Phosphate solubilization mechanisms in alkaliphilic bacterium *Bacillus marisflavi* FA7

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This study reports the mechanisms of phosphate solubilization present in alkaliphilic *Bacillus marisflavi* FA7. The strain obtained from sediment samples of mangrove ecosystem exhibited different mechanisms to solubilize inorganic phosphate and mineralize organic phosphate under alkaline conditions. It reduced the pH of the medium that showed near perfect correlation with tri-calcium phosphate solubilization. Organic acids produced by the strain were detected in broth. Maximum decrease in pH of the medium was observed with NH_4Cl as an inorganic nitrogen source. This indicated involvement of proton extrusion mechanism toward phosphate solubilization during ammonium uptake. It produced exopolysaccharide, but failed to produce siderophore. *Bacillus marisflavi* FA7 produced extracellular alkaline phosphatase having molecular weight of 175–200 kDa. The pH optimum for maximum enzyme activity was 10.1 and K_m of 1.13 μM *p*-nitrophenolphosphate. This is the first study to report the highest tri-calcium phosphate solubilization by an alkaliphilic bacterium.

Keywords: Alkaliphilic bacterium, *Bacillus*, mechanism, phosphate solubilization, phosphatase.

PHOSPHORUS, one of the major essential macronutrients required for plant growth, is taken up by plants as an orthophosphate ion[1]. Phosphate containing compounds present in soil can be classified into three groups, viz. soluble orthophosphate, insoluble inorganic phosphate and insoluble organic phosphate. Orthophosphate ions react with numerous organic and inorganic constituents of soil and therefore become least mobile and unavailable for uptake by plants. Hence, a large quantity of phosphate fertilizer is applied to achieve maximum plant productivity. However, it is observed that orthophosphate ions of the applied fertilizer react with cations of the soil to precipitate into inorganic tri-calcium phosphate, iron phosphate and aluminium phosphate. Phosphorus containing organic compounds enter the soil by decomposition of plant, animal and microbial matter. Different forms of organic phosphorus compounds include nucleic acids, phospholipids, phosphoproteins, inositol phosphates, phosphonates, etc.[2]. These forms are not taken up by plants due to their high molecular weight. Transformation of phosphate from orthophosphate ions into inorganic or organic phosphate occurs in a cyclic form.

The global cycling of insoluble inorganic and organic soil phosphates is attributed to microorganisms[3]. Several mechanisms responsible for solubilization of inorganic phosphates and mineralization of organic phosphates have been reported[4–15]. Organic acid production is the most reported mechanism for solubilization of inorganic phosphate[4,5]. Production of sulphuric, nitric and carbonic acids has been reported to solubilize inorganic phosphate[6]. Acidification of the medium as a result of H^+ excretion originating from NH_4^+ has been suggested as an alternate mechanism of inorganic phosphate solubilization[7,8]. The high molecular weight microbial exopolysaccharides have been shown to play an indirect role in phosphate solubilization[9,10]. The rate of dissolution due to exopolysaccharide appears to be dependent on the microbial source and concentration of polymer. Since siderophores chelate iron, it has been attempted to correlate the amount of siderophore released to the amount of phosphate solubilized from insoluble iron phosphates[11–13]. However, siderophore production has not been widely implicated as a phosphate solubilization mechanism. Other postulated mechanisms include production of hydrogen sulphide by sulphur reducing bacteria. H_2S reacts with phosphate containing minerals, thus releasing phosphate[14]. Organic phosphate solubilization is called mineralization of organic phosphorus[15]. It occurs due to production of extracellular or membrane bound enzymes like phosphatase, phytase, phosphonatase, and C–P lyase by microorganisms in soil. Phosphatase enzymes render high molecular weight organic phosphate into low molecular weight compounds by the hydrolysis of ester-phosphate bonds, leading to the release of phosphate ions. Based on their pH optima, these enzymes are classified as acid, neutral and alkaline phosphatase[16,17]. Phytase enzyme hydrolyses phytic acid or myo-inositolphosphate compounds. Phosphonatase and C–P lyase hydrolyse ester bonds of phosphonates (e.g. phosphoenol pyruvate, phosphonoacetate) and converts phosphonates into hydrocarbons and phosphate ions for assimilation[18].

