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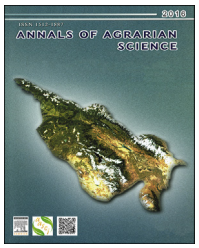

Isolation and characterization of phosphate solubilizing bacterium *Pseudomonas aeruginosa* KUPSB12 with antibacterial potential from river Ganga, India

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ABSTRACT

In the present study, the antibacterial potential of a phosphate solubilizing bacterium isolated from the river Ganga, West Bengal, India was investigated. Experimental studies found that the strain KUPSB12 was effective in phosphate solubilization with phosphate solubilization index of 2.85 in Pikovskaya's agar plates along with very high soluble phosphate production of 219.64 ± 0.330 μg mL^{-1} in liquid medium. The phosphate solubilizing bacterium was identified using physiological, morphological and biochemical characters as well as 16S rRNA gene sequencing. The phosphate solubilizing bacterium was identified as a strain of *Pseudomonas aeruginosa*. The antibacterial activity of the cell-free filtrates of this isolate was evaluated against three Gram negative bacteria (*Escherichia coli* MTCC 443, *Shigella flexneri* MTCC 1457 and *Vibrio cholerae* MTCC 3904) and three Gram positive bacteria (*Bacillus subtilis* MTCC 441, *Micrococcus luteus* MTCC 1538 and *Staphylococcus aureus* MTCC 3160). *P. aeruginosa* KUPSB12 strain showed the wide inhibitory spectrum against all tested pathogenic bacterial stains. Among the bacteria tested *M. luteus* MTCC 1538 was found to be most susceptible (19.33 ± 0.33 mm) to the cell-free filtrates of this isolate. These findings suggest that the identified strain may be utilized for screening the antibacterial substances to formulate new treatments for infections caused by pathogenic bacteria.

Introduction

Natural solubilization of mineral phosphates is an important phenomenon exhibited by different microorganisms, known as phosphate solubilizing microorganisms (PSM). Bacteria are the predominant microorganisms that solubilize mineral phosphate in nature, as compared to other microorganisms [1]. Phosphate solubilizing bacteria (PSB) play an important role in biogeochemical phosphorus cycling in both terrestrial

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and aquatic environments [2]. A small number of studies investigated on the occurrence of phosphate solubilizing bacteria in the marine environment and fresh water environment like lake ecosystem [3,4]. But similar studies in freshwater environments especially riverine ecosystems, which tend to be more phosphate deficient, are meagre [5,6].

Interest in replacement of synthetic chemicals by biological alternatives is a current trend in the area of medical science. Synthetic chemicals can negatively affect the environment, human and animal health [7]. The antimicrobial properties of biologically active products from nature such as plant [8], algae [9], sponge [10], fungi [11], actinomycetes [12] and bacteria [13] have been studied worldwide by a number of researchers. It is a general phenomenon that most bacteria produced few or large number of antibacterial compounds. One of the genera having the potential to produce bioactive compounds against pathogens is *Pseudomonas*. The *Pseudomonas* (γ-Proteobacteria) are motile (one or several polar flagella), non-sporulating rods with Gram negative reaction [14]. A wide spectrum of antimicrobial components against pathogenic bacteria and fungi are produced [15]. Pseudomonades usually produced several metabolites from different groups such as 2,4-diacetylphloroglucinol, 2-acetamidophenol, hydrogen cyanide, indoles, phenazines, phenazine-1-carboxylic acid, pseudotrienic acids, pyocyanin, pyoluteorin, pyrrolnitrin, tenzin and viscosinamide [16–18].

Despite the large number and diversity of biologically active compounds isolated from various microorganisms, since the penicillin era, new infectious diseases and pathogens still represent a serious problem for human life [19,20]. With the increase of continual bacterial resistance against common antibiotics in global public health, it is necessary to sort out new sources of antimicrobials [21]. Recently there has been a lot of attention focused on producing medicines and products from natural origin. The present investigation focuses to establish the phylogenetic diversity of phosphate solubilizing bacterial isolate and closely related bacteria and evaluate the potential of activity of the antibacterial compound.

