The Pharma Innovation Journal 2018; 7(11): 56-62

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(11): 56-62
© 2018 TPI
www.thepharmajournal.com
Received: 01-09-2018
Accepted: 03-10-2018

Deepika Mazumdar
Department of Biotechnology, University of North Bengal, Siliguri, West Bengal, India

Shyama Prasad Saha
Department of Microbiology, University of North Bengal, Siliguri, West Bengal, India

Shilpi Ghosh
Department of Biotechnology, University of North Bengal, Siliguri, West Bengal, India

Correspondence
Shilpi Ghosh
Department of Biotechnology, University of North Bengal, Siliguri, West Bengal, India

Klebsiella pneumoniae rs26 as a potent PGPR isolated from chickpea (*Cicer arietinum*) rhizosphere

Deepika Mazumdar, Shyama Prasad Saha and Shilpi Ghosh

Abstract
In this study, chickpea plant (*Cicer arietinum*) rhizobacteria were screened for plant growth promoting traits and among the isolated PGPR, a potent strain RS26 was selected for further studies. The strain was non-pathogenic to human as determined by its inability to produce hemolysin and was identified by morphological, biochemical and 16S rRNA analysis as *Klebsiella pneumoniae* RS26. RS26 was found to be capable of N_2 fixation, ammonia production, phosphate solubilisation and IAA production. Time dependent analysis of ammonia production revealed that RS26 produced 15.21 µg/ml of NH_3 at 72 h of incubation. IAA production by the strain enhanced in presence of tryptophan and was maximum (15 µg/ml) at 48 h of incubation. Phosphate solubilisation was negatively correlated with the medium pH and maximum phosphorus solubilisation (29µg/ml) was observed after 7 days of incubation.

Keywords: PGPR, *Klebsiella pneumoniae* RS26, N_2 fixation, IAA, phosphate solubilisation

1. Introduction
The rhizospheric bacteria that can promote or enhance the plant growth through wide variety of mechanism such as N_2 fixation, IAA production, soil phosphate and zinc solubilisation, siderophore production, controlling the plant pathogens etc., are known as plant growth promoting rhizobacteria (PGPR) (Bhattacharyya and Jha, 2012) [1]. Hence, the use of chemical fertilizers, pesticides and other supplements are being replaced by the PGPR due to their great potency and environment friendly nature (Bharadwaj *et al.*, 2017) [31].
The use of chemical fertilizers in agriculture fields leads to various problems such as environmental pollution, health hazards, interruption of natural ecological nutrient cycles and destruction of biological communities. Among the micronutrient required for plant health phosphorus is an essential element. About 80% of phosphorus applied as chemicals to the agriculture field are reported to form complex with Ca^{2+}, Fe^{3+} and Al^{3+} and remains in soil as insoluble mineral form (Qureshi *et al.*, 2012) [2]. The soil insoluble phosphate can be made available to plants by utilisation of PGPR with phosphate solubilisation capacity as a sustainable and viable approach (Vessey, 2003) [3]. Several reports noted that PGPR enhance plant growth by inducing the synthesis of plant auxin (Kloepper *et al*., 2004; Yao *et al.*, 2006) [4, 5]. PGPR may also release metal-chelating siderophores into the rhizospheric soil. These siderophore then stimulate the uptake of various metal ions, including Fe, Zn, and Cu by the plants (Carrillo-Castaneda *et al.*, 2005; Egamberdiyeva 2007; Dimkpa *et al.*, 2008; Dimkpa *et al.*, 2009; Gururani *et al.*, 2012) [6-10]. In addition, PGPR also play an important role in protection of plants from pathogen by enhancing the generation of plant induced systemic resistance (ISR) (Ramamoorthy *et al.*, 2001; Kirankumar *et al.*, 2008) [11, 12]. In past, several numbers of PGPR belonging to the genera *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus and Serratia* have been isolated (Kloepper *et al.*, 1989; Okon and Labandera-Gonzalez 1994; Glick 1995; Gururani *et al.*, 2012) [9, 13-15].
We herein report the characterization of a potent PGPR, *Klebsiella pneumoniae* RS26**,** isolated from chickpea (*Cicer arietinum*) for its functional trait associated with plant growth promotion and its pathogenic nature against human.

2. Materials and methods
2.1. Isolation of PGPR strains
Soil samples from the rhizosphere of plants in the agriculture field near University of North Bengal was collected in sterilized zip bags and brought immediately to the laboratory for further processing.

