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RESEARCH PAPER

Alkaline phosphatase activity of a phosphate solubilizing *Alcaligenes faecalis*, isolated from Mangrove soil

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Abstract Microorganisms are capable of converting insoluble phosphate into a bioavailable form through solubilization and mineralization processes. Hence in the present study a phosphate solubilizing bacterium, PSB-26, was isolated from mangrove of the Mahanadi delta using NBRIP-agar and NBRIP-BPB broth containing tricalcium phosphate as the phosphate source. Based on phenotypic and molecular characterization, the strain was identified as *Alcaligenes faecalis*. The maximum phosphate solubilizing activity of the strain was found to be 48 μg/ml with decrease in pH of the growth medium from 7.0 to 3.2. During phosphate solubilization, various organic acids, such as oxalic acid (289 mg/L), citric acid (0.2 mg/L), malic acid (0.3 mg/L), succinic acid (0.5 mg/L) and acetic acid (0.4 mg/L) produced in the broth culture were detected through HPLC analysis. Crude alkaline phosphatase activity of the strain was determined by *p*-nitrophenyl phosphate assay and optimized with different growth parameters to obtain maximum enzyme production. Under optimized sets of conditions, maximum alkaline phosphatase activity of 93.7 U/ml was observed. Partially purified alkaline phosphatase exhibited three protein bands of sizes approximately 45 kDa, 25 kDa and 17 kDa. Partially purified alkaline phosphatase during characterization showed maximum activity at pH 9.0 (96.53 U/ml), temperature of 45 °C (97.99 U/ml) and substrate concentration of 1.75 mg/ml (96.51 U/ml). The effect of the bacterium on growth of *Arabidopsis thaliana* plant showed that inoculation of bacterial culture exhibited better growth in comparison to the control. Hence the phosphate solubilizing and alkaline phosphatase production activity of the bacterium may have probable use for future biotechnological application.
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Introduction

Mangrove forests inhabit the tropical and subtropical regions of the world, thriving in the transitional regions between the land and the sea and offering a unique environment for diverse groups of organisms (Flores-Mireles, Winans, & Holguin, 2007). These ecosystems are characterized by periodic tidal flooding, which results in a highly saline soil profile, with variable levels of nutrients. Muddy mangrove soil has a strong capacity to absorb nitrates and insoluble phosphates carried out by the tides (Vazquez, Holguin, Puente, Lopez Cortes, & Bashan, 2000). Phosphorus usually precipitates in mangrove sediment due to its binding with various cations available in the interstitial water (De, Sarkar, Maity, Mukherjee, & Das, 2011). As a result, phosphorus becomes largely unavailable to plants which is detrimental as phosphorous is vital to plant growth, especially in nutrient-limited mangrove environments. The developments of stalk, stem strength, root, flower, seed formation, crop maturity and resistance to plant diseases are factors associated with phosphorus nutrition (Khan, Jilani, Akhtar, Naqvi, & Rasheed, 2009). Deficiency of soil P is one of the most important chemical factors restricting plant growth in soils. Phosphate solubilizing bacteria as potential suppliers of soluble phosphorus should confer a great advantage for plants through solubilization and mineralization (Hong, Geun, Mi, & Moon, 2006; Rodriguez & Fraga, 1999). Solubilization of mineral phosphate by phosphate solubilizing bacteria is generally associated with the release of low molecular weight organic acids (Goldstein, 1995). Their hydroxyl and carboxyl groups are able to form complexes with the iron and aluminium of corresponding phosphate compound in soil, thereby releasing bioavailable phosphate into the soil which can be utilized by plants (Gyaneshwar, Kumar, Pareka, & Podle, 2002). Solubilization of phosphate-rich compounds is also carried out by the action of a phosphatase enzyme called alkaline phosphatase (AlPase). In all bacteria, this enzyme catalyzes the hydrolysis of a wide variety of phosphomonoesters and catalyzes a transphosphorylation reaction by transferring the phosphoryl group to alcohol in the presence of certain phosphate acceptors (Coleman, 1992). Hence, application of phosphate solubilizing bacteria (PSB) with triple super phosphate can increase plant height, growth, yield, number of tillers and mineral nutrient content in tissues (Chen, 2006; Panhwar et al., 2013; Sarkar et al., 2012).

In recent years, different screening programs have been performed in saline habitats in order to isolate and characterize novel enzymatic activities with different properties to those of conventional enzymes. Besides being intrinsically stable and active at high salt concentrations, halophilic enzymes offer important opportunities in biotechnological applications, such as food processing, environmental bioremediation and biosynthetic processes. In this sense, the finding of novel enzymes showing optimal activities at various ranges of salt concentrations, temperatures and pH values is of great importance (Gomez & Steiner, 2004). With the above notions in consideration, this study focuses on phosphate-solubilizing bacterium which have been identified and characterized from saline mangrove soils of the Mahanadi river delta, Odisha, India. Further attempt has been made to purify and characterize the alkaline phosphatase enzyme produced by the bacterial isolates which may have potential biotechnological application. In addition, the efficiency of the isolated and identified bacterium was also evaluated in relation to its plant growth promotion.

Materials and methods

Isolation of PSB

Phosphate solubilizing bacteria were isolated from different locations of mangrove forest soil of the Mahanadi river delta by culturing strains on plates containing National Botanical Research Institute's phosphate agar, (NBRIP) contained L^{-1}: glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2{\cdot}6H_2O$, 5 g; $MgSO_4{\cdot}7H_2O$, 0.25 g; KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g; pH 7.0 (Nautiyal, 1999). Colonies of phosphate-solubilizing bacteria were recognized by the formation of clear halos around them. They were also screened for their ability to change the blue colour of NBRIP–bromophenol blue broth medium (pH 7.0) to colourless state by the formation of organic acid and lowering of pH (Mehta & Nautiyal, 2001).

