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Isolation of a phosphate solubilizing bacterial strain *bacillus Tequilensis* mcc 3872 from the rice field of burdwan district and characterization of its plant growth promoting traits

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Abstract
Excessive use of phosphatic chemical fertilizers hampers agricultural productivity. Plant absorb few amount of applied phosphate-based chemical fertilizer, rest amount is converted into insoluble complexes and stored in the soil which plants are unable to take. This has led to search for an eco-friendly and economically reasonable option for developing crop production. Phosphate solubilizing bacteria (PSB), capable of solubilize insoluble forms of phosphate into plant-utilizable soluble forms. Nine bacterial strains, possess phosphate solubilizing activity were isolated from the rice field of Burdwan and their phosphate solubilizing activity as well as other plant growth promoting traits were determined. Among them, CP6 strain, identified as Bacillus tequilensis based on 16S rDNA sequencing and phenotypic characterization, selected as best-performing strain. The cell density and Phosphate solubilizing ability of CP6 was also checked with time and in different pH to ensure CP6 strain can be introduced as a potent phosphate Biofertilizer.

Keywords: Phosphate solubilizing bacteria, plant growth promoting rhizobacteria, phosphate Biofertilizer, *Bacillus tequilensis*, plant growth promoting traits

1. Introduction
Phosphorus is least mobile and least available micronutrient, plays vital role in different developmental process in plants like nutrient uptake, cell division, photosynthesis etc. [1]. Phosphorus present in soil in very low concentration, so, to support crop production, different phosphate-based chemical fertilizers are introduced to agricultural field [2]. Excessive use of these chemical fertilizers reduces soil fertility as very few amount of the applied fertilizer is taken up by plants, rest of all amount are converted into insoluble phosphate and stored in the soil [3, 4]. The present scenario has led to search for an eco-friendly and economically reasonable option for developing crop production in phosphate deficient soil. Rhizosphere is a rich niche of microbes and should be explored for obtaining potential plant growth promoting rhizobacteria (PGPR), which can be useful in developing in bio-inoculants for enhancement of growth and yield of crop plants [5]. Phosphate solubilizing bacteria (PSB) are a group of PGPR, capable of solubilizing insoluble forms of phosphate into plant-utilizable soluble forms, perhaps by chelation, producing organic acid and exchanging ions [6, 7, 8]. A large number of PSB inhabits in soil rhizosphere but the amount of phosphate solubilized by them is not sufficient for plant growth enhancement [9]. These novel PSB strains also exhibits different plant growth promoting traits such as production of phytohormones, nitrogen fixation, siderophore production, HCN production, Ammonia production, exo-polysaccharide production, thereby promote plant growth [9]. Considering these novel properties of PSB, in respect to agricultural and ecological viewpoints, the main objective will be to improve plant productivity for incoming population explosion, to increase food quality and to maintaining environmental quality for sustainable agriculture.

2. Material and Methods
2.1. Collection of Rhizosphere Soil sample
Soil samples were collected from the rhizosphere of rice plant of Burdwan rice field. (Latitude- N 23°14'20.86", Longitude- E 87°51'45.743"). The plants were uprooted and the soil attached to the roots was collected in sterile polythene bags.

2.2. Isolation of Phosphate-Solubilizing Bacteria (PSB)
Pikovskaya's agar media [Yeast extract- 0.5g/ltr, Dextrose-10.0g/ltr, Calcium phosphate-5.0g/ltr, Ammonium phosphate-0.5g/ltr, Potassium chloride-0.2g/ltr, Magnesium sulphate-0.1g/ltr, Manganese sulphate-0.0001g/ltr, ferrous sulphate-0.0001g/ltr, Agar-20.0g/ltr] was used to obtain pure culture by dilution plate method. 1g of soil was diluted to 10 ml of water in a test-tube which served as stock solution. Remaining 10 test tubes were filled with 9 ml of water. Transferring of 1 ml of water from the stock solution to 9 ml of sterilized distilled water with the help of pipettes yielded 10^{-1} dilutions and the series continued up to 10^{-10} dilutions.

2.3. Selection of isolates having phosphate solubilizing property
Isolates were selected on the basis of phosphate solubilizing capacity as they exhibit a clear halo zone around them while growing on Pikovskaya's agar medium containing insoluble tri-calcium phosphate [10].

