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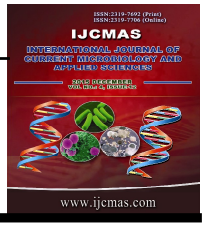


Original Research Article

Role of *Lysinibacillus sphaericus* SNCh5 Bacterial Strain as Bio-inoculant for Agriculture Practice

Nisha Sharma and Baljeet Singh Saharan*

Department of Microbiology Kurukshetra University, Kurukshetra 136119, India
**Corresponding author*

Keywords

Bio-inoculant, IAA, PGPB, Phyllosphere, Seed germination, Siderophore

A B S T R A C T

Lysinibacillus sphaericus SNCh5 is a gram positive rod shaped and mesophilic bacterium. In the present study we investigated that *L.sphaericus* SNCh5 has important properties as it solubilised 10g/L of Ca_3PO_4 and pH value of SNCh5 inoculating broth was decreased to 2.18. It also produced maximum 142.7 µg/mL ammonia and 56.8 µg/mL of IAA. About 87.4% of *in vitro* BNF was also performed in 4 days and 89.9% after utilization of 8% sucrose in nitrogen free broth medium. The effect of SNCh5 on *Trigonella feonum-graecum* (methi) and *Vigna radiata* (mung beans) seeds after treatment was observed. Growth parameters under natural condition in the pot experiment were also estimated in SNCh5 treated *Vigna radiate* seeds. Genome sequence of SNCh5 bacteria was matched with *Lysinibacllius sphaericus* and the sequences were submitted to NCBI (Accession No. KT380157).

Introduction

The phyllosphere is an excellent environment for microorganisms. It is the leaf and stem surface of plants where large number of microorganisms have been recorded that are either beneficial or harmful for plants. The beneficial bacteria perform their role by establishing close relationship with plants and provide nutrients in form of minerals (through phosphate solubilization, ammonia, phyto-hormone production, iron chelation, nitrogen fixation *etc.*) and prevention from pathogenic micro-organisms. The pathogenic micro-organisms decrease the crop yield and also effect the plants growth.

Plant growth promoting bacteria (PGPB) are one such type of beneficial bacteria which promote the plant growth by suppressing the pathogen and produce useful compounds and ultimately increase crop yield. Thus the use of chemical fertilizers can be reduced (Saharan and Nehra, 2011; Glick, 2012).

Chemical fertilizers are synthetically produced chemicals that help to promote the yield and growth of plants by providing nutrient to them. But these chemicals have various harmful effects *e.g.* cause water pollution and decrease mineralization.

Moreover, they also affect the human and animals health when these foods formulated with chemical fertilizers are eaten as raw. On the other hand, PGPB are eco-friendly (Saharan and Verma, 2015).

PGPB helps to provide phosphate that is present in insoluble form in soil. Ammonia production and *in vitro* biological nitrogen fixation (BNF) help in providing nitrogen that helps in plant growth. PGPB also promote the growth and yields of plants in the form of phyto-hormones production. Indole acetic acid (IAA) is precursor for auxin hormone that helps in seed germination, growth of seedling, vascular system development and flowering *etc.* (Zhao, 2010).

Siderophores are the iron binding agents that help the plant to provide iron molecules from soil and allow it to bind with plant root so that it can be easily utilized by plants for growth and defence mechanism against pathogenic microbes. Hydrogen cyanide helps in protection from pathogenic microorganisms by inhibiting their metabolic system.

Spinach (*Spinacia oleracea*) is a useful bony medicinal plant having nutritional and medicinal importance.

In the present investigations, studied PGPB properties (phosphate solubilization, ammonia production, IAA production, HCN, *in vitro* biological nitrogen fixation, siderophore production and enhancement in seed germination of methi and mung bean seeds) exhibited by *L.sphaericus* SNCh5 bacterial strain isolated from spinach phyllosphere. The results suggest that SNCh5 seems to be useful bio-inoculant and may be used in promising agriculture development process.

Materials and Methods

Sample Collection

The fresh leaves of spinach plant were collected from different regions of Haryana (Shahabad, Karnal, Chika, Kurukshetra) and Punjab (Kapiyal and Sangroor). The leaves were stored in sterile polythene bags and then used within 24 hours for the isolation of phyllospheric bacterial strains (Ceballos *et al.*, 2012).

Isolation of Bacterial Strains

The fresh leaves were surface sterilized with 70 % alcohol for 20-30 seconds and then washed with sterile distilled water followed by vortex for 15-20 min. The serial dilution of samples were prepared in range of 10^{-1}-10^{-5} dilution and 100 µL of diluted samples were spreaded in separate nutrient agar plates and then allowed to incubate at 30-37^{o}C for 24-48h. Isolates were preserved in 50 percent (%) glycerol containing nutrient agar slants at 4^{o}C (Ceballos *et al.*, 2012).

Morphological and Bio-chemical Characterization

The bacterial strains were examined for their morphology by visualising the colony characteristics including shape, surface, elevation, margin, pigmentation and colony colour *etc*. Gram staining procedure was also performed according to the Bergy's manual edited by Holt, 1994.

Various Biochemical tests *i.e.* catalase, IMVIC, sugar fermentation, gelatin liquefaction, urease, simmon citrate agar utilization and nitrate reduction were performed using standard available protocols (Dubey and Maheshwari, 2006).