Materials and methods

Isolation of phosphate solubilizing bacterial strain

Phosphate solubilizing bacterial strain isolated from a jute mill effluent mixing area of Ganga river water at Bansberia (22°58′17″N and 88°24′03″E), West Bengal, India. The bacterium was isolated and screened on Pikovskaya's agar (PKA) medium consisting of constituents: glucose 10 g; tri-calcium phosphate (TCP) 5 g; yeast extract 0.5 g; ammonium sulphate 0.5 g; potassium chloride 0.2 g; sodium chloride 0.2 g; magnesium sulphate 0.1 g; ferrous sulphate trace; manganese sulphate trace; agar agar 15 g; distilled water 1 L; the pH was adjusted to 7.0 ± 0.2 before sterilization, by pour plate technique [22]. After 48 h of incubation at 28 ± 2 °C discrete colony showing halo zones were picked up, sub-cultured in PKA slants and preserved.

Estimation of phosphate solubilization efficiency

The quantitative estimation or abilities of the isolated phosphate solubilizing bacterium to solubilize TCP on Pikovskaya's agar media was determined in terms of solubilization index (SI). Phosphate solubilization index was calculated by measuring the colony diameter and the halo zone diameter and the colony diameter, using the following formula of Edi-Premono et al. [23].

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

The qualitative analysis of phosphate solubilization potential of selected PSB isolate was measured *in vitro* by determining available soluble phosphate in the Pikovskaya's broth supplemented with 0.5% TCP. The broth medium was inoculated in triplicate with phosphate solubilizing bacterial strain KUPSB12. The flasks were incubated at 28 ± 2 °C for 5 days on rotary shaker at 180 rpm and centrifuged at 10,000 rpm for 10 min. Phosphomolybdate method was used for determination of available soluble phosphate in culture supernatant [24]. The pH of the broth medium was also measured with a digital pH meter (Jenway 3510) after regular intervals.

Identification of bacterial strain

Different morphological, physiological and biochemical tests of the selected phosphate solubilizing bacterial isolate were carried out for identification as per the methods defined in Bergey's Manual of Determinative Bacteriology [25].

16S rRNA gene sequencing and analysis

To determine the phylogenetic relationship, the 16S rRNA gene sequence of isolated bacterial strain was obtained using the 16S rRNA gene specific universal primers: 8F and 1492R (Table 1). The 16S rRNA gene sequence of the isolated strain was analyzed at NCBI GenBank (http://www.ncbi.nlm.nih.gov) using BLAST (N) program [28]. Phylogenetic tree was constructed by neighbour joining method for this alignment using the MEGA 6 (Molecular Evolutionary Genetics Analysis) software [29]. The final sequence was submitted at GenBank [30].

Preparation of extract and preliminary screening for antibacterial activity

Pure colony of bacterial isolate was transferred to Erlenmeyer flasks containing nutrient broth and was incubated on a rotary shaker (150 rpm) at 28 °C to produce secondary metabolites. After 5 days, the broth culture first was centrifuged at 10,000 rpm for 20 min at 4 °C, and then supernatant was extracted using the ratio of 1:1 (v/v) of ethyl acetate [31]. Solvent was removed at 37 °C by evaporation. Then the crude extract was weighed and dissolved in ethyl acetate to achieve a concentration of 100 μg μl^{-1}.

Table 1 – Details of the primers used in the present study.

Primer name	Sequence	References
8F	5′-AGAGTTTGATCCTGGCTCAG-3′	[26]
1492R	5′-GGTTACCTTGTTACGACTT-3′	[27]

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Antibacterial activity of cell free extracted supernatant was tested by agar well diffusion method [32]. Test bacterial strains used in this study include three Gram negative bacteria (*Escherichia coli* MTCC 443, *Vibrio cholerae* MTCC 3904 and *Shigella flexneri* MTCC 1457) and three Gram positive bacteria (*Bacillus subtilis* MTCC 441, *Micrococcus luteus* MTCC 1538 and *Staphylococcus aureus* MTCC 3160). All of the test bacterial cultures were grown in Mueller Hinton broth at 37 °C for 24 h. Final inoculum concentrations were adjusted to 10^8 CFU/ml [33]. Approximately 7 mm diameter of well was made on Mueller Hinton agar plate using sterile cork borer. The pathogenic tested bacterial cultures were uniformly spread on Mueller Hinton agar with the help of sterilized glass spreader. About 25 μl of extracted samples of bacteria were introduced to each well, and then the Petriplates were incubated at 37 °C for 24 h in incubator, the diameter of inhibition zones were measured.