Morphological and biochemical characterization of bacteria

Culture characteristics such as colony appearance, spore formation and motility of each strain were determined according to standard methods. Cell shape and size were determined by scanning electron microscopy (SEM) (Zeiss, Sigma). Several biochemical characteristics such as production of catalase, urease, indole, nitrate reduction, citrate utilization, acid-gas production from sugar, Voges–Proskauer (V–P) reaction, hydrolysis of tributyrin, tween-80, cholesterol, gelatine, casein, pectin and chitin etc. were also determined. The results were compared with Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974).

Molecular identification of bacteria

The 16S rRNA gene of the bacterial isolate was amplified using universal 27F forward primer (5′-AGGCCTAACACATGCAAGTC-3′) and 1492R reverse primer (5′-GGGCGGWGTGTACAAGGGC-3′) described by Das et al. (2014). PCR product of 16S rRNA gene was purified using QIA quick gel extraction kit (Qiagen GmbH, Germany) and nucleotide sequences were determined using the Big dye terminator v 3.1 cycle sequencing kit in an automated 3130xl 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 genetics analysis software (MEGA5.0; Tamura et al., 2011) using Clustal-W. The phylogenetic tree was prepared with the help of Neighbour Joining method and Kimura-2 as the model taking boot strap value as 1000.

Quantitative estimation of soluble phosphate

Erlenmeyer flasks containing 100 ml of NBRIP broth without bromo phenol blue were inoculated with bacterial culture in triplicate. Uninoculated medium served as a control. The flasks were incubated in a shaker at 30 °C up to 264 h at a shaking speed of 100 rpm. The pH of the culture medium was measured at specific time intervals. 5 ml of bacterial culture were collected at every 24 h and centrifuged at 10,000 rpm for 10 min. The supernatant was separated from the bacterial cells by successive filtration through Whatman No. 1 filter paper followed by a 0.22 μm millipore membrane and used to estimate phosphate release spectrophotometrically (880 nm) according to the method of Murphy and Riley (1962).

Organic acid production by the bacterium

Analysis of organic acids was carried out by inoculating the bacterial culture in 250 ml conical flask containing 50 ml of NBRIP broth. The flasks were incubated in an orbital shaker at 100 rpm and 30 °C for 144 h. One millilitre of incubated sample was centrifuged at 10,000 rpm (Mikro-200, Hettich Zentrifugen, Germany) for 15 min and filtered through 0.2 mm nylon membranes (Pall India Pvt. Ltd.) to obtain cell-free culture supernatant. 20 μl of filtered supernatant was injected for HPLC (LC-10AT, Shimadzu). The organic acids separation was carried out on an ion exclusion column, Aminex® HPX-87H, 300 mm × 7.8 mm (Bio- Rad Laboratories, Inc.) with 0.008 M H_2SO_4 as mobile phase at a constant flow rate of 0.6 ml/min and at operating temperature of 30 °C. The retention time of each signal was recorded at a wavelength of 210 nm (SPD 10A, Shimadzu) and compared with organic acid analysis standard kit (Bio-Rad Laboratories, Inc.) following the methods of Yadav, Gothwal, Nigam, Sinha-Roy, and Ghosh (2013).

Alkaline phosphatase assay

Extraction of the enzyme was carried out by inoculating 100 μl of bacterial culture in a 250 ml of conical flask containing 100 ml of sterilized NBRIP broth. The inoculated flasks were incubated up to 192 h at 37 °C. The samples were drawn at every 24 h and centrifuged at 10,000 rpm for 10 min at 4 °C. The cell-free supernatants were assayed for crude alkaline phosphatase activity following the method of Tabatabi and Bremner (1969). Alkaline phosphatases were assayed using *p*-nitrophenyl phosphate (pNPP), a colourless substrate that produces a colorimetric end-product *p*-nitrophenol (pNP) (yellow colour). 1 ml of bacterial cell free culture supernatant was mixed with 4 ml of modified universal buffer (pH 11.0). Further 1 ml of 0.025 mM disodium *p*-nitrophenyl phosphate (tetrahydrate) was mixed with the culture supernatant and incubated at 37° C for 1 h. One drop of toluene was added to stop the microbial growth. After 1 h of incubation, the reaction was stopped by adding 4 ml of 0.5 M NaOH and 1 ml of 0.5 M $CaCl_2$. The contents were filtered through Whatman No. 42 filter paper. The concentration of *p*-nitrophenol was measured in triplicates by taking the absorbance at 420 nm using UV–Vis spectrophotometer and values were extrapolated using a standard curve determined by using serially diluted solutions of *p*-nitrophenol as standard. One unit (U) of phosphatase activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol/ml/min from di-Na *p*-nitrophenyl phosphate (tetrahydrate) under the assay conditions.

Optimization of growth condition parameters for maximum crude alkaline phosphatase production

Optimization of growth condition for maximum crude alkaline phosphatase production was carried out by inoculating the bacterial isolates in NBRIP broth for 48 h under different conditions: pH 3.0–10.6; temperatures 25–65 °C at 10 °C intervals; shake rates of 0, 60, 80, 100 and 120 rpm; carbon sources such as lactose, sucrose, maltose and glucose and nitrogen sources such as ammonium molybdate, potassium nitrate, urea and ammonium sulfate.

