

Research Article

A Study on Isolation of Phosphate Solubilizing Bacterial (PSB) Strain from Vermicomposted Soil and Their Phosphate Solubilizing Abilities

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ABSTRACT:

Phosphate-solubilizing bacteria (PSB) function in soil phosphorus cycle, increasing the bioavailability of soil phosphorus for plants and increase their growth. Isolation and application of such phosphate solubilising bacteria will felicitate the growth of plants in soil-based agriculture. A phosphate solubilising bacterium was isolated from the vermicomposted soil in Chennai, which also had the ability to utilize Tricalcium phosphate at different concentrations. The bacterium was studied for its biochemical characteristics. The 16S rRNA gene sequencing proved that the bacterial strain SK1 belongs to *Bacillus flexus*. The bacterial strain SK1 showed maximum growth when they were cultured at temperature 35°C and at pH 4. The bacterial strain was able to utilize up to 125 mg/L of Tricalcium phosphate in the medium.

Keywords: Phosphate solubilizing bacteria , 16S rRNA gene sequencing, Tricalcium phosphate, Vermicomposting, *Bacillus flexus*

[I] INTRODUCTION

Phosphorus is the second most important nutrient after nitrogen for the growth of plants and microorganisms. Out of added phosphorus fertilizer only 10-20 % is available for the plants. The rest remains in the soil as insoluble phosphate in the form of rock phosphate and tricalcium phosphate. Phosphate solubilizing Bacteria (PSB) significantly helps in the release of this insoluble inorganic phosphate and makes it available to the plants. PSB are a group of beneficial bacteria capable of hydrolysing organic and inorganic phosphorus from insoluble compounds. The metabolic activities of microorganisms (production of acids) solubilize phosphate from insoluble

calcium, iron and aluminum phosphates, in addition to it microbial degradation of organic compounds like nucleic acids which release phosphates. These biochemical changes that take place in the soil prove that microorganisms perform numerous essential functions that contribute to the productivity of soil.

Phosphorus is a major growth-limiting nutrient, and unlike the case for nitrogen, there is no large atmospheric source that can be made biologically available for root development, stalk and stem strength, flower and seed formation, crop maturity and production, N-fixation in legumes, crop quality, and resistance to plant diseases are the attributes associated with phosphorus

nutrition. However, a greater part of soil phosphorous, approximately 95-99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the living plant [1]. It helps plants to survive winter rigors and also contributes to disease resistance in some plants. Phosphorous availability is low in soils because of its fixation as insoluble phosphates of iron, aluminium and calcium [2]. Release of Phosphorus by PSB from insoluble and fixed / adsorbed forms is an import aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant [3]. Organic acid exudation from roots is considered an important mechanism for plants to adapt in P-deficient environments[4]. Since deficiency of Phosphorus is the most important chemical factor restricting plant growth, chemical phosphatic fertilizers are widely used to achieve optimum yields. Soluble forms of Phosphorus fertilizer used are easily precipitated as insoluble forms, this leads to excessive and repeated application of Phosphorus fertilizer to cropland [5]. Phosphorus is a plant macronutrient that plays a significant role in plant metabolism, ultimately reflected on crop yields. It is estimated that about 98% of Indian soils contain insufficient amounts of available phosphorus, which is necessary to support maximum plant growth [6]. Hence the present study focuses on the isolation of the phosphate solubilising bacteria from vermicomposted soil and chromium removal by the earthworms by open-vermicomposting method.

[II] MATERIALS AND METHOD

2.1. Media and Chemicals used

Pikovskay's medium, National Botanical Research Institute's phosphate growth medium devoid of yeast extract (NBRIY), Tricalcium

phosphate and all other chemicals used in mineral salt medium (MSM) preparation were of analytical grade and purchased from Merck, India.