1 g soils suspended in 10 ml sterilized saline water and then serially diluted up to 10^{-7}. The serially diluted samples were then spread plated into 1X nutrient agar plates and the plates were incubated at 37ºC for 24 h. The colonies were then isolated depending on the distinctive colony morphology.

2.2. Identification of the strain RS26

2.2.1. Morphological and biochemical characteristics

Morphology characterization of the bacterial isolate RS26 was evaluated by gram staining, growth pattern, motility test, and spore formation test. To separate the organism in distinguished genera gram staining was performed. Aerobic/anaerobic nature of the isolate was confirmed by growing the isolates in nutrient agar in presence of O_2, motility test was performed in the sulphide indole motility agar medium; spore formation capability was checked by malachite green staining. The biochemical characterisation studies included catalase test, Voges-Proskaur 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 and fermentation of sugars (glucose, fructose, mannitol, lactose, sucrose, maltose, starch, xylose, sorbitol, mannose) (Smibert and Kreig 1994) [16].

2.2.2. Molecular identification of the RS26

2.2.2.1. Genomic DNA isolation

Genomic DNA was isolated by Murmur's method (Murmur 1961) [17]. RS26 culture was centrifuged at 8,000 rpm for 10 min at 4 ºC to collect the cell pellet. The cell pellet was washed with 0.1M EDTA: 0.15M NaCl solution (1:1) followed by centrifugation at 10,000 RPM for 5 min. The pellet was resuspended in 3 ml of 0.1 M EDTA: 0.15 M NaCl and stored at -20 ºC for 4 h. Frozen cells were then incubated at 55 ºC water bath and mixed with 50 µg/ml solution of lysozyme (prepared in 0.1 M Tris-HCl pH 8) and the mixture was incubated at 37 °C for 30 min. SDS was added to the cell lysate and incubated at 55 °C for 15 min. The resulting mixture was treated with proteinase K (4 µg/ml) 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) [18]. DNA was separated from the aqueous phase by adding double volume of 100 % ethanol followed by centrifugation at 10,000 RPM for 10 min at 4°C. The DNA pellet was air dried and dissolved in TE buffer [10 mM Tris HCl and 1 mM EDTA (pH 8.0)].

2.2.2.2. PCR amplification of 16S rRNA

Genomic DNA of RS26 was used as template for PCR amplification of 16S rRNA gene. The reaction mixture in total volume of 25 µl contained; 9.5µl ultrapure water, 5µl 5X PCR buffer (100 mM Tris-HCl, 500 mM KCl pH 8.3), 2µl $MgCl_2$ (2mM), 1µl dNTP's (10 mM), 1µl forward primer (10 µM) 27 F (5'AGAGTTTGATCCTGGCTCAG3'), 1µl reverse primer (10 µM) 1492R (5'TACGGTTACCTTGTTACGACTT3'), 5µl genomic DNA (20ng) and 0.50 µl DNA polymerase enzyme (5 U/µl). PCR condition was initial denatuaration step at 94 ºC for 5 min followed by 25 cycles of denaturation at 94 ºC for 30 sec, annealing at 58 ºC for 30 sec and extension at 72 ºC for 1 min and then a final extension at 72 ºC for 7 min. The PCR product was separated on 1% agarose TAE gel, cut from the gel, and then extracted and purified using gel extraction kit (QIAGEN, India). The purified PCR product was sequenced by Sanger dideoxy method.

2.2.2.3. Phylogenetic Analysis

The phylogenetic relationship of the strain RS26 was determined by comparing the 16S rRNA sequence with the sequences retrieved from the Gen Bank database of the National Center for Biotechnology Information (NCBI), via BLAST search (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul *et al.,* 1990) [19]. Tree was constructed by neighbour joining method using the MEGA 6 (Tamura *et al.,* 2007) [20].

2.2.3. Plant growth promoting trait of RS26

2.2.3.1. N_2 Fixation

RS26 was cultivated in Asbhy's N-free agar plates containing (g/l) mannitol, 15; $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.2; $CaCl_2$, 0.2; $FeCl_3$,0.05, 10 µl Na_2MoO_4 (10% w/v) and agar-agar 20 at 37 ºC for 24 h. Ammonia production ability of the isolate was estimated by the method described by Goswami *et al.,* 2014 [21]. RS26 was inoculated and incubated in 50 ml of Asbhy's N-free liquid medium at 37 ºC for 24, 48, 72 and 96 h in separate 100 ml Erlenmeyer Flasks. After the respective time of incubation the culture broth was centrifuged at 8000 rpm for 10 min and 0.2 ml supernatant was added with 1ml Nessler's reagent and total volume was made 8.5ml by adding doubled distilled water. The mixture was incubated at 37 ºC for 30 min. Brown to yellow colour was developed indicate the ammonia production and the concentration of the ammonia was estimated by measuring the optical density at 450 nm against the standard curve prepare with 0.1-10 µmol ammonium sulphate.