2.4. Morphological characterization of nine selected strains
Morphological characters like shape, surface, margin, colour, odor, pigmentation are recorded of the 3-day old colony. (Table-1).

2.5. Qualitative estimation of phosphate solubilization
To determine phosphate solubilization ability, the isolated bacterial strains were streaked into Pikovskaya agar medium [10] and incubated at 28°C for 3 days. After 3 days, the bacterial colonies showing the clear halo zone considered as phosphate solubilizing bacteria. Phosphate Solubilization Index (PSI) was measured using following formula [11] (Table-2).

PSI = (Colony diameter + Halozone diameter) / Colony diameter

2.6. Quantitative estimation of phosphate solubilization
This was done following ammonium-molybdate method [12]. 3 days old bacterial strain grown in Pikovskaya broth media were used. After centrifugation, 1 ml of culture filtrare was taken out and mixed with 2.5 ml Barton's reagent [solution A: Ammonium molybdate (25 gms) was dissolved in 400 ml distilled water. Solution B: Ammonium metavanadate (1.25 gms) in 300 ml boil distilled water and cooled then 250 ml conc.HNO_3 was added. Solution A and B were mixed and the volume was made-up upto 1000ml with distilled water] and volume made up to 5 ml. After 15 minutes, OD was taken at 430nm wave length and concentrations of solubilized phosphates were determined with the standard curve of KH_2PO_4. (Fig 1)

2.7. *In vitro* characterization of plant growth promoting traits
2.7.1. Estimation of Indole Acetic Acid production
Bacterial strains were allowed to grow in Nutrient broth [Peptone- 5g/ltr, NaCl- 5g/ltr, Yeast extract-1.5g/ltr, Beef extract-1.5g/ ltr] supplemented with 0.5% L-Tryptophan for 48 hours then centriguation was done at 6000 rpm for 10 minutes. 2 ml of supernatant was taken out from each sample and 3-4 drops of orthophosphoric acid was added to it along with 2 ml of Salkowsky reagent [1%0.5 M FeCl3 in 35% perchloric acid]. Then the reaction mixture was kept at dark condition for 20 minutes. Appearance of pink color in the test-tube indicated the production of IAA. After that, absorbance was taken at 533 nm [13]. (Fig 2)

2.7.2. Nitrogen fixing ability
The ability to fix atmospheric nitrogen was tested by inoculating the isolate in the Asbay's Mannitol Agar media [Mannitol-20.0g/ltr, Dipotassium phosphate-0.2g/ltr, Magnesium sulphate-0.2g/ltr, Sodium chloride-0.2g/ltr, Potassium sulphate-0.1g/ ltr, Calcium carbonate-5.0g/ltr, Agar-20.0g/ltr, Final pH (at 25°C) 7.4±0.2] without ready source of nitrogen i.e. Ammonium sulphate and they are allowed to incubate at 30±2º C temperature for 3 days. Occurrence of bacterial growth indicates its ability to fix atmospheric nitrogen. (Table-3)

2.7.3. Ammonia production
Bacterial isolates were inoculated in peptone water and after incubation period of 48 hours, 0.5ml of Nesseler reagent was added to aml of each culture. The development of yellow to dark orange color indicated the positive result of ammonia production [14]. (Table-3)

2.7.4. Hydrogen cyanide (HCN) production
HCN production test was done using Nurient agar media [Peptone- 5g/ltr, NaCl- 5g/ltr, Yeast extract-1.5g/ltr, Beef extract-1.5g/ltr, Agar-20.0g/ltr] supplemented with 4.4g/ltr glycine. Bacterial strains were streaked into the glycine supplemented plates. Whatman filter paper no.1 was dipped in 2% sodium carbonate in 0.5% picric acid solution which was impregnated at the inner surface of the lid of the Petri dishes. Plates were carefully covered with parafilm and incubated at 30°C for 4-7 days. Development of orange to brown color indicated that the bacterial strain have the capability to produce hydrogen cyanide [15]. (Table 3)