2.2. Bacterial strains and culture conditions

The bacterial strains were isolated from soil samples from three different regions of non-cropped, undisturbed site that of vermicomposted soil. For acclimatization 1 gram of the soil sample was weighed and inoculated in 100 ml containing nutrient agar with 100mg/L and 150mg/L of soil in a sterilized 250 ml conical flask in a shaker at room temperature. Sub culturing was done every 24 h of interval. The serially diluted soil samples were plated on standard agar medium (pH 6.8–7.0) containing 5 g of tricalcium phosphate (TCP) as sole phosphorus source for selectively screening the bacteria which have the ability to release inorganic phosphate from tricalcium phosphate [7]. Uninoculated plates and E. coli inoculated plates served as controls. After 3-days of incubation at 30 8C, PSB developed clear zones around colonies. Colonies with clear zones were further purified by replating on agar medium supplemented with TCP. Individual bacterial isolates were obtained from the enriched culture by plating on nutrient agar medium containing 100 mg/L of TCP. The selected isolates were then purified by streaking on nutrient agar added with 100 mg/L of the dyes. The single colony pure cultures were stored in 15 % glycerol at 20°C. The isolated bacterial strains were incubated for 24-48 hours under the room temperature.(Morphological characteristics) of isolates viz. shape, size, surface form, color were observed for their characterization (Bacterial strains were tested by plate assay using PVK and NBRIP media supplemented with 1.5% of bacto-agar. Four strains were stabbed individually on the agar plate using sterile toothpicks in triplicates. The halo zone formed and colony diameters were measured after 14 days of incubation of the inoculated petriplates at 28⁰ C.

The halo zone size was measured and calculated by subtracting colony diameter from total diameter).

2.3. Quantitative Estimation of Phosphate Solubilization by Broth Assay

The quantitative bioassay was carried out using Erlenmeyer's flasks (150 ml) containing 10 ml of NBRIP broth medium inoculated with the bacterial cells of about 10^8 - 10^9 CFU/ml. Autoclaved un-inoculated NBRIY medium served as control. The flasks were incubated for 2 days at 30° C. The cultures were harvested by centrifugation at 10,000 rpm for 10 minutes. Supernatant was decant and autoclaved at 121°C for 20 minutes. Autoclaved samples were then filtered through at 4.5µm membrane. Phosphorus content in the culture supernatant as well as control (supernatant obtained from no bacteria inoculation) was estimated using the UV spectrophotometer by measuring the absorbance at a wave length of 420nm.

2.4. Effect of Tricalcium Phosphate in Pikovskay's Medium

To study the effect of TCP in pikovskay's medium different concentrations tricalcium phosphate was used (25mg, 50mg, 75mg, 125mg) in the medium. The above mentioned concentrations were individually suspended in pikovskay's medium and sterilized. Further effect of tricalcium phosphate was studied by measuring the growth OD values at 540nm and to check for pH and temperature at every day interval for the isolated bacterial strain.

2.5. Antagonistic Activity

Antagonistic activity was performed by the agar diffusion method with the indicator organisms. The zone of inhibition was measured after the incubation period.

2.6. Morphological Identifications of the Bacterial Isolate

Gram Staining and the slides were examined under a light microscope. Motility test was done and (motile/non-motile) of the bacteria was determined visually for morphological

identifications of the bacterial isolates. Catalase test, Urease test, IMViC Test, Indole production test, Methyl Red-Voges Proskauer (MR-VP) Test, Citrate utilization test, Triple Sugar –Iron Agar Tests were done for the identification of the bacterial isolates. Antibiotic sensitivity of bacterial species was tested by Kirby Bauer's method to find out the resistant pattern to various antibiotics. Then the antibiotic discs like Erythromycin, Amoxicillin, Co-trimoxazole, Azithromycin, Tetracyclin, Penicillin, Ampicillin, and Gentamicin. The sensitivity and resistance of each of them were determined.

2.7. 16S rRNA Partial Gene Sequencing

Chromosomal DNA was isolated from the pure strains of the consortium by the standard phenol/chloroform extraction method [8]. The 1.5 kilo base partial sequence of the 16S rRNA gene was amplified from the chromosomal DNA using polymerase chain reaction (PCR) with universal Eubacteria- specific primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1525XP (5'-TTC TGCAGT CTA GAA GGA GGT GWT CCA GCC-3') [9]. The PCR conditions used were an initial denaturation at 94°C for two minutes, followed by 35 cycles of denaturation at 95°C for one minute, annealing at 55°C for one minute, and extension at 72°C for one minute, and a final extension at 72°C for 10 minutes and sequenced on an ABI310 automated DNA sequencer using the Big Dye terminator kit (Applied Biosystems 3730 x 1 DNA Analyzer). The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification by precipitation with polyethylene glycol and NaCl. The primers used to obtain the complete sequence of 16S rRNA gene of the isolates were the same as for PCR amplification (16F27N and 16R1525XP). Sequence data analysis was done using ChromasPro and Sequencing Analysis software. The output file of sequence alignment was used to compute phylogenetic trees for aligned sequences of 16s rRNA sequencing results of the three bacterial

strains. Neighbour joining method was used for tree building with MEGA 6 software. To access the reliability of the phylogenetic tree, MEGA provides bootstrap test which used the bootstrap resampling strategy. The user has to input the number of replicates. In this experiment, 500 replicates were used.

