



(RESEARCH ARTICLE)



Isolation and characterization of phosphate solubilizing bacteria in saline soil from Costal Region of Odisha

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GSC Biological and Pharmaceutical Sciences, 2021, 16(03), 109–119

Publication history: Received on 13 July 2021; revised on 14 September 2021; accepted on 16 September 2021

Article DOI: <https://doi.org/10.30574/gscbps.2021.16.3.0273>

Abstract

The present study was conducted to isolate phosphate solubilizing bacteria (PSB) from rhizospheric saline soils of coastal Odisha, India and evaluated their phosphate solubilizing ability. Total four PSB were isolated based on the halo zone formation (solubilizing index 2.63-3.14) on PVK agar medium and were characterized based on biochemical and molecular characteristics as *Bacillus subtilis* (B1), *B. megaterium* (B2), *Sphingomonas paucimobilis* (P2) and *Kocuria kristinae* (P6). The inorganic phosphate released by PSB ranged from 18.532 to 38.250 $\mu\text{g/ml}$ with decreasing the pH PVK broth up to 3.9. Acid phosphatase activity for PSB were recorded 84.237-98.658 $\mu\text{mol/min}$. Glucose was found to be the best carbon source for *B. subtilis*, *Sphingomonas paucimobilis* and *Kocuria kristinae* whereas mannitol for *B. megaterium*. Optimum acid phosphatase activity was observed for all the four PSB isolates in presence of ammonium sulphate as nitrogen source in PVK broth at 30 $^{\circ}\text{C}$ and pH 7.0.

Keywords: Phosphate solubilizing ability; Halo zone; PSB; Solubilizing index; Acid phosphatase

1. Introduction

Soil is rich source of nutrients. Among the soil nutrients, phosphorus (P) is the second most important nutrient after nitrogen [4]. Phosphate is a structural component of many coenzymes, phospho-proteins, phospholipids and nucleic acids of all living organisms [24]. Phosphorus is especially important in photosynthesis, carbon metabolism and membrane formation [40]. 95-99% of soil P presents as insoluble form [36] both in organic and inorganic form, which makes it a major growth limiting factor for plant [14]. This unavailability of P is due to P-fixation, either it is absorbed by the soil minerals or get precipitated by free Al^{3+} and Fe^{3+} in the soil solution [32, 15]. To increase the availability of P to plants for better yield, a requisite amount of phosphate as chemical fertilizer is applied to soil, whose large portion is quickly transformed into insoluble form [23] and very little percentage of it is available to plants that increase the necessity of continuous application of chemical fertilizer [1].

Phosphate solubilizing microbes have the potential of making these phosphates available to the plants. Bacteria solubilize phosphate more effectively than fungi [3]. Rhizodeposition of various exudates provide important substrates for the soil microbial community which helps them for releasing various compounds for the growth of plant [17, 27]. The mechanisms by which plant growth promoting rhizobacteria (PGPR) promote plant growth, include the ability to produce phytohormones, asymbiotic nitrogen fixation against phytopathogenic microorganisms by production of siderophores, the synthesis of antibiotics, enzymes and/or fungicidal compounds and also solubilization of mineral phosphates and other nutrients [12].

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2. Material and methods

2.1. Isolation of phosphate solubilizing bacteria (PSB)

Rhizosphere soil samples were collected from coast-line of Puri, Baleshwar and Ganjam districts, Odisha, India crowded with casuarina plants. Approximately 2kg of soil sample was taken from the upper 15-30cm of the soil profile. Soil pH, Electrical conductivity and available phosphorous were determined by the method of Jackson ML. Soil Chemical Analysis [16]. Available nitrogen was estimated by alkaline $K_2Cr_2O_7$ method as outlined by a rapid procedure for the determination of available nitrogen in soil. [33]. Organic Carbon was measured by Walkley and Black method [39].