2.2.3.2. Production of Indole acetic acid (IAA)

IAA production was evaluated by the method of Patten and Glick, 2002 [22]. RS26 was inoculated and incubated LB broth at 37 °C for 96 h, either in presence or absence of tryptophan (1mg/ml). After specified incubation time, the culture was centrifuged at 8000 rpm for 10 min. The culture supernatant (1ml) was mixed with 2ml of Salkowski's reagent (150 ml 98 % H_2SO_4, 7.5 ml 0.5M $FeCl_3.6H_2O$ and 250 ml distilled water) and incubated for 30 min at 25 ºC. IAA production was indicated by the development of pink colour and the concentration of the IAA was estimated my measuring the optical density of the mixture at 530 nm using the standard curve prepared with 1-20 µg/ml of standard IAA.

2.2.3.3. Inorganic Phosphate solubilisation

Qualitative estimation of phosphate solubilising ability of the isolate was done by the method of Katznelson and Bose, 1959 [23]. Bacterial culture grown in Pikovskaya broth for 24 h was spot inoculated on Pikovskaya agar plate (PAP) containing tricalcium phosphate (TCP). The plates were incubated at 37ºC for 9 days and then observed for the appearance of clear zone around the colonies at specified time period. Phosphate solubilisation index (PSI) was calculated from the following equation.

$$\text{Phosphate solubilisation index} = \frac{\text{Diameter of phosphate solubilization zone}}{\text{Growth diameter of spot inoculant}}$$

Quantitative estimation of the solubilised phosphate content was done by vanadomolybdophosphoric acid method (Barton, 1948) [24]. Bacterial culture was grown in Pikovskaya broth containing TCP for 9 days. Concentration of soluble phosphate was estimated at various incubation time using the standard curve of KH_2PO_4 (10-100 µg/ml).

2.2.4. Pathogenicity test of RS26
Pathogenecity test was done by following the method of Chahad *et al.,* 2012 [25]. The bacterial culture was grown on blood agar base supplemented with 5% (v/v) sheep blood to determine their ability to produce different types of hemolysins. Plate was incubated at 37ºC for 24 h. The results were recorded. Positive control strain was used for comparison and a clear zone on the blood agar plate was considered as a positive result.

3. Results and Discussion
The group of beneficial bacteria that enhances the plant growth and acts as biocontrol agent by wide variety of mechanisms are called plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1978) [26]. The utilization of PGPR for agriculture practices can minimize chemical inputs into soil and is also beneficial in context of increasing global concern for food and environmental quality (Verma and Shahi, 2015) [27]. Plants growth is stimulated by PGPR with various direct and indirect mechanisms. Direct mechanisms includes the acquisition of nutrient i.e. solubilisation of phosphate and zinc, nitrogen fixation, iron accumulation by siderophore and modulating the level of plant hormones etc (Patten and Glick, 1996, Glick *et al.,* 1998) [28, 29].
In the present study, *Klebsiella pneumoniae* RS26, a potent PGPR strain isolated from rhizosphere of chickpea were screened for plant growth promoting (PGP) traits and its pathogenic nature was also evaluated.

3.1. Isolation and Identification of PGPR strain RS26
Total number of bacteria isolated from the rhizospheric soil sample was 3.8 $X10^7$ CFU/ml. Among the microorganism 90 strains were further isolated depending on their distinctive colony morphology and successively purified in NA plates using streak plate methods. These isolated strains were further evaluated for their potential PGP activity. Among the bacterial isolates strain RS26 was selected for further evaluation in relation to its biochemical and molecular identification as well as its PGP activities. The strain RS26 was morphologically characterized as gram negative, rod shaped, non motile bacteria. The colony of the isolate was white, opaque, slimy, glossy, having entire margin and was round in shape. The bacterium was characterized biochemically as positive in citrate utilization, Voges-Proskauer tests, nitrate reduction, lipase and amylase production test (Table 1). Phylogenetic analysis based on 16S rRNA gene sequence comparison showed the isolate RS26 (Gen Bank accession number MH819506.1) belonging to the branch encompassing members of genus *Klebseialla* and was most closely related to *Klebsiella pneumoniae* ATCC 13884 with 99% 16S rDNA sequence similarity (Fig. 1) and hence identified as *Klebsiella pneumoniae* RS26. There are several reports on *Klebsiella pneumoniae* as potent PGPR strain, such as 4 species of *Klebsiella* having the phosphate solubilisation and auxin production activity were isolated Ji *et al.,* 2014 [30], Bhardwaj *et al.,* 2017 [31] isolated *Klebsiella pneumoniae* from the rhizosphere of *Saccharum officinarum* that was found to solubilise 17.4 µg/ml of inorganic phosphate and produced 45.32 µg/ml of IAA after 96 h of incubation. In another report the researchers isolated four PGPR strains from maize rhizosphere and identified as *Klebsiella* sp. Br1, *Klebsiella pneumoniae* Fr1, *Bacillus pumilus* S1r1 and *Acinetobacter* sp. S3r2 and all of which were found positive for N_2 fixation, phosphate solubilisation and auxin production (Kuan *et al.*, 2016) [32].