2.7.5. Siderophore production
The siderophore activity of selected isolates was tested following the Chrome Azural S (CAS) agar plate method. To prepare 1L of blue agar, 60.5mg CAS was dissolved in 50ml of distilled water and mixed with 10ml of iron (III) solution (1mM FeCl3, 6H2O in 10mM HCl) with stirring. This solution was slowly added to 72.9mg HDTMA dissolved in 40ml water. The resultant dark blue liquid was autoclaved. Also, autoclaved was a mixture of 750ml H2O, 100ml (10 mM9 salts), 15g agar, 30.24g PIPES, 12.0g of a 50% (w/w) NaOH solution to raise the pH of PIPES (6.8) and 25% NaCl. After cooling to 50oC, 30ml of filter - sterilized casamino acids (10%), the carbon source and other required supplements, like vitamins and antibiotics, were added to sterile solution, along the glass wall, with enough agitation to achieve mixing without generation of foam. Each plate received 30ml of blue agar. Interaction of the dye with agar extends the applicability of the method to higher pH values, although a change to green was observed above pH 7. [16] (Table 3).

2.7.6. Exo-polysaccharide production
It is estimated following Phenol sulphuric acid method [17]. Bacterial isolates were grown in Nutrient broth, after incubation of 48 hours, cultures were centrifuged at 6000 rpm for 10 minutes. Then the precipitate were again re precipitated with 3 ml of Acetone and re centrifuged, this process were repeated for 3-5 times. After that this precipitate was

suspended with distil water. 1 ml of 5% aquas phenol was added to 1 ml of this suspension along with 5 ml concentrated Sulphuric acid and kept for 20 munities. Development of orange red colour indicates that it is polysaccharide. OD was taken at 490 nm and the concentration was determined using the standard curve of glucose. (Fig 3)

2.8. Selection of best-performing srain

On the basis of phosphate solubilizing ability and other plant growth promoting characters, CP6 was selected as best performing strains and further experiments were carried out with CP6 only. Phenotypic and Biochemical characterization of CP6 was carried out in National Centre for Microbial Resource (NCMR), Pune (Table-4)

2.9. Optimization of different parameters influencing phosphate solubilization ability

2.9.1. Cell density and amount of phosphate solubilization

The cell density [18] and phosphate solubilization was spectrophotometrically [12] measured in every 6 hours upto 48 hours. (Fig 4)

2.9.2. Amount of phosphate solubilization in different pH

In different pH condition (pH 4, 5, 6, 7, 8) phosphate solubilization was determined by ammonium molybdate method [12] (Fig 5).

2.10. Identification of the strain CP6 by 16S rDNA sequencing

The bacterial strain CP6 was sent to NCMR-NCCS, Pune for molecular identification using 16S rDNA method. (Table 6). Genomic DNA was isolated by the standard phenol/ chloroform extraction method [19] followed by PCR amplification of the 16S rRNA gene using universal primers 16F27 [5'-CCA GAG TTT GAT CMT GGC TCA G-3'] and 16R1492 [5'-TAC GGY TAC CTT GTT ACG ACT T-3']. Jukes and Cantor's methods was used for constructing phylogenetic tree in MEGA 7.0 software (Fig 7). This strain was sent to National Centre for Micribial Resource (NCMR), Pune for general deposition.

2.11. Plant growth promotion test on Mung bean using CP6 strain

Mature mung bean *(Vigna radiata* L. Wilczek.) seeds were surface sterilized with 0.1% $HgCl_2$ for 3 min, followed by successive washing with sterile distilled water and then the water was decanted. The seeds were kept for 10 minutes in 48h old bacterial cultures. The seeds were then kept on sterilized moist blotting paper and incubated in dark at 25±2°C for 2-3 days and then those are transferred in a growth room [RH- 70-80%, temp. 30±2 °C]. 2mL of bacterial culture at a cell density of 1×10^8 cfu/mL was added in one set of petridishes (treated set). Seeds treated with sterilized medium instead of bacterial culture were considered as control.