The serially diluted soil samples were allowed to grow on the Pikovaskaya's agar media [25] containing tricalcium phosphate as the sole source of phosphate. The phosphate solubilizing activity was measured on the basis of halo zone formation around the inoculated area after 7 days of incubation. Phosphate solubilizing index (SI) was determined by measuring both colony and halo zone diameters using Edi-Premono [11] formula: Phosphate SI = (colony diameter + halo zone diameter)/colony diameter. Phosphate solubilization efficiency (PSE) was also determined by the formula: PSE= halo zone diameter/colony diameter \times 100 [22].

2.2. Biochemical characterization of Phosphate Solubilizing Bacteria

Characterization of isolates such as colony appearance, shape, size gram staining was determined according to the standard methods. Biochemical characteristics such as carbohydrate fermentation test were done by Hicarbohydrate™ kit as described by the manufacturer (Himedia Laboratories, Mumbai, India). Methyl red, Voges-Proskaur, catalase, urease and starch hydrolysis reactions were done by standard methods. The results were compared with Bergey's Manual of Determinative Bacteriology [7].

2.3. Molecular identification of Phosphate Solubilizing Bacteria

The 16S rDNA of PSB was amplified by using universal 27F forward primer (5'-AGGCCTAACACATGCAAGTC-3') and 1492R reverse primer (5'-GGGCGWGTGTACAAGGGC-3') [10]. The amplified PCR product was purified by HiPura™ PCR product purification kit (HIMEDIA) and nucleotide sequences were determined using Big dye terminator v 3.1 cycle sequencing kit using an automated 3500 genetic analyzer system (Applied Biosystems, Hitachi, USA) and submitted to gene bank. The sequences were finally aligned in the alignment explorer tool of the Molecular Evolutionary genetic analysis software (MEGA 6; [35]) by using Clustal-W. The phylogenetic tree was prepared with the help of neighbor joining method with boot strap value of 1000.

2.4. Quantitative estimation of soluble phosphate

In Erlenmeyer flask, 100ml of PVK broth with tricalcium phosphate as the sole phosphate source were prepared and sterilized. To each flask the bacterial culture was inoculated and the uninoculated one was taken as control. The flasks were incubated in a rotary shaker at 100 rpm and 28°C for 120h. At a regular interval of 24h, sample was collected from each flask and pH was measured by pH meter and centrifuged at 10,000g for 15 minutes followed by filtration through Whatman No.1 filter paper having pore size 0.22 μ m to obtain cell-free supernatant. The amount of soluble phosphate released was measured by spectrophotometer at 600nm by Murphy and Riley method, [21]. The value of OD was compared against the standard curve prepared by taking known concentration of orthophosphate (20, 40, 60, 80 and 100 μ g/ml).

2.5. Acid Phosphatase

Extra cellular acid-phosphatase extraction was carried out by inoculating 100 μ l of each Phosphate solubilizing bacterial culture into 100ml of PVK broth in 250ml Erlenmeyer flask in triplicates and incubated at 28°C. At a regular interval of 24h the samples were centrifuged at 10,000rpm for 10 min at 4°C. The cell-free supernatant was assayed for crude acid phosphatase activity by the method of Tabatabai and Bremner [34]. 1ml of supernatant was mixed with 4ml of MUB (pH 6.5). Further 1ml of 0.025mM disodium *p*-nitrophenyl phosphate tetrahydrate (Sigma) and 0.25ml toluene were added to the above mixture and incubated for 1h. After 1h of incubation the reaction was stopped by adding 4ml of 0.5M NaOH and 1ml of 0.5M $CaCl_2$. The contents were then filtered through Whatman No. 42 filter paper. The concentration of *p*-nitrophenol was measured at 420nm using UV-VIS spectrophotometer and the values were extrapolated on standard curve. A standard curve was prepared from serially diluted samples of *P*- nitro phenol solution. Acid

phosphatase activity was defined as the amount of enzyme required to release 1 μ mol of *p*-nitrophenol /ml/min from di-sodium *p*-nitrophenyl phosphate (tetrahydrate) under the assay conditions.