Phosphate solubilizing bacterial bio-fertilizers have been developed for plants of agriculture, horticulture and forestry importance. These bio-fertilizers work best in

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neutrophilic soil conditions. Due to extensive agricultural practices, soil becomes alkaline and saline. In India, 7 million hectares of land is approximately alkaline and saline, most of which occurs in the Indo-Gangetic plane[19]. Alkalinity and salinity increase the precipitation of available soluble phosphates. With a growing world population, there is limited opportunity for opening up new land for agriculture. In order to combat alkalinity and salinity, farmers resort to mulching. However, over a period of time, repeated use of mulching adds salt to the already alkaline–saline soil, making it unsuitable for agriculture. Alkalinity and salinity limit the yield and profits of agricultural crops due to osmotic effects, toxicity of salt ions and the changes in the physical and chemical properties of soil[20,21]. Over the years, farmers have been successful in cultivating salt-tolerant crops[22]. For such crops, bio-fertilizers have not been formulated. Therefore, alkaliphilic bacteria which can thrive in alkaline saline environment and are also capable of solubilizing phosphate are useful in increasing the fertility of alkaline and saline soils. Such bacteria may be found in ecosystems that are exposed to different levels of alkalinity and salinity. Attempts have been made to characterize halotolerant and alkalitolerant phosphate solubilizing bacteria[23,24]. However, data regarding alkaliphilic bacteria in phosphate solubilization and the mechanisms involved is lacking. This study aims at investigating the possible mechanisms employed by alkaliphilic bacteria for phosphate solubilization under alkaline conditions.

Materials and methods

Bacterial strain and growth medium

Alkaliphilic bacterium used in this study was isolated from sediment samples of mangrove ecosystem located in Quellossim, Goa, India. The medium used for isolation and maintenance of this strain was polypeptone yeast extract glucose agar (PPYG)[25] (composition in g/l: peptone: 5.0, yeast extract: 1.5, glucose: 5.0, Na_2HPO_4: 1.5, NaCl: 10.5, $MgCl_2$: 0.1, Na_2CO_3: 5.0, Agar: 15, pH: 10.5). It was identified as *Bacillus marisflavi* FA7 based on morphological, cultural, biochemical and 16S rRNA gene sequence (GenBank Accession Number KX098478).

Inoculum development

Bacillus marisflavi FA7 was inoculated in modified Pikovskaya's broth[26] (composition in g/l: glucose: 10.0, $(NH_4)_2SO_4$: 0.5, KCl: 0.2, NaCl: 10.5, $MgSO_4$: 0.1, $MnSO_4$: 0.0001, $FeSO_4$: 0.0001, yeast extract: 0.5, pH: 10.0). The culture broth was incubated on 150 rpm shaker at 30°C till the optical density at 600 nm reached 0.700. This was used to inoculate media in experiments at 1% (v/v). For time course, flasks were inoculated with 5% of inoculum.

Tri-calcium phosphate solubilization in shaker flask

Bacillus marisflavi FA7 was inoculated into 50 ml modified Pikovskaya's broth containing tri-calcium phosphate (5.0 g/l). Culture flasks were incubated at 30°C in a rotary shaker at 150 rpm. After the desired incubation time, the culture broth was analysed to determine pH, solubilized phosphate and biomass for five days.

Mechanisms of inorganic phosphate solubilization

Cell-free culture broth of day 1 and day 5 from time course experiment was analysed for the presence of organic acids by high performance liquid chromatography (HPLC)[27].

The effect of different inorganic nitrogen sources on growth and solubilization of tri-calcium phosphate was studied by inoculating *Bacillus marisflavi* FA7 into 50 ml modified Pikovskaya's broth amended with different nitrogen salts (0.5 g/l), viz. NH_4Cl, $(NH_4)_2SO_4$, $NaNO_3$, KNO_3 and NH_4NO_3. Inoculated flasks were incubated under 150 rpm shaker condition at 30°C for 48 h. Final pH, solubilized phosphate and biomass were estimated.

Siderophore production was detected by the standard Chrome Azurol-S (CAS) plate assay at pH 10 (ref. 28). *Bacillus marisflavi* FA7 was spot inoculated on to the medium and incubated at 30°C for 24 h. Development of orange to yellow halo around the colony indicated production of siderophores.

Exopolysaccharide (EPS) production by the strain was evaluated by spot inoculating it on to Congo red nutrient agar containing 5% sucrose[29]. Development of brown colouration around the colony indicates EPS production.

Mechanism of organic phosphate solubilization

Bacillus marisflavi FA7 was inoculated in mineral salts medium (MSM) (composition in g/l: $NaNO_3$: 2.0, $MgSO_4$: 0.2, $MnSO_4$: 0.02, $CaCl_2$: 0.02, $FeSO_4$: 0.02, glucose: 5, pH: 10.0) containing disodium-*p*-nitrophenyl phosphate (*p*-NPP, 1 mM) as the sole source of phosphate[30]. The inoculated flask was incubated at 150 rpm, 30°C for 24 h. Protein content and phosphatase enzyme activity were determined in the cell-free culture broth.

Characterization of alkaline phosphatase

Chilled acetone was added to the cell-free culture broth in the ratio of 1 : 3 (supernatant : acetone) and the

precipitate obtained was collected by centrifugation at 10,000 rpm for 15 min at 4°C. Precipitate was dissolved in 1.5 ml of carbonate–bicarbonate buffer (0.1 M, pH 10.1). This crude enzyme extract was used for characterization.

