

## EVALUATION OF PHOSPHATE SOLUBILIZING ACTIVITY OF BACTERIA ISOLATED FROM EARTHWORM GUT AND SEAWEED VERMICOMPOST

ANANTHAVALLI. R<sup>1</sup> & RAMADAS. V<sup>2</sup>

<sup>1</sup>Research Scholar, Department of Zoology, Manonmaniam Sundaranar  
University, Tirunelveli, Tamil Nadu, India

<sup>2</sup>Assistant Professor, Department of Zoology, Raja Doraisingam Government Arts  
College, Sivagangai, Tamil Nadu, India

### ABSTRACT

Five bacterial strains were isolated for primary screening to test the phosphate solubilizing activity from seaweed fed earthworm gut and seaweed vermicompost. The selected three bacteria were tested for phosphate solubilizing activity in Pikovskaya's broth amended with 250mg of TCP. Among the bacteria, maximum phosphate solubilization was observed in *B. licheniformis* i. e.,  $795.3 \pm 10.5 \mu\text{g/ml}$ . 16S rRNA sequence of most efficient bacteria, *Bacillus licheniformis* strain APSAC 04 was deposited in the NCBI Gen bank with the following accession number KY886136 for further retrieval and comparison.

**KEYWORDS:** Gut Bacteria, Phosphate Solubilization, *Bacillus Licheniformis* & 16S rRNA Sequencing

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### INTRODUCTION

The actions of earthworms during vermin composting include not only digestion and release of easily assimilable substances, such as mucus for microbiota (Brown and Doube, 2004) but also the transport and dispersal of microorganisms through casting.

Bacterial diversity in earthworm guts can be quite high, several hundred bacterial strains have been isolated from earthworm guts. Gut bacteria are also functionally diverse, digesting a wide range of compounds including amino acids, sugars, cellulose, chitin, lignin, starch, polylactic acids (Makulec, 2002).

Phosphate-solubilizing microorganisms (PSM) may play an important role in developing sustainable phosphate fertilizer systems (SubbaRao, 1982 and Kucey et al., 1989). The increase in number and diversity of microorganisms and their interaction lead to increase in number and diversity of effective organic acids through the solubility process of insoluble phosphorous (Arpana et al., 2002).

Phosphorous exists in nature in a variety of organic and inorganic forms, primarily in either insoluble or very poorly soluble inorganic forms (Nasreen and Shailesh, 2005). Soluble forms of phosphate fertilizers applied to the soil are easily precipitated as insoluble forms. Phosphate solubilizing microorganisms solubilize insoluble P by producing various organic acids. Plants take up this available P. Hence this study is aimed to screen an efficient phosphate solubilizing bacteria from the earthworm gut and seaweed vermicompost.

## MATERIALS AND METHODS

### Isolation of Phosphate Solubilizing Microbes

Seaweed vermicompost and gut content of *Perionyxexcavatus* were the sources for isolation of microorganisms for screening phosphate solubilizing activity. Vermicompost and the gut contents were serially diluted on sterile tubes from  $10^{-1}$  to  $10^{-6}$  and plated on standard agar medium (Pikovskaya's medium) which containing 250mg of tricalcium phosphate (TCP) as a sole source of phosphate which selectively for screening the organisms, which have the ability to release inorganic phosphate from TCP. The inoculated plates were incubated at 30°C, after 3 days the different colonies were enumerated. Then, the pure cultures were subcultured on standard agar medium which was selected for preliminary screening (Sundararao and Sinha, 1963). After 3 days of incubation, phosphate solubilizing microorganisms were selected by the appearance of clear zones around the colonies. The selected phosphate solubilizing microorganisms were identified based on standard procedure.

### Measurement of Phosphate Solubilization, Biomass and pH

Phosphate solubilizing microbes were inoculated into 100ml Pikovskaya's broth containing 250mg of TCP. In the total 15 days of incubation, once in 3 days pH of the medium was noted with a pH meter equipped with Glass electrode, microbial biomass was measured by using methods and amount of soluble phosphate was measured by chloromolybdate method (Jackson, 1973 and Sujatha *et al.*, 2004). To the supernatant of the culture medium 10ml of chloromolybdic acid, reagent was added along the sides of the flask. The contents were diluted with 40ml distilled water and 5 drops of chlorostannous acid reagent were added and mixed gently. The appearance of blue colors was read at 600nm and the OD was recorded. The unknown samples also detected using the same procedure.

