelaere et al., 2003; Kennedy et al., 2004). In the present study the rhizobacteria isolated from mustard plant were preliminarily screened for N-fixation attribute. Screening method involving N_2-fixation ability allows further selection of strains with more survival activity in N-deprived soil (Piromyou et al., 2011). Altogether twenty-seven isolates showed positive growth on Asbhy's N-free medium, a differentiating media screening for PGPR with potential biological N_2-fixation ability (Wu et al., 2005; Arruda et al., 2013). They belonged to various genera, like *Bacillus*, *Cedecea*, *Pseudomonas*, *Rhizobium*, *Klebseilla*, *Acinetobacter* and *Burkholderia*. In previous studies several bacterial species belonging to genera like

Paenibacillus, *Bacillus*, *Azospirillum*, *Arthrobacter*, *Achromobacter*, *Burkholderia*, *Chryseobacterium*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Cedecea*, *Pseudomonas*, *Rhizobium*, *Pantoea*, *Herbaspirillum* etc. have been found in the plant rhizosphere and they have been reported to exhibit the potential to promote plant growth and yield (Malik et al., 1997; Mirza et al., 2006; Perin et al., 2006; Beneduzi et al., 2012; Jha and Kumar, 2009, Yadav et al., 2019). Similar to findings of this study, N_2-fixing bacterial strains such as *Bacillus* sp., *Klebsiella* sp., *Azospirillum* sp., *Azotobacter* sp. and *Pantoea* sp. were isolated from rhizospheric soil sample and most of them belongs to class gamma-proteobacteria (Wu et al., 2005; Montanez et al., 2009). In addition, several scientific reports have cited the ability to fix atmospheric N_2 by *Cedecea* sp. (Jeong et al., 2017), *Pseudomonas* sp. (Li et al., 2017; Mirza et al., 2006), *Rhizobium* sp. (Ludwig, 1984), *Acenitobacter* sp. (Kuan et al., 2016), *Burkholderia* sp. (Santos et al., 2002). Further, all N_2-fixer were screened for other PGP traits; and all of them exhibited PGP traits viz. inorganic phosphate solubilisation, organic phosphate solubilisation, zinc solubilisation, IAA production, ACC deaminase production. The distribution of PGP trait among the bacterial genera is presented in the Fig 2.16. Majority of our isolates belong to the class gamma-proteobacteria and few isolates fall under class beta-proteobacteria and firmicutes.