Factors affecting phosphatase enzyme activity: Different concentrations of substrate p-NPP were used to determine kinetic parameters K_m and V_{max}. Enzyme activity was assayed at different alkaline pH (8.8, 9.1, 9.6, 10.1, 10.6 and 10.8). To study the effect of sodium ions on enzyme activity, carbonate–bicarbonate buffer of different molarity (0.01–2 M) was used. Effect of various additives like metal ions [Mg^{+2}, Zn^{+2} (1 mM)], detergents [sodium dodecyl sulphate (SDS) (1 mg/ml), Triton (0.10%)], chelating agent [ethylenediaminetetraacetic acid (EDTA) (1 mM)], and denaturing agents [urea (0.5 mM), β-mercaptoethanol (1 mM)] was studied on activity enzyme.

Native polyacrylamide gel electrophoresis (PAGE) and zymography: Native PAGE of crude enzyme extract was carried out by a method developed by Laemmli[31]. Crude enzyme concentrate was mixed with sample loading *Tris* buffer (62.5 mM, containing 0.05% bromophenol blue, pH 6.8) and resolved on a 12% separating gel topped with a 1% stacking gel. Crude enzyme sample was loaded in two wells separated by a well in which native protein marker was loaded. Following electrophoresis, the gel was cut vertically into two parts. The first part containing native protein marker and crude enzyme sample was stained by silver staining method to visualize protein bands[32,33]. The second part containing only a crude enzyme sample was used for zymography. The vertical gel strip was cut into 2 mm horizontal bands for preparation of zymography. Each cut portion was added in an Eppendorf tube containing 1 ml *p*-NPP (5 mM) substrate, 0.4 ml carbonate-bicarbonate buffer (0.1 M, pH 10.1). The Eppendorf tubes were incubated in dark and monitored for colour change. After 4 hours of incubation, absorbance of the sample was recorded at 405 nm.

Analytical methods

Estimation of biomass and residual phosphate: Culture broth was centrifuged at 10,000 rpm for 10 min to obtain a pellet containing cells and residual tri-calcium phosphate[34]. The pellet was suspended in chilled HCl (1N) to dissolve residual tri-calcium phosphate. The mixture was centrifuged at 10,000 rpm for 10 min to separate the cell pellet from dissolved residual phosphate. The supernatant was collected in a separate tube and NaOH (1N) was added to re-precipitate tri-calcium phosphate. Pellets of cells and tri-calcium phosphate were washed in distilled water and centrifuged at 10,000 rpm. Pellets were dried in an oven to estimate biomass and residual phosphate.

Organic acid detection: Detection of organic acids was performed using HPLC (Waters) filled with XBridge C8 column (4.6 × 250 mm). Samples were passed through 0.2 μm filter and 10 μl of the filtered sample was injected into the column. Elution was performed with an isocratic solvent consisting of 10.8% acetonitrile (HPLC grade) in 0.01% phosphoric acid with a flow rate of 1 ml/min and organic acids were detected at 210 nm (ref. 27). Standard organic acids (1 mg/ml) used were acetic acid, citric acid, formic acid, gluconic acid, succinic acid, lactic acid, oxalic acid and malic acid.

Phosphatase enzyme assay: Phosphatase enzyme assay was determined by measuring the amount of liberated para-nitrophenol (*p*-NP) from *p*-NPP[35]. The reaction was carried out in a total volume of 3 ml at pH 10.1 using carbonate–bicarbonate buffer (0.1 M). The reaction mixture consisted of 2 ml *p*-NPP substrate (5 mM), 0.95 ml buffer and 50 μl enzyme sample. The contents were incubated at 30°C; time course of 510 sec was followed at wavelength 405 nm using spectrophotometer. For the cell free culture broth, enzyme reaction was carried out using 200 μl broth with 0.8 ml buffer. One enzyme unit is defined as liberation of 1 μM of *p*-NP in 1 sec. All solutions of substrates, additives, etc. were prepared in the carbonate–bicarbonate buffer.

Protein estimation: The protein content of the supernatant and crude enzyme extract was determined using Folin Lowry's method[36]. Bovine serum albumin was used as standard.

Statistical analysis: Linear regression was carried out for time courses of enzyme activity. One way analysis of variance (ANOVA) and Student Newman Keuls method was performed to detect significant difference among different treatments. All statistical analysis was carried out using Sigma Stat Ver 4.0.

Results

Analysis of culture supernatant

Bacillus marisflavi FA7 started to grow in the exponential phase within 12 h of incubation in the modified Pikovskaya's medium (Figure 1). Stationary phase was observed within 72 h of incubation. *Bacillus marisflavi* FA7 showed no growth but was found to survive and produce organic acids after the broth attained neutral pH. Decrease in pH of the medium was observed from initial 10 to 5.12 over a period of 120 h of incubation. Tri-calcium phosphate solubilization started within 12 hours of incubation and continued through all the days with a final solubilized amount of 0.57 g/l of tri-calcium phosphate. The pH of the medium had negative correlation

with tri-calcium phosphate solubilization by *Bacillus marisflavi* FA7 ($r = -0.954$). Biomass and tri-calcium phosphate solubilization had a positive correlation ($r = 0.8378$).