[III] RESULTS

3.1. Isolation of Phosphate Solubilizing Bacteria

Vermicomposts were prepared by using different composition of saw dust, rice straw and sugarcane waste by inoculating earthworm species viz., *E. eugeniae*. Initial phase during the screening several phosphate solubilising bacterial strains existed in the vermicomposted soil. Further only three bacterial strains survived and produced zone of inhibition. Three different bacterial strains (SK1, SK2, SK3) were isolated and identified based on their colony morphology. The Phosphate solubilizing bacteria SK1 strain produced zone of inhibition (15 mm) SK2 (10 mm) and SK3 (12 mm) respectively. As it is noted that clearance of zone in phosphate containing medium determines the nature of phosphate solubilizing bacterial strain which are considered to fix higher content of phosphate in the soil. As SK1 strain produced maximum zone of inhibition, for the further study on phosphate solubilisation, bacterial strain SK1 was used. [Figure 1] illustrated SK1- Phosphate solubilizing bacteria showing halozone of inhibition.

3.2. Growth of Phosphate Solubilizing Bacteria

To study the growth of phosphate solubilizing bacterial strain it was inoculated in pikovskay's broth and growth was measured by observing the optical density at 24 hours interval. [Figure 2] illustrated the Growth pattern of the Phosphate solubilising Bacteria SK1 strain. It was observed SK1 strain showed a maximum growth of 0.040 (OD) on the 2nd day of incubation by the end of 4th day it reached the decline phase which

showed that the PSB SK1 bacterial strain was able to survive in the medium for 5 days.

3.3. Effect of TCP at different concentrations on the growth of SK1

It was observed from the graph that Phosphate Solubilising Bacteria- SA1 was able to utilize TCP in the medium from 25 mg/L to 125 mg/L. It was observed from [Figure 3] that maximum growth SK1 was observed on the 2nd day with 125 mg/L of TCP as the substrate showing 0.052 as the growth OD value. While it reduced to 0.043 with 75 mg/L of TCP. The growth was observed at least with 25 mg/L of TCP 0.005 which showed that SK1 bacterial strain was phosphate solubilizing bacteria which could utilize maximum amount of TCP in 125 mg/L.

3.4. Effect of pH on the growth of SK 1 strain

The Phosphate solubilising bacteria was allowed to grow at different pH to study the effect of pH in PVK medium containing 125 mg/L of TCP. It was observed from the graph that PSB-SK1 strain was able to utilize TCP at a lower pH from pH 4- pH 6 at 125 mg/L. It was observed from the [Figure 4] that maximum growth of the bacterial strain SK1 was observed at pH 4. While at the pH 6 and pH 7, the growth of the bacterial strain was comparatively less. This shows the bacterial strain would have produced acids during the utilization of TCP and the growth was maximum at lower pH of 4 and 5 respectively.

3.5. Effect of temperature on the growth of SK 1 strain

The effect of temperature was analyzed at 25 °C, 35 °C, and 45 °C. The temperature 35 °C enhanced the growth of the bacterial strain and showed maximum utilization of TCP by the end of the 5th day. This was followed by 45 °C and at temperature 25 °C showed the least growth on the 5th day [Figure 5].

3.6. Antagonistic Activity

Antagonistic activity was performed with phosphate solubilising SK 1 strain against the indicator organisms. SK 1 strain showed about 1.2 cm with *E.coli* [Figure 6]. While the other

organisms (*Pseudomonas aeruginosa*, *Klebsilla pneumonia*, *Proteus* sp.) showed less than 1 cm. These results also proved that such isolated PSB SA1 strain had antagonist activity against most pathogenic strains tested.

