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Research Article

PHOSPHATE SOLUBILIZING ACTIVITY OF SOME BACTERIAL STRAINS ISOLATED FROM JUTE MILL EFFLUENT EXPOSED WATER OF RIVER GANGA

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ABSTRACT
Phosphate solubilizing bacteria (PSB) are known to be able to solubilize different forms of inorganic phosphates. A total of twenty four phosphate solublizing bacterial colonies were isolated on the Pikovskaya's (PKV) agar medium, containing insoluble tri-calcium phosphate (TCP), from the jute mill effluent exposed area of river Ganga at Bansberia, West Bengal, India.. The colonies showing clear halo zones around the bacterial growth were considered as phosphate solubilizers. Out of 24 bacterial isolates, 5 isolates showing highest phosphate solubilisation index (SI) ranged from 2.50-3.14 were selected for the further study as qualitative as well as quantitative estimation of phosphate. Among these 5 potent isolates, *Bacillus* sp JPSB16 showed the maximum phosphate solubilization index of 3.14 in Pikovskaya's agar plates along with highest soluble phosphate production of 203.64 µg P ml^{-1} in broth culture. Isolates JPSB15, JPSB17, JPSB18 and JPSB19 belong to genera *Pseudomonas* sp, *Azotobacter* sp, *Flavobacterium* sp and *Bacillus* sp as identified by their morphological, physiological and biochemical characteristics respectively. In all the phosphate solubilizing bacterial isolates, decrease in pH was observed in liquid medium ranging from 3.53 to 4.23 from initial pH of 7.0±0.2. The decrease in pH of the culture medium indicated the production of various organic acids by the culture.

Key Words: *Phosphate Solubilizing Bacteria, Jute Mill Effluent, River Ganga*

INTRODUCTION
Phosphorus is known as one of the major nutrients required by living organisms involved in major physiological processes. It is well recognized that the majority of phosphates in the sediments are present as insoluble organic and inorganic forms. The phosphate concentration in water depends upon various factors, of which the bottom deposits play a significant role (Promod and Dhevendaran, 1987). Previous studies have indicated the mechanisms of phosphate exchange between the sediments and water, suggesting that sediments act as buffer on the concentration of phosphate in the overlying water column (Carritt and Goodgal, 1954; Gessner, 1960; Pomeroy *et al.,* 1965; Promod and Dhevendaran, 1987). Therefore, the major part of available phosphate is locked up as insoluble inorganic and organic phosphorous compounds in the sediments.
Microorganisms play an important role for transformation of phosphorous in water and sediments and the phosphate ions are reported to be strongly adsorbed by sediments with a high content of silt and clay (Seshadri *et al.,* 2002). Bacteria are the predominant microorganisms that can solubilize phosphate compared to the fungi and actinomycetes (Yin, 1988). There are some species of bacteria which have potential to mineralize and solubilize organic and inorganic phosphorus (Khiari and Parent, 2005). Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez and Fraga, 1999).
It is generally accepted that the mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; Kim *et al.,* 1997), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabai, 1994). However, phosphorus solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Reyes *et al.,* 1999).
A few reports are available on the occurrence and distribution of phosphate solubilizing microbes in the marine environment (Ayyakkannu and Chandramohan, 1971; Venkateswaram and Natarajan,

1983; De Souza *et al.*, 2000; Seshadri *et al.*, 2002). Till now, information on phosphorous solubilizing bacteria of the riverine environment is scanty (Paul and Sinha, 2013). Therefore, the present experiment was designed to study the phosphate solubilizing bacteria from jute mill effluent exposed water of the river Ganga and to identify the potential ones using conventional methods of identification and the potential PSBs solubilisation index and phosphate solubilization were studied *in vitro*.

MATERIALS AND METHODS

Sampling Site

Water samples were collected from the jute mill effluent exposed area of river Ganga at Bansberia (22°58'17"N and 88°24'04"E), West Bengal, India (Figure 1). Water samples collected in sterilized McCartney bottles were transported to the laboratory in an icebox immediately after collection for further studies.