Screening for PGP attributes

Ammonia Production

The freshly grown bacterial culture was inoculated in 10 mL peptone water broth and incubated for 48-72h at 30-37ºC. About 0.5 mL of Nessler's reagent was added Appearance of yellow/ brown colour indicated the ammonia production (Cappuccino and Sherman, 2010).

Quantitatively ammonia production was analysed by inoculating the fresh bacterial culture into peptone water for 1-7 days at 35±2 °C and then centrifuged for 15 min. at 10,000 rpm. The supernatant was collected after every 24 h and1mL of Nessler's reagent was added to 1mL of supernatant and then volume was made up to 10 mL with distilled water. Brown/ yellow colour was developed and absorbance was measured spectrophotometrically at 630nm. The amount of ammonia was estimated by relating with the standard curve of ammonium sulphate in the range of 0.1-1 mol/mL (Demutskaya and Kalinichenko, 2010).

Phosphate Solubilization (PS)

The phosphate solubilization ability of bacterial strain was observed by streaking it on pikovskaya's agar media along with bromothymol blue dye and incubated at 35-37°C for 3-5 days (Nautiyal, 2000).The diameter of solubilization zone was measured after 5-7 days of inoculation.

Quantitatively, PS was estimated by inoculating bacterial culture in Pikovskaya's broth with known amount of tri-calcium phosphate (Ca_3PO_4) *i.e.* 5g/L and incubated at 35-37°C for upto 10 days (Nautiyal, 1999). Inoculated culture broth was centrifuged and supernatant was collected. About 10mL supernatant was mixed with 10mL ammonium molybdate solution by constantly shaking to remove CO_2. When frothing was completely removed, 2mL distilled water was added along with 0.25mL of freshly prepared $SnCl_2$ solution. The change in colour intensity was observed at 630nm and simultaneous change in pH was also analysed. Effect of various conc. of phosphate in the range of 2-20g/L was also estimated with incubation time up to 5 days.

IAA Production

The bacterial culture was inoculating in Luria broth (LB) containing known conc. of L-tryptophan and incubated at 35-37^{o}C for 48-72 h (Brick *et al.,* 1991). Bacterial culture was centrifuge at 10,000 rpm for 10 min. and 2 mL of supernatant was mixed with two drops of o-phosphoric acid along with 4 mL salkowski reagent (50 mL, 35% of perchloric acid, 1 mL of 0.5m $FeCl_3$ solution). It was observed for pink colour formation after the incubation of 0.5-2h at room temp.

Quantitatively it was estimated by inoculating the bacterial culture in different conc. of L-tryptophan (20-500 µg/mL) at 35-37^{o}C for 2-3 days. It was also analysed by maintaining the tryptophan conc. constant (200 µg/mL) and incubation was performed for upto 7 days. After centrifugation 1 mL of supernatant was mixed with 4 mL salkowski reagent and absorbance was taken after 30 min. at 530nm in UV/Visible spectrophotometer (Brick *et al.,* 1991).

In-vitro Biological Nitrogen Fixation

In vitro BNF was observed by streaking the bacterial culture on nitrogen free Jensen's media along with addition of bromothymol blue (BTB) stain as an indicator dye and

incubated at 35-37 ºC for 24-48h (Jensen, 1942). Yellow coloured zone around the colony indicated the positive result of *in vitro* BNF.

Quantitatively it was estimated by inoculating the inoculum in nitrogen free broth medium with constant sucrose conc. (3%) and allowed to incubate the bacterial culture at 35-37 ºC for 7 days. The sucrose was also varied from 2-10% in order to check the effect of sugar conc. on *in vitro* BNF and O.D. was taken at 630nm (Jensen, 1942). *In vitro* BNF (%) was calculated by the formula shown as

$$\% \textit{ in vitro} \text{ BNF} = \frac{\text{O.D. of Reference} - \text{O.D. of Inoculated culture}}{\text{O.D. of Reference}} \times 100$$

Seed Germination Assay

Seeds of *Vigna radiate* (mung bean) and *Trigonella foenum-graecum* (methi) were collected and surface sterilized by soaking in sodium hypochlorite solution for 2-3 min. and washed with sterile distilled water for three times (Abdul and Anderson, 1973). Seeds were treated with bacterial culture, kept in 1% soft agar plates and then incubated for 3-5 days in the dark. The seed germination was evaluated and untreated seeds were taken as control. Germination efficiency along with seedling height, root/shoot length, wet/dry weight, % seed germination and vigor index were analysed.

$$\text{Germination (\%)} = \frac{\text{No. of seeds germinated} \times 100}{\text{Total no. of seeds}}$$

$$\text{Vigor index} = \text{Germination\%} \times \text{Seedling length (cm)}$$

Pot Experiment for PGPB Activity of SNSr7

Pot experiment was performed under natural environmental conditions applied in pot for 21 days. In this experiment, *Vigna radiata* (mung bean) seeds were sown into pots with 3 m^2area filled with soil. About 50 sterilized seeds were treated with I mL of 24h old bacterial culture(10^8 CFU/mL)into nutrient broth for 1h and then sown in each pot (in triplicate). After 15-21 days seed germination rate and other growth parameters were observed. Treated and un-treated (control) pots were irrigated with water after every 24h.

