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# Nutrient Modulated Alkaline Phosphatase and Associated Processes in Diazotrophic Cyanobacteria

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Abstract

Nutrient regulation of alkaline phosphatase (phosphomonoesterase – PMEase) was studied in some diazotrophic cyanobacterial strains like *Anabaena variabilis*, *Anabaena torulosa*, *Calothrix brevissima*, *Scytonema javanicum* and *Hapalosiphon intricatus*, in response to the macronutrients (Phosphate, Calcium and Magnesium) and the micronutrients (Zinc, Copper, Iron and Manganese). The phosphate grown cells of cyanobacterial strains when transferred to the phosphate deficient medium, showed expression of cellular PMEase activity and released the enzyme extracellularly. The increased concentration of phosphate inhibited enzyme activity severely in a concentration dependent manner. The phosphate depletion stimulated spore formation in *A. variabilis* and *H. intricatus*, whereas its addition enhanced spore's differentiation in *A. torulosa*. The switch-on time detected for both cellular and extracellular PMEase varies among the strains. The increase in ionic concentration of $Ca^{2+}$ enhanced the PMEase activity more profoundly than $Mg^{2+}$ in diazotrophic strains. The lower level of micronutrients either promoted or had no effect on photosynthetic inhibitors (DCMU) and respiratory electron transport chain inhibitor (sodium azide). It revealed that the energy for the synthesis of PMEase enzyme is mostly derived from photosynthesis and the role of respiratory energy is marginal. The Phosphate (Pi) uptake function in the strains was found to be substrate concentration dependent.

Key words: Alkaline phosphatase, diazotrophic cyanobacteria, inhibitors, nutritional regulation, sporulation

## Introduction

Cyanobacteria flourish remarkably in the environment with widely fluctuating nutrient availability. Deprivation of nutrient results in signalling the synthesis of new proteins which contribute to the survival of the organism. Certain enzymes synthesised during nutrient limitation become the part of their unique survival strategy. Diazotrophic strains in response to nutrient limitation synthesise new enzymes that may mobilise the availability of a particular nutrient (Grossman *et al.*, 1994). During the nitrogen limitation they fix molecular nitrogen into ammonia by enzyme nitrogenase (Stewart, 1980). This group of organisms have devised a number of measures to deal with phosphate deprivation. During P-stress, the strains scavenge P from other sources since they are unable to harness large organic-P molecules. They are known to cleave $P_i$ moiety from a number of substrates with the help of extracellular phosphatases (Whitton and Potts, 2000). Moreover, the P-stress results into several morphological modifications, such as hair formation in *Calothrix parietina* (Livingstone and Whitton, 1983) and spore formation in several cyanobacteria (Adams and Carr, 1981).

Diazotrophic cyanobacteria are oxy-photoautotrophic microorganisms and they grow on the mere expense of solar energy, water and $CO_2$. Their nutritional requirement is very simple, however they play significant role in the regulation of many metabolic processes. Calcium has been implicated in a variety of cellular processes in *Anabaena* PCC 7120 (Onek and Smith, 1992). The requirement of cyanobacterial photosystem II for $Ca^{2+}$ was demonstrated by England and Evans (1983) and *Synechococcus* has been reported to possess several $Ca^{2+}$ binding sites (Brahamsha, 1996). Also $Mg^{2+}$ as a divalent cation is known to be required for the activity of several enzymes, though other divalent cations can substitute for magnesium (Merida *et al.*, 1990). Even cyanophages, most frequently require $Ca^{2+}$ or $Mg^{2+}$ in 5–10 μM concentration for their absorp-

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tion (Ackermann and Dubow, 1987). The EDTA-modulated inhibition of alkaline phosphatase enzyme activity can be restored by the addition of $MgCl_2$ in *Anacystis nidulans* and it has been implicated that $Mg^{2+}$ is either required for phosphatase activity or substitutes for another ion bound to the enzyme (Block and Grossman, 1988). Zinc is the structural component of many proteins (Berg and Shi, 1996). Iron stress results in major physiological and ultastructural changes and induces the synthesis of several proteins including a novel chlorophyll binding protein and flavodoxin which acts as a replacement for the iron-containing ferredoxin (Mann, 2000). The cyanobacterial enzymes like sucrose phosphate synthase have a specific requirement for $Mn^{2+}$ or $Mg^{2+}$ for their catalytic activities (Porchia and Salerno, 1996). Copper containing plastocyanin is a novel protein in cyanobacteria which functions as an electron carrier between PSII and PSI (Carr and Whitton, 1982).

Various cyanobacteria belonging to Section IV and V (Rippka *et al.*, 1979), like orders Nostocales and Stigonematales (Desikachary, 1959) differentiate spores (akinetes) which serve as perennating structures (Adams and Carr, 1981). Limitation of inorganic nutrients although is not very important but the phosphate limitation is found to have direct effect on spore differentiation in *Anabaena cylindrica* (Simon, 1977), *Cylindrospermum licheniforme* (Fisher and Wolk, 1976) and *Anabaena doliolum* (Pandey and Kashyap, 1987). Pandey *et al.* (1991) observe an increased alkaline phosphatase activity and its induction period during sporulation in a wild type *A. doliolun* which suggest that the enzyme activity has some functional linkage with sporulation rather than the phosphate starvation. The enzyme APase is formed during sporulation in *Bacillus subtilis* even in the presence of inorganic phosphate (Warren, 1968).

Cyanobacteria, either in natural or laboratory conditions are often exposed to P-stress. The macro and micronutrients present outside the cell environment (either under stress condinations or in abundance) may influence the phosphatase activity in cyanobacteria, however, the literature available on the nutritional regulation of alkaline phosphatase activity are stochastic and nonsystematic. In view of the above, the present study