2.6. Optimization of growth conditions for maximum crude acid phosphatase production

The PSB isolates were analysed for maximum production of crude acid phosphatase by inoculating them in Pikovaskaya's broth media for 96h under different parameters like carbon sources: glucose, sucrose, fructose and mannitol; nitrogen sources: ammonium sulphate, ammonium molybdenum, urea and potassium nitrate; pH: 5.0-8.0 and temperature: 25°C-40°C [5].

2.7. Statistical analysis

DMRT for multiple comparisons was performed using SAS 9.1.3. $\alpha=0.05$ was considered to be significant.

3. Results and discussion

3.1. Solubilizing Index (SI) and Phosphate solubilizing efficiency (PSE) of PSB

After seven days of incubation, clear halo zone was observed surrounding the colonies on the PVK agar plates (fig 1) which is measured and the data is given in table 1.

Table 1 Solubilizing Index (SI) and Phosphate solubilizing efficiency (PSE) of PSB

Name of PSB	Halo zone diameter (mm)	Colony diameter (mm)	Solubilization index (SI)	PSE (%)
P2	12.3	5.9	3.09 \pm 0.006 ^a	209.0
P6	4.3	2.7	2.63 \pm 0.022 ^b	162.5
B1	8.6	4.2	3.13 \pm 0.022 ^a	213.2
B2	12.0	5.6	3.14 \pm 0.027 ^a	213.7

*=Mean of triplicates \pm standard error, same alphabets in a column are not significantly different by DMRT at 0.05% probability level.



Figure 1 Halo zone formation by PSB

3.2. Identification of PSB

The four efficient PSB isolates were identified up to generic level based on biochemical tests which are presented in Table 2. The PSB belonged to the genera *Bacillus* (P2, B1 and B2) and *Kocuria* (P6).

Table 2 Biochemical characteristics of PSB

Biochemical tests	PSB isolates			
	P2	P6	B1	B2
Lactose	-	-	+	+
Xylose	-	-	+	+
Maltose	-	+	+	+
Fructose	+	+	+	+
Dextrose	-	+	+	+
Galactose	-	-	-	+
Raffinose	-	+	-	+
Trehalose	-	+	+	+
Melibiose	-	+	+	+
Sucrose	+	+	-	+
l-arabinose	+	+	+	+
Mannose	+	+	+	+
Innulin	-	+	+	+
Sodium gluconate	-	+	+	+
Glycerol	-	+	-	+
Salicin	-	+	-	+
Dulcitol	-	+	-	+
Inositol	-	-	-	+
Sorbitol	-	+	+	+
Mannitol	-	+	+	+
Adonitol	-	-	+	+
Arabitol	-	-	+	+
Erythritol	-	-	+	+
α -Methyl-D-glucoside	-	-	-	+
Rhamnose	-	-	-	+
Cellobiose	-	-	+	+
Melezitose	-	+	-	+
α -Methyl-D-mannoside	-	-	-	+
Xylitol	-	-	-	+
ONPG	-	-	-	+
Esculin hydrolysis	-	-	+	+
D-Arabinose	-	-	-	-
Citrate utilization	+	+	+	-
Malonate utilization	-	+	+	-
Sorbose	-	-	-	-
Methyl red	-	-	-	-
VP	-	+	-	-
Starch hydrolysis	-	-	-	+
Catalase	+	+	+	+
Urease	+	-	-	-

Molecular identification of four PSB isolates by 16S rDNA sequencing is given in table-3. Phylogenetic tree of the identified strains is represented in fig 2.