Table 1: Morphological and biochemical properties of the strain RS26

Test	Inference
Colony morphology	White, opaque, slimy, Glossy, Entire margin, round in shape
Shape	Rod
Gram Staining	-ve
Capsule	+ve
Gelatin Hydrolysis	-ve
Motility	-ve
H_2S production	-ve
Indole	-ve
MR (Methyl Red)	-ve
VP	+ve
Citrate utilization	+ve
Nitrate reduction	+ve
Fermentation of	
Arabinose	+ve
Cellobiose	+ve
Glucose	+ve
Glycerol	+ve
Lactose	+ve
Maltose	+ve
Mannitol	+ve
Mannose	+ve
Sorbitol	+ve
Sucrose	+ve
Xylose	+ve
Production of Lipase	+ve
Production of amylase	-ve
Production of Catalase	+ve
Probable Genera of RS26	*Klebseilla* sp

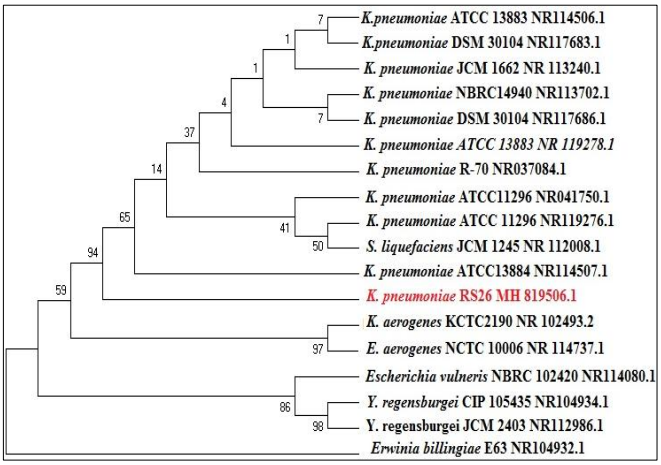


Fig 1: Phylogenetic tree construction based on 16S rRNA gene sequences reflecting the position of strain RS26 with the other *Klebsiella* species. Bar represents 1 nucleotide substitution per base. Numbers at nodes represent bootstrap values. Accession numbers are given at extreme left of each strain.

3.2. N_2 fixation and ammonia production by RS26

Symbiotic or non-symbiotic N_2 fixation by the microorganisms contribute to the N-uptake and hence to the N content of the crops (Goswami *et al.*, 2014) [21]. In our study the isolated strain RS26 was found to grow well in N-free Asbhy's media indicating their ability to fix N to ammonia. The results in Fig 2 show that RS26 produced 10.13 µg/ml of ammonia after 24 h of incubation and maximum ammonia production by the strain was recorded as 15.21 after 72 h of incubation. The strain also formed pellicle in the JNFb semi-solid N-free medium and hence was categorized as free living N fixer. Previously, various diazotrophic bacteria were isolated and characterized. They belong to the genera of *Azospirillum, Azoarcus, Enterobacter, Klebsiella and Zoogloea*. (Bilal and Malik 1987; Malik *et al.*, 1991) [33, 34]. Regulation of nitrogen fixation in *Klebsiella pneumonia* had been well documented by isolation and characterization of strains with nif-lac fusions (Douglas *et al.*, 1981) [35].