Measurement of Chlorophyll Concentration

The chlorophyll content was observed by mixing 0.08 g of fresh leaves samples of treated and untreated (*Vigna radiata*) plant in 10 mL acetone (90%) and incubated overnight at -4^{o}C. One mL of dimethyl sulfoxide (DMSO) was added by continuous mixing for 2 min and then centrifuged at 10,000 rpm for 10 min. Supernatant was removed and the process was repeated again with the addition of 1 mL DMSO. Further, process was repeated again to get the maximum chlorophyll content. This absorption process for chlorophyll content was performed within 20 min. after centrifugation by taking DMSO as blank. chlorophyll a (ChlA), chlorophyll b (ChlB) and total chlorophyll content was observed by taking O.D at 645 and 663 nm (Arnon *et al.*, 1949).

$$\text{ChlA (mg/L)} - 0.0127A_{663} - 0.00269A_{645}$$
$$\text{ChlB (mg/L)} - 0.0029A_{663} - 0.00802A_{645}$$
$$\text{Total Chl (mg/L)} - 0.0202A_{663} + 0.00802A_{645}$$

Statistical Analysis

Results were expressed in the form of mean ± Standard deviation (SD) by using SPSS software version 16.00.

Microbial Identification of SNCh5

DNA was isolated from bacterial sample by using Biopure™ kits for genomic DNA isolation. 16S rRNA gene was amplified using PCR by extracting DNA from bacterial isolate. The primers used for the amplification of 16S rRNA gene are as shown below, where F= forward sequence; R= reverse sequence

F AGAGTTTGATCHYGGYTYAG
(where Y and H are mixed base pair *i.e.* Y= Y=C/T, H=A/C/T)
R ACGGCTACCTTGTTACGACTT

PCR condition

94°C for 5 min. (Initial denaturation); 35 cycles including: 94°C for 60 sec, (denaturation); 53°C for 45 sec. (annealing); 68°C for 90 sec. (extension) and then final extension was done at 68°C for 10 min.

The amplified PCR products were electrophoresed by using 1% Agarose gel in TAE buffer and visualized by staining with ethidium bromide. PCR product was then purified by washing with sodium acetate and 70% of ethanol and eluted from the gel. Forward and reverse sequencing reactions of PCR amplicon were carried out on the sequencer (ABI 3730XL) for obtaining the sequence. The assembled DNA sequence was then submitted to NCBI and Phylogenetic tree was prepared.

Results and Discussion

Isolation

About 225 different bacterial colonies were obtained from spinach phyllosphere from 19 samples. Out of these, 15 bacterial isolates exhibited PGP properties. Further the isolate *L. sphaericus* SNCh5 was studied in detail for various properties.

Morphological and Biochemical Information

L.sphaericus SNCh5 bacterial strain was found to be greenish to brownish coloured with spherical, flat, entire margin and with pungent smell (Fig.1.1). Gram staining indicated it as gram positive, small sized and rod shaped bacterium (Fig.1.2). It showed positive test for nitrate reduction, H_2S production, citrate utilization.

Results of Plant Growth Promoting Attributes by *L. Sphaericus* SNCh5

Phosphate Solubilization (PS)

PS was positively observed in *L.sphaericus* SNCh5 by the formation of clear zone around the colony in Pikovskaya's agar media (Fig.2.1). Qualitatively PS zone was estimated by measuring the halo zone around the spotted colony of SNCh5 bacterial strain (Fig.2.2). The colony diameter was found to be8mm of size with halo zone size15.3mm. The Solubilization efficiency was 191.25 and solubilization index (SI*) was around 2.91 (Fig 2.3).

SNCh5exhibitedmaximum solubilization at 10g/L of Ca_3PO_4in 5 days at 37^oC and pH of culture inoculated broth was decreased to2.18 (Fig. 2.4). Maximum phosphate solubilization efficiency was also analysed by varying the incubation time from 1-10 days at the same incubation temp with constant conc. of Ca_3PO_4. It was found that maximum phosphate solubilization in 7 days at 37^oC (Fig. 2.5) decreased in pH value by 2.It may be due to the formation of some acidic compounds in Pikovskaya broth during phosphate solubilization (Fig.2.6).

Ammonia Production

Ammonia production by *L. sphaericus* SNCh5was confirmed by the formation of orange colour in peptone water broth (Fig. 3.1) and quantitatively it was estimated by incubating the SNCh5 bacterial strain for time incubation of 1-7 days at 37^{o}C.It was observed that SNCh5 produced 142.7 µg/mL of ammonia in4daysat 37^{o}C (Fig. 3.2).

In Vitro Biological Nitrogen Fixation (BNF)

L. sphaericus SNCh5 helps in promoting the growth of plants by *in vitro* BNF that was qualitatively confirmed by formation of yellow zone around the colony in nitrogen free Jensen's media (Fig. 4.1). About 18mm of yellow zone size was observed indicating nitrogen fixation ability of SNCh5 (Fig. 4.2).

Quantitatively it was observed that as the conc. of sugar (sucrose) varied from 2-10%, about 91.6% of *in vitro* BNF was estimated after inoculation of SNCh5 in 10% of sucrose containing nitrogen free broth media at 37^{o}C from 3-5 days having O.D. value 0.039 (Table 1). Along with this, 87.4% of *in vitro* BNF was also estimated at constant conc. of 3% in 4days at 37^{o}C with0.20 (reference) and 1.59 (inoculated culture) O. D. value, respectively (Fig. 4.3).