Organic acid profile

HPLC analysis of day 1 and day 5 culture supernatants of *Bacillus marisflavi* FA7 showed four peaks (Figure 2). Retention time of the peaks was compared with standard organic acid peaks (Table 1). Two peaks were identified as oxalic acid and formic acid and the remaining two were unidentified. Absorbance of acids produced on day 5 was five times higher when compared to day 1.

Effect of inorganic nitrogen sources

Bacillus marisflavi FA7 grew in presence of all the tested inorganic nitrogen sources (Figure 3). The biomass obtained with addition of inorganic nitrogen sources in the medium was higher than the control ($p < 0.001$). Maximum growth was observed with NH_4NO_3 and $NaNO_3$ when compared to other treatments ($p < 0.001$). Fall in the pH of culture broth was noted with all the substrates. Least decrease in pH was observed with $NaNO_3$ and

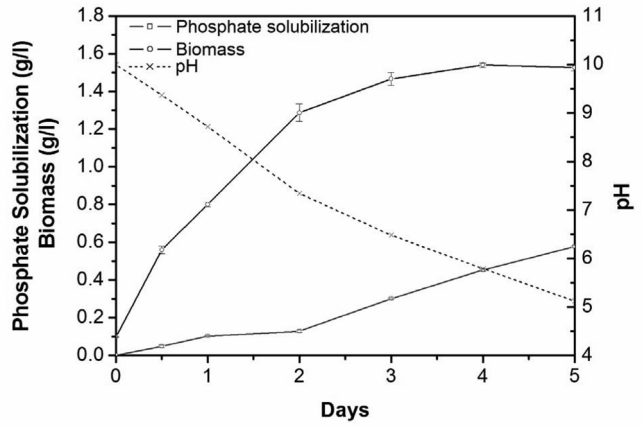

Figure 1. Time course of *Bacillus marisflavi* FA7 in Pikovskaya's medium in shaker flask condition with tri-calcium phosphate as sole source of phosphate.

Table 1. Organic acid produced by *Bacillus marisflavi* FA7 in modified Pikovskaya's broth under shaker conditions on day 1 and day 5 (Figure 2)

Organic acid	Retention time	Day 1	Day 5
Gluconic acid	2.8	–	–
Oxalic acid	2.97	+	+
Malic acid	3.1	–	–
Citric acid	3.2	–	–
Formic acid	3.3	+	+
Lactic acid	3.5	–	–
Succinic acid	3.7	–	–
Acetic acid	3.8	–	–
Unidentified	3.95	+	+
Unidentified	4.1	+	+

+, Present, –, Absent.

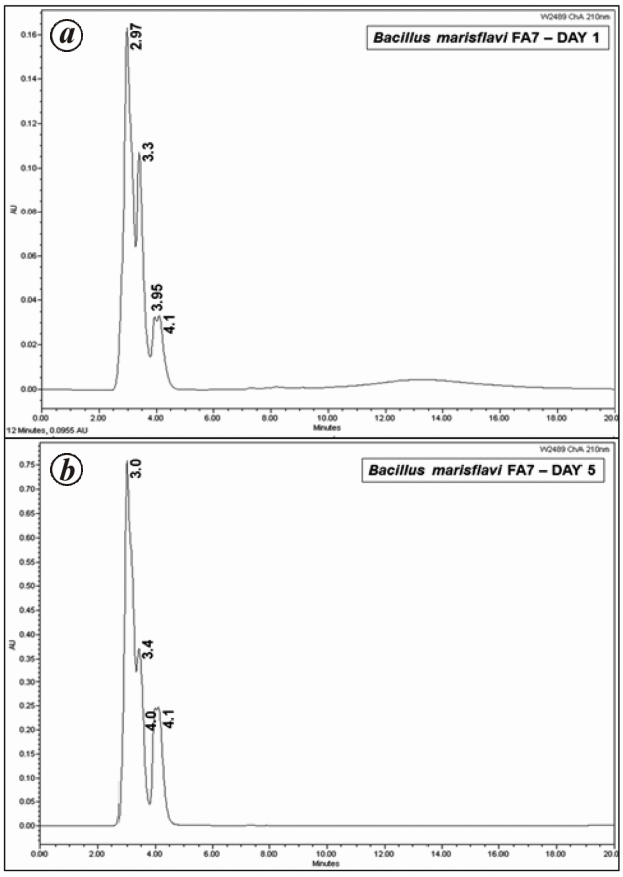

Figure 2. HPLC analysis of culture supernatant of *Bacillus marisflavi* FA7 on day 1 (***a***) and day 5 (***b***) for determination of organic acids (Table 1).