Statistical analyses

All experiments were performed in triplicate, and the results were expressed as the mean. Means and standard errors (SE) were analyzed by using the SPSS 13.0 software package.

Results and discussion

In the present study, the collected river water samples were plated in Pikovskaya's (PKV) agar plate for PSB. The PSB isolate KUPSB12 was found to be potent phosphate solubilizer showing clear halo zone around its colony. The PSB isolate KUPSB12 showed 13.00 mm phosphate solubilizing halo zone around its colony (Table 2). The solubilization index (SI) of the isolated strain KUPSB12 was also calculated at the end of the incubation period and observed phosphate solubilization index (SI) of 2.85. The halo zone formation around the bacterial colonies could be due to the production of organic acids or due to the production of polysaccharides or due to the activity of phosphatase enzymes of phosphate solubilizing bacterial strains [34–37].

The phosphate solubilizing efficiency of isolated PSB strain KUPSB12 in Pikovskaya's broth indicated that the strain efficiently solubilized inorganic phosphate in the medium containing 0.5% tri-calcium phosphate (Table 3). PSB isolate KUPSB12 was produced 219.64 μg mL^{-1} soluble phosphate in the PKV broth after 96 h of incubation period. Banerjee et al. [38] also reported that maximum phosphate solubilization efficiency of the isolated *Bacillus* sp. was after incubation of 96 h. But some other researchers have reported 3 days, more than 10 days and even up to 15 days to be the optimum incubation period for phosphate solubilization by various bacterial isolates [39,40].

Table 2 – Qualitative estimation of phosphate solubilization efficiency of KUPSB12.

PSB isolate no.	Colony diameter (mm)	Halo zone diameter (mm)	Solubilization index (SI)
KUPSB12	7.00 ± 0.577	13.00 ± 0.288	2.85

Table 3 – Quantitative estimation of phosphate solubilization efficiency of KUPSB12.

PSB isolates	Incubation period (h)	P-solubilization (μg mL^{-1})	pH after incubation
KUPSB12	96	219.64 ± 0.330	5.21 ± 0.030

Table 4 – Characterization of the isolated bacterial strain (*Pseudomonas* sp. KUPSB12).

Characteristics	Bacterial isolate
Cell morphology	Rod
Gram staining reaction	–
Motility	+
Bacterial growth at 5% NaCl	+
Bacterial growth at 6 °C	–
Bacterial growth at 37 °C	+
Bacterial growth at 42 °C	+
Bacterial growth on MacConkey agar	+
Fluorescence on King's B medium	+
Oxidase activity	+
Catalase activity	+
Indole production test	–
Methyl red test	–
Voges–Proskauer test	–
Citrate utilization test	+
H_2S production test	–
Urease activity	–
NO_3^- reduction test	+
Gelatine liquefaction	+
Hydrolysis of starch	–
Hydrolysis of casein	+
Hugh–Leiffson (O/F) test	O/F
Utilization of carbon source	
Glucose	+
Lactose	–
Fructose	+
Arabinose	+
Rhamnose	–
Sucrose	+
Maltose	–
Raffinose	–
Cellobiose	–
Xylose	+
Mannitol	–
Sorbitol	–
Dulcitol	–

+ indicates presence or positive; – indicates absence or negative; O = Oxidation; F = Fermentation.

In liquid medium, the solubilization of tri-calcium phosphate by phosphate solubilizing bacterial strain KUPSB12 was accompanied by a significant decline in pH of culture supernatant from an initial pH of 7.0 ± 0.2 after 96 h of incubation period were recorded (Table 3). The maximum drop in pH value was correlated with elevated levels of phosphate solubilization, PSB strain KUPSB12 where pH was declined to 5.21 from initial pH. Park et al. [41] also reported that in case of *Pseudomonas fluorescens* RAF15, the maximum drop of pH was recorded at pH 4.0. The acidification of culture supernatants clearly indicated the production of organic acid seemed to be generally the main mechanism for phosphate solubilization [42,43]. Rodriguez and Fraga [44] reported that various