Table 3 Accession No. of identified strain of Gen bank, NCBI

PSB isolates	Identification	Accession No.
P2	<i>Sphingomonas paucimobilis</i>	MK091525
P6	<i>Kocuria kristinae</i>	MK201658
B1	<i>Bacillus subtilis</i>	MK091526
B2	<i>Bacillus megaterium</i>	MK105917

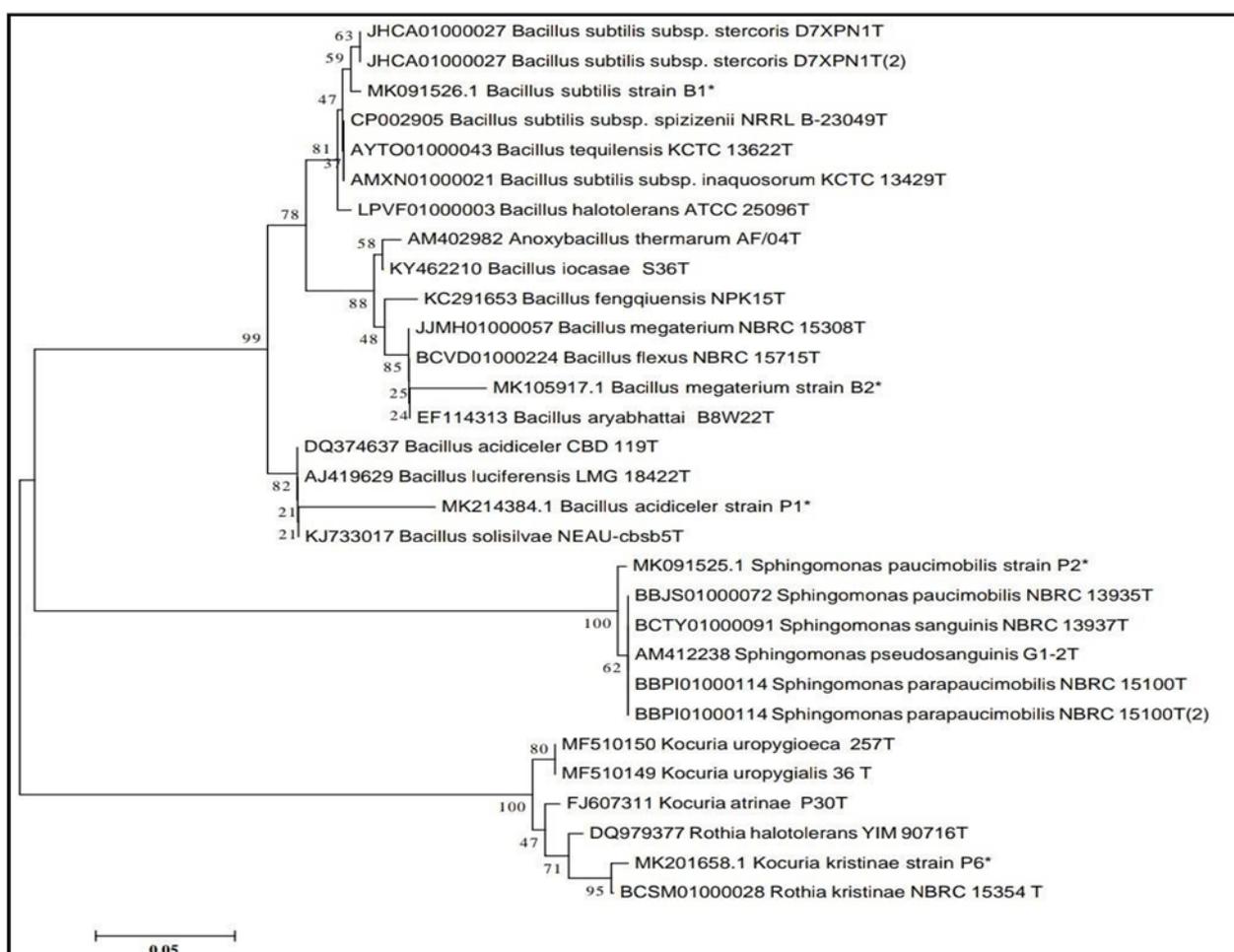


Figure 2 Phylogenetic tree based on 16S rRNA gene sequences by Neighbor Joining method (using MEGA 6.0), showing the relationship between strains P2, P6, B1, B2 and other members of the *Bacillus* sp, *Sphingomonas* sp and *Kocuria* sp. The Genbank nucleotide accession numbers are listed next to the strain names. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The scale bars represent 0.05 substitution /site.