IAA production

SNCh5 exhibited maximum IAA production ability by converting the transparent coloured LB broth into the deep pink colour after utilization of L-tryptophan as substrate for this enzymatic reaction (Fig. 5.1). It was observed that SNCh5 produced56.8 µg/mL of IAA by utilizing 80µg/mL of L-tryptophan (Table 2) and maximum of 54.4 µg/mL of IAA was produced by it in 5daysat 37^{o}C at constant conc. (200µg/mL) of L-tryptophan in LB (Fig. 5.2).

In Vitro Seed Germination Assay

Seed germination assay was performed on two important crops *i.e. Trigonella feonum-graecum* (methi)(Fig. 6.1) and *Vigna radiata* (mung bean) seeds (Fig. 6.2). It was observed that maximum germination of SNCh5 treated *Vigna radiata* (mung bean) seeds in the form of seedling height, % seed germination, root/shoot length, wet/dry weight and vigor index was observed in comparison to untreated control seeds. All these growth parameters were found to be more in treated *Trigonella feonum-graecum* (methi) seeds in comparison to untreated seeds (Table 3).

Pot Experiment under Natural Conditions

It was observed that treatment of *Vigna radiata* (mung bean) seeds with SNCh5 bacterial strains showed maximum effect on all the growth parameters in comparison to untreated seeds (Fig. 7). We observed that SNCh5 treated *Vigna radiate* showed 100% seed germination while in control it was only 60% (Table 4). Seedling height, root and shoot length, wet/dry weight, vigor index of *L. sphaericus* SNCh5 treated (*Vigna radiata*) mungi plants were observed very high in comparison to that of untreated seeds (Table 4).Chlorophyll content ofSNCh5 treated mung bean plants leaves were high in comparison to untreated plants (Fig. 8).

Microbial Identification of SNCh5

Molecular sequencing of SNCh5 was performed after polymerase chain reaction (PCR) by using DNA marker that was 100 base pair (bp)of size and PCR products were found to be 400 bp size.These 16S rRNA

gene sequences were compared with other bacterial sequence using NCBI mega Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and phylogenetic tree was constructed with the help of MEGA 5.05 software (Fig. 9).These16S rRNA sequence obtained showed 100% identity to the sequence of *Lysinibacillus sphaericus.*

In present study we investigated the PGP properties of *L. sphaericus* SNCh5 spinach phyllospheric bacterium and its important role in agriculture. Its morphological studies revealed that it is gram positive, rod shape, mesophilic bacterium and forms greenish to brown colored colonies on nutrient agar medium. Biochemically, it shows positive reaction for citrate utilization, H_2S production and nitrate reduction.

During our research we observed that this bacterial strain exhibited the property of phosphate solubilization by formation of 18mm of halo zone around the colony with the solubilization efficiency of 191.25. Quantitatively it was analysed that SNCh5 solubilizes 10g/L conc. of Ca_3PO_4 in 3 days of incubation with decrease in pH value of broth to 2. Maximum solubilization was also observed after7 days of incubation at 37^oC by solubilizing Ca_3PO_4 (5g/L) and pH was decreased to value 2.6, which is supported byfindings of Nico *et al.* (2012). They reported that certain PGPB like *Pseudomonas* sp. (PAC) and *Serratia* sp. solubilize phosphate along with different time incubation. This study outcome is also supported by literature available (Khan *et al.*, 2013). They stated that certain multifactor processes were performed by bacteria (*Rhizobia* and *Azotobacter*) in phosphate solubilization and they were important for crop production. Similarly, Goswami *et al.* (2013) reported in accordance to our findings by describing that *Pseudomonas* sp. strain OG isolated from marine water solubilized 34µg/mL of phosphate.

Ammonia production by SNCh5 was also observed. SNCh5 produced 142.7µg/mL of ammonia in 4 days at 37 ^oC and these findings are favored by literature (Damodaran *et al.,* 2013; Kumar *et al.*, 2013). *In vitro* BNF ability was also analysed by formation of 18mm size of yellow zone around the *L. sphaericus* SNCh5 bacterial colony in nitrogen free medium at 37 ^oC after 3-5 days of incubation. Quantitatively, 91.6% *in vitro* BNF was estimated after utilization of 10% of sucrose at the same incubation time and temp. and 87.4% was observedby SNCh5 at constant sucrose conc. (3%) after 4 days of incubation at 37 ^oC that is accordance to the literature (Husen, 2013).Mehta *et al.* (2015) also supported our findings by reporting that *A. vinelandii* Mac259 produced 85.21% of siderophore unit.

L. sphaericus SNCh5 produces 56.8µg/mL of IAA by utilizing 80µg/mL of L-tryptophan in LB at 37 ^oC after incubation for 5 days and it also produces maximum of 54.4µg/mL of IAA at the constant conc. of L-tryptophan after incubation of 5 days at the same incubation temp. This IAA production was in accordance with the report of Ahmad*et al.* (2008).

They stated that15 µg/mL and 22.02 µg/mL of IAA was produced by *Azotobacter* sp.AZT_{26} and *Pseudomonas* sp. Ps_g by utilizing 500 µg/mL of L-tryptophan isolated from different sources. Similarly, Kumar *et al.* (2012), also supported our findings by reporting that 213.15µg/mL of IAA was produced by FBK3 bacterial strain while 29µg/mL of IAA was produced by *Pseudomonas* sp. isolated from Olive green (Goswami *et al.*, 2013).Our outcomes are also favored by Husen *et al.* (2013) in which they revealed that only 30µg/mL of IAA was produced by PGPR traits.