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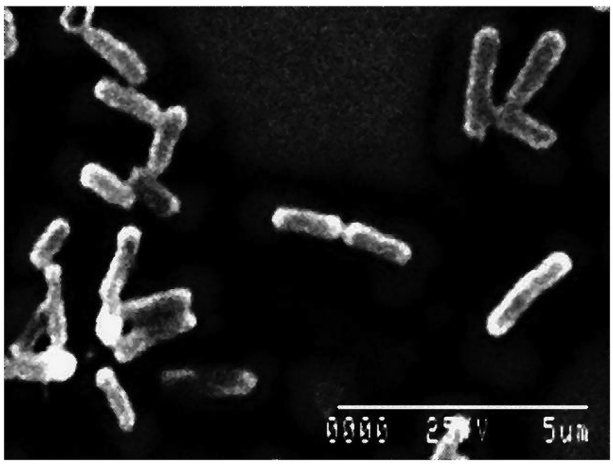

Fig. 1 – Scanning electron micrograph of PSB isolate KUPSB12.

phosphate solubilizing microorganism produced gluconic acid, 2-ketogluconic acid, lactic acid, isovaleric acid, isobutyric acid, acetic acid, oxalic acid, citric acid etc for solubilization of insoluble phosphates.

To characterize the isolated phosphate solubilizing bacteria, several biochemical tests were performed. All these results are consistent with the many phenotypic characteristic of the genus *Pseudomonas*. On the basis of morphological, physiological and biochemical characteristics the phosphate solubilizing bacterial isolate was identified as *Pseudomonas* sp. This bacterial genera was well known identified as phosphate solubilizer by several authors [45,46]. Physiological, morphological and biochemical characteristics of isolate *Pseudomonas* sp were outlined in Table 4 and Fig. 1.

To identify the isolated organism, 16S rRNA sequencing was performed. BLAST search analysis was carried out for the 16S rRNA sequences thus obtained using NCBI-Gene Bank database that showed a sequence identity of 99.0% with *Pseudomonas aeruginosa* (Table 5). The FASTA sequence was submitted to GenBank with the accession number KJ131180 (Fig. 2). Since the BLAST analysis revealed the alignment of the 16S rRNA sequence with a number of species of the genus *Pseudomonas*, we used the most similar sequences to construct a phylogenetic tree using neighbour joining method (Fig. 3). It was evident from the phylogenetic tree that the isolated organism *P. aeruginosa* lies in gamma-proteobacteria order of *Pseudomonas* and exhibited a close relation with its nearest neighbours. Therefore, the isolated organism was assigned as *P. aeruginosa* KUPSB12.

The antibacterial compound produced by *P. aeruginosa* KUPSB12 was effective against of all the six tested pathogenic bacteria (Fig. 4). The bacterial extract exhibited bactericidal activity against both Gram negative and Gram positive bacteria. Highest inhibition zone of 19.33 ± 0.33 mm diameter was formed against *M. luteus* and the lowest of 15.66 ± 0.33 mm was produced against *Vibrio cholerae*. Similar results for being less effective against Gram negative bacteria than that on Gram positive ones were previously reported by Haba et al. [47]. Gram negative and Gram positive bacteria differ in their structures of cell wall. Onbasli and Aslim [48] also obtained similar results which might be due to the presence of permeable characteristic of Gram negative bacterial membranes to hydrophobic and amphipathic molecules.

Table 5 – Molecular characterization of PSB strain to genomic level.

PSB isolate	Genus and species	Accession no.	Nucleotides length (bp)	16S rRNA identity (%)
KUPSB12	*Pseudomonas aeruginosa*	KJ131180	1382	99

>KUPSB12
GTCGAGCGCATGAAGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGG
AATCTGCCTGGTAGTGGGGGATACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAG
GGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGTTAGCTAGTT
GGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCAC
ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAT
GGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCAC
TTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAATA
AGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCA
ACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCT
GTGTAGCGGTGAAATCGTAGATATAGGAAGGAACACCAGTGGGAAGGCGACCACCTGGACT
GATACTGACACTGAGGTGCGAAACGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCAACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCG
ATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTT
GACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGC
ATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTT
GTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACA
ATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTA
GTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAG
AATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGG
TTGCTCCAGAAGTAGCTAGTCTAACCGCAAG

Fig. 2 – FASTA sequences of the PSB isolate KUPSB12.