3.3. Quantitative estimation of soluble phosphate released by the PSB isolates

The amount of soluble phosphate released from TCP in the PVK broth by PSB and their effects on pH of PVK broth are presented in Table 4.

Table 4 Incubation period optimization of phosphate solubilizing bacteria

PSB	Conc. of soluble phosphate ($\mu\text{g/ml}$)*					pH				
	24h	48h	72h	96h	120h	24h	48h	72h	96h	120h
<i>Spingomonas paucimobilis</i>	31.027 \pm 0.004 ^c	33.521 \pm 0.009 ^b	35.581 \pm 0.011 ^a	23.286 \pm 0.019 ^d	18.546 \pm 0.009 ^e	5.0 \pm 0.003 ^b	4.8 \pm 0.007 ^c	4.3 \pm 0.003 ^d	4.4 \pm 0.003 ^e	5.0 \pm 0.003 ^a
<i>Kocuria kristinae</i>	23.002 \pm 0.011 ^e	30.882 \pm 0.007 ^c	32.269 \pm 0.009 ^b	32.655 \pm 0.032 ^a	29.396 \pm 0.005 ^d	4.9 \pm 0.007 ^a	4.6 \pm 0.012 ^b	4.5 \pm 0.003 ^c	4.3 \pm 0.009 ^d	4.6 \pm 0.003 ^a
<i>B. subtilis</i>	30.704 \pm 0.019 ^e	32.224 \pm 0.025 ^d	36.959 \pm 0.007 ^a	36.810 \pm 0.012 ^b	33.190 \pm 0.015 ^c	4.7 \pm 0.012 ^a	4.5 \pm 0.006 ^b	4.1 \pm 0.007 ^c	4.2 \pm 0.006 ^d	4.5 \pm 0.003 ^e
<i>B. megaterium</i>	27.358 \pm 0.001 ^e	34.080 \pm 0.017 ^d	38.138 \pm 0.002 ^c	39.659 \pm 0.012 ^c	38.250 \pm 0.002 ^b	4.9 \pm 0.009 ^a	4.4 \pm 0.006 ^b	4.3 \pm 0.006 ^c	3.9 \pm 0.006 ^e	4.2 \pm 0.007 ^d

*=Mean of triplicates \pm standard error, same alphabets in a column are not significantly different by DMRT test at 0.05% probability level

Table 5 Incubation period optimization for acid phosphatase activity

Incubation period (h)	Acid phosphatase activity ($\mu\text{mol/min}$)*			
	<i>Spingomonas paucimobilis</i>	<i>Kocuria kristinae</i>	<i>B. subtilis</i>	<i>B. megaterium</i>
24	47.395 \pm 0.091 ^e	47.033 \pm 0.094 ^e	52.846 \pm 0.035 ^d	51.056 \pm 0.041 ^e
48	75.302 \pm 0.054 ^c	62.495 \pm 0.054 ^d	72.565 \pm 0.041 ^c	65.337 \pm 0.093 ^d
72	85.302 \pm 0.061 ^a	71.419 \pm 0.051 ^c	93.454 \pm 0.130 ^a	82.097 \pm 0.122 ^b
96	89.676 \pm 0.082 ^b	84.237 \pm 0.062 ^a	84.249 \pm 0.035 ^b	98.658 \pm 0.062 ^a
120	67.395 \pm 0.104 ^d	76.144 \pm 0.073 ^b	72.565 \pm 0.081 ^c	81.033 \pm 0.062 ^c

*=Mean of triplicates \pm standard error, same alphabets in a column are not significantly different by DMRT test at 0.05% probability level.

