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Original Article

Isolation and characterization of a phosphate solubilizing heavy metal tolerant bacterium from River Ganga, West Bengal, India

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Abstract

Phosphates solubilizing bacterial (PSB) strains were isolated from the jute mill effluent discharge area of the Ganga river water at Bansberia, West Bengal, India. Experimental studies found that the strain KUPSB16 was effective in solubilization of phosphate with phosphate solubilization index (SI) = 3.14 in Pikovskaya's agar plates along with maximum solubilized phosphate production of 208.18 µg mL^{-1} in broth culture. Highest drop in pH value was associated with maximum amount of phosphate solubilization by the PSB strain KUPSB16 where pH decreased to 3.53 from initial value of 7.0±0.2. The isolated PSB strains were tested for tolerance against four heavy metals such as cadmium (Cd), chromium (Cr), lead (Pb) and zinc (Zn) at concentrations 1-15 mM. The results showed that most of the PSB isolates grew well at low concentrations of heavy metals and their number gradually decreased as the concentration increased. Isolated PSB strain KUPSB16 was tested for its multiple metal resistances. Minimal inhibitory concentrations (MIC) for Cd^{2+}, Cr^{6+}, Pb^{2+} and Zn^{2+} in tris-minimal broth medium were 4.2, 5.5, 3.6 and 9.5 mM respectively. The MIC values for the metals studied on agar medium was higher than in broth medium and ranged from 4.8-11.0 mM. The isolated bacterial strain KUPSB16 was subjected to morphological, physiological and biochemical characterization and identified as the species of the genus *Bacillus*. The phosphate solubilizing bacterium possessing the properties of multiple heavy metal tolerance in heavy metal contaminated areas might be exploited for bioremediation studies in future.

Keywords: phosphate solubilizing bacteria, heavy metal, River Ganga

1. Introduction

Pollution of the biosphere by different pollutants is a global threat that has increased dramatically since the beginning of industrial revolution. Among the pollutants toxic metals are of serious concern, because they accumulate through the food chain and create environmental problems (Praveena *et al.*, 2010). Higher concentrations of heavy metals can form harmful complex compounds, which critically effect different biological functions (Rajbanshi, 2008). Resistance to heavy metals, disinfectants, detergents, antibiotics, and other toxic materials were noted in a wide group of bacteria, particularly in the genus *Bacillus* (Velusamy *et al.*, 2011). Microbial survival in high concentrations of heavy metals depends on intrinsic structural and biochemical characteristics, physiological, and/or genetic adaptation (Ehrlich, 1997). Microorganisms exert various kinds of tolerance mechanisms against heavy metals encoded by chromosomal genes or plasmids (Guo and Mahillon, 2013; Mallick *et al.*, 2014). Generally, they conduct various resistance mechanisms to deal with heavy metal toxicity, such as extracellular precipitation and exclusion, binding to the cell surface, intracellular sequestration and volatilization (Silver, 1992; Vijayaraghavan and Yun, 2008).

Phosphate solubilizing bacteria (PSB) are capable of solubilizing different forms of inorganic phosphates by acidi-

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fication, chelation and exchange reactions in the periplasm, which function as an indicator for general isolation and selection procedure of phosphate solubilizers (Illmer and Schinner, 1992). Various domains of phosphate-solubilizing microorganisms, generally bacteria and fungi, have been reported to solubilize inorganic phosphate compounds to soluble (Sharma *et al.*, 2013). Bacteria belonging to the genera *Bacillus, Pseudomonas, Enterobacter, Erwinia Azotobacter, Rhizobium, Mesorhizobium*, *Acinetobacter, Flavobacterium, Klebsiella,* and *Micrococcus* have been reported as efficient phosphate solubilizers (Villegas and Fortin, 2002; Paul and Sinha, 2013a).