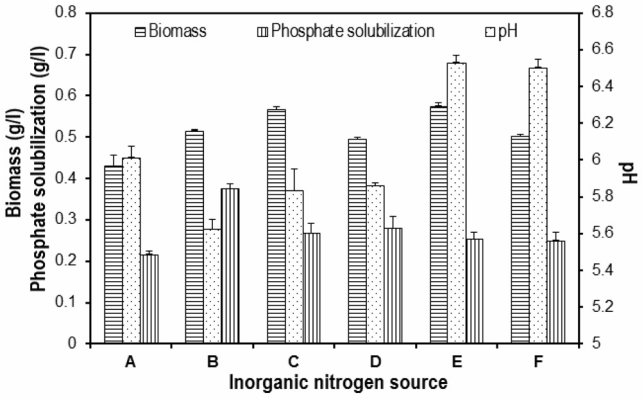

Figure 3. Effect of different inorganic nitrogen sources on solubilization of tri-calcium phosphate. A, Control (no inorganic nitrogen source); B, NH_4Cl, C, NH_4NO_3; D, $(NH_4)_2SO_4$; E, $NaNO_3$ and F, KNO_3.

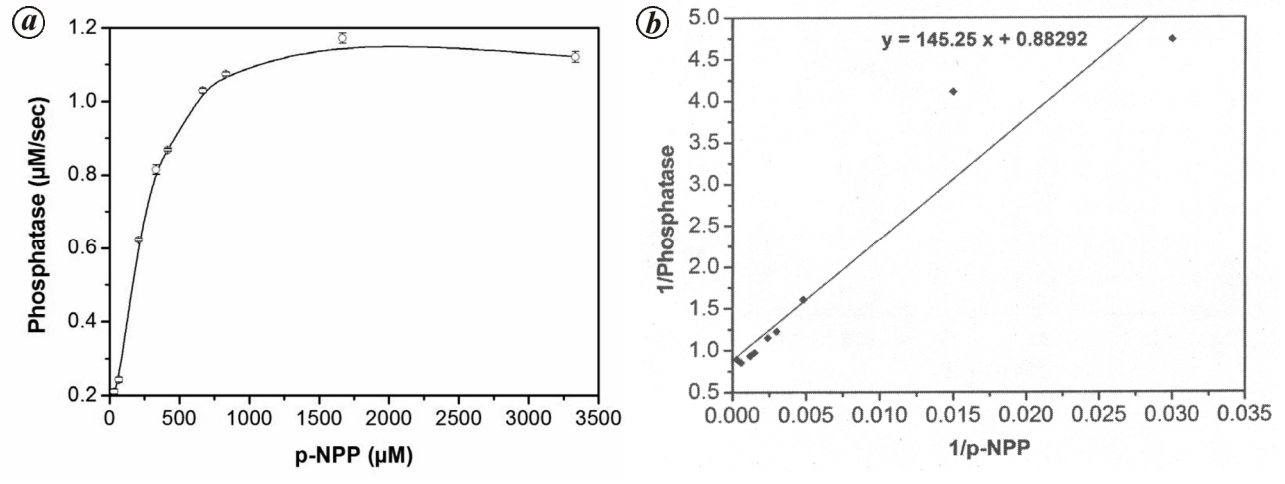

Figure 4. Effect of different concentrations of *p*-NPP on phosphatase enzyme activity (***a***) along with Lineweaver–Burk plot for determination of K_m and V_{max} (***b***).

KNO_3 ($p < 0.001$). Significant decrease in pH was observed with NH_4Cl ($p < 0.001$) when compared to other treatments. It was noted that the final pH of control, $(NH_4)_2SO_4$ and NH_4NO_3 were similar ($p > 0.01$). *Bacillus marisflavi* FA7 could solubilize tri-calcium phosphate in all the treatments. Interestingly, highest tri-calcium phosphate solubilization was obtained with NH_4Cl when compared to other combinations ($p < 0.01$).

EPS and siderophore production

Bacillus marisflavi FA7 showed production of EPS on Congo red agar and failed to produce siderophores on CAS agar.

Characterization of phosphatase enzyme

Bacillus marisflavi FA7 produced phosphatase in MSM containing *p*-NPP as the source of organic phosphate. Phosphatase enzyme activity and protein content of the cell free culture broth was found to be 5.14 U/ml and 368.5 µg/ml respectively. After acetone precipitation, phosphatase enzyme activity of the crude extract was 22.428 U/ml and protein concentration was 1000.5 µg/ml. Recovery during concentration step was 4.34% and 4% for enzyme and protein respectively.

Activity of crude phosphatase enzyme extract was observed to increase with increase in concentration of *p*-NPP substrate (Figure 4 *a*). However, after 1666 µM, no effect on the rate of reaction was observed. The K_m and V_{max} values for the enzyme were 1.13 µM and 164.51 U/sec respectively (Figure 4 *b*).