2.12. Estimation of Different plant growth parameter:

Several plant growth parameters like seedling vigor index, germination percentage, amount of chlorophyll were measured in 16 days seedling in each set. (Table 5)

2.12.1. Germination percentage (GP) was calculated following this formula:

$$GP = \frac{Total\ no.of\ seedgerminated}{total\ no\ of\ seeds\ in\ all\ replicates}\ X\ 100$$

2.12.2. Seedling vigor Index (SVI) was calculated by this following formula [20]:

SVI= [(mean root length + mean shoot length) × GP]

2.12.3. For extraction of chlorophyll, 3g of fresh leaf tissue was taken from each set and ground with 80% acetone and then centrifuged at 10,000 rpm for 6 minutes, this process was repeated for 3-4 time until the precipitate become colourless. Then supernatant was taken out and absorbance was recorded against acetone at 645nm and 663nm [21]. The amount of chlorophyll a, chlorophyll b and total chlorophyll was calculated by this formula:

Total Chlorophyll: 20.2(A645) + 8.02(A663)
Chlorophyll a: 12.7(A663) – 2.69(A645)
Chlorophyll b: 22.9(A645) – 4.68(A663)

3. Result

Nineteen bacterial colony with distinct morphology were obtained in dilution plate method. Among them, nine bacterial colony [CP1, CP2, CP3, CP4, CP5, CP6, CP7, CP8, CP9) were selected as they exhibits halo zone around the colony indicating phosphate solubilizing ability.

Table 1: Morphological characterization of nine selected strains

Isolates	Shape	Surface	Margin	Colour	Pigmentation	Odour
CP1	Round	Smooth-shiny	Smooth	Brownish	None	Odorless
CP2	Round	Smooth-shiny	Smooth	Whitish	None	Odorless
CP3	Round	Smooth-shiny	Smooth	Whitish	None	Odorless
CP4	Round	Smooth-shiny	Smooth	Off-white	None	Odorless
CP5	Round	Smooth-shiny	Smooth	Yellowish	None	Odorless
CP6	Round	Smooth-shiny	Smooth	Off-white	None	Odorless
CP7	Round	Smooth-shiny	Smooth	Off-white	None	Odorless
CP8	Round	Smooth-shiny	Smooth	Whitish	None	Odorless
CP9	Round	Smooth-shiny	Smooth	Brownish	None	Odorless

Table 2: Phosphate Solubilization Index (Psi) Of Nine Isolates

Phosphate Solubilization Index (PSI)	CP1	CP2	CP3	CP4	CP5	CP6	CP7	CP8	CP9
	1.04	1.29	2.18	1.45	1.11	2.85	1.89	2.22	2.38

Table 3: Characterization of plant growth promoting traits of nine isolates

Isolates	Nitrogen fixing ability	Ammonia producing ability	HCN producing ability	Siderophore producing ability
CP1	P	N	P	P
CP2	N	P	N	N
CP3	N	P	N	P
CP4	N	N	N	N
CP5	P	P	P	N
CP6	P	P	P	P
CP7	P	P	N	P
CP8	N	P	N	N
CP9	P	P	P	N

'P' represents positive result whereas 'N' represents negative result. All data are mean value of three replicates.

Table 4: Phenotypic Characterization of Cp6

Production of catalase	P	Fermentation of glucose	N
Production of oxidase	N	Fermentation of mannose	N
Hydrolysis of casein	P	Fermentation of inositol	N
Hydrolysis of starch	P	Fermentation of sorbitol	N
Hydrolysis of Urea	N	Fermentation of sucrose	N
Citrate Utilization	P	Fermentation of rhamnose	N
Indole Production	N	Gelatin hydrolysis	P

Table 5: Growth parameters in control and treated set

Growth parameter	Control	Treated
Germination percentage (%)	96.66±1.20	99.33±0.66
Seedling Vigor Index (SVI)	265±1.21	370±0.50
Chlorophyll a (mg/ml)	78±2.32	190±3.11
Chlorophyll b (mg/ml)	57±0.66	165±1.12
Total chlorophyll (mg/ml)	133±1.78	339±3.09