The heavy metals are generally stable, persistent and are not easily degradable biologically to more or less toxic products and hence, persist in the environment (Chairgulprasert *et al.*, 2013). For heavy metal bioremediation over other conventional physicochemical processes, biological processes are found to be cost-effective, eco-friendly methods for long time (Congeevaram *et al.*, 2007). However, microbes can transform a wide variety of multivalent metals which pose major threat to the environment. Heavy metal pollution for last few decades poses a great threat in various aquatic environments particularly in riverine ecosystem. Previous study reported that most of the water samples of river Ganga in lower stretch were found polluted in terms of heavy metals contamination such as cadmium, chromium, zinc, and lead (Paul and Sinha, 2013b). The higher concentration of heavy metal load in the river surface water can be attributed to the discharge of industrial effluents to the river water. Phosphate solubilizing bacteria are able to solubilize phosphorus from insoluble phosphate sources through the release of organic acids. Such phosphate compounds generally have been used for immobilization of heavy metals in contaminated environments. Remediation of metal-polluted sites using biological systems such as microorganisms is an emerging area of interest and has shown a substantial progress *in situ*. The application of bacteria specifically adapted to high heavy metals concentrated area increase the ability to remediate heavy metal contaminated sites. Hence, the present study focuses to isolate and characterize phosphate solubilizing bacterial strain from jute mill effluent discharge area of the Ganga river water containing heavy metals such as cadmium, chromium, zinc and lead which could tolerate high concentration of different heavy metals.

2. Materials and Methods

2.1 Sampling and isolation of phosphate solubilizing bacteria

Collection of water samples were made from the jute mill effluent discharge area Ganga river water at Bansberia (22°58′17″N and 88°24′04″E), West Bengal, India (Figure 1). Five water samples at different point of this area were collected in sterilized McCartney bottles (capacity 28 ml) and transported immediately to the laboratory in an ice bucket after collection for future studies. The sample was serially diluted, plated on Pikovskaya's (PKV) agar media consisting of ingredients in g/l: glucose 10 g; ammonium sulfate 0.5 g; tri-calcium phosphate (TCP) 5 g; yeast extract 0.5 g; sodium chloride 0.2 g; potassium chloride 0.2 g; magnesium sulfate 0.1 g; manganese sulfate trace; ferrous sulfate trace; agar agar 15 g; the pH was adjusted to 7.0±0.2 (Pikovskaya, 1948) and plates were incubated at 28±2°C for 48-96 h. Colonies of bacteria showing clear zone around them in PKV agar were considered as phosphate solubilizing bacteria (De Freitas *et al.*, 1997). Repeated sub-culturing for 2-3 times on fresh PKV agar plate were done to obtain pure culture, which were maintained on PKV agar slants at refrigerator temperature.

2.2 Qualitative estimation of phosphate solubilization

The qualitative analyses of phosphate solubilizing activity of the selected PSB isolates were estimated by plate screening method. To measure the ability of the bacteria to solubilize insoluble phosphate, the bacterium was spot inoculated on the center of Pikovskaya's agar plate aseptically. Incubation was done at 28±2°C and was analyzed for the zone of clearance up to five days. A clear zone surrounding a growing colony indicated phosphate solubilization and was measured as phosphate solubilization index (SI). Phosphate solubilization index (SI) was determined by formula (Edi-Premono *et al.*, 1996) given below:

Solubilization Index (SI) =
(Colony diameter + Halo zone diameter) / Colony diameter.

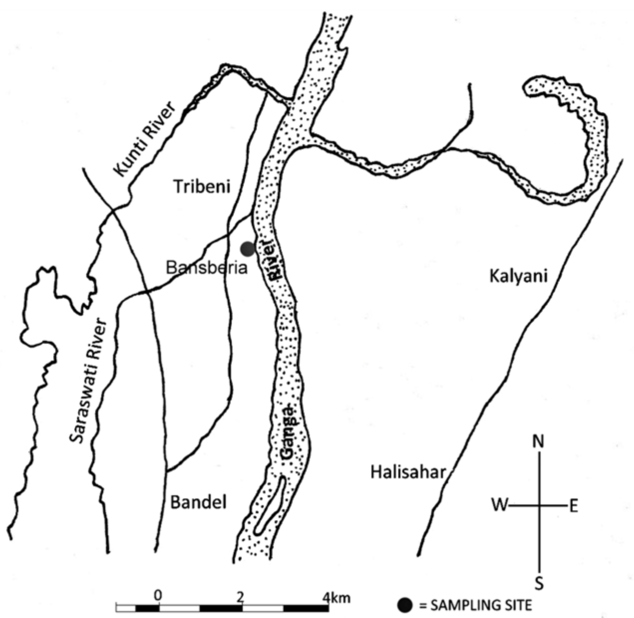

Figure 1. Map of the river Ganga showing sampling site.