Phosphatase enzyme showed activity over a pH range 8.8–10.8 (Figure 5 *a*). Maximum activity was observed at pH 10.1. However, at pH 10.6 enzyme activity decreased drastically ($p < 0.001$).

Activity of phosphatase enzyme increased with increase in molarity (Figure 5 *b*). Further, from 0.1 M to 2 M of Na^+, the activity increase was gradual ($p < 0.001$). No significant difference was observed in enzyme activity at molarities 0.1, 0.5 and 1.0.

Phosphatase enzyme activity was significantly inhibited with different additives ($p < 0.001$) (Figure 5 *c*). β-mercaptoethanol caused maximum inhibition (69.58%) in activity compared to other additives. SDS, EDTA, Urea and Triton X100 showed reduction in activity but only to an extent of 10–26%. Interestingly, phosphatase enzyme activity significantly increased in the presence of metal ions Zn^{+2} and Mg^{+2} when compared to other treatments ($p < 0.001$).

Zymography of the protein gel demonstrated enzyme activity in the 3rd and 4th fractions of the gel. Silver staining of the gel replicate showed 4 dark bands and 3 faint bands. Activity fraction showed that the protein is present in the molecular weight range 175–200 kDa (Figure 6).

Discussion

Alkaliphilic *Bacillus marisflavi* FA7 isolated from Quellossim mangrove ecosystem solubilized 0.57 g/l tri-calcium phosphate in five days of incubation. Bacterial strains belonging to *Bacillus*, *Pseudomonas* and *Azotobacter* isolated from Chollangi, Godavari mangrove, solubilized phosphate in the range of 0.080–0.1 g/l (ref. 37). Pramod and Dhevendaran[38] isolated phosphate solubilizing *Vibrio* and *Pseudomonas* from the mangrove ecosystem of Cochin, India. They could solubilize tri-calcium phosphate in the range of 0.5–0.55 mg/l. So far, the highest tri-calcium phosphate solubilization activity of 0.4 g/l was reported by the *Vibrio proteolyticus* isolated from the mangrove ecosystem of Mexico[39].

Bacillus marisflavi FA7 showed correlation between decrease in pH and tri-calcium phosphate solubilization. Similar findings have been reported in *Pseudomonas fluorescens*[40], *Burkholderia cepacia* DA23[41], *Pseudomonas trivialis* BIHB747[42], *Enterobacter intermedium*[43] and *Serratia marcescens* GPS-5[44]. *Bacillus marisflavi* FA7 showed 4 units decrease in the pH of the medium which is similar to the findings in phosphate solubilizing *Serratia* sp.[45].

Decrease in pH has been attributed to the production of organic acids. The biochemical basis of gluconic acid and 2-ketogluconic acid has been well studied in Gram-negative bacteria by Goldstein[46]. *Bacillus marisfavi* FA7 released organic acids in the medium identified as oxalic acid, formic acid and two unidentified peaks. *Pseudomonas* strains produced gluconic acid, 2-keto gluconic acid, malic acid, succinic acid, formic acid and citric acid during solubilization of tri-calcium phosphate[42]. Citric and lactic acids were produced by *Bacillius megaterium* and *Bacillus circulans* from glucose in Pikovskaya's medium[47]. Vazquez[39] reported production of lactic acid, valeric acid, iso-butyric acid and acetic acid during the solubilization of tri-calcium phosphate by *Bacillus licheniformis*. Another *Bacillus* strain produced oxalic acid, 2-ketogluconic acid, succinic acid, acetic acid, lactic acid and isovaleric acid[39]. Several reports suggest that gluconic acid and alpha-ketogluconic acid are major organic acids responsible for tri-calcium phosphate solubilization[42,43,48].

Other reported mechanisms, which decrease the pH, are production of inorganic acids and H+ ion excretion due to ammonia assimilation. *Bacillus marisflavi* FA7 showed maximum decrease in pH with ammonium compared to nitrate salts. When only nitrate salts were used, decrease in pH was least. In line of this, maximum tri-calcium phosphate solubilization was seen only with NH_4Cl. This suggests that proton extrusion mechanisms