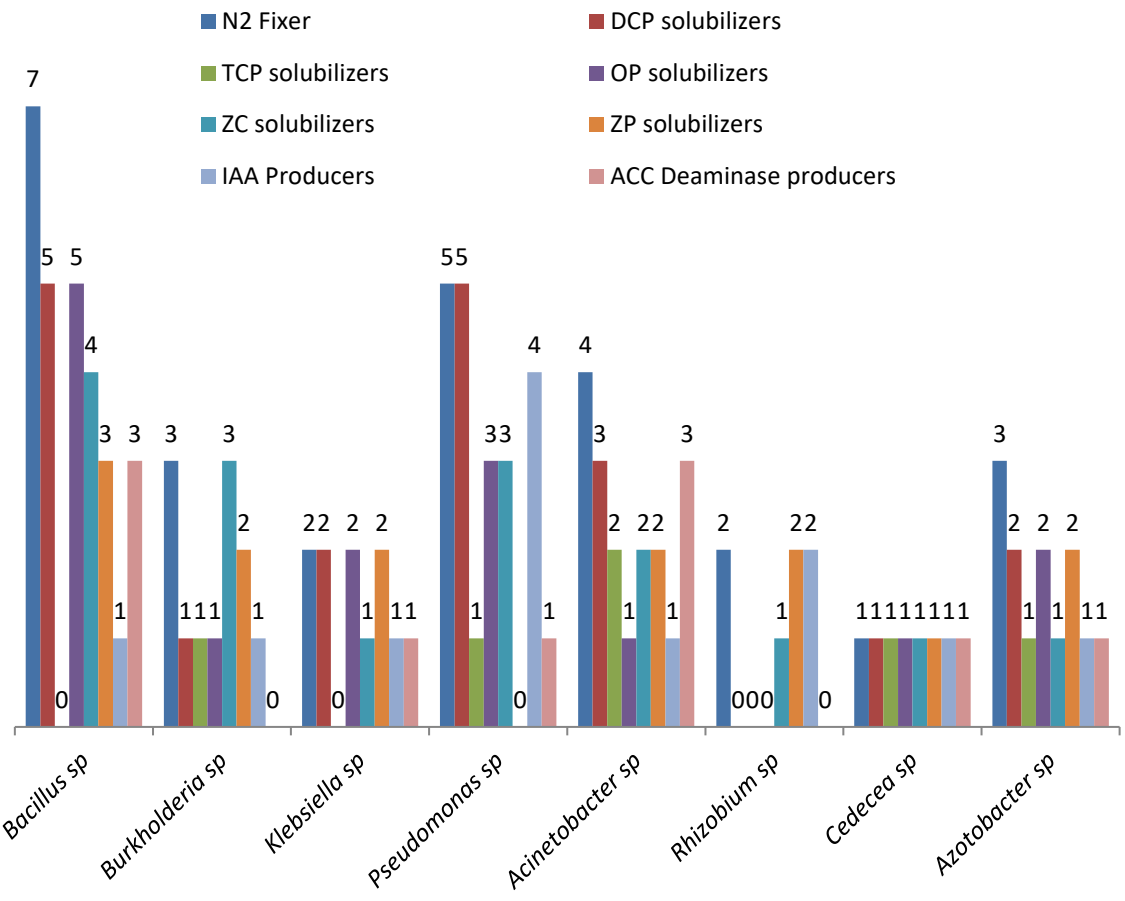


Fig 2.16. Distribution of PGP traits in various bacterial genera. The number of strains obtained for each trait and their respective genera are shown.

Due to its various roles in photosynthesis, respiration, macromolecules biosynthesis and energy transfer, phosphate is a key essential nutrient for plant growth (Huang et al., 2008). In soil the inorganic and organic phosphate compounds are tightly complexed, which are water insoluble and thus poorly available to the plants. Phosphate solubilisation by rhizobacteria is very essential because plant can absorb and assimilate only inorganic soluble phosphate. Release of mineral dissolving compounds, organic acids, protons and extracellular secretion of phosphate hydrolyzing enzymes such as phytases and other phosphatases, are the key to phosphate solubilisation by the phosphate solubilising bacteria (PSB) (Richardson et al., 2011; Pal Roy et al., 2016). In present investigation, bacterial isolates RS3, RS7, RS10, RS23, RS26 and RS49 showed the property of phosphate solubilisation with PSI ≥2 (Fig 2.6a). The Pi release by these bacteria was found to be associated with decrease of media pH from neural to acidic range, which could be due to secretion of organic acids commonly associated with phosphate solubilisation like citric, propionic, gluconic, succinic, oxalic, acetic, formic and lactic acids (Chen et al., 2006; Wei et al., 2018). Furthermore, the phosphate solubilising capability was recorded for most of the isolates belonging to the genera *Bacillus* (Fig 2.9). Similarly, Maheswar and Sathiyavani (2012) reported solubilisation of insoluble phosphates by *B. subtilis* and *B. cereus*. The PGPR strain *Pseudomonas aeroginosa* KUPSB12, showed the PSI index of 2.85 on DCP containing Pikovskaya's agar (Paul and Sinha, 2016) and *Klebsiella pneumoniae* SM6 and SM1 also showed the phosphate solubilisation ability by producing organic acids (Rajput et al., 2015). This is probably the first report on phosphate solubilising capability of *C. davisae*.

Organic phosphate in soil is mainly present as phytic acid and calcium or sodium phytate that are unavailable to plants and therefore, it becomes necessary to determine rhizospheric bacteria with the capability of phytate hydrolysis (Alori et al., 2017). The phophatase family enzyme phytase (myo-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8) catalyses the hydrolysis of phytate (myo-inositol hexakisphosphate) to myo-inositol and soluble Pi. Among the isolated microorganisms RS3, RS23, R26 and RS49 produced extracellular phytase with substantial activity in the pH range 4.5–7.5 indicating their ability to hydrolyze phytate in soil with similar pH values. A wide group of phytase-producing bacteria have already been reported, including *Bacillus subtilis* (Keruvuo et al., 1998; Kumar et al., 2012; Reddy et al., 2015), *Bacillus* sp. (Kim et al., 1998; Choi et al., 1999; Choi et al., 2001), *Enterobacter* sp. (Yamata et al., 1968; Yoon et al., 1996; Kim et al., 2003), *Pseudomonas* sp. (Richardson and Hadobas, 1997), *Advenella* sp. (Singh et al., 2014), *Bacillus* sp. (Zaheer et al., 2019).