Table.1 *In Vitro* BNF (%) by SNCh5 in the Nitrogen Free Jensen's Media

Sr. No.	Conc. of Sucrose (%)	% *in vitro* BNF by SNCh5
1.	2	59.14
2.	4	64.73
3.	6	86.24
4.	8	89.9
5.	10	91.6

Table.2 IAA Production by SNCh5

Sr. No.	L-Tryptophan conc. (µg/mL)	IAA production (µg/mL)
1	20	49
2	40	49.4
3	60	49.9
4	80	56.8
5	100	55.2
6	150	50.3
7	200	49.9
8	250	48.6
9	300	48.6
10	500	41

Table.3 Effect of *L. sphaericus* SNCh5 Bacterial Strain on Seed Germination in Treated *Trigonella feonum-graecum*(methi) and *Vigna Radiata*(mung bean) Seeds

Name of Plant's seed	% Seed germination	Seedling height (cm)±SD	Root length (cm) ±SD	Shoot length (cm) ±SD	Wet weight (g) ±SD	Dry weight (g) ±SD	Seedling vigor index
Mung bean seeds treated	98.33	17.5±0.70	7.7±1.00	10.46±3.40	0.300±0.10	0.078±0.10	1720.7
SNCh5 untreated mung bean seeds	70.22	2.1±1.60	0.3±0.61	0.8±0.68	0.122±0.056	0.046±0.061	147.46
Methi seeds treated	91.66	11.64±0.68	4.5±1.00	7.11±1.20	0.426±0.45	0.063±1.00	1067
SNCh5 untreated methi seeds	69.11	2.1±1.00	0.5±0.34	1.1±0.64	0.07±0.04	0.011±0.00	145.13

Table.4 Effect of SNCh5 on all Growth Parameters in Mung Bean Seeds after Treatment in Comparison to Control under Natural Environmental Condition in Pot Experiment

Name of Plant's seed	Seed germination %	Seedling height (cm) ±SD	Root length (cm) ±SD	Shoot length (cm) ±SD	Wet weight (g) ±SD	Dry weight (g) ±SD	Seedling vigor index
L. sphaericus SNCh5 treated Mung bean seeds	100	66.72 ±1.00	26.88 ±0.23	32 ±0.01	2.2 ±0.10	0.63 ±0.00	6672
Untreated mung bean seeds	60	20.2 ±0.91	5.1 ±0.61	11.89 ±1.13	0.55 ±0.05	0.08 ±0.00	1212

Fig.1.1 SNCh5 Culture **Fig.1.2** Gram Staining

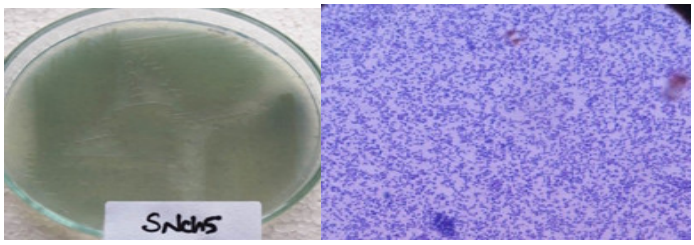


Fig.2.1 Comparison of PS Zone Around SNCh5 Colony with Control

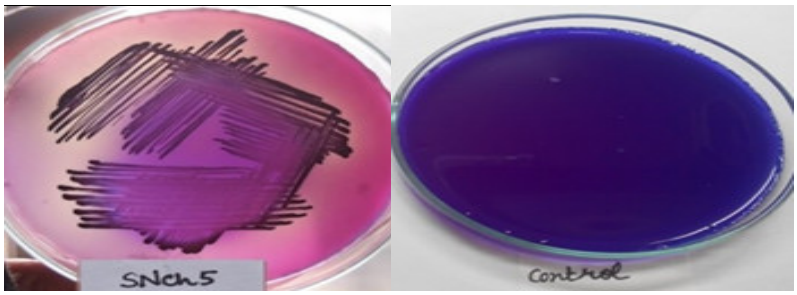


Fig.2.2 Zone of Solubilization Formed Around the SNCh5 Colony

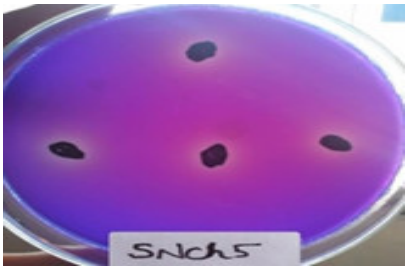


Fig.2.3 Effect of Ca_3PO_4 Conc. on Solubilization Efficiency of Phosphate by *L. sphaericus* SNCh5

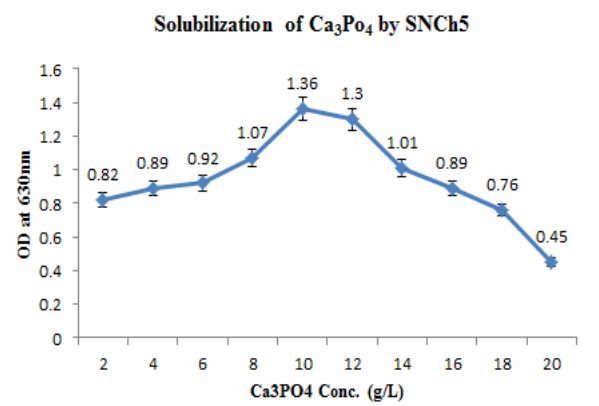


Fig.2.4 Effect of P Solubilization along with Varying Conc. of Ca_3PO_4 on pH of Pikovskaya Broth