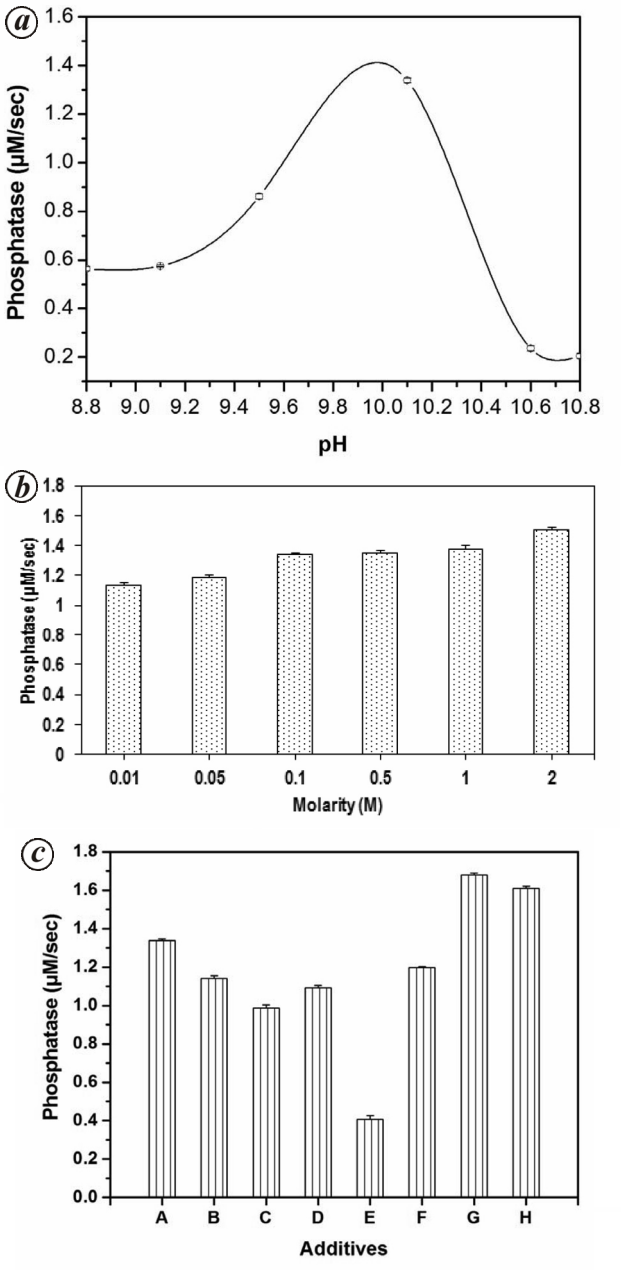

Figure 5. Effect of pH (***a***), molarity of sodium ions (***b***) and additives (A, Control; B, SDS; C, Urea; D, EDTA; E, β-mercaptoethanol; F, Triton X-100; G, Zn^{+2} ions, H, Mg^{+2} ions) (***c***) on phosphatase enzyme activity.

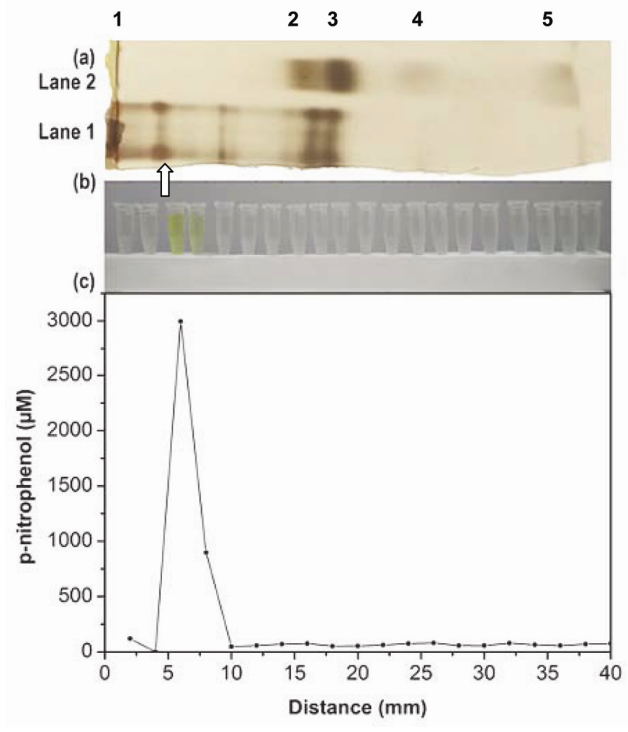

Figure 6. Native PAGE and Zymography of crude alkaline phosphatase enzyme. Silver-stained replicate of native gel (a); Lane 1 shows crude enzyme extract, Lane 2 shows native PAGE molecular weight marker; 1, 240 kDa; 2, 67 kDa; 3, 43 kDa; 4, 20 kDa, 5, 18 kDa. Phosphatase enzyme activity of 2 mm gel pieces as release of p-nitrophenol; b, pictorial; c, *p*-nitrophenol released.

involved during ammonium assimilation were responsible to some extent for phosphate solubilization by *Bacillus marisflav*i FA7. The amount of protons released into the external medium is often significantly influenced by nitrogen supply. In general, a greater reduction in pH, together with more solubilized tri-calcium phosphate has been observed with NH_4^+ as the sole nitrogen source compared to NO_3, due to the extrusions of protons to compensate for NH_4 uptake[1,7,49]. Illmer and Schinner[1] reported that a species of *Pseudomonas* did not produce any organic acid even though the bacterium solubilized inorganic tri-calcium phosphate. In *Pseudomonas fluorescens*, the type of carbon source (e.g. glucose versus fructose) rather than nitrogen source (e.g. NH_4^+ versus NO_3) had the greatest effect on proton release[8]. This indicates that for different species, different mechanisms are responsible for proton release.