2.3 Quantitative estimation of phosphate solubilization

The PSB isolates, positive for TCP solubilization, were further analyzed in liquid medium for their ability to solubilize. Hundred milliliter of Pikovskaya's broth contained in 250 mL of Erlenmeyer flasks were inoculated with bacterial isolates of 1% (v/v) ($1x10^6$ cfu mL^{-1}) and incubation was done for five days at 28±2°C in a rotary shaker incubator at 200 rpm. Each treatment was done in triplicates. After incubation the bacterial cultures were clarified after incubation by centrifugation for 10 minutes at 10,000 rpm. Uninoculated broth was used as control. The pH of the filtrate was recorded with a digital pH meter (model: Jenway 3510) and amount of soluble phosphate was determined by Molybdenum blue method (Watanabe and Olsen, 1965).

2.4 Screening for heavy metals tolerance

Tolerance to heavy metals was measured through agar dilution method (Sabry *et al.*, 1997). The plates contained 20 ml of tris-minimal medium along with different concentrations (1-15 mM) of heavy metals (cadmium, chromium, lead and zinc) studied. The plates were inoculated with cultures and incubated at 37°C for 48 hrs.

2.5 Determination of the minimal inhibitory concentration

The lowest concentration of heavy metals that cause total inhibition of growth is known as minimal inhibitory concentration (MIC) (Yilmaz, 2003). MICs for different heavy metals were determined on agar plates containing tris-minimal medium using agar dilution methods and were confirmed in broth medium. Five ml of tris-minimal medium containing different concentration of heavy metals salts $Cd(NO_3)_2$, $2H_2O$; $K_2Cr_2O_7$; $Pb(NO_3)_2$ and $(CHCOO)_2Zn$, $2H_2O$ was prepared for broth medium and inoculated with 200 µl (OD_{600}=0.5) of an 24 hr-old culture of the studied PSB strain at 37°C for 48 hrs.

2.6 Identification of bacterial strain

Morphological, physiological and biochemical tests of the selected PSB strains were conducted by the methods outlined in Bergey's Manual of Determinative Bacteriology along with Gram and endospore staining properties for their identification (Holt *et al.*, 1994).

2.7 Statistical analyses

All experiments were performed in triplicate, and the results were expressed as the mean. Means and standard deviations (SD) were analyzed by using the Origin 8.5 data analysis and graphing software.

3. Results and Discussion

In the present study, the collected water samples were studied *in vitro* for isolation of PSB in Pikovskaya's (PKV) agar plates. The PSB isolates were found to be potent phosphate solubilizers indicating clear halo zone around theirs colonies. Twelve PSB isolates were screened from five water samples collected. So based on collected samples the percentage of PSB isolates was 240%. Further, near about 42% (5 out of 12 PSB) isolates were potent phosphate solubilizers on the basis of halo zones around their colonies on PKV plates. Among those five potent PSB isolates, strain KUPSB16 showed the maximum phosphate solubilizing halo zone (15.00 mm) (Table 1). The solubilization index (SI) of the isolated strain KUPSB16 was calculated at the end of the incubation period. The strain KUPSB16 with higher phosphate solubilization index (SI=3.14) was also tested for phosphate solubilization in broth culture medium. The halo zone formation around the bacterial colonies could be due to the production of organic acids by the PSB isolates. It appears that numerous strains of bacteria which produce organic acid from sugar metabolism are capable of dissolving TCP (Trivedi and Sa, 2008).

The phosphate solubilizing efficiency of isolated PSB strains in Pikovskaya's broth indicated that the strains solubilized inorganic phosphate efficiently in the medium containing 0.5% tri-calcium phosphate (TCP) (Table 2). Phosphate solubilizing bacterial isolate, KUPSB16 was produced 208.18 µg mL^{-1} soluble phosphate in the PKV broth after 96 hrs of incubation period. Narveer *et al.* (2014) found that maximum phosphate solubilization efficiency of the isolated strain *Bacillus* sp. NPSBS 3.2.2 was after 96 hrs of incubation. However some other authors have reported three days, more than ten days, and even up to 15 days to be the optimum

Table 1. Qualitative estimation of phosphate solubilization efficiency of PSB isolates.

PSB isolates	Colony diameter (mm)	Halo zone diameter (mm)	Solubilization Index (SI)
KUPSB15	8.00	12.00	2.50±0.043
KUPSB16	7.00	15.00	3.14±0.017
KUPSB17	7.00	12.00	2.71±0.030
KUPSB18	8.00	13.00	2.62±0.026
KUPSB19	6.00	11.00	2.83±0.026

Table 2. Quantitative estimation of phosphate solubilization efficiency of isolates.