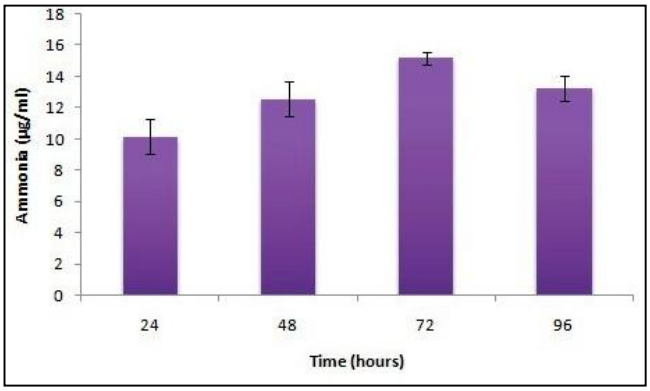


Fig 2: Ammonia production by *K. pneumoniae* RS26 in N-free Asbhy's nitrogen media. Data were represented as triplicate of mean ± SD.

3.3. IAA Production

Phytohormone IAA produced by the plants endogenously, is responsible for the root shoot elongation as well as induction of cambial cell division [22]. However, IAA produced can be effective when applied exogenously. In the present study, isolated rhizospheric bacteria were found to be producing IAA. The results in Fig 3 suggest that RS26 synthesized IAA either in presence or absence of tryptophan. Moreover, the production was more in the presence of the amino acid. Maximum IAA production of 15 and 7 µg/ml, respectively, was observed in presence and absence of tryptophan at 48 h incubation. On 96 h of incubation the production declined to 4 ug/ml and 8 ug/ml without and with tryptophan, respectively. Several bacteria have been reported to produce IAA. *Pseudomonas alcaligenes* and *Mycobacterium phlei* were found to be producing 0.3 µg/ml and 0.5 µg/ml of IAA (Egamberdiyeva *et al*. 2007) [7]. *K. pneumoniae* strains were reported for the highest production of 27.5 µg/ml (Sachdev *et al.*, 2009) [38]. Kuan *et al.*, 2016 [32] reported the production of 12.99 µg/ml of IAA by *K. pneumoniae* Fr1 which was significantly higher among the other isolated strains such as *Bacillus subtilis* UPMB (10.10 µg/ml), *Klebsiella* sp. Br1 (4.91 µg/ml), *Bacillus pumilus* S1r1 (4.55 µg/ml) and *Acinetobacter* sp. S3r2 (10.70 µg/ml). In the present research elevated production of IAA on tryptophan supplementation could be due to the amino acid being the precursor of IAA and as root exudates contain tryptophan due to the transammination and decarboxylation reactions operated in plant roots, which can stimulate PGPR strains to produce more amount of IAA (Patten and Glick, 2002) [22].

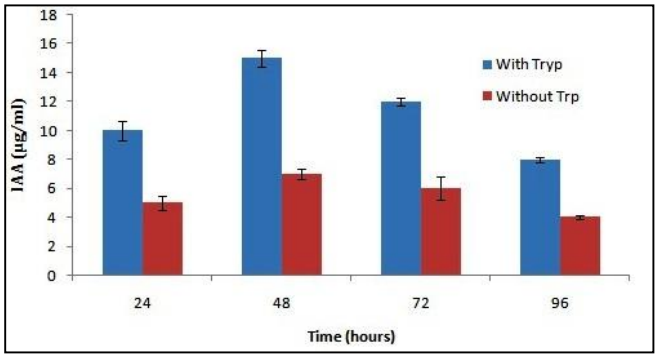


Fig 3: IAA production by *K. pneumonia* RS26 in LB broth media in presence or in absence of tryptophan. Data were represented as triplicate of mean ± SD.

3.4. Phosphate solubilisation

Qualitative and quantitative estimation of inorganic phosphate solubilisation ability of RS26 was evaluated by growing the bacterial strain in Pikovskya agar and broth media respectively, for 2, 4, 6, 7, 8 and 9 days respectively. The results in Fig 4 represent the qualitative estimation of phosphate solubilisation by the isolated strain in terms of phosphate solubilisation zone and phosphate solubilisation index (PSI). PSI was found to increase gradually from day 2 to 7 and was maximum at day 7 with a PSI of 2.09. Although phosphate solubilisation zone was maximum (11 mm) at day 8, but PSI ratio was found to be 1.77. The result also suggested that the phosphate solubilisation zone was positively correlated (r=0.91) with the diameter of the spot inoculants of the strain.

Quantitative estimation revealed that the solubilisation of the phosphate by RS26 was also increased gradually from 2 to 7 days (10-29 µg/ml) and was correlated with the decrease in medium pH from 6.4 to 5.5, further incubation decreased the amount of soluble phosphate (Fig 5). The result suggests that the strain RS26 could solubilise the phosphate by secreting some organic acids in the media as reflected by the decreased medium pH. Organic acids such as citric, propionic, gluconic, succinic and lactic acids are reported to be the most common in phosphate solubilisation (Glick *et al.*, 1998) [29]. Several scientific report suggested that the *Klebsiella* sp can solubilise the inorganic phosphates such as *Klebsiella* sp. Br1, *Klebsiella pneumoniae* Fr1 (Kaun *et al.*, 2016), *Klebsiella pneumoniae* VRE36 (Bhardwaj *et al.*, 2017) [32, 31].