Exopolysaccharides and biosurfactants are produced by micro-organisms largely in response to biofilm formation and stress. Microbial exopolysaccharides have shown their ability to form a complex with metals in soil[50]. This suggests that exopolysaccharides play a possible role in the release of phosphates from metal compounds. *Bacillus marisflavi* FA7 showed the production of exopolysaccharide. However, its role in phosphate solubilization needs further investigation. In *Enterobacter* sp., exopolysaccharide contributed to the dissolution of tricalcium phosphate synergistically with organic acids[51].

Siderophores are chelating agents that have a high affinity for iron and are produced by micro-organisms in response to iron deficiency. *Bacillus marisflavi* FA7 did not show production of siderophores. Many studies have reported the production of siderophores from phosphate solubilizing microorganisms[11–13]. Reid *et al.*[52] studied the ability to increase iron and phosphate diffusion of two siderophores (desferrioxamine-B, desferriferrichrome) compared to water using a root simulation technique. They found that desferriferrichrome increased phosphate diffusion 13-fold compared to water, whereas different concentrations of desferrioxamine-B exhibited only a small effect. Siderophore production could be useful in solubilizing iron containing phosphate compounds.

Bacillus marisflavi FA7 produced alkaline phosphatase enzyme. The major source of phosphatase activity in soil is considered to be of microbial origin. Bacteria belonging to the genera *Bacillus*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Proteus* and *Pseudomoans*, *Rhizobium* and *Serratia* have been reported to produce phosphatase enzyme[53].

Cell membrane bound phosphatase enzyme has been reported to be responsible for phosphate solubilization in *Bacillus subtilis* and *Bacillus megaterium*[54]. Phosphatase enzymes of these cultures are not detected in the culture broth. Extracellular alkaline phosphatase has been reported in *Bacillus amyloliquefaciens* and *Bacillus cereus*[55]. Extracellular phosphatases hold promise for toxic metal bioremediation and bio-recovery through metal phosphate precipitation. Liang *et al.*[56] observed that phosphatase from *Aspergillus niger* and *Paecilomyces javanicus* was able to precipitate lead when grown in medium containing glycerol-2-phosphate as the sole source of phosphorus. Similar results have been reported with copper metal[57]. Isolate *Bacillus marisflavi* FA7 demonstrated production of extracellular phosphatase enzyme with molecular weight of approximately 175–200 kDa in its native protein form. The molecular weight of extracellular alkaline phosphatase from *Bacillus licheniformis* was reported to be 121 kDa and was composed of four subunits[55]. Alkaline phosphatase from *Vibrio* was recorded to be 60 kDa (ref. 58).

The K_m and V_{max} values for the phosphatase enzyme of *Bacillus marisflavi* FA7 were 1.13 μM and 164.51 μM/sec. The alkaline phosphatase of *E. coli* has been reported to be an intracellular zinc metallo-enzyme with the molecular weight of 80 kDa, composed of two identical subunits. Its K_m was found to be 1 mM with an optimum pH of 10.0 (ref. 59). Aziz *et al.*[60] determined K_m and V_{max} for phosphatase enzyme of *Pyrobaculum calidifontis* as 60 μM and 4.0 μmol/min/mg respectively.

Considerable variations have been observed in alkaline phosphatase activity in the presence of environmental factors such as pH, salt concentration, moisture, temperature, heavy metals, precipitation and nutrients in various ecosystems[61]. Phosphatase enzymes are pH sensitive. Phosphatase enzyme of *Bacillus marisflavi* FA7 was highly active at alkaline pH 10.1. It showed activity at higher molar concentration of sodium ions. Sodium ions are essential for growth of marine, alkaliphilic bacteria that live in sodium rich habitats[62]. Phosphatase enzyme of *Bacillus marisflavi* FA7 showed decrease in activity in the presence of additives like beta-mercaptoethanol, SDS, EDTA, Urea and Triton × 100. Aziz *et al.*[60] observed that in the presence of EDTA, phosphatase enzyme activity was inhibited as it requires metal ions for activity.

Alkaliphilic *Bacillus marisflavi* FA7 has shown inorganic tri-calcium phosphate solubilization due to production of organic acids and H^+ ion excretion during ammonia assimilation. It produced exopolysaccharide. It could mineralize organic phosphate by secretion of extracellular alkaline phosphatase enzyme. This is the first report on mechanisms of inorganic and organic phosphate solubilization by an alkaliphilic bacterium. Since *Bacillus marisflavi* FA7 contains various mechanisms of phosphate solubilization and mineralization under alkaline conditions, it could be a potential phosphate biofertilizer candidate in alkaline soil.

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