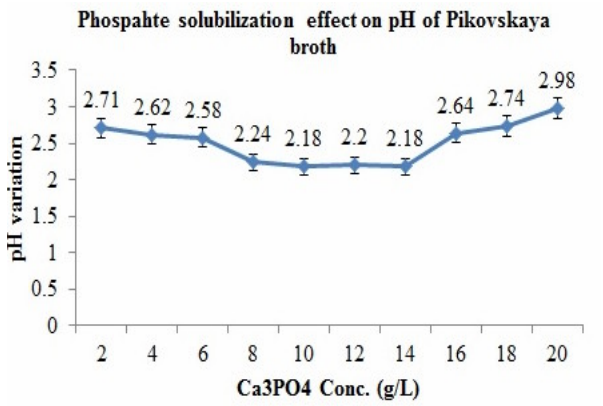


Fig.2.5 Phosphate Solubilization by SNCh5 During Different Time Period

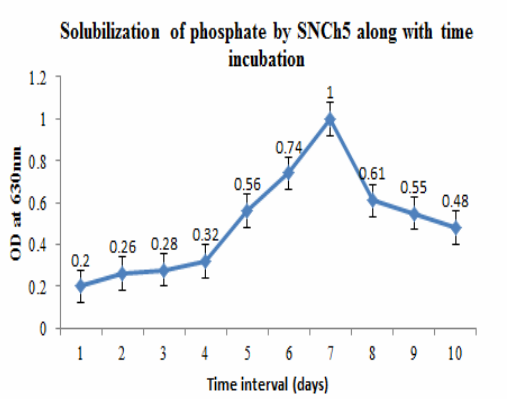


Fig.2.6 Effect of Solubilization of Phosphate by SNCh5 with Time on pH of Broth

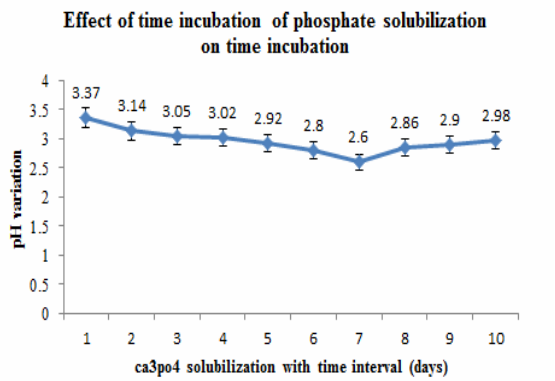


Fig.3.1 Ammonia Production Indicated by Orange Colour Formation in Peptone Broth

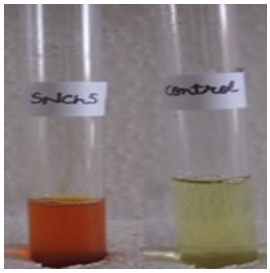


Fig.3.2 Ammonia Production by *L. sphaericus* SNCh5

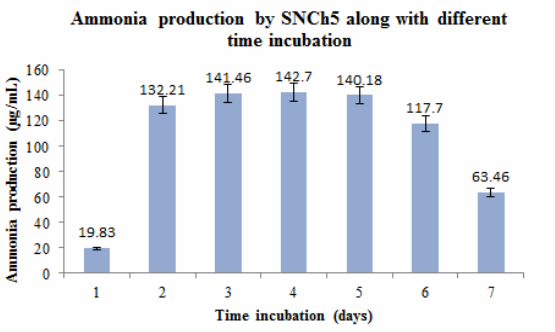


Fig.4.1 *In vitro* Biological Nitrogen Fixation Indicated as Yellow zone Formation Around the SNCh5 Colony

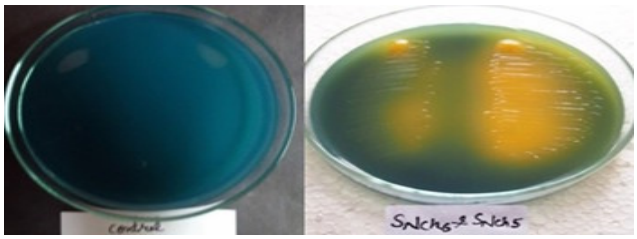


Fig.4.2 Yellow Coloured zone Formation used for Measurement of *In vitro* BNF in term of zone Size (mm)

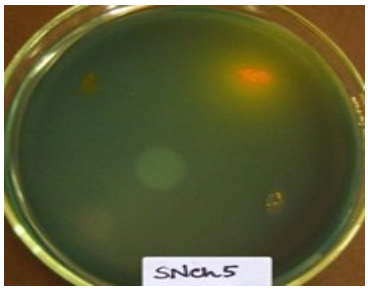


Fig.4.3 % *in vitro* BNF by SNCh5

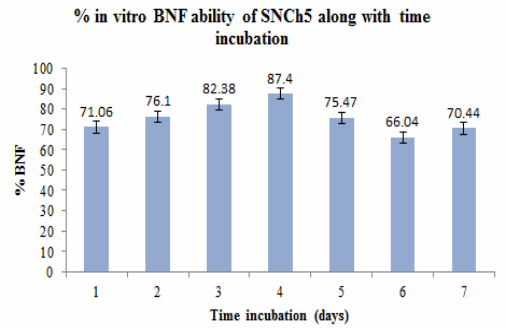


Fig.5.1 IAA Production shown by *L. sphaericus*SNCh5 due to Formation of Pink Colour in L-Tryptophan Amino Acid Containing LB Broth