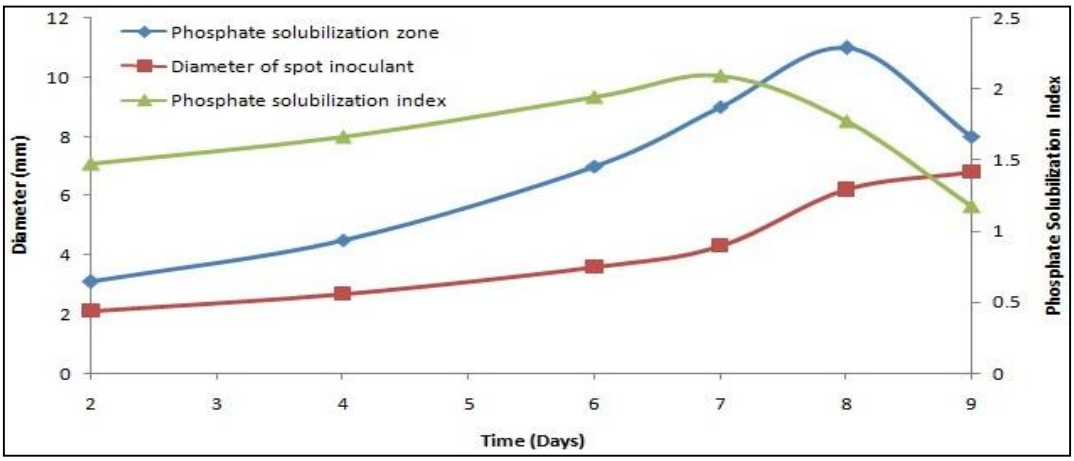


Fig 4: Evaluation of phosphate solubilization index (PSI) created by *K. pneumoniae* RS26 on Pikovskaya' sagar medium after 2, 4, 6, 7, 8 and 9 days of incubation.

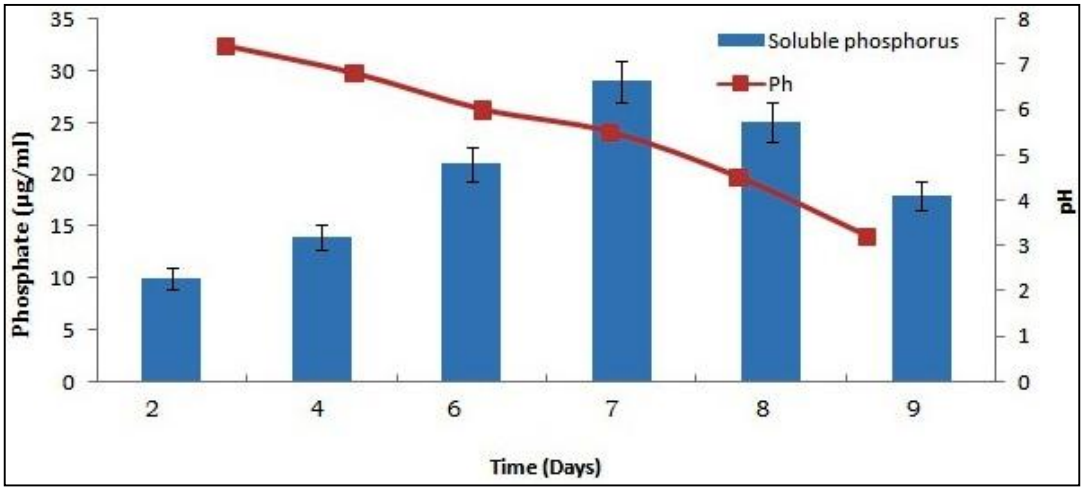
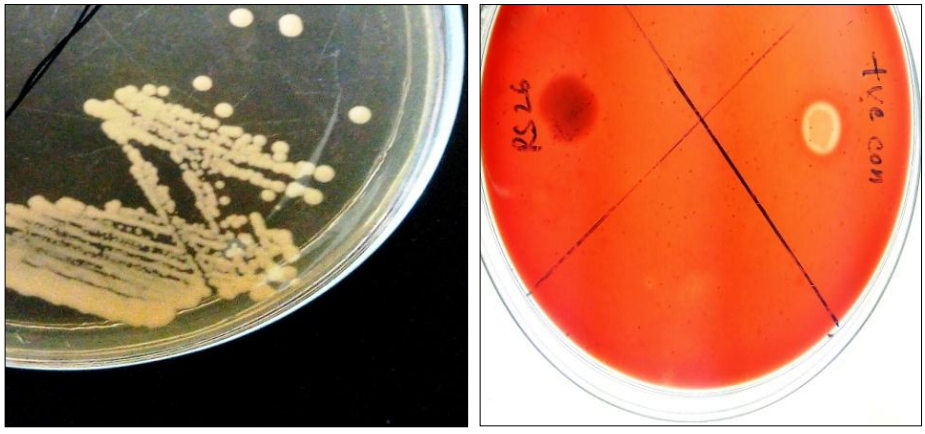