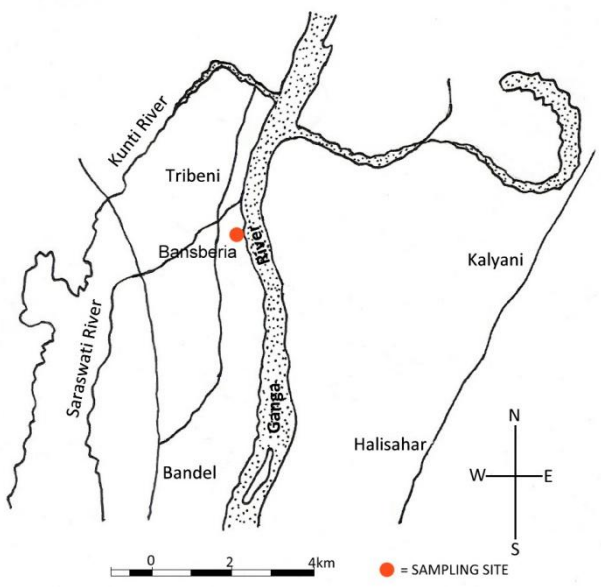

Figure 1: Map of the river Ganga showing sampling site

Isolation of Phosphate Solubilizing Bacteria (PSB)

Water and sediment samples were aseptically transferred to the laboratory and serially diluted water samples were plated on Petridishes containing Pikovskaya's (PKV) agar medium consisting of ingredients in g/l: Glucose 10g; tri-calcium phosphate (TCP) 5g; ammonium sulphate 0.5g; sodium chloride 0.2g; potassium chloride 0.2g; magnesium sulphate 0.1g; yeast extract 0.5g; manganese sulphate trace; ferrous sulphate trace; agar 15 g; the pH was adjusted to 7.0 ± 0.2 before sterilization (Pikovskaya, 1948) by pour plate technique and incubated at 28 ± 2°C for 48-96h. The bacterial colonies showing clear zone around them were considered as phosphate solubilising bacteria (PSB) (De Freitas *et al*, 1997). Pure culture of the isolates were made by repeated subculturing for 2-3 times on fresh PKV plate and were maintained on PKV slants at refrigerator temperature. A total of 24 phosphate solubilizing bacterial colonies were isolated.

Identification of Bacterial Strains

Identification of phosphate solubilizing bacterial strains was performed by physiological, morphological characteristics and biochemical analysis comparing with standard references (Aneja, 2002). Physiological, morphological and biochemical tests of the PSB isolates were carried out for their identification as per the procedures outlined in Bergey's Manual of Systemic Bacteriology as in (Krieg and Holt, 1984; Sneath *et al.*, 1986).

Qualitative Estimation of Phosphate Solubilisation

Out of 24 bacterial isolates, 5 isolates having larger halo zones were selected for further study. The qualitative analysis of phosphate solubilizing activity of the selected isolates was conducted by plate

screening method. Bacterial isolates were screened for their tri-calcium phosphate (TCP) solubilizing activity on PKV plates. Isolates were spot inoculated on the centre of agar plate aseptically. All the plates were incubated at 28 ± 2°C for 3 days. A clear zone around a growing colony indicated phosphate solubilisation and was measured as phosphate solubilisation index (SI). Phosphate solubilization index was determined by the following formula (Edi-Premono, 1996),

$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}}$$

All the observations were recorded in triplicate. Strains developing clear zones around their colonies could easily be identified as PSBs (Gyaneshwar *et al.*, 1999; Sundara-Rao and Sinha, 1963).

Quantitative Estimation of Phosphate Solubilization

The bacteria, found to be positive for TCP solubilization were further analyzed for their ability to solubilize it in liquid medium. Bacterial isolates were inoculated in Pikovskaya's broth (100 mL) in 250 mL of Erlenmeyer flasks and incubated at 28 ± 2°C for 5 days in rotary shaker at 200 rpm. Triplicates were maintained for each treatment. After incubation the bacterial cultures were filtered through Whatman No.1 filter paper and were clarified by centrifugation at 10,000 rpm for 10 minutes. Uninoculated broth served as control. After that pH of the filtrate was recorded with a digital pH meter (Jenway 3510) and amount of soluble phosphate was measured by Molybdenum blue method (Strickland and Parsons 1972). Potassium di-hydrogen phosphate was used as standard. The intensity of blue colour was measured by uv-vis spectrophotometer (CECIL CE7200) at 690 nm.