Partial purification of crude alkaline phosphatase

For partial purification, the crude alkaline phosphatase was subjected to 70% of ammonium sulfate precipitation up to saturation followed by overnight dialysis at 4 °C. The molecular weight of the partially purified enzyme was carried out by SDS-PAGE, using 5% stacking gel and 10% resolving gel and electrophoresis was performed with a 15 mA fixed current (Laemmli, 1970; Sasirekha, Bedashree, & Champa, 2012). The gel was stained with coomassie brilliant blue R250 and destained with destaining solution (methanol:acetic acid:water: 30:10:10) for 8–10 h. Quantification of the protein contents of both crude and partially purified alkaline phosphatase was carried out following the method of Lowry, Rosebrough, Farr, and Randall (1951), with bovine serum albumin as a standard (Sigma, Germany).

Characterization of partially purified alkaline phosphatase

After partial purification, the purified enzyme was characterized with different parameters, such as pH from 3 to 10.6 by adjusting the pH of the buffer, temperature at 10 °C intervals from 25 to 65 °C and different substrate concentration of 0.5–2.5 mg ml^{-1} of *p*-nitrophenyl phosphate following the method of Tabatabi and Bremner (1969).

Effect of phosphate solubilizing bacteria on plant growth promotion

The effect of phosphate solubilizing bacteria on the growth of *A. thaliana* (Col-0) was studied by growing the surface sterilized seeds on a planton tissue culture container (7.5 mm × 7.5 mm × 10 mm) containing 40 ml of Murashige and Skoog (MS) agar medium (Murashige & Skoog, 1962). Three seeds were taken in each planton with three replicas for each observation. The original MS media was kept as the positive control (MS media + sterile seed) where as in the test; the original soluble P source (KH_2PO_4) was replaced with insoluble tricalcium phosphate (TCP) and the modified medium was named as modified MS + TCP media. Overnight grown bacterial culture (10 μl) were inoculated

with the modified MS + TCP media containing sterilized seeds of *A. thaliana* and considered as test planton. The MS + TCP media inoculated with sterilized seed (modified MS + TCP media + sterile seed) without bacterial inoculation was considered as the negative control. All test plantons, positive control plantons and negative control plantons were incubated at 4 °C for 2–3 days for cold stratification. After stratification, all the plantons were placed into the long day (16 h day and 8 h night) light cabinet (J.K.G. Bioscience Pvt. Ltd., Ohio) and kept at 22 °C and humidity of 65% for 30 days.

Statistical analysis

ANOVA for multiple comparisons was performed using Graph Pad Prism version 5.01. A p-value $\leq$0.05 was considered to be significant.

Results

Isolation and identification of phosphate solubilizing bacteria

Forty-eight phosphate solubilizing bacteria (PSB-1–PSB-48) isolated in terms of formation of halo zones on NBRIP-agar medium and change of the intensity of the colour of bromophenol blue of NBRIP-BPB broth medium. Out of the 48 bacterial isolates, PSB-26 showed the maximum halo zone (1.7 cm) on NBRIP-agar medium as well as a maximum colour change of the bromophenol blue (1.188 O.D.) of the NBRIP-BPB broth medium. Thus, the bacterium PSB-26 was selected as the most efficient phosphate solubilizing bacterium and used for further study. Based on the morphological and biochemical analysis, the bacterium PSB-26 was found to be rod-shaped with cell diameter 0.99 μm, positive for motility, aerobic growth, catalase, citrate utilization, oxidase, where as negative for gram staining, spore, urease, MRVP, indole, nitrate, protease, lipase, acid and gas production, fimbriae, starch and biofilm production test, hence tentatively assigned to the genus *Alcaligenes*. Further confirmation of genus *Alcaligenes* was achieved by BLAST analysis data of the 16S rRNA gene sequence which showed 100% similarity with the genus *Alcaligenes*. The gene bank accession number of the strain is KR632642. A phylogenetic tree was constructed by comparing the nucleotide sequences of 16S rRNA sequences of the isolate, PSB-26 with different *Alcaligenes species*, submitted to the NCBI database. We found that our isolate is most closely related to *Alcaligenes faecalis* subsp. *phenolicus* (Fig. 1).

Solubilization of tricalcium phosphate

The phosphate solubilizing ability of the strain increased from 0.0 μg/ml to 48 μg/ml as the pH decreased from 7.0 to 3.2. The pH of the medium decreased steadily after 48 h of incubation and increased again after 144 h of incubation. The maximum phosphate solubilization was observed at 144 h of incubation with a maximum drop in pH (3.2) of the medium (Fig. 2).