Fig 5: Quantitative estimation of soluble phosphorus by *K. pneumoniae* RS26 on Pikovskaya's broth medium after 2, 4, 6, 7, 8 and 9 days of incubation.

3.5. Pathogenecity test of RS26

Hemolysin production test of RS26 was evaluated by growing the organism in sheep blood agar medium. The result (Fig 6B) indicates the absence of the zone of hydrolysis suggesting its non-pathogenic nature. Therefore, from this experiment it can be concluded that strain RS26 can be used as PGPR for plant growth promotion without causing any human pathogenicity.



A **B**

Fig 6: Plates showing the colony morphology of RS26 on nutrient agar plate (A) and the non-haemolytic nature of RS26 as compared to the positive control (B).

4. Conclusion

In the present research a potent bacterial strain was isolated and identified as *K. pneumoniae* RS26. The strain was able to fix atmospheric N_2, produce considerable amount NH_3 and IAA, solubilize insoluble inorganic phosphate. Moreover, nonpathogenic nature of RS26 along with PGP activity makes

it as good and promising candidate for bio-fertilizer formulation. Further detailed investigations are required to check its ability of plant growth promotion in field or pot experiments.

5. References

1. Bhattacharyya P, Jha D. Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microb. Biot. 2012; 28(4):1327-1350.
2. Qureshi MA, Ahmad ZA, Akhtar N, Iqbal A. Role of phosphate solubilizing bacteria (PSB) in enhancing P-availibility and promoting cotton growth. J Animal-Plant Sci. 2012; 22:204-210.
3. Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. Plant and soil. 2003; 255(2):571-586.
4. Kloepper JW, Ryu CM, Zhang S. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathol. 2004; 94:1259-1266.
5. Yao AV, Bochow H, Karimov S, Boturov U, Sanginboy S, Sharipov K. Effect of FZB24 *Bacillus subtilis* as a biofertilizer on cotton yields in field tests. Arch Phytopathol. Plant Protect. 2006; 39:1-6.
6. Carrillo-Castaneda G, Munoz JJ, Peralta-Videa JR, Gomez E, Gardea-Torresdey JL. Modulation of uptake and translocation of iron and copper from root to shoot in common bean by siderophore-producing microorganisms. J Plant Nutr. 2005; 28:1853-1865.
7. Egamberdiyeva D. The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. Appl. Soil Ecol. 2007; 36:184-189.
8. Dimkpa C, Svatos A, Merten D, Buchel G, Kothe E. Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote growth in cowpea (*Vigna unguiculata* L.) under nickel stress. Can. J Microbiol. 2008; 54:163-72.
9. Dimkpa C, Merten D, Svatoˇs A, B¨uchel G, Kothe E. Siderophores mediate reduced and increased uptake of cadmium by Streptomyces tendae F4 and sunflower (*Helianthus annuus*), respectively. J Appl. Microbiol. 2009; 107:1687-96.
10. Gururani MA, Upadhyaya CP, Baskar V, Venkatesh J, Nookaraju A, Park SW. Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. J Plant Growth Regul, 2012. http://dx.doi.org/10.1007/s00344-012-9292-6.
11. Ramamoorthy V, Viswanathan R, Raguchander T, Prakasam V, Samiyappan R. Induc-tion of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and disease. Crop Protect. 2001; 20:1-11.
12. Kirankumar R, Jagadeesh KS, Krishnaraj PU, Patil MS. Enhanced growth promotion of tomato and nutrient uptake by plant growth promoting rhizobacterial isolates in presence of tobacco mosaic virus pathogen. Karnataka J Agric. Sci. 2008; 21:309-311.
13. Kloepper JW, Lifshitz R, Zablotowicz RM. Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol. 1989; 7:39-43.
14. Okon Y, Labandera-Gonzalez CA. Agronomic applications of *Azospirillum*: an evaluation of 20 years word wide field inoculation. Soil Biol. Biochem. 1994; 26:1591-1601.