PSB isolates	Incubation period (hrs)	P-solubilization (μg mL^{-1})	pH after incubation
KUPSB15	96	185.76±1.521	5.26±0.051
KUPSB16	96	208.18±1.959	3.53±0.045
KUPSB17	96	190.68±1.990	4.84±0.030
KUPSB18	96	188.56±1.279	5.04±0.052
KUPSB19	96	190.92±0.930	4.74±0.026

incubation period for phosphate solubilization by various bacterial isolates (Sridevi and Mallaiah, 2009; Banerjee *et al.*, 2010).

In the liquid the solubilization of $Ca_3(PO_4)_2$ medium by phosphate solubilizing bacterial strain KUPSB16 was accompanied by a significant fall in pH from an initial pH of 7.0±0.2 after 96 hrs were recorded (Table 2). The maximum drop in pH value was correlated with elevated levels of phosphate solubilization, PSB strain KUPSB16 where pH was declined to 3.53 from initial pH. Tripti *et al.* (2012) also reported that in case of *Bacillus* sp, the maximum drop of pH was recorded at pH 3.5. The decline in pH might indicate the production of organic acid, which suggested that acidification of culture supernatants might be the principal mechanism for phosphate solubilization (Chen *et al.*, 2006; Sharma *et al.*, 2013).

The potential five isolates of PSB strains were tested for tolerance against four heavy metals such as cadmium (Cd), chromium (Cr), lead (Pb) and zinc (Zn) at concentrations 1-15 mM. The numbers and percentage of the tolerance of the isolated PSB strains at different concentrations of heavy metals are shown in Table 3. The results showed that most of the PSB isolates grew well at low concentrations of heavy metals and their number gradually decreased as the concentration increased. For different heavy metals studied, most of the PSB isolates able to grow at concentration 2 mM, except in the case of lead (1 mM) and zinc (3 mM).

On contrast all of the isolates tolerate up to 2 mM of cadmium and chromium, and drastically decreased by 60% at 3 mM, reached null above 6 mM and 7 mM respectively. In other wise lead is consider the most toxic heavy metals since 80% of the isolates were inhibited by 4 mM and since there no isolate can grow above this concentration. In case of zinc all of the isolates tolerate up to 3 mM of zinc, and reached null above 11 mM. Among the five PSB isolates, KUPSB16 showed high tolerance to the cadmium (Cd), chromium (Cr), lead (Pb) and zinc (Zn) and it tolerates 5 mM, 6 mM, 4 mM, and 11 mM Cd^{2+}, Cr^{6+}, Pb^{2+} and Zn^{2+} respec-

Table 3. Numbers of heavy metal tolerant PSB isolates at different concentrations of tested heavy metals.

Heavy metals concentration (mM)	Heavy metals			
	Cadmium (Cd)	Chromium (Cr)	Lead (Pb)	Zinc (Zn)
1.00	5 (100)	5 (100)	5 (100)	5 (100)
2.00	5 (100)	5 (100)	4 (80)	5 (100)
3.00	3 (60)	3 (60)	2 (40)	5 (100)
4.00	1 (20)	2 (40)	1 (20)	4 (80)
5.00	1 (20)	1 (20)	-	4 (80)
6.00	-	1 (20)	-	3 (60)
7.00	-	-	-	3 (60)
8.00	-	-	-	2 (40)
9.00	-	-	-	1 (20)
10.00	-	-	-	1 (20)
11.00	-	-	-	1 (20)
12.00	-	-	-	-
13.00	-	-	-	-
14.00	-	-	-	-
15.00	-	-	-	-

Values in parenthesis indicated % of heavy metal tolerant isolates tively. The order of toxicity of heavy metals towards the isolates of KUPSB16 are $Pb^{2+} > Cd^{2+} > Cr^{6+} > Zn^{2+}$. The ability to grow at high metal concentrations is found in many bacterial species and may be the result of induced or intrinsic mechanisms, as well as of other environmental factors (Zouboulis *et al.*, 2004; Guo *et al.*, 2010).

The minimal inhibitory concentration (MIC) of the isolated phosphate solubilizing bacterial strain KUPSB16 to heavy metals were carried out in tris-minimal agar and on broth medium were shown in Figure 2. The values MIC in agar medium were 5.0, 6.8, 4.8 and 11.0 mM for Cd^{2+}, Cr^{6+}, Pb^{2+} and Zn^{2+} respectively. On the other hand the MIC value in broth medium were 4.2, 5.5, 3.6, 9.5 mM for Cd^{2+}, Cr^{3+}, Pb^{2+} and Zn^{2+} respectively. The MIC values on agar medium for the metals studied was found to be greater than in broth medium and ranged from 4.8-11.0 mM on agar medium, while in the broth from 3.6-9.5 mM.