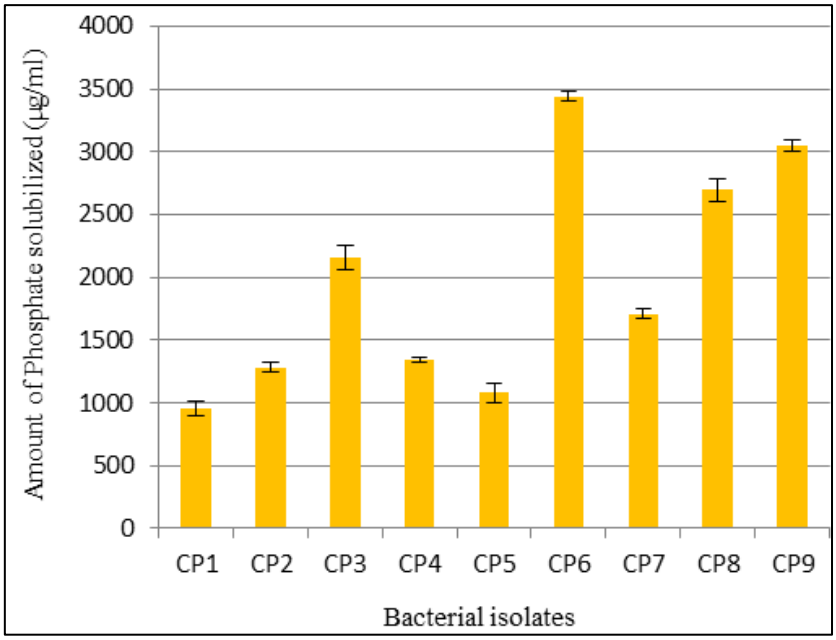

Fig 1: Quantitative Estimation of Amount of Phosphate Solubilized (Tri-Calcium Phosphate) By the PSB Isolates