15. Glick BR. The enhancement of plant growth by free living bacteria. Can. J Microbiol. 1995; 41:109-114.
16. Simbert RM, Krieg NR. Phenotypic characterization, in Methods for General and Molecular Bacteriology. Gerhardt, P, Ed, Amer. Soc. Microbiol, Washington, D.C, 1994, 607-654.
17. Murmur J. A Procedure for the Isolation of Deoxyribonucleic Acid from Micro-Organisms. J of Mol. Biol. 1961; 3:208-218.
18. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Habor, NY, 1989.
19. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol. Biol. 1990; 215:403-410.
20. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. Mol. Biol. Evol. 2011; 28:2731-2739. doi: 10.1093/molbev/msr121 PMID: 21546353
21. Goswami D, Dhandhukia P, Patel P, Thakker JN. Screening of PGPR from saline desert of Kutch: growth promotion in *Arachis hypogea* by *Bacillus licheniformis* A2. Microbiol. Res. 2014; 169:66-75.
22. Patten CL, Glick BR. Role of *Pseudomonas putida* Indoleacetic Acid in Development of the Host Plant Root System. Appl. Environ. Microbiol. 2002; 68:3795-3801.
23. Katznelson H, Bose B. Metabolic activity and phosphate-dissolving capability of bacterial isolates from wheat roots, rhizosphere, and non-rhizosphere soil, Can. J Microbiol. 1959; 5:79-85.
24. Barton CJ. Photometric analysis of phosphate rock. Anal. Chem. 1948; 20:1068-1073.
25. Chahad OB, Bour ME, Calo-Mata P, Boudabous A, Barros-Velazquez J. Discovery of novel biopreservation agents ` with inhibitory effects on growth of food-borne pathogens and their application to seafood products. Res. in Microbiol. 2012; 163(1):44-54.
26. Kloepper JW, Schroth MN. Plant growth-promoting rhizobacteria on radishes, Proceedings of the International conference on plant pathogenic bacteria. 1978; 2:879-882.
27. Verma P, Shahi SK. Characterization of plant growth promoting rhizobacteria associated with potato rhizosphere. Int. J Adv. Res. 2015; 3:564-572.
28. Patten CL, Glick BR. Bacterial biosynthesis of indole-3-acetic acid. Can. J Microbiol. 1996; 42:207-220.
29. Glick BR, Penrose DM, Li J. A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. J Theor. Biol. 1998; 190:63-68.
30. Ji SH, Gururani MA, Chuna SC. Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol. Res. 2014; 169:83-98.
31. Bhardwaj G, Shah R, Joshi B, Patel P. *Klebsiella pneumoniae* VRE36 as a PGPR isolated from *Saccharum officinarum* cultivar Co 99004. J Applied Biol. Biotechnol. 2017; 5(01): 047-052.
32. Kuan KB, Othman R, Abdul Rahim K, Shamsuddin ZH. Plant Growth-Promoting Rhizobacteria Inoculation to Enhance Vegetative Growth, Nitrogen Fixation and Nitrogen Remobilisation of Maize under Greenhouse Conditions. PLoS One. 2016; 11(3):1-19. e0152478. Doi:

10.1371/ journal.pone.0152478
33. Bilal R, Malik KA. Isolation and identification of a N_2-fixing Zoogloea-forming bacterium from kallar grass histoplane. J Appl. Bacteriol. 1987; 62:289-294.
34. Malik KA, Rasul G, Hassan U, Mehnaz S, Ashraf M. Role of N_2-fixing and growth hormones producing bacteria in improving growth of wheat and rice. In Nitrogen Fixation with non-legumes. Eds. N A Hegazi, M Fayez and M Monib. The Am. Univ. in Cairo Press, Cairo, 1993, 409-422.
35. Douglas M, Zhu J, Winston JB. Regulation of Nitrogen Fixation in *Klebsiella pneumoniae*: Isolation and Characterization of Strains with nif-lac Fusions. J Bacteriol. 1981; 145:348-357.
36. Sachdev D, Agarwal V, Verma P, Shouche Y, Dhakephalkar P, Chopade B. Assessment of microbial biota associated with rhizosphere of wheat (*Triticum aestivum*) during flowering stage and their plant growth promoting traits. Internet J Microbiol. 2009. http: //dx.doi.org /10.5580/21a7