### DNA Sequencing

#### Extraction of Genomic DNA

Biochemically identified promising bacterial strains were grown in 100ml nutrient broth for 24h at  $28 \pm 2^\circ\text{C}$  and then centrifuged at 10,000 rpm for 10 minutes at  $4^\circ\text{C}$ . The pellets obtained were then individually resuspended in TE buffer. Then lysozyme was added to a final concentration of  $8 \text{ mg ml}^{-1}$  and incubated for 1hour at  $37^\circ\text{C}$ . To which,  $100\mu\text{l}$  of 0.5M EDTA (pH 8.0),  $60\mu\text{l}$  of 10% SDS and  $3\mu\text{l}$  of proteinase-K ( $20\mu\text{g ml}^{-1}$ ) were added and incubated at  $55^\circ\text{C}$  overnight. The supernatant was extracted twice with phenol: chloroform (1:1) and once with chloroform: isoamyl alcohol (24:1) and precipitated with ethanol. Finally, the genomic DNA was resuspended in sterile distilled water for further analysis (Sambrook *et al.*, 1989).

#### Amplification of 16S rRNA Gene

Bacterial 16S rRNA of promising bacterial strains was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: Forward primer 5' AGAGTTTGATCCTGGCTCAG 3' and Reverse primer 5' GGTTACCTTGTTACGACTT 3'. Polymerase chain reaction was performed in 50  $\mu\text{l}$  of reaction mixture containing 2  $\mu\text{l}$  (10 ng) of DNA as template, each primer at a concentration of  $0.5\mu\text{M}$ , 1.5 mM  $\text{MgCl}_2$ , and each deoxynucleoside triphosphate (DNTP) at a concentration of  $50\mu\text{M}$ , as well as 1U of *Taq* polymerase and buffer as recommended by the manufacturer (MBI Fermentas). After the initial denaturation for 3 minutes at  $95^\circ\text{C}$ , there were 40 cycles consisting of denaturation at  $95^\circ\text{C}$  for 1 minute followed by annealing at  $55^\circ\text{C}$  for 1 minute and extension at  $72^\circ\text{C}$  for 2 minutes and then a final extension of 5 minutes at  $72^\circ\text{C}$ . The amplification of 16S rRNA was performed using

Mastercycler Personal (Eppendorf, Germany) and the final PCR product was sequenced using the Genetic Analyzer (Applied biosystems, USA).

### Nucleotide Sequence Analysis

The sequence data were obtained and matched with previously published sequences available in NCBI using BLAST (Altschul *et al.*, 1997) and then the processed sequences were submitted to NCBI Gen Bank. The sequences of promising bacterial strains along with its closely related sequences were retrieved from the NCBI database and aligned through multiple sequences alignment programmes by using CLUSTAL-X software (Thompson *et al.*, 1997).

## RESULTS AND DISCUSSIONS

Microbial biomass in the worm casts was found to be high and their activity was essential for the release of nutrients into the medium so as to be taken by the plants (James, 1991) Enhanced nutrients (N, P, K, S, Ca, Mg, Mn, Fe, Zn) in the casts of earthworm, compared to the surrounding soil, was shown to be due to mineralization taking place in the gut as well as in the casts (Parthasarathi and Ranganathan, 1999).

The phosphate solubilizing microbial strains were isolated from earthworm gut and vermicompost of seaweed by serial dilution method. Five bacterial strains were isolated for primary screening to test the phosphate solubilizing activity. Among the bacteria, *B. licheniformis* produced a maximum zone of clearance (1.2 cm) with 400 percentage of solubilizing efficiency (Table 1). *Rhizobium* sp. and *Azospirillum* sp. were not able to produce solubilization zone around the culture. The zone of clearance was high due to the solubilization of insoluble phosphates by acidification associated with either proton extrusion or organic acid secretion (Bardiya and Gaur, 1974 and Darmalet *et al.*, 1989). The production of the clear zone around the colonies of the organism is an indication of phosphate solubilization. The efficiency was observed from the clear zone diameter which around their colonies in Pikovskaya's medium. Phosphate solubilizing activity could be due to a mechanism of organic acid production (Mehta and Nautiyal, 2001).