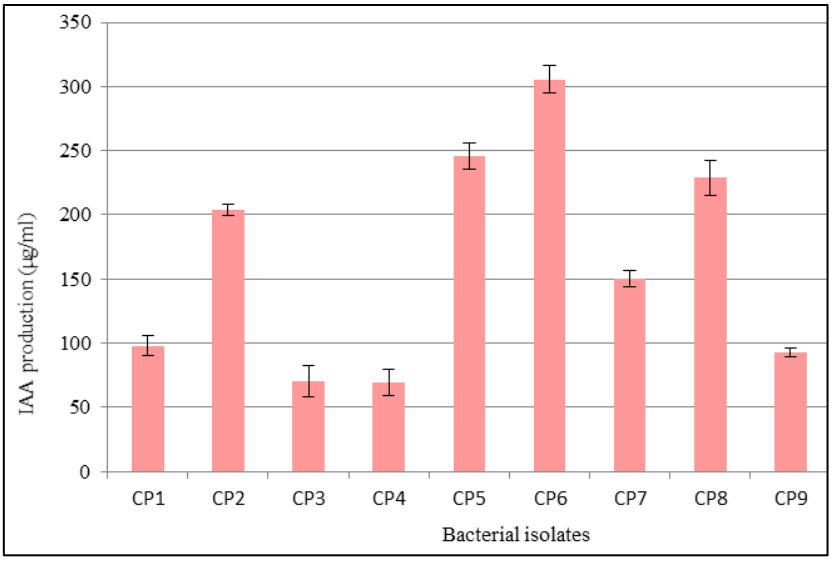

Fig 2: Quantitative estimation of IAA production by PSB isolates

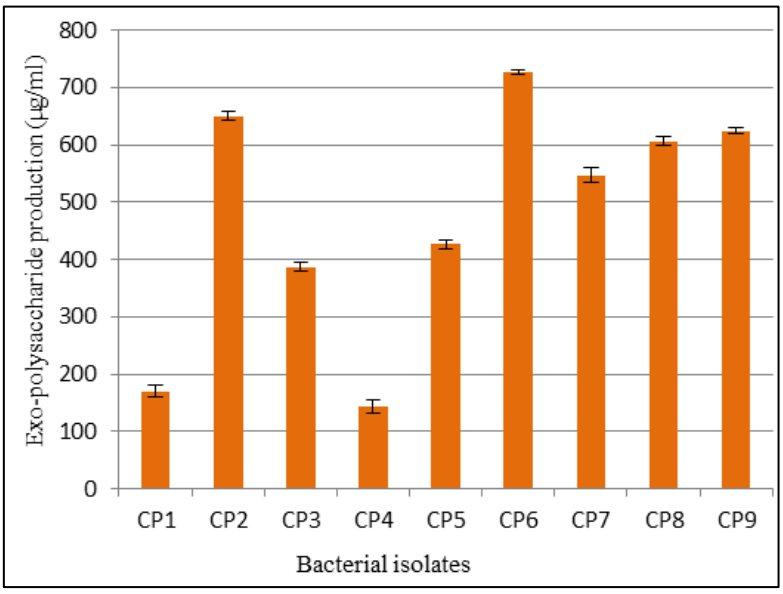

Fig 3: Quantitative estimation of exo-polysaccharide production by PSB isolates

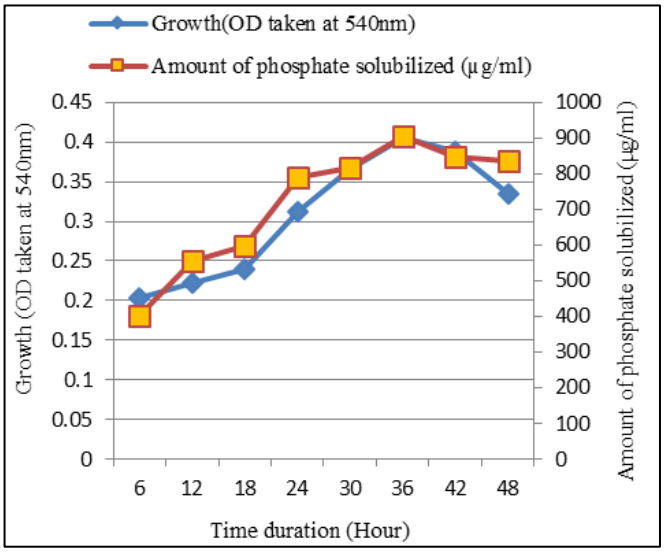

Fig 4: Cell density and amount of phosphate solubilization by CP6 at 6 hours time interval upto 48 hours

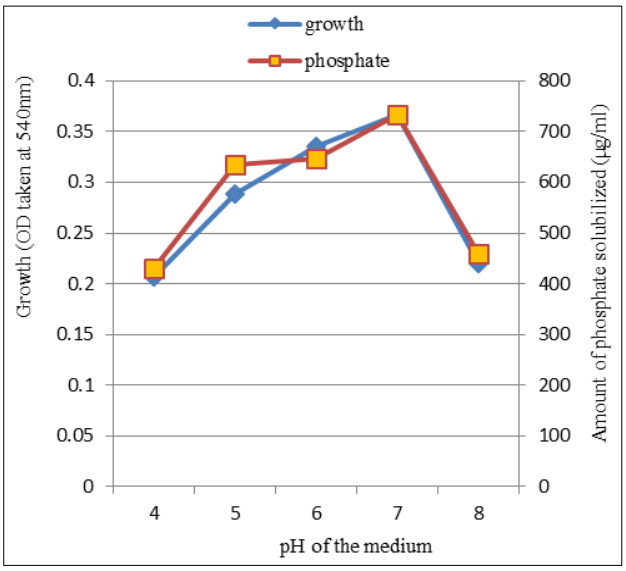

Fig 5: Effect of media pH on phosphate solubilization

Table 6: Molecular Identification

Closest similarity	Pairwise similarity (%)	16 Sr DNA fragment Length (bp)	Strain accession no.
Bacillus Tequilensis AYTO01000043	99.84	1273	MCC 3872

Sequence Text of CP6 in FASTA Format

CAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACT
GGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCA
CTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT
GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTG
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CGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTC
CGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGG
AAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAG
TGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGT
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CTG.