RESULTS AND DISCUSSION

In the present investigation, the collected water samples were evaluated *in vitro* for phosphate solubilising bacteria in Pikovskaya's (PKV) agar plates. Initially, 24 isolates were isolated on the basis of halo zone around their colonies on PKV plates. Out of 24 bacterial isolates, 5 isolates showed higher phosphate solubilisation index ranged from 2.50-3.14 were selected for the further studies. These isolates were further characterized, by a series of biochemical reactions and identified as genus *Pseudomonas* sp, *Bacillus* sp, *Azotobacter* sp and Flavobacterium sp (Table 1). These bacteria were well known identified as phosphate solubilizer by many researchers (Rodriguez and Fraga, 1999; Kumar *et al.*, 2001).

All the selected isolates were found to be potent phosphate solubilizers showing clear halo zone around their colonies. Zone of solubilization around the bacterial colony on PKV agar plates after 3 days of incubation at temperature 28±2°C ranged from 11 to 15 mm, the size of the bacterial colony varied from 6 to 8 mm. Among these 5 potent isolates, strains JPSB16 showed the maximum phosphate solubilization activity as visualized by the size of halo zone developed around the colony, which showed solubilization index of 3.14 followed by JPSB19 (2.83) (Figure 2). The zone formation could be due to the activity of phosphatase enzyme in bacterial isolates (Goldstein, 1995).

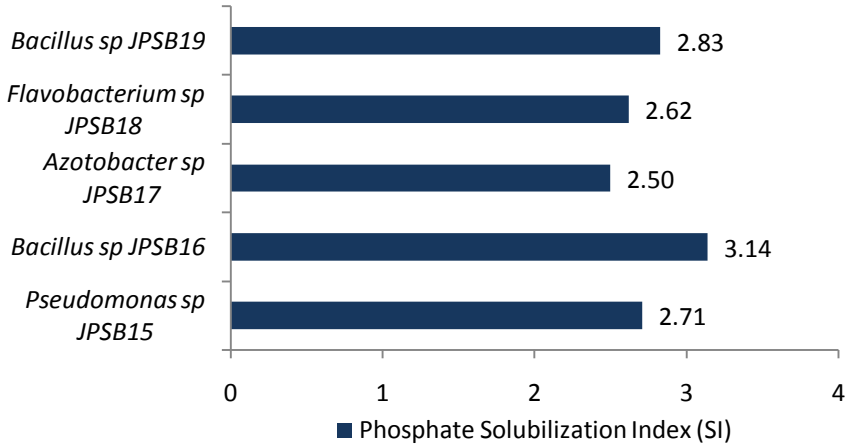

Figure 2: Phosphate solubilising index (SI) of the PSB isolates

Table 1: Morphological and Biochemical characteristics of the PSB isolates

Test	PSB isolates				
	***Pseudomonas* sp JPSB15**	***Bacillus* sp JPSB16**	***Azotobacter* sp JPSB 17**	***Flavobacterium* sp JPSB18**	***Bacillus* sp JPSB19**
Cell shape	Rod	Rod	Rod	Rod	Rod
Gram reaction	-	+	-	-	+
Motility	+	+	+	+	+
Growth at 5% NaCl	+	+	-	-	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
IMViC test					
Indole	-	-	+	-	-
Methyl red	-	-	+	-	-
Voges-Proskauer	-	+	+	-	+
Citrate	+	+	+	-	+
Urease	-	-	+	-	-
H_2S production	-	-	-	+	-
NO_3^- reduction	-	-	+	+	-
Gelatine liquefaction	+	+	-	+	+
Starch hydrolysis	-	+	+	-	+
Hugh-Leiffson (O/F) reaction	O/F	F	F	-	F
Utilization of carbon source					
Glucose	+	+	+	+	+
Fructose	+	+	+	-	+
Sucrose	+	+	+	-	+
Lactose	+	+	-	-	-
Raffinose	-	+	+	-	+
Cellobiose	-	+	-	-	-
Xylose	+	-	+	-	-
Mannitol	-	+	+	-	+
Sorbitol	-	+	+	-	+