**Table 1: Screening of Bacteria for Tricalcium Phosphate Solubilization and Evaluation of Solubilization Efficiency (E) By Plate Assay Method**

Strain Name	Micro Organism	Colony Diameter (cm)	Zone of Clearance (cm)	Solubilization Efficiency(E) (%)
B1	<i>Pseudomonasfluorescens</i>	0.2	0.6	300
B3	<i>Rhizobium</i> sp.	0.4	-	-
B6	<i>Bacillus licheniformis</i>	0.3	1.2	400
B8	<i>Streptomyces</i> sp.	0.2	0.5	250
B10	<i>Azospirillum</i> sp.	0.3	-	-

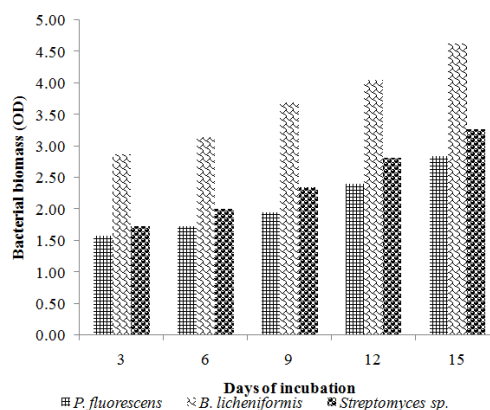
The selected three bacteria were tested for phosphate solubilizing activity in Pikovskaya's broth amended with 250mg of TCP. Phosphate solubilization efficiency of bacterial cultures was gradually increased from day 1 to day 15. The phosphate solubilization ability of the isolated microorganisms varied from 388.3 ±2.8 to 795.3 ±10.5 µg/ml. Maximum phosphate solubilization was obtained from the culture flask inoculated with *B. licheniformis*(795.3 ±10.5 µg/ml) followed by *P. fluorescens*(634.7±8.0 µg/ml) at 15<sup>th</sup> day of incubation is given in Table 2. The solubilization of phosphates in a liquid medium depends on the nature of the phosphate source, the organic incubation period and the nature

and quantity of the organic acids secreted into the medium (Sujatha *et al.*, 2004). The number of days taken for the maximum activity varied for various species. Fourteen days of incubation period was favorable for maximum phosphate solubilization by microorganisms in a liquid medium. Narsianet *et al.* (1995) observed that the phosphate solubilizing activity of *Aspergillus aculeatus* was the highest after 48 h of fungal growth and that the fungal biomass production was more in the case of tricalcium phosphate than other phosphate sources.

**Table 2: Quantitative Estimation of Tricalcium Phosphate Solubilization by Phosphate Solubilizing Bacteria**

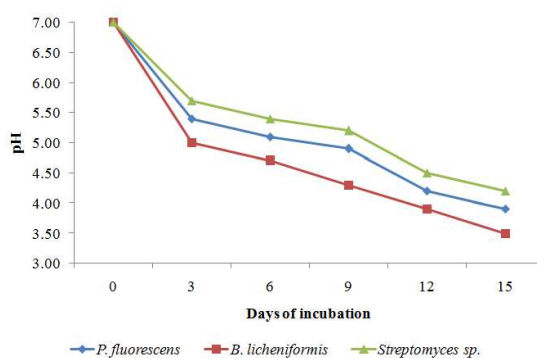
Strain Name	Bacterial Species	Phosphate Solubilizing Activity ( $\mu\text{g/ml}$ )				
		Day of Incubation				
		3	6	9	12	15
B1	<i>Pseudomonas fluorescens</i>	431.4 $\pm$ 3.5	468.2 $\pm$ 4.8	493.1 $\pm$ 5.3	584.7 $\pm$ 6.2	634.7 $\pm$ 8.0
B6	<i>Bacillus licheniformis</i>	573.6 $\pm$ 4.2	608.3 $\pm$ 6.8	659.4 $\pm$ 7.5	725.6 $\pm$ 9.0	795.3 $\pm$ 10.5
B8	<i>Streptomyces sp.</i>	388.3 $\pm$ 2.8	412.8 $\pm$ 3.7	486.7 $\pm$ 5.0	522.3 $\pm$ 5.4	561.0 $\pm$ 6.7