3.4. Acid phosphatase activity

The results of acid phosphatase activity of four PSB isolates are presented in Table 5.

3.5. Optimization of growth conditions for acid phosphatase production

The efficacy of acid phosphatase production by *Sphingomonas paucimobilis*, *Kocuria kristinae* and *Bacillus subtilis* was maximum in PVK medium containing glucose mannitol as the sole source of carbon whereas by *Bacillus megaterium* it was maximum in PVK medium containing mannitol as the sole source of carbon (Fig 3A). The efficacy of acid phosphatase production by all the four PSB was maximum in PVK medium containing ammonium sulphate as the sole source nitrogen (Fig 3B). All the four PSB showed their efficacy for optimum acid phosphatase activity at pH 5.0 and temperature 30°C (Fig 3C and 3D).

4. Discussion

4.1. Isolation of phosphate solubilizing bacteria

The electrical conductivity of soil samples collected from Puri, Baleswar and Ganjam district was 4.1 and 4.3; 3.9 and 4.0; 4.5 and 4.8 respectively.

pH of soil samples collected from Puri, Baleswar and Ganjam district was 7.0; 6.4 and 6.6; 6.8 and 6.9 respectively. According to the USDA system, saline soils have electrical conductivity > 4 dS/m and pH < 8.5 [6 and 8], which indicates that the soil samples of coastal Odisha are coming in the range of salinity.

In the present investigation, PSB were screened from the rhizosphere saline soil of coastal Odisha based on the halo zone formation in the PVK agar medium. Halo zone formation was due to the solubilization of insoluble phosphate by acidification of either proton extrusion or organic acid secretion [9]. The present experiment showed that highly efficient phosphate solubilizers inhabit the rhizosphere saline soil of Casuarina in coastal Odisha having SI ranging from 2.5 to 4.3 and PSE ranging from 162.5% to 213.7% in the Pikovaskaya's agar medium having TCP as the insoluble source of phosphate (table-1). This result is supported by [19], who reported that PSB isolated from the rhizosphere of *Agave angustifolia* was showing SI in the range from 1.4 to 4.5 and PSE 42.8 to 350% on the medium having TCP as insoluble phosphate source. They have also reported that *Sphingomonas paucimobilis* has SI 1.5 and PSE 57% in their study, which is lower than the present result where SI and PSE are 2.62 and 162.5% respectively (table-1). The solubilisation efficiency of *Pseudomonas fluorescens* and *B. megaterium* were recorded as 200 and 128.57% respectively [31]. [20] reported that PSE of *B. megaterium* and *B. subtilis* was 140% and 120% respectively, but the observations from present investigation showed the PSE for *B. subtilis* and *B. megaterium* were 213.2% and 313.7% respectively (table-1). This variation might be due to the physical, chemical and biological characteristics of soil samples.