+ indicates presence or positive reaction; - indicates absence or negative reaction;
O = Oxidation; F= Fermentation

The solubilization levels of TCP varied with deferent isolates, all the 5 isolates were capable of solubilizing tri-calcium phosphate (TCP) in broth medium containing 0.5% of TCP. The soluble-P concentration in the medium ranged between 150.46 to 203.64 µg P ml^{-1} with variations among different isolates. PSB strain JPSB16 (*Bacillus* sp.) produced highest soluble phosphate of 203.64 µg ml^{-1} followed by JPSB19 (*Bacillus* sp.) which produce 187.78 µg ml^{-1} of soluble phosphate in the PKV broth (Table 2).

The solubilization of $Ca_3(PO_4)_2$ in the liquid medium by different strains was accompanied by a significant drop in pH (3.53 to 4.23) from an initial pH of 7.0±0.2 after 120 h were recorded (Figure 3). Maximum drop in pH was associated with higher levels of P-solubilization, *Bacillus* sp JPSB16 where pH was decreased to 3.53 from initial pH 7.0±0.2. The decrease in pH clearly indicates the production of organic acid and phosphatase, which is considered to be responsible for phosphate solubilization activity (Vassilev *et al.,* 2006; Perez *et al*., (2007) suggested that acidification of culture supernatants can be the main mechanism for P solubilization.

Table 2: Phosphate solubilising activities of PSB isolates

Isolates	Phosphate solubilization		
	Phosphate solubilization zone (mm)	Soluble phosphate concentration (µg P ml^{-1})	Incubation period (h)
JPSB15	12	172.32	120
JPSB16	15	203.64	120
JPSB17	12	150.46	120
JPSB18	13	158.16	120
JPSB19	11	187.78	120

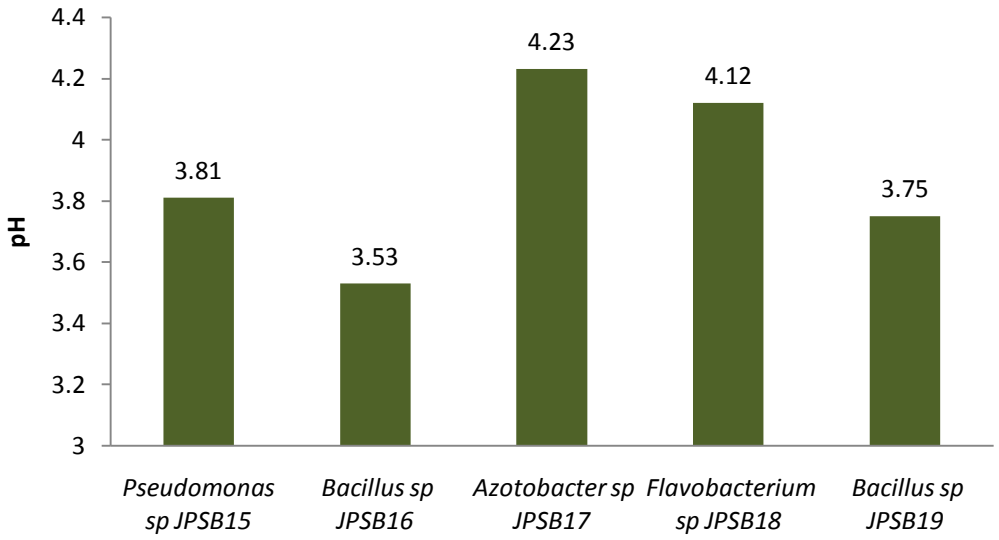

Figure 3: Change of pH of the liquid medium after 120h incubation

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