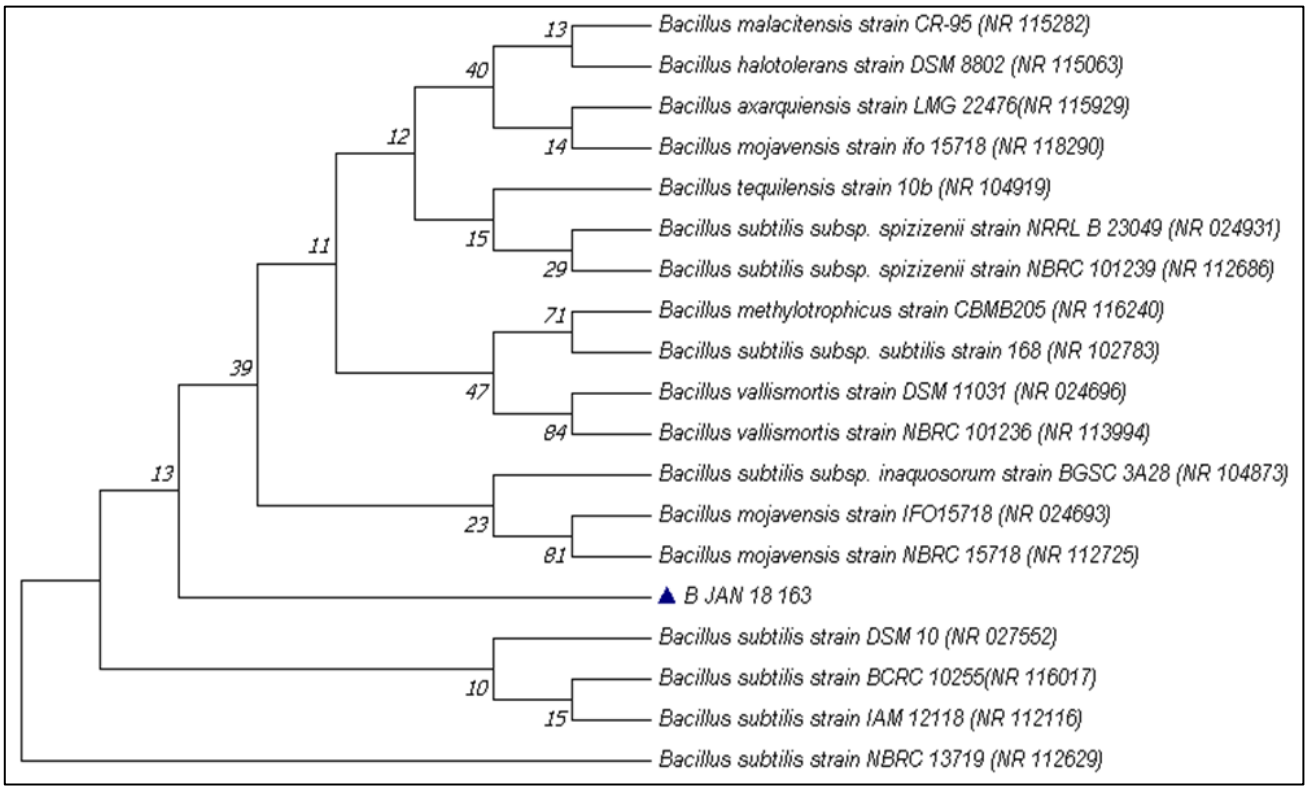

Fig 7: The tree was generated using MEGA 7.0 software with Jukes and Cantor's correction (1969). Bootstrap values of 1000 replications expressed as percentages are given at branch points.

4. Discussion

In the diltution plate method, nineteen bacterial colony with distinct morphology has been isolated, among which nine bacterial isolates were selected after screening for phosphate solubilization test [10]. After studying the colony characteristics, some of the plant growth promoting (PGP) traits of those nine isolates were determined. Based on phosphate solubilizing ability and PGP traits, CP6 was found to be the best-performing strain among the nine isolates. CP6 is a gram positive, motile, thin rods having ability to produce catalase, citrate, can hydrolyse starch, casein and gelatin. The optimum temperature for growth of CP6 is 30°C-37°C, though it can grow upto 50°C. CP6 can grow in pH range of 6-9, also it has salt (NaCl) tolerance property as it showed growth upto 8% NaCl supplemented media. From the growth curve of CP6, it has been showed that, both the cell density and phosphate solubilization capacity is highest in 36 hour, then both started to decline. The optimum media pH for highest growth and phosphate solubilization is pH 7. Considering CP6 as a very efficient PSB, we have checked its plant growth promoting ability in Mung bean seeds and from the result, it was found that, germination percentage and seedling vigor index and total chlorophyll were much higher in CP6 treated set than that of control set. Lastly, the strain CP6 was identified by 16S rDNA sequencing method from NCMR, Pune. The closest neighbor of CP6 is Bacillus tequilensis which has 99.84% pairwise similarity.

5. Conclusion

From the above study, it can be concluded that, CP6 strain is very efficient phosphate solubilizer as well as have different PGP traits. So, the isolate CP6 have the potential to be used as biofertilizer in the place of phosphate-based chemical fertilizers for plant growth and to offer an environmentally sustainable approach to increase crop production.

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