4.2. Quantitative estimation of inorganic phosphate released by PSB

The findings of present study resulted in the production of soluble phosphate were 39.659µg/ml by *B. megaterium*, 36.959µg/ml by *B. subtilis*, 35.581µg/ml by *Sphingomonas paucimobilis* and 32.542µg/ml by *Kocuria kristinae*. According to [28] *Bacillus* sp. TRSB16 consistently showed high solubilization rates of tricalcium phosphate (144 µg/ml). [19] reported that *sphingomonas paucimobilis* produced 39.5 µg/ml inorganic phosphates. [38] reported phosphate solubilisation by *Kocuria kristinae* from the sample collected from Northern hill zones of India. The drop in the pH of Pikovskaya's broth was also examined in this investigation which is associated with the amount of phosphate solubilization. All the PSB isolates were observed to lower the pH of the medium. The pH of the broth medium was dropped from initial pH 7 to a lower value of 3.9 by PSB at the incubation period where they solubilized maximum phosphate and after which again pH was increased consistently in the acidic medium which is associated with the decreasing amount of soluble phosphate (table-4). Hypothetically it could be concluded that the rise in pH in the medium could be due to the utilization of organic acid or the production of some alkaline compounds [2]. *Sphingomonas paucimobilis* solubilised maximum phosphate after 72h of incubation by decreasing the pH of the medium from 7.0 to 4.3. [19] Also reported that the pH of the medium was reduced to 4.5 by *Sphingomonas paucimobilis*. The inverse relationship between pH and soluble-phosphate concentration observed in the present study indicates that organic acid production by the strain plays a significant role in the acidification of the medium, facilitating the phosphate solubilization. This decrease in pH appears to indicate the production of organic acid by PSB [36].

4.3. Acid phosphatase activity of PSB

In the present study the optimum acid phosphatase production of *Sphingomonas paucimobilis* and *B. subtilis* was observed at 72h of incubation whereas *Kocuria kristinae* and *B. megaterium* showed their maximum activity for the production of acid phosphatase at 96h of incubation. [5] observed the optimum acid phosphatase activity at 48 h of incubation (76.808 U/ml) by *Serratia* sp.

4.4. Optimization of growth conditions for acid phosphatase activity of PSB

Acid phosphatase production is greatly influenced by growth parameters such as pH, temperature, carbon source and nitrogen source. In order to optimize the crude enzyme production, there is a need to evaluate optimum conditions of these parameters under laboratory conditions. For the production of cost-effective industrial enzymes one of the alternative ways is to use low-cost substrate. The amount of enzyme produced by each substrate differs depending on the type of carbon and nitrogen source used by organisms. In the present findings, the bacterial isolates *B. subtilis*, *Sphingomonas paucimobilis* and *Kocuria kristinae*, showed maximum acid phosphatase production in the medium supplemented with glucose as a carbon source and ammonium sulphate as nitrogen source whereas optimum production was found in medium having Mannitol as carbon source for *B. megaterium*.