Zinc is an essential element for optimum plant growth. The plant Zn deficiency leads to reduced carbohydrates, auxins, nucleotides, cytochromes, and chlorophyll synthesis; reduced integrity of membrane, and increased susceptibility to high temperature (Singh et al., 2005). Bacterial isolates capable of solubilizing Zn in soil are considered as potential alternates to Zn fertilization. There are several mechanisms for zinc solubilisation that are used by microorganisms, one of which is acidification. Microbes sequester the soil zinc cations through the production of organic acid and reduce the pH of the nearby soil (Alexander, 1997). Production of siderophores and oxidoreductive systems on cellular membrane, and chelating ligands are the other mechanisms for zinc solubilisation used by

microbes (Wakatsuki, 1995; Chang et al., 2005; Saravanan et al., 2011). Present study found some PGPR belonging to genera *Klebsiella*, *Cedecea*, *Bacillus*, *Burkholderia* and *Acinetobacter* as Zn solubilizers with highest ZSI value for *Cedecea* species. Our findings are consistent with previously reported literature where numerous PGPR have shown to improve the plant growth and intracellular zinc content at agriculture scale, which include *Bacillus aryabhattai* (Ramesh et al., 2014), *Bacillus* sp. and *Azospirillum* (Hussain et al., 2015), *Pseudomonas*, Rhizobium strains (Deepak et al., 2013; Naz et al., 2016). In laboratory scale numerous PGPR strains have been reported to solubilise Zn, including *Burkholderia cenocepacia* (Pawar et al., 2015), *Bacillus* sp., *Pseudomonas striata*, *P. fluorescence*, *Klebsiella pneumoniae*, *Serratia liquefaciens*, *S. marcescens*, and *Bacillus thuringiensis* (Ullah et al., 2015), *Cedecea davisae*, *P. aeruginosa* (Fasim et al., 2002), *Gluconacetobacter diazotrophicus* (Saravanan et al., 2007).

Due to its ability to enhance root development and improve mineral uptake, IAA is considered as an important PGP trait (Santoyo et al., 2016). The rhizobacteria mainly use tryptophan as precursor for IAA synthesis, however tryptophan independent pathways have also been described in a report (Ribeiro and Cardoso, 2012), which explains the synthesis of IAA in absence of tryptophan by soil rhizobacteria. Greater than 80% of IAA producing soil bacteria colonize plant root surface and in conjunction with plant endogenous IAA stimulate the root system to increase size, increase number of adventitious roots and making them enable to up take more nutrient from soil for plant growth (Patten and Glick, 2002). Out of various PGPR isolated from mustard rhizosphere few genera of *Pseudomonas*, *Rhizobium*, *Cedecea*, *Azotobacter*, *Acinetobacter*, *Klebsiella*, *Burkholderia* and *Bacillus*, produced IAA. Moreover, within the IAA producers *Klebsiella pneumoniae* RS26 was found to produce highest amount of IAA both in presence and absence of tryptophan, which were, 14.51 ± 1.1 and 7.13 ± 0.94 µg mL^{-1}, respectively. Another strain, *Cedecea* RS3 also showed significant amount of IAA production and recorded yield in presence and absence of tryptophan were 10.13 ± 1.01 and 5.63 ± 0.89 µg mL^{-1}, respectively. IAA biosynthesis has previously been reported for various N-fixing bacteria that include *Azospirillum* sp., *Bacillus* sp., *Gluconacetobacter* sp., *Burkholderia* sp., *Paenibacillus* sp., *Sphingomonas* sp., *Herbaspirillum* sp., and *Pseudomonas* sp (Dobbelaere et al., 2003, Islam et al., 2009). It has been reported that phytohormones produced by rhizobacteria are more effective by virtue of their continuous and slow release (Mohite, 2013).

The role of rhizobacteria in modulating ethylene phytohormone synthesis has been widely studied. Many soil bacteria are known to produce the ACC deaminase that degrade ACC, the direct precursor of ethylene, into ammonia and α-ketobutyrate and thus impart a positive effect on stimulating root and plant growth (Glick et al., 2007; Franche et al., 2009). In this study, a wide range of N-fixing PGPR, such as *Bacillus*, *Klebsiella*, *Pseudomonas*, *Acinetobacter*, *Cedecea* and *Azotobacter* exhibited ACC deaminase activity. Similar results were also observed by Glick (2012), wherein they found the production of ACC deaminase by *Bacillus* sp., *Pseudomonas* sp., *Klebsiella* sp., *Cedecea* sp., *Burkholderia* sp. and *Serratia* sp.

The proper selection of potent microorganisms is a kind of risk-benefit analysis which requires a study at strain level to analyse the outputs of each isolates and select the most potent strains. At this point, in our study a multivariate analysis (Principal component analysis; PCA) was performed as a tool to achieve insight into the complexity of the 11 isolates (those showing at least 4 PGP traits) quantitative PGP traits to reduce their number and to select the most potent strains. There are many techniques and approaches for data clustering and classification such as k-means, principal component analysis, cluster analysis and multiple correspondences and which have their own benefits and limitations (Di Benedetto et al., 2019). However, PCA is the most appropriate and suitable approach because (i) PCA reduces many variables to a smaller number, while losing very little information as possible (reduction of the complexity), (ii) PCA can segregate the samples into stratum or homogenous group (clustering) and (iii) PCA can gives an overview of the important variables which perform a crucial role in clustering (leading variables). Therefore, in this study PCA was employed for selection of bacterial strains for plant application. Two microbial strains RS3 and RS26 were found to have significant, positive, contributions to most PGP attributes and selected for the pot trial experiment for improving the mustard plant growth. A summary of the strategy for selection of PGPR is depicted in Fig 2.17.