3.7. Morphological and Biochemical Characterization

There were about three bacterial strains which were isolated from the vermicomposted soil. The biochemical characteristics of three bacterial strains showed that they belong to the phyla Firmicutes. These groups of bacteria are commonly present in the contaminant soil, water and wastewater. Table 1 summarizes the results on the biochemical tests of the isolated bacterial strains. All the three strains were round in shape with smooth and had irregular morphology on the Pikovskay's medium agar plate.

3.8. Antibiotic Sensitivity Test

SA1 was sensitive to Ampicillin and Tetracycline and was resistant to Azithromycin, Amoxycillin, Erythromycin and Penicillin. SA2 shows resistance to Ampicillin, Amoxycillin, Erythromycin and Penicillin G and it was sensitive to Azithromycin, Gentamycin and Tetracycline. SA3 was resistant to Ampicillin, Co-Trimaxazole and Penicillin G and it was sensitive to Azithromycin, Amoxycillin, Erythromycin and Tetracycline.

3.9. Description of *Bacillus flexus* and phylogenetic analysis

Phylogenetic analysis based on nucleotide sequences from SK1 strain showed a maximum of 99% identity towards *Bacillus flexus* strain NBN2. SA1 was identified as *Bacillus flexus*. Domain: Bacteria, Phylum: Firmicutes, Class: Bacilli, Order: Bacillales, Family: Bacillaceae, Genus: *Bacillus*. This bacterial strain which belongs to phylum firmicutes, is a motile gram positive organism commonly present in the contaminated soil. According to biochemical test, this strain showed Gram positive, motile Bacilli and biochemical results for triple sugar iron agar, catalase, methyl red were positive and

negative result for urease, indole. Voges proskauer and citrate utilization. Phylogenetic tree of the bacterial strain was constructed from 16s rDNA sequencing of *Bacillus flexus*. [Figure 7] showing the Phylogenetic analysis of *Bacillus flexus* strain B53.

[IV] DISCUSSION

Phosphorus is an important limiting factor in agriculture production, and microbial activation seems to be an effective way to solve the solidified phosphorus in soil. Total soil Phosphate has accumulated from Phosphate fertilization and mismanagement; whereas soluble Phosphate has become less available with the increase in Phosphate fertilization in soil. Many bacterial strains with P-solubilizing abilities have been examined in previous studies [10].

The formation of the clear zones is concerned with the P-solubilization of the strain [Figure 1]. It may secrete some substances into surroundings in the course of growing, which can solubilize phosphate or organophosphate. P-solubilization result may vary depending on kinds of the metabolism, how it is released, and spreads in to the medium. Therefore, observational method of P-solubilizing zone can only be used to qualitative assays [11]. The PVK medium was used in the present study because it acts as specific isolation medium for phosphate solubilizing bacteria due to the presence of tricalcium phosphate, which is known for halo zone formation [12].

The most effective Phosphate solubilizing strains isolated from P-amended and Pb-contaminated soils were putatively identified as *Pantoea* sp. and *Enterobacter* sp. The major mechanism of Phosphate solubilization by these PSB is considered to involve pH reduction through the production of organic acids [13].

A number of theories have been proposed to explain the mechanism of phosphate solubilization, and their acid production.

According to the acid production, the process of phosphate solubilization by PSB is due to the production of low molecular weight organic acids that was accompanied by the acidification of the medium [14], and those organic acids can chelate the cation with their hydroxyl and carboxyl groups [15]. A decrease in the pH of the medium from the initial value of 7.0 to a final value of 2.0 was reported by many researchers [16] and in the present study, the decrease in the pH of the medium to 4 containing TCP showed increase in growth of the bacterial strain SK1 which supported the previous work.

There was significant positive correlation found between P solubilization with plant uptake, dry mass, photosynthesis, chlorophyll and root length. This indicated that with addition of PSB more Phosphate was solubilized and positively affected plant growth. PSB inoculation with *Bacillus* strains PSB 9 and PSB 16 can effectively enhance Phosphate solubility of applied RP fertilizers, maintain a favourable soil P pool and increase productivity of aerobic rice. In the long term, this approach would ensure a cost effective, sustainable and environmental friendly production system for aerobic rice[17].