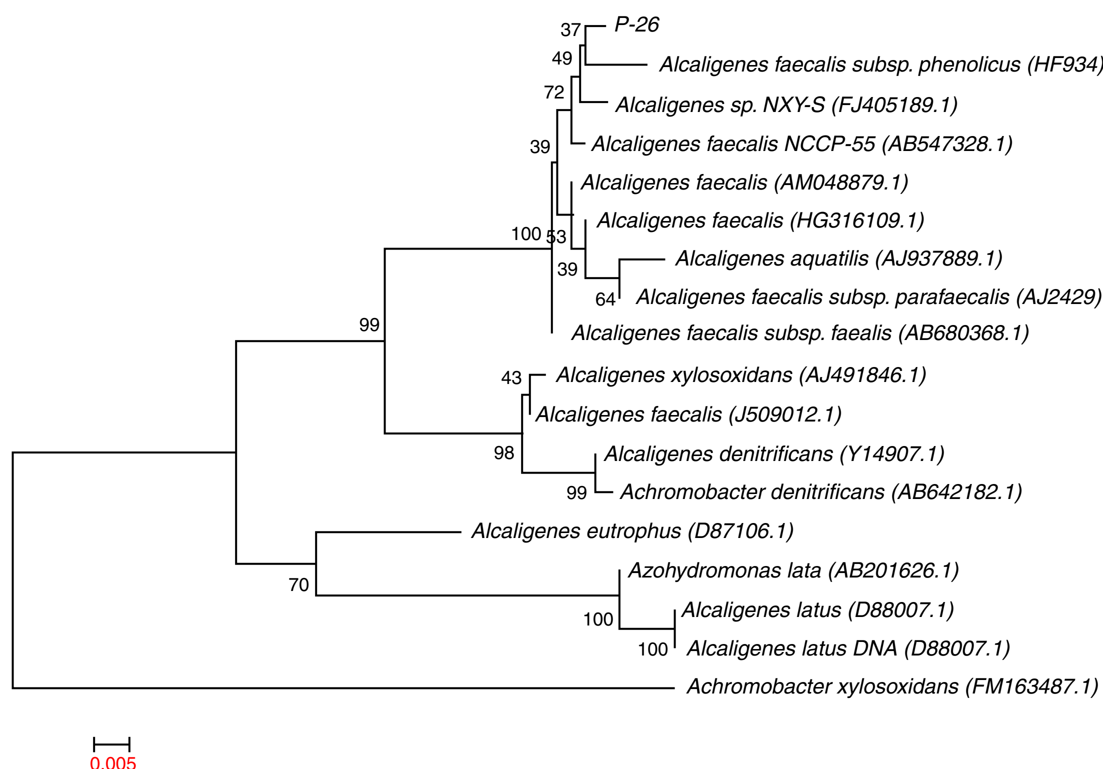

Figure 1 Phylogenetic tree based on 16S rRNA gene sequences by Neighbour Joining method (using MEGA 6.0), showing the relationship between strain PSB-26 and other members of the *Alcaligenes* sp. The Genbank nucleotide accession numbers are listed next to the strain names. The scale bars represent 0.005 substitution/site.

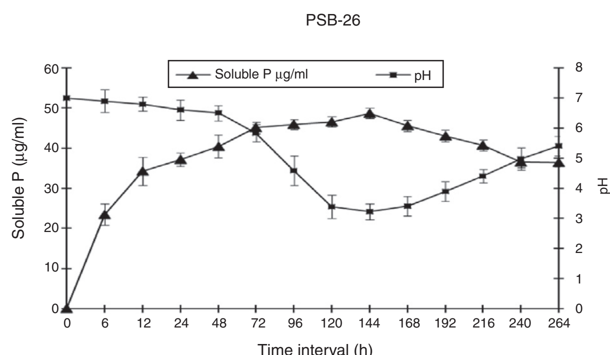

Figure 2 Effect of incubation period on tri-calcium phosphate solubilization and on drop of pH of the NBRIP broth medium by the bacterial isolates PSB-26.

Detection of organic acid

Five different organic acids were detected from the culture medium of the isolate, PSB-26 (Fig. 3) including three unknown peaks (5.5, 8.2 and 10.1). Out of the five different organic acids, oxalic acid was produced in largest quantity (289 mg/L), followed by succinic acid (0.5 mg/L), acetic acid (0.4 mg/L), malic acid (0.3 mg/L) and citric acid (0.2 mg/L).

Alkaline phosphatase production

Maximum alkaline phosphatase production (71.531 U/ml) by the bacterial isolates was recorded at 48 h of incubation and decreased upon further incubation up to 192 h (data not shown). The NBRIP broth medium without inoculation did not exhibit any presence of the enzyme.

Optimization of growth condition parameters for maximum of alkaline phosphatase production

The effect of the incubation period on crude alkaline phosphatase production showed that maximum enzyme production (71.531 U/ml, $p < 0.001$) was observed after 48 h of incubation. Effect of pH on alkaline phosphatase showed a gradual increase in enzyme production with increase in pH from 5.0 to 9.0 (82.91 U/ml, $p < 0.001$) and decreased thereafter (Fig. 4a). In shaken cultures, 45 °C was found to be the optimum temperature for maximum alkaline phosphatase production (84.99 U/ml, $p < 0.001$) (Fig. 4b). Fig. 4c shows that highest alkaline phosphatase production was obtained (87.085 U/ml) when agitated at 100 rpm. It was observed that among the various carbon supplements, the medium containing glucose yielded maximum alkaline phosphatase production (87.66 U/ml, $p < 0.001$) followed by lactose, sucrose and maltose with alkaline phosphatase value of 69 U/ml, 62.23 U/ml and 45.83 U/ml respectively (Fig. 4d). It was observed that maximum production of alkaline phosphatase occurred (88.66 U/ml, $p < 0.001$) when ammonium sulphate was supplied to the medium followed by potassium nitrate (66.16 U/ml), urea (49.5 U/ml) and ammonium molybdate (34.83 U/ml) respectively (Fig. 4e).

Under optimized sets of condition (incubation period 48 h, initial pH of 9.0, growth temperature of 45 °C, shaking velocity of 100 rpm, glucose as a carbon source and ammonium sulphate as a nitrogen source), the bacterial isolate showed the maximum alkaline phosphatase production of 93.7 U/ml.