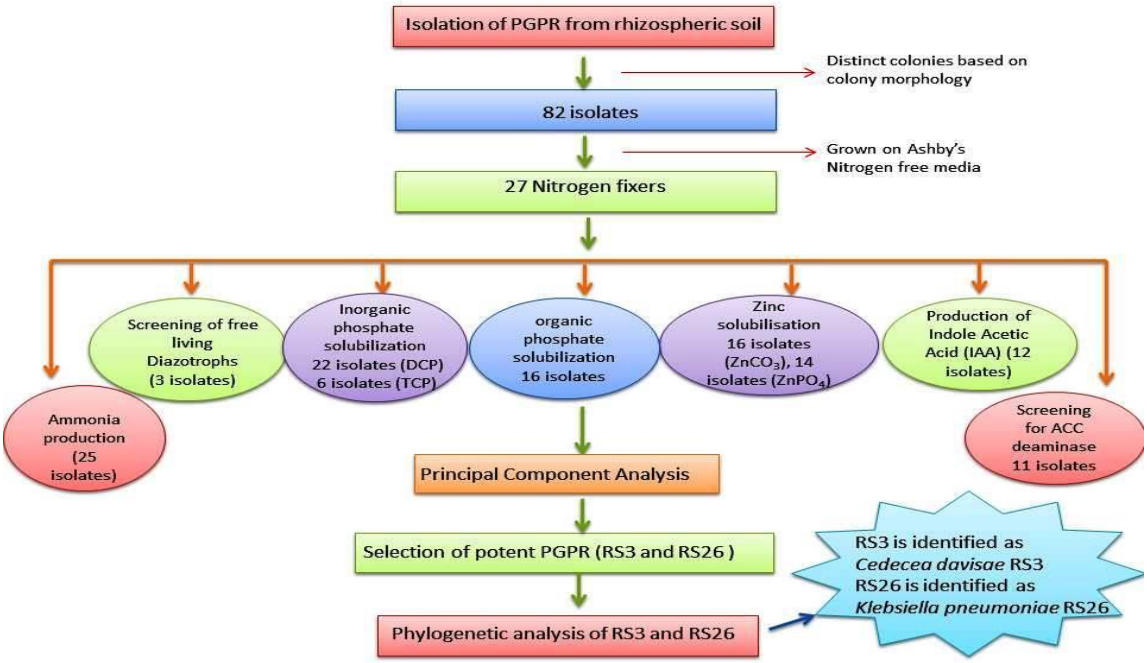


Fig. 2.17. Flow diagram of the isolation, screening and identification of PGPR isolates

In conclusion, all the 27 N_2-fixing strains were evaluated for multiple PGP traits, including, ammonia production, phosphate solubilisation, phytase production, zinc solubilisation, IAA production and ACC deaminase production. Most of the bacterial strains tested possessed at least 4 or more PGP traits and *C. davisae* RS3 and *K. pneumoniae* RS26 were selected as the two most potent PGPR strains using PCA of their PGP traits.

Supplementary Table: **Details of PGPR with shared PGP attributes as shown by the in venn-diagram**			
Figure No.	**PGP attributes shared**	**No. of isolates**	**Name of the isolates sharing the PGP attributes**
2.5 a	N_2 fixation, IAA production, ACC deaminase production	3	RS3, RS10, RS26
2.5 a	N_2 fixation, IAA production	9	RS7, RS9, RS11, RS23, RS31, RS46, RS48, RS49, RS51
2.5 b	N_2 fixation, DCP solubilization, TCP solubilization	2	RS3, RS7
2.5 b	N_2 fixation, DCP solubilization, TCP solubilization, OP solubilization	2	RS3, RS20
2.6 a	N_2 fixation, ZC solubilization, ZP solubilization	7	RS2, RS3, RS9, RS14, RS26, RS61, RS74
2.6 a	N_2 fixation, ZC solubilization	9	RS6, RS23, RS39, RS46, RS48, RS49, RS53, RS65, RS77
2.6 b	ZC solubilization, ZP solubilization, DCP solubilization, TCP solubilization, OP solubilization	1	RS3
2.6 b	ZC solubilization, ZP solubilization, OP solubilization	3	RS3, RS14, RS26
2.7 a	IAA production, ACC deaminase production, DCP solubilzation,	3	RS3, RS10, RS31
2.7 a	IAA production, ACC deaminase production, DCP solubilzation, OP solubilisation	1	RS3
2.7 b	IAA production, ACC deaminase production, ZC solubilization, ZP solubilization	1	RS3
2.7 b	IAA production, ACC deaminase production, ZP solubilization	1	RS10