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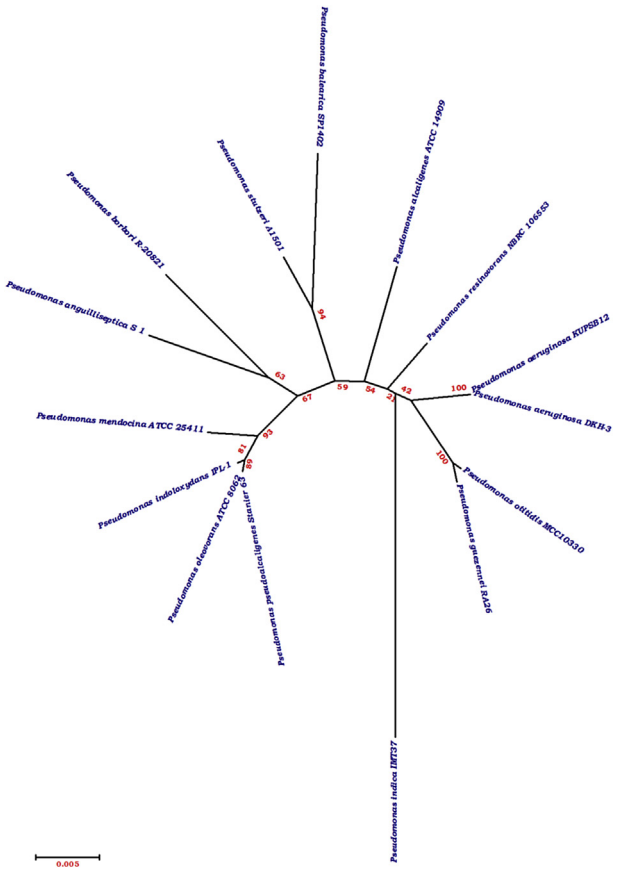

Fig. 3 – Phylogenetic tree showing the evolutionary position and relationship of *Pseudomonas aeruginosa* KUPSB12 with other bacterial isolates. The tree was constructed neighbour joining method with 1000 bootstrap replicates.

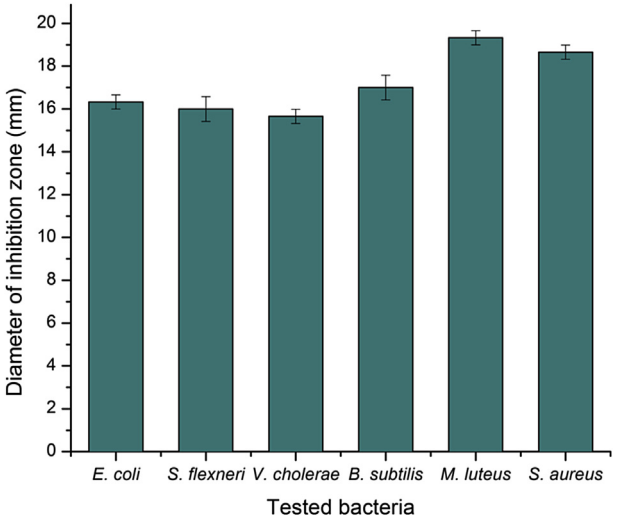

Fig. 4 – Antibacterial activity of as *Pseudomonas aeruginosa* KUPSB12 extracts against tested pathogenic bacteria (mean ± SE).

Synthetic antibacterial products are generally associated with high costs and various side effects. Many different antibacterial substances from natural origin have been extensively studied as an alternative to prevent bacterial infections. The antibacterial activity has been extensively reported for extracts of various groups of microorganisms [12,49]. Recently, numerous compounds were isolated from bacteria with antimicrobial activities [50,51]. The discovery of new classes of antibacterial compounds is essential due to the increased incidence of multiple resistances among pathogenic microorganisms to drugs which are currently in clinical use [52–54]. Microorganisms such as bacteria provide such significant opportunity for study as they are the sink of many such bioactive compounds. Accordingly, the discovery of natural, efficient and cost effective drugs from natural origin against these pathogenic bacteria may be an important solution for this global problem [55]. Our study indicates that the use of a secondary metabolite from bacteria *P. aeruginosa* KUPSB12 could be effective against these pathogenic strains that cause diseases.

Conclusion

Microorganisms especially bacteria are promising resources for biologically active compounds. In the present study, the crude extracts of *P. aeruginosa* KUPSB12, a phosphate solubilizing bacterial isolate exhibited broad antibacterial activities. The isolated bacterial strain seemed to be highly potent in controlling bacterial pathogens regarding health significance. Hence, sufficient studies are needed to isolate and purify the individual compounds behind such activities and elucidate their structures.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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