In a literature study conducted they have shown that all the PSB isolates in their study except three produced multiple organic acids followed by a decrease in the pH of the culture medium

there by solubilizing the insoluble tricalcium phosphate[18]. Use of these PSB as bioinoculants will increase the available P in soil, helps to minimize the P-fertilizer application, reduces environmental pollution and promotes sustainable agriculture. In addition to P-solubilization, PSB also produced the other secondary metabolites like IAA and siderophore. Several evidences related to plant growth promotion by PSB through the production of IAA [19] and siderophore [20] makes the PSB more suitable as Biofertilizers.

[V]CONCLUSION

It is concluded from the present study that the PSB isolate followed by a decrease in the pH of the culture medium there by solubilizing the insoluble tricalcium phosphate. The bacterium was studied for its biochemical characteristics and 16S rRNA gene sequencing proved that the bacterial strain SK1 belongs to *Bacillus flexus*. The bacterial strain SK1 showed maximum growth when they were cultured at temperature 35°C , at pH 4 and the strain was able to utilize up to 125 mg/L of Tricalcium phosphate in the medium. Use of these PSB as bioinoculants will increase the available P in soil, helps to minimize the P-fertilizer application, reduces environmental pollution and promotes sustainable agriculture

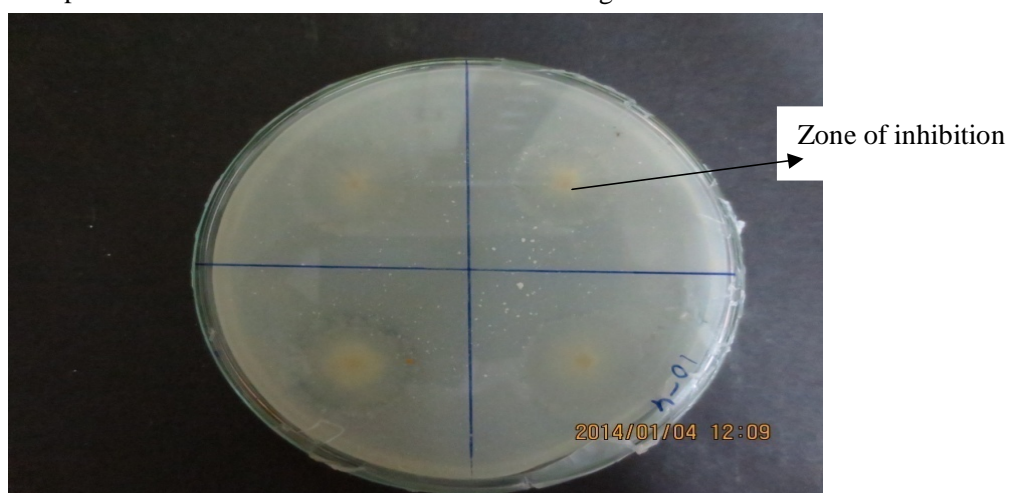


Figure 1 SK1- Phosphate solubilizing bacteria showing halozone of inhibition

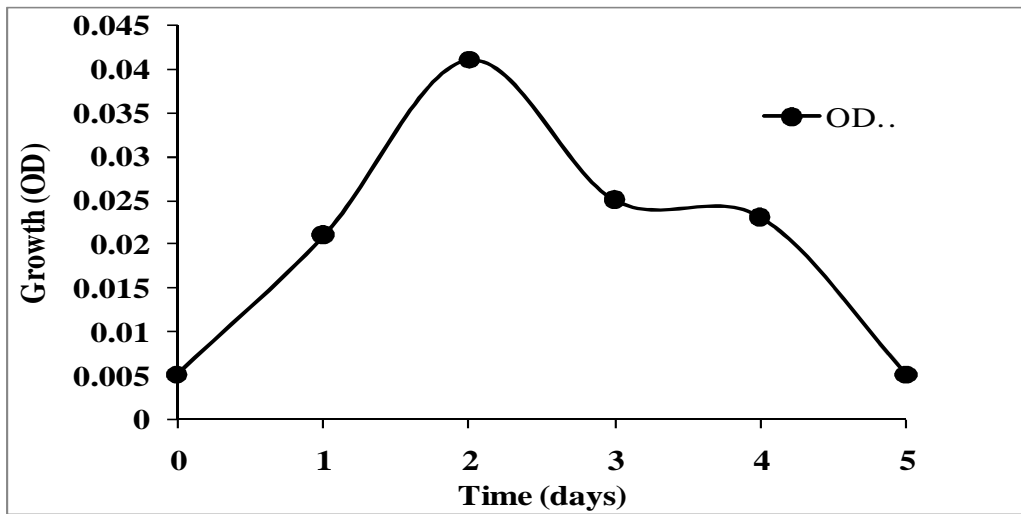


Figure 2 Growth pattern of Phosphate Solubilising Bacteria- SK1 strain

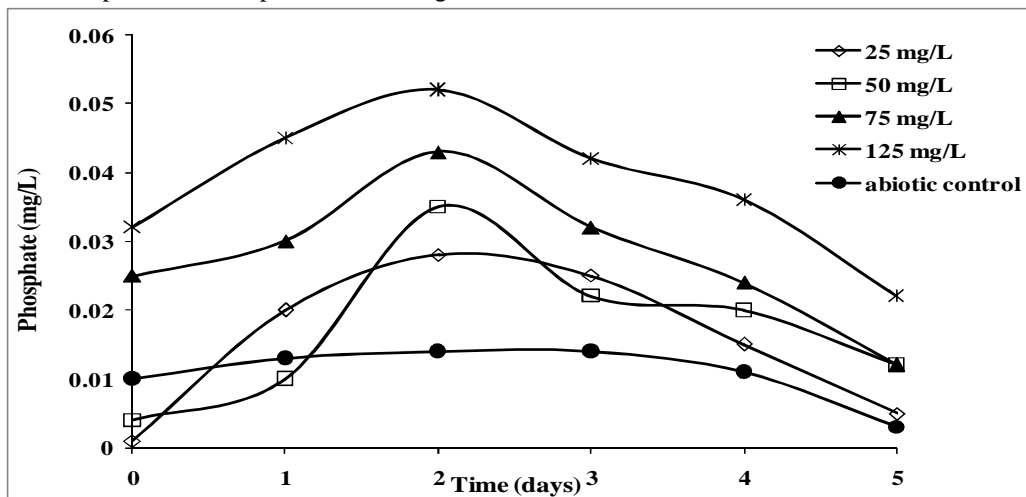