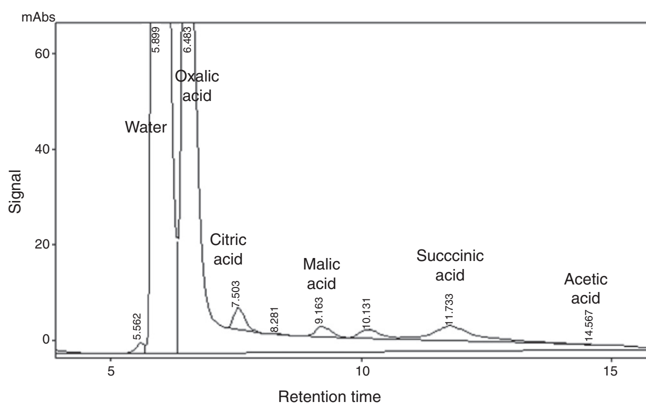

Figure 3 HPLC analysis of organic acids detected in the culture supernatant of isolate PSB-26 at 144 h of incubation in NBRIP broth. The corresponding peaks detected in culture medium were of oxalic, citric, malic, succinic and acetic acids including three unknown peaks (5.5, 8.2 and 10.1).

Partial purification of alkaline phosphatase

After partial purification, the enzyme showed band sizes of approximately 45 kDa, 25 kDa and 17 kDa on SDS-PAGE (Fig. 5a). Results showed that the partially purified alkaline phosphatase from the bacterial isolate, PSB-26 exhibited protein content of 6 mg/ml with a specific activity of 16.33 U/mg which corresponds to 1.82-fold purification and 41.09% yield (Table 1).

Characterization of partially purified alkaline phosphatase

The activity of partially purified alkaline phosphatase was checked with different parametric conditions such as in different pH (3.0–10.6), temperature 25–65 °C and substrate concentration ranged from 0.5 to 2.5 mg/ml. The maximum alkaline phosphatase activity of the bacterial isolate, PSB-26 was recorded at pH 9.0 (96.53 U/ml, $p < 0.001$) (Fig. 5b). The optimum temperature for enzyme activity was found to be 45 °C with maximum enzyme activity of 97.99 U/ml, $p < 0.001$ (Fig. 5c). It has been observed that the enzyme activity increased with increasing in substrate concentration up to 2 mg/ml of *p*-nitrophenyl phosphate concentration with a maximum activity of 96.51 U/ml, $p < 0.001$ (V_{max} value 213.70 ± 42.43 and K_m value 2.61 ± 0.87) and remained constant thereafter (Fig. 5d).

Effect of phosphate solubilizing bacteria on *Arabidopsis thaliana* plant growth

Comparative analysis of vegetative and reproductive plant growth patterns i.e., the date of sowing, date of germination, number of plants germinated, number of leaves, length of root, length of shoot and number of flower were

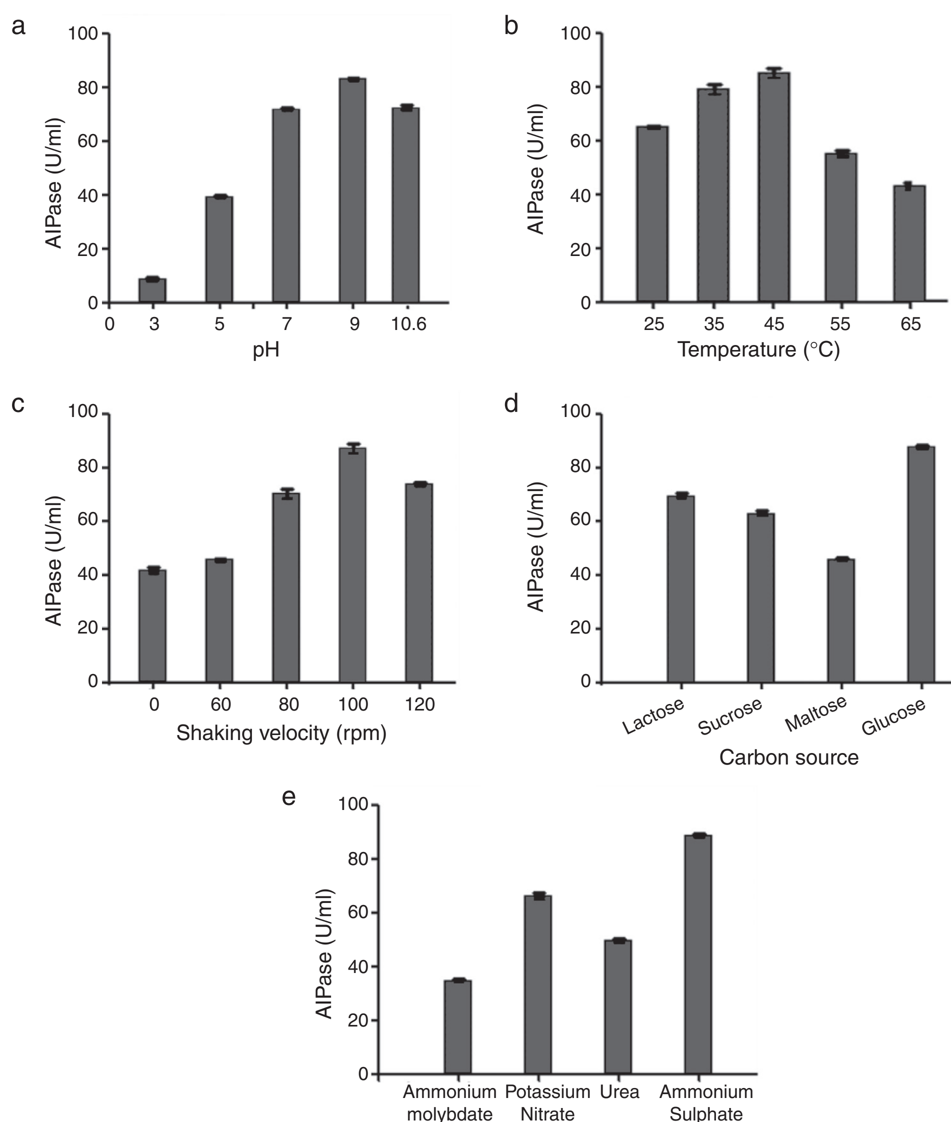

Figure 4 Effect of different growth parameters such as (a) pH, (b) temperature, (c) shaking velocity, (d) carbon sources and (e) nitrogen sources on crude alkaline phosphatase production by the isolate, PSB-26.