Fig.5.2 IAA Production by SNCh5 at Constant Conc. of L-Tryptophan (200 µg/mL) in LB with Varying Time Incubation

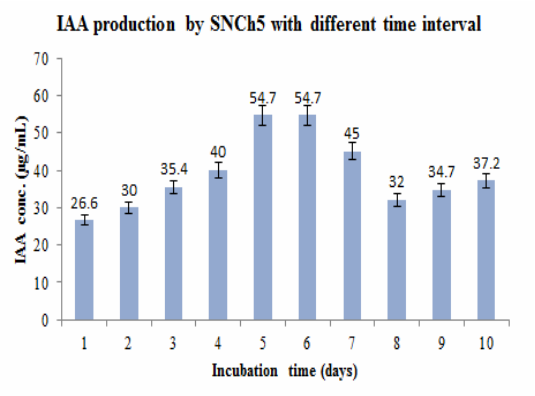


Fig.6.1 Mung Bean Seeds Treated with *L. sphaericus* SNCh5 Bacterial Strain and in Comparison to Control

Fig.6.2 *In Vitro* Seed Germination of Methi Seeds Treated with SNCh5

Fig.7 Pot Experiment under Natural Environmental Conditions Showing Effect on Growth Rate of *Vigna Radiata* (mungi seeds) after Treatment with SNCh5

Fig.8 Detection of Chlorophyll Content of *Vigna radiate* plant's Leaves after Treatment with SNCh5 Bacterial Strain

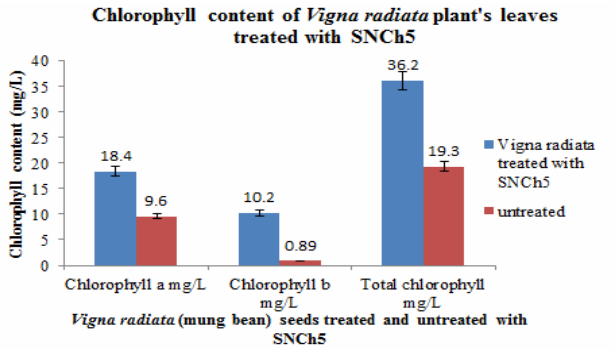
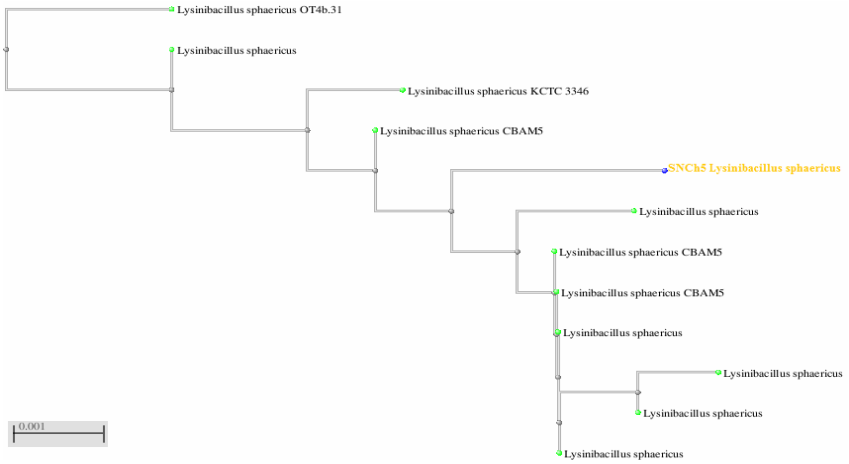


Fig. 9 Phylogenetic Tree Showing Resemblance of SNCh5 with *Lysinibacillus sphaericus* Bacterial Strain



The present study descriptions of IAA production are also supported by the earlier literature report of Mehta *et al.* (2015). They stated that *B. circulans* CB7 bacterial strain produced 26.2 µg/mL of IAA. SNCh5 also has the ability to perform seed germination *i.e.* seed germination of *L. sphaericus* treated SNCh5 methi and mung bean seeds were observed to be more in comparison to untreated seeds. It included all the growth parameters of SNCh5 treated methi and mung bean seeds were high in the form of seedling height, shoot/root length, wet/dry weight and in vigor index in comparison to untreated seeds. All these growth parameters were also observed more when performed using SNCh5 treated *Vigna radiata* seeds (mung bean) in pot experiment under natural environmental conditions as compared to untreated seeds. The chlorophyll content of SNCh5 treated seeds were also more in comparison to untreated mung bean seeds. The present study outcomes are also favored by Kumar *et al.* (2015). They reported shoot length (13.82%),root length (25.07%), shoot dry weight (29.47%), root dry weight (33.33%) and vigor index 2455.69 of seeds treated with plant growth-promoting traits of phosphate solubilizing bacteria(*Bacillus subtilis* CKS1)isolated from *Hippophae rhamnoides* L.(Sea-buckthorn).

These findings are also supported by Teotia *et al.* (2012). They reported the role of PGPR in the growth of Pigeon pea by IAA production, cellulose, amylase, chitinase, siderophore, HCN *etc.* So these findings suggest this phyllospheric bacterial strain may be useful for agriculture in the form of important bio-inoculant.

Reference

Abdul, B. A. A., and Anderson, J. D., 1973. Vigor determination in soybean seed by multiple criteria. Crop Sci.13:630-633.