Figure 3 Effect of TCP at different concentrations on the growth of SA1 strain

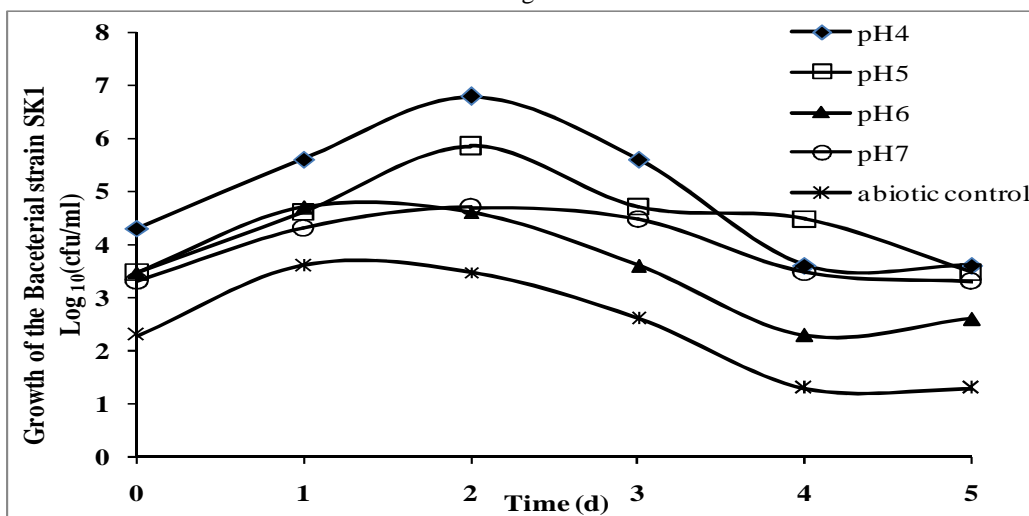


Figure 4 Effect of pH on TCP (125 mg/L) by SA1 strain

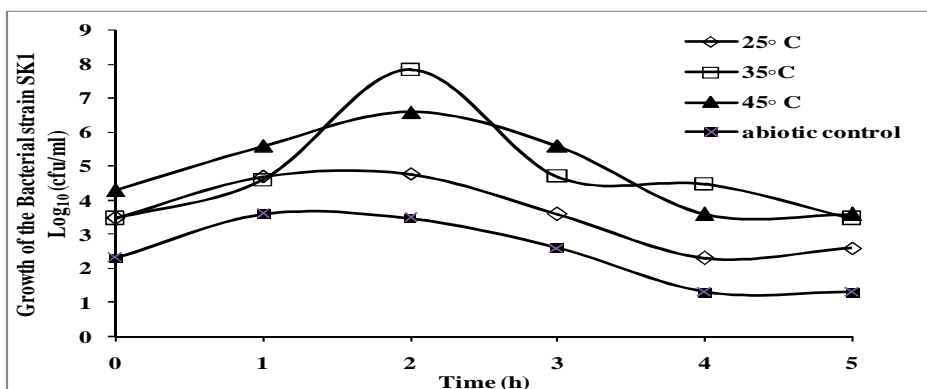


Figure 5 Effect of Temperature on TCP(125 mg/L) by SK1 strain

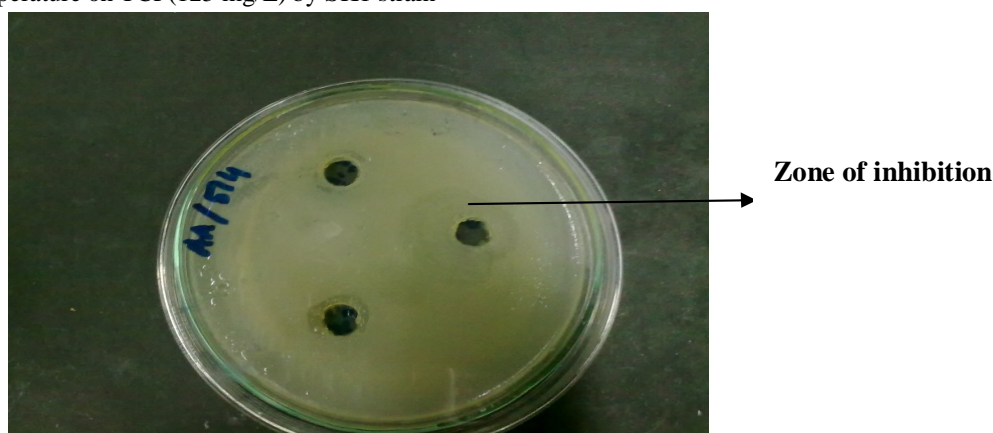


Figure 6 PSB strain SK1 showing the maximum zone of inhibition with *E.coli*

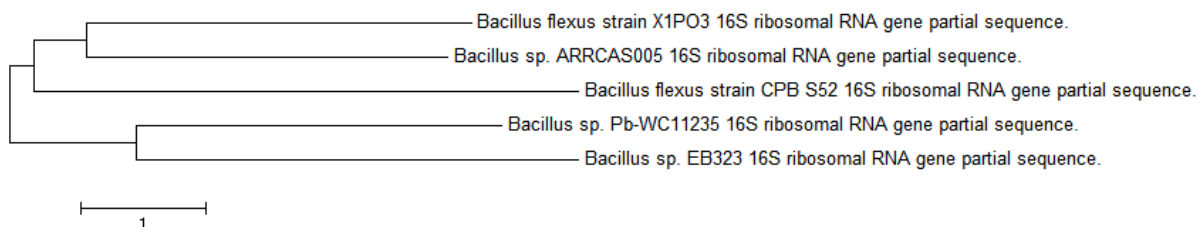


Figure 7 Phylogenetic analysis of Bacillus flexus strain B53 [Table-1].

1.	CHARACTERISTICS	SA1	SA2	SA3
2.	Gram staining	+	-	+
3.	Motility	+	+	+
4.	Triple sugar iron agar	+	-	+
5.	Catalase	+	-	+
6.	Urease	-	+	-
7.	Indole	-	+	-
8.	Methyl red	+	-	+
9.	Vogesproskauer	-	+	-
10.	Citrate utilization	-	+	-

Table:1. Biochemical and morphological characterization of bacterial strains

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