recorded in triplicate from germination of seeds up to maturation of the plant. Plant development data were collected and presented in Table 2. Minimum shoot/root ratio was observed in the negative control planton (modified MS + TCP media + sterile seed without bacterial inoculation), which showed very stunted growth (Fig. 6a) in comparison to the test (modified MS + TCP media + sterile seed + 10 μl bacterial inoculation) (Fig. 6c) and positive control planton (MS media + sterile seed without bacterial inoculation) (Fig. 6b). Higher plant growth was observed in the planton containing modified MS + TCP media inoculated with bacterial culture (Fig. 6c).

Table 1 Partial purification of crude alkaline phosphatase.

Isolates PSB-26	Total volume (ml)	Protein concentration (mg/ml)	Total protein content (mg)	AlPase activity (U/ml)	Total AlPase activity (U)	Specific activity (U/mg)	Fold of purification	Total yield (%)
Crude extract	50	8.0	400	71.53	3576.55	8.94	1.0	100%
70% $(NH_4)_2SO_4$ precipitation and dialysis	15	6.0	90.0	7.99	1469.85	16.33	1.82	41.09%

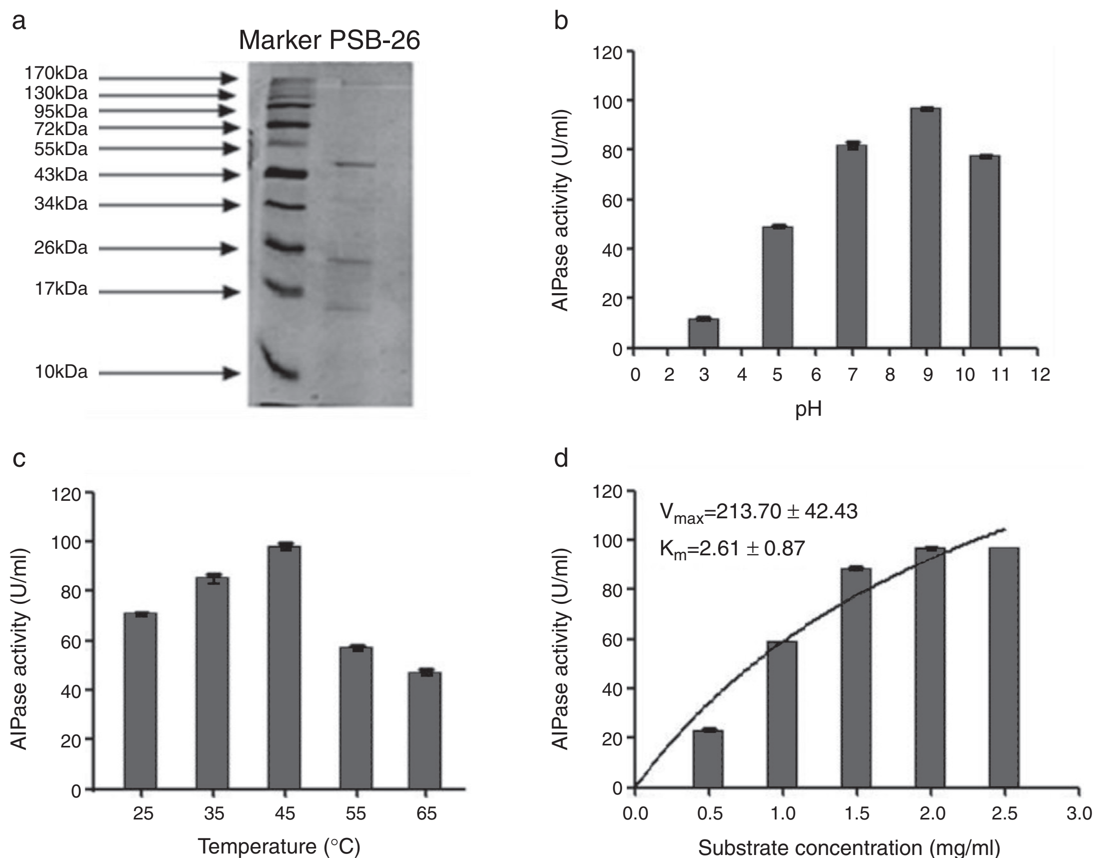

Figure 5 (a) SDS-PAGE of partially purified AlPase from PSB-26 (proteins were visualized with Coomassie blue staining. Lanes 1: molecular weight standards; Lane 2: cell supernatant after partial purification), (b) effect of pH, (c) temperature, (d) substrate concentration on activity of partially purified alkaline phosphatase from PSB-26.

Discussion

Phosphate solubilizing bacteria forming halo zones were isolated from mangrove soil of Mahanadi river delta using NBRIP-agar medium. However, the result of the halo based technique is not always reliable. Many isolates which did not produce any visible halo zones on agar plate could also solubilize various types of insoluble inorganic phosphate in liquid medium (Das, 1963). This may be because of various diffusion rates of different organic acids secreted by an organism (Johnson, 1959). Therefore, phosphate solubilizing bacteria were further screened in NBRIP-BPB broth medium to evaluate their phosphate solubilizing efficiency. NBRIP medium contains bromophenol blue (BPB), a pH indicator dye which changes its colour due to the decrease in pH of the medium. Hence, the phosphate solubilizing efficiency can be easily screened based on this visual observation (Mehta & Nautiyal, 2001).