Ahmad, F., Ahmad, I., and Khan, M. S., 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res.163(2):173-181.

Arnon, D. I., 1949. Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant physiol. 24(1): 1.

Brick, J. M., Bostock, R. M., and Silverstone, S. E., 1991. Rapid *in situ* assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. Appl. Environ. Microbiol.57:535-538.

Cappuccino, J., and Sherman, N., 2010.Microbiology: A Laboratory Manual. Benjamin Cummings Publishing Company, USA, 560.

Ceballos, I., Mosquera, S., Angulo, M., Mira, J. J., Argel, L. E., Uribe-Velez, D., Romero-Tabarez, M., Orduz-Peralta, S., and Villegas, V., 2012. Cultivable bacteria populations associated with leaves of banana and plantain plants and their antagonistic activity against *Mycosphaerella fijiensis*. Microb. Ecol.64:641-653.

Damodaran, T., Sah, V., Rai, R. B., Sharma, D. K., Mishra, V. K., Jha, S. K., and Kannan, R., 2013. Isolation of salt tolerant endophytic and rhizospheric bacteria by natural selection and screening for promising plant growth-promoting rhizobacteria (PGPR) and growth vigor in tomato under sodic environment. African J. Microbiol. Res. 7:5082-5089.

Demutskaya, L. N., and Kalinichenko, I. E., 2010. Photometric determination of ammonium nitrogen with the nessler reagent in drinking water after its chlorination. J. Water Chem. Tech.32(2):90-94.

Dubey, R. C., and Maheshwari, D. K., 2006. Practical microbiology and microbial physiology.

Glick, B. R., 2012.A review: Plant Growth-Promoting Bacteria: Mechanisms and Applications. Hindawi, 1-12.

Goswami, D., Vaghela, H., Parmar, S., Dhandhukia, P., and Thakker, J. N., 2013. Plant growth promoting potentials of *Pseudomonas* sp. strain OG isolated from marine water. J. Plant Interact.8 (4):281-290.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T., 1994.Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore Maryland (9th*ed.*).

Husen, E., 2013. Screening of soil bacteria for plant growth promotion activities *in vitro*. Indonesian J. Agri. Sci. 4(1).

Jensen, H. L., 1942. Pro Line Soc. NSW57:205-212.

Khan, M. S., Ahmad, E., Zaidi, A., and Oves, M., 2013. Functional aspect of phosphate-solubilizing bacteria: importance in crop production. In: Bacteria in Agrobiology: Crop Productivity. Springer Berlin Heidelberg, 237-263.

Kumar, A., Guleria, S., Mehta, P., Walia, A., Chauhan, A., and Shirkot, C. K., 2015. Plant growth-promoting traits of phosphate solubilizing bacteria isolated from *Hippophae rhamnoides* L.(Sea-buckthorn) growing in cold desert Trans-Himalayan Lahul and Spiti regions of India. Acta Physiologiae Plantarum,37(3):1-12.

Kumar, A., Kumar, A., Devi, S., Patil, S., Payal, C., and Negi, S., 2012. Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: an in vitro study. Recent Res. Sci. Technol.4(1).

Kumar, V., Singh, P., Jorquera, M. A., Sangwan, P., Kumar, P., Verma, A. K., and Agrawal, S., 2013. Isolation of phytase-producing bacteria from Himalayan soils and their effect on growth and phosphorus uptake of Indian mustard (*Brassica juncea*). World J. Microbiol. Biotechnol.29(8):1361-1369.

Mehta, P., Walia, A., Kulshrestha, S., Chauhan, A., and Shirkot, C. K.,2015. Efficiency of plant growth-promoting P-solubilizing *Bacillus circulans* CB7 for enhancement of tomato growth under net house conditions. J. Basic Microbiol. 55(1):33-44.

Nautiyal, C. S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett.170(1):265-270.

Nautiyal, C. S., Bhadauria, S., Kumar, P., Lal, H., Mondal, R., and Verma, D., 2000. Stress induced phosphate solubilization in bacteria isolated from alkaline soils. FEMSMicrobiol. Lett.182(2):291-296.

Nico, M., Ribaudo, C. M., Gori, J. I., Cantore, M. L., and Curá, J. A., 2012. Uptake of phosphate and promotion of vegetative growth in glucose-exuding rice plants (*Oryza sativa*) inoculated with plant growth-promoting bacteria. Appl. Soil Ecol.61:190-195.

Teotia, A., and Goel, G., 2012. Role of PGPR under different agroclimatic conditions. Bacteria in Agrobiology: Plant Probiotics, 169-183.

Saharan, B. S., and Nehra, V., 2011. Assessment of Plant growth promoting attributes of cotton (*Gossypium hirsutum*) rhizosphere isolates and their potential as bio-inoculants. J. Environ Res. Dev. 5(3):575-583.

Saharan,B. S., and Verma, S., 2015.Evaluation of rhizospheric bacteria from *Ocimum* sp. as potential PGPR. J. Microb. Biochem. Technol.7(2):088-095.

Singh, A. V., Chandra, R., and Goel, R., 2013. Phosphate solubilization by *Chryseobacterium* sp. and their

combined effect with N and P fertilizers on plant growth promotion. Arch. Agro. Soil Sci. 59(5):641-651.

Zhao, Y., 2010.Auxin biosynthesis and its role in plant development. Annu. Rev. Plant Biol.61:49-64.