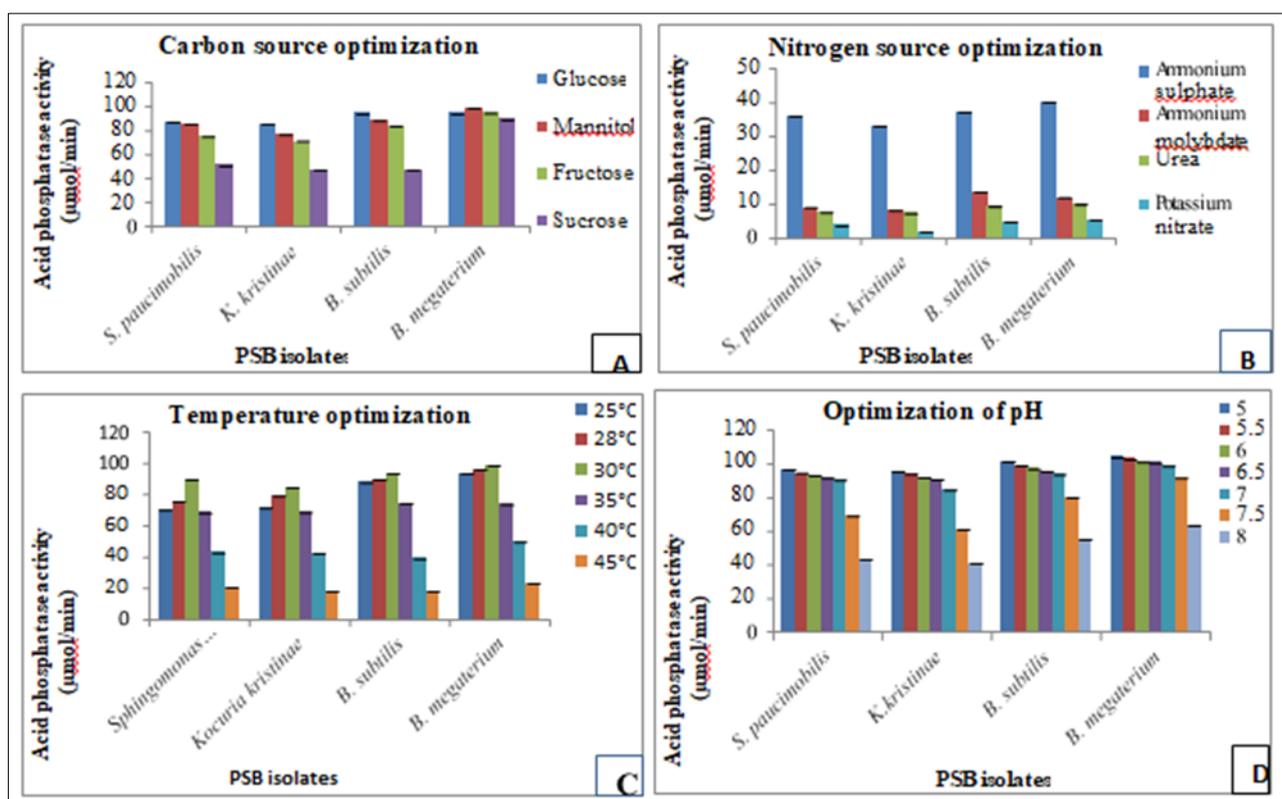


Figure 3 (A) Carbon source optimization for acid phosphatase activity (B) Nitrogen source optimization for acid phosphatase activity (C) Temperature optimization for acid phosphatase activity (D) pH Optimization for acid phosphatase activity

The effect of temperature on enzyme activity is a critical parameter which usually varies from organism to organism [18]. As production of extra cellular enzymes is influenced by temperature, their secretion is possibly influenced by changing the physical properties of the cell membrane [26]. In the present study, maximum acid phosphatase activity was observed at 30°C in the four isolates. Acid phosphatase activity in the same range was also reported by [5] and [13]. Increase or decrease in pH beyond the optimum value can affect the active site of the amino acids as the enzyme is unable to form an enzyme substrate complex and thus there is decrease in enzyme activity [30]. All the four PSB isolates under study were found to optimally synthesize acid phosphatase at pH 5.0 (Fig 3D). [5] Also reported maximum acid phosphatase activity at pH 5.0 by *Streptococcus equisimilis* and *Serratia marcescens* respectively. [29] Detected more acid phosphatase activity in acidic medium.

5. Conclusion

It is concluded from the above study that PSB like *Spingomonas paucimobilis*, *Kocuria kristinae*, *B. subtilis* and *B. megaterium* inhabit the saline rhizosphere soil of coastal Odisha. Due to phosphate solubilizing and acid phosphatase activity those bacteria can be used as bio- inoculants to increase soil fertility, hence reducing the application of chemical fertilizers.

Compliance with ethical standards

Acknowledgments

Authors are grateful to the funding agency DST, Govt. of Odisha for providing Biju Patnaik Research Fellowship. Authors are also thankful to the Department of Botany and Biotechnology, Ravenshaw University, Cuttack. Fund support under FIST program by DST Government of India to Department of Botany, Ravenshaw University, Cuttack is gratefully acknowledged.

Disclosure of conflict of interest

All authors have read, understand and agreed to the submission guidelines, policies and submission declaration of the journal. All authors have seen and approved the manuscript as submitted. All authors participated in the work in a substantive way and are prepared to take public responsibility for the work. All authors of the manuscript have no conflict of interests to declare. All the data taken from other sources is written in authors own language and properly cited. The text, illustrations, and any other materials included in the manuscript do not infringe upon any existing copyright or other rights of anyone.

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