From biochemical tests and phylogenetic analysis based on partial 16S rRNA gene sequences, it is determined that the bacterial isolate, PSB-26, shares 100% homology with *A. faecalis*. The detection of *A. faecalis* in mangrove soil from the Mahanadi river delta, Odisha is a novel observation.

The bacterial isolate, PSB-26, exhibited maximum phosphate solubilization ability of 48.70 μg/ml after 144 h of incubation. Phosphate solubilization efficiencies in lower and higher range were also reported from bacteria isolated from other mangrove ecosystems. Phosphate solubilizing

Table 2 Effect of *A. faecalis* on plant growth.

Plant specification	Number of leafs	Number of flowers	Length of shoot (cm)	Length of root (cm)	Shoot/root
MS media + plant	6 ± 1.41	4 ± 1.41	6.76 ± 0.52	3.16 ± 0.11	2.14
(Modified MS + TCP media) + plant	4 ± 0	0	0.42 ± 0.11	0.25 ± 0.05	1.67
(Modified MS + TCP media) + plant + 10 μl bacteria	7 ± 1.41	30 ± 4.24	15.11 ± 1.46	1.64 ± 0.08	9.24

Modified MS + TCP media: MS media in which KH_2PO_4 replaced with TCP.

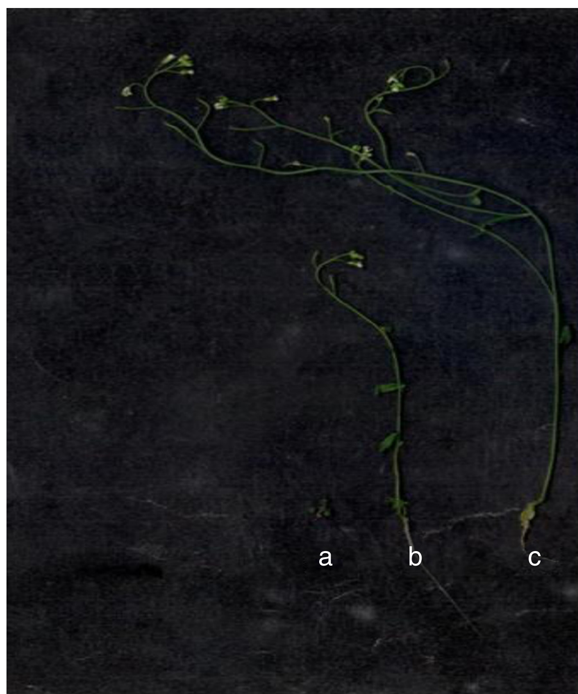

Figure 6 Effect of *A. faecalis* on *A. thaliana* plant growth promotion. The plantons are (a) modified MSTCP media + plant without bacterial inoculation as negative control, (b) MS media + plant without bacterial inoculation as positive control, (c) modified MSTCP media + plant with 10 μl of bacterial inoculation as test planton.

bacteria, IPSB *Vibrio* sp. and *Pseudomonas* sp. isolated from Cochin mangrove of India, could solubilize 0.55 μg/ml of phosphate (Pramod & Dhevendaran, 1987). Kathiresan and Selvam (2006) reported 24 phosphate solubilizing bacteria from mangrove soils of Vellar estuary at Parangipettai, southeastern coast of India, with phosphate solubilizing efficiency in the range of 0.012—0.141 μg/ml. Much higher phosphate solubilizing activity (400 μg/ml) was also reported by the bacterial population in an arid mangrove ecosystem in Mexico (Vazquez et al., 2000). Similarly, seven bacterial sp. such as, two *Bacillus subtilis*, three *Pseudomonas* sp. and two *Azotobacter* sp. isolated from mangrove soil of Chollangi, East Godavari exhibited solubilizing activity of 80—100 μg/ml of phosphate (Audipudi, Pradeep Kumar, & Sudhir, 2012).

In the present study, the isolate PSB-26, induced 3.5 to 4 units of decrease in pH of the medium which is similar to the findings of Perez, Sulbarn, Maria Ball, and Yarzabal (2007), who reported 3.2—4 units decrease in pH of the medium during phosphate solubilization. Again the rise in pH could be due to the utilization of organic acids or the production of alkaline compounds (Abusham, Rahman, Salleh, & Basri, 2009). A similar inverse relationship between pH and soluble phosphate has been previously observed (Illmer & Schinner, 1995).

For the solubilization of inorganic phosphate, application of oxalic acid has been proven to be effective, which is more capable of solubilizing inorganic phosphate than other organic acids (Wei, Chen, & Xu, 2009). Strong binding abilities of oxalic and citric acids have led to these acids being established as the most competent agents to solubilize soil P (Jones, 1998). In the present study HPLC analysis showed that a much higher amount of oxalic acid was observed in the broth culture of the *A. faecalis* than other organic acids (citric acid, malic acid, succinic acid, lactic acid, acetic acids) (Fig. 3).

Additionally, we observed that maximum production of alkaline phosphatase (71.531 U/ml) by *A. faecalis* occurred after 48 h period of incubation. A significant amount of phosphatase activity has also been reported earlier from different bacterial genera (Prasanna, Joshi, Rana, Shivay, & Nain, 2011; Walpola & Yoon, 2013).