The isolated bacterial strain KUPSB16 was morphologically, physiologically and biochemically characterized. The PSB strain was found to be Gram positive, rod shaped, and motile, which demonstrates physiological properties primarily indicative of the genus *Bacillus* (Table 4). Based on these characters, the bacterial isolates were tentatively identified as *Bacillus subtilis*. This bacterium was well known recognized as phosphate solubilizer by many authors (Swain *et al.*, 2012; Tallapragada and Seshachala, 2012). Chihomvu *et al.* (2014) also isolated multiple heavy metal tolerant *Bacillus subtilis* from heavy metal contaminated water of Klip river of South Africa.

Heavy metal resistant microorganisms play a significance role in heavy metal contaminated environments for bioremediation (Sinha *et al.*, 2011; Sinha and Paul, 2014). The variation in the heavy metal toxicity toward the bacterial isolates might be explained by the conditions of isolating bacteria and the nature as well as physiological characteristics of bacterial isolates (Hassan *et al.*, 2008; Wei and Wee, 2011). Evaluation of the potential influence of the various abiotic factors upon the heavy metal pollutant and the impact of the heavy metal pollutant under such conditions upon microorganisms would be useful for clear understanding of the interaction of heavy metals on biogeochemical cycles in aquatic environment. Phosphate solubilizing bacterial strains generally remediate heavy metal contaminated environment largely through facilitating phytostabilization (by transforming metal species into immobile forms to decrease toxicity of metals). In this backdrop, PSB are found to be very promising agents due to their solubilization of insoluble and biologically unavailable metals such as Zn by secreting low molecular weight organic acids (He *et al.*, 2013). Several organic acids such as citric, formic acid, glycolic, 2-ketogluconic, lactic, malic, malonic, oxalic, piscidic, succinic, tartaric and valeric have been identified, which possess chelating properties (Panhwar *et al.*, 2013). In general, these acids are capable of chelating cations (mainly Ca^{2+}) bound to phosphate through their carboxyl and hydroxyl groups or solubilize them by the protons liberation, thus transforming

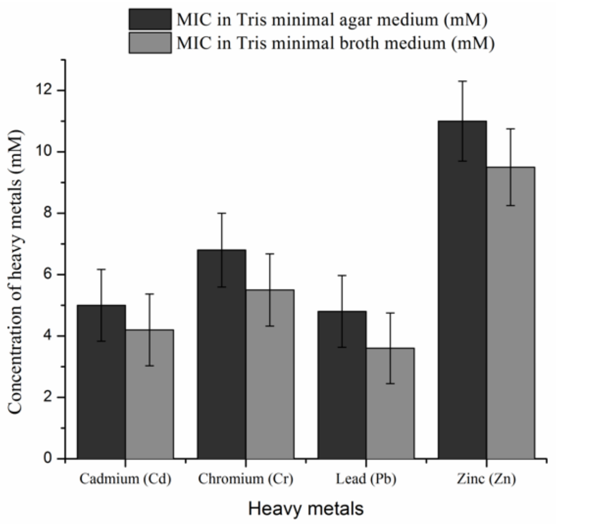

Figure 2. Minimum inhibitory concentrations (MIC) for different heavy metals of KUPSB16.

Table 4. Morphological, physiological and biochemical characteristics of the PSB isolate KUPSB16.

Test/ Characters	*Bacillus* sp KUPSB16
Cell shape	Rod
Gram reaction	+
Endospore formation	+
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-Leifson (O/F) reaction	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.

insoluble phosphate into soluble forms (Kpomblekou and Tabatabai, 1994). PSB can chelate several heavy metals such as, arsenic cadmium, chromium, copper, lead, nickel and zinc with variable affinities (Schalk *et al.*, 2011). Hence, PSB might be unique alternatives to aggravate this process as organic acids secreted by these microorganisms solubilize sparingly-soluble heavy metal complexes. Thus, PSB *Bacillus subtilis* KUPSB16 might be the better choice in assisting the bio-remediation process in heavy metal contaminated environment.

4. Conclusions

The results of the present investigation suggested that *Bacillus subtilis* KUPSB16 can be useful to remediate heavy metal contaminated sites. Use of these phosphate solubilizing bacteria as bio-inoculants will increase the available phosphorus, reduces heavy metal pollution and promotes sustainable agriculture.

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