Microbial biomass produced from bacterial and fungal cultures in Pikovskaya's broth was observed at 3 days interval. With the increase in the number of incubation days the microbial biomass also increased. Among the bacterial cultures, maximum growth (Optical Density - OD) of 4.63 was achieved by *B. licheniformis* (Figure 1). Comparatively less growth was attained by *P. fluorescens*.



**Figure 1: Bacterial Biomass Production during the Phosphate Solubilizing Activity**

Changes in the medium pH were also observed during the incubation days and shown in Figure. 2. Among the bacterial cultures, great reduction of pH was observed in *B. licheniformis* (3.5) and least reduction was found in *Streptomyces sp.* (4.2) from the initial pH of 7.



**Figure 2: Measurement of pH during Phosphate Solubilization by Bacteria**

The fall in the pH during the initial days was due to the production of organic acids like citric, acetic, fumaric and malic in the growth. The mechanisms used by microorganisms for inorganic phosphate solubilization have been attributed mainly to acidification, chelation and exchange reactions in the growth environment (Molla and Chowdhry, 1984 and Cunningham and Kuyack, 1992).

In the present study solubilization efficiency of each microorganism varied with the reduction in pH of the medium during the incubation period. A correlation between final pH and soluble phosphorus level has been reported by Dave and Patel, (1999). There is no correlation between microbial biomass and phosphate solubilization efficiency was reported.

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GenBank: KY886137.1
FASTA Graphics
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DEFINITION  Bacilluslicheniformis strain APSAC 04 16S ribosomal RNA gene,
partial sequence.
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VERSION     KY886137.1
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ORGANISM    Bacillus licheniformis
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1 (bases 1 to 1511)
AUTHORS     Ananthavali,R., Ramadas,V., Arockia John Paul,J. and Siva
Kumar,P.
TITLE       Utilization of seaweed for vermicomposting and preparation of
consortium of effective microbial biofertilizer - link no.
MRP-5760/15 (SERO/UGC)
JOURNAL     Unpublished
REFERENCE   2 (bases 1 to 1511)
AUTHORS     Ananthavali,R., Ramadas,V., Arockia John Paul,J. and Siva
Kumar,P.
TITLE       Direct Submission
JOURNAL     Submitted (05-APR-2017) Department of Zoology, Arumugam Pillai
SeethaiAmmal College, Tiruppattur, Sivagangai, Tamil Nadu 630211,
India
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121  tgggataaatccgggaaacgggggttaataccggatgcttgattgaacgcgatgggtcaa
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301  ctgggactgagacacggcccgactcctacgggagggcagcagtagggaatcctccgcaat
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1501 cggaaaggtgcg
//

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Figure 3: Bacillus Licheniformis Strain APSAC 04 16S Ribosomal RNA Gene, Partial Sequence

Among the isolated bacterial strains, the strain B6 (*Bacillus licheniformis*) showed maximum phosphate solubilizing activity; besides, morphological, biochemical and physiological analysis the promising bacterial isolates B6 was further subjected to 16S rRNA analysis for identification. The sequence obtained and submitted to GenBank of NCBI is given in Figure 3. The candidate strain B6 showed highest sequence similarity values (99%) with *Bacillus licheniformis* strain DSM 13 (Accession number – NR118996.1) in the NCBI database. The 16S rRNA sequence of B6 was then deposited in the NCBI Gen bank data under the name *Bacillus licheniformis* strain APSAC 04 with the following accession number (KY886137).

## CONCLUSIONS

An effective phosphate solubilizing bacteria, *B. licheniformis* has been isolated based on the enzyme activity and maximum utilization of tricalcium phosphate. Further confirmation was also done through 16S rRNA sequencing and submitted to the NCBI Gen bank for easy retrieval. Further, this bacterial strain may be utilized for the preparation of biofertilizer with an added advantage of phosphate solubilization.

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