In order to evaluate the maximum crude enzyme production, there is a need to evaluate the optimal growth conditions required. The optimum pH for most aquatic bacteria is between pH 7 and 8.5 (Padan, Bibi, Ito, & Krulwich, 2005). This corresponds to the pH range of most of the water bodies. The bacterium under study was found to grow best and synthesize alkaline phosphatase at pH 9.0. Temperature is a critical parameter which needs to be controlled and this usually varies from organism to organism (Kumar & Takagi, 1999). The temperature for growth and maximum phosphatase production for *A. faecalis* was found to be optimum at 45 °C. In the present study optimal alkaline phosphatase production for *A. faecalis* was obtained at 100 rpm and variation in agitation or shaking speed has been found to influence the extent of enzyme production (Nascimento & Martins, 2004). The use of low-cost substrates for the production of industrial enzymes is one of the ways to reduce significant production costs. The amount of enzymes produced by each substrate differs depending on the type of carbon and nitrogen source utilized by the organisms. Investigations on the impact of carbon and nitrogen supplements revealed that not all carbon and nitrogen sources would act as an enhancer for the production of enzymes. In the present finding, *A. faecalis* showed maximum alkaline phosphatase production in medium supplemented with glucose as a carbon source and ammonium sulfate as nitrogen source. In the present study, optimization was carried out using different parameters with consideration of the previous optimized parametric conditions established to maximum enzyme production. Hence final optimization was carried out by using all of the optimized conditions which exhibited maximum enzyme production and it has been observed that under optimized sets of conditions higher crude enzyme production was achieved in comparison to only using the individual parameters.

After partial purification, the enzyme was purified up to 1.82 fold with a yield and specific activity of 41.09% and 16.33 U/mg respectively. Our finding is lower than the findings of Poirier and Holt (1983), who recorded a 64% yield of alkaline phosphatase after partial purification from *Capnocytophaga ochracea*. González et al. (1994) also observed 48.8% yield and of 108.3 U/mg specific activity of purified alkaline phosphatase from *Myxococcus coralloides D*.

The SDS-PAGE analysis of the partially purified alkaline phosphatase of *A. faecalis* showed three consecutive bands of approximately 45 kDa, 25 kDa and 17 kDa. Our results are in accordance with the finding. Alkaline phosphatase of band size 40 kDa and 46 kDa has also been reported from *Escherichia coli* and *Bacillus intermedius* respectively (Bradshaw et al., 1981; Sharipova et al., 1998).

In the present study purified alkaline phosphatase activity of *A. faecalis* was characterized with different parameters such as pH, temperature and substrate concentrations. The highest alkaline phosphatase activity by *A. faecalis* was obtained at pH 9.0. Maximum alkaline phosphatase activity at pH 9.0 was also reported from *Micrococcus sodonensis* (Glew & Heath, 1971). An increase in temperature accelerates the velocity of an enzyme-catalyzed reaction until an optimum is reached, after which the velocity decreases and finally results in the denaturation of the enzyme. In the present study, the highest alkaline phosphatase activities were observed at 45 °C. The effect of temperature on enzyme activity varies from organism to organism. González, Esther Fárez-Vidal, Arias, and Montoya (1994) reported that 37 °C was the optimum temperature for the alkaline phosphatase activity, purified from *Myxococcus coralloides D.* whereas alkaline phosphatase purified from *Pseudomonas aeruginosa* exhibited its optimum activity at 41 °C temperature (Day & Ingram, 1973). The effect of different pNPP (substrate) concentrations (0.5–2.5 mg/ml) on alkaline phosphatase activity revealed that alkaline phosphatase activity was increased with an increase in substrate concentration up to 2 mg/ml. Further, the activity was found to be constant at higher concentrations. The present study was also in agreement with studies reported by Ul qader, Iqbal, and Niazi (2009).

The effect of *A. faecalis* on the growth of *Arabidopsis thaliana* plant revealed that in comparison to positive and negative controls the test planton inoculated with *A. faecalis* (modified MS + TCP media + sterile seed + 10 μl of bacterial culture) showed better vegetative growth, hence can be used as a beneficial and efficiency boosting microorganism in organic agriculture. Also there are several reports published earlier on the application of *A. faecalis* as an efficient plant growth promoter which accelerates significant growth of plant height, higher yield, more numbers of leaves, higher numbers of pods, better auxin production, antagonistic activity against plant pathogen etc. (Nandini, Preethi, & Earaana, 2014; Sayyed, Gangurde, Patel, Joshi, & Chincholker, 2010).

Conclusion

The above study provides evidence for the existence of agriculturally important bacteria in saline and relatively unexplored mangrove environment which will likely aid taxonomists, enzymologists and even some agriculturalists in their own research. Our observation that the bacterium *A. faecalis* initially promoted plant growth in vitro is interesting as the species is known to act as human pathogens, indicating that a bacterium may not strictly be a pathogen in all contexts (Egamberdieva et al., 2008). Although, the bacterium is isolated from mangrove soil sample and reported to cause sepsis, peritonitis, meningitis and chronic suppurative otitis earlier but this organism was also reported as harmless saprophyte in the alimentary tract in 5%–19% of the normal population (Kavuncuoglu et al., 2010; Tena, Fernandez, & Lago, 2015). From the biochemical characterization, it is also clear that the strain *A. faecalis* (PBS-26) is non-pathogenic in nature due to negative for virulence factors such as fimbriae, protease, gelatinase, amylase, lipase and biofilm formation ability which are necessary for establishment of infection, host-pathogen interaction and invasion; however, a more detailed analysis involving its virulence potential is required prior to its application in agriculture. Moreover, due to the phosphate solubilizing ability, alkaline phosphatase production and plan growth promotion activity of the bacterium, it may have a promising use as a bio-inoculants to increase soil fertility by minimizing fertilizer application which can promote sustainable agriculture to help meet future needs.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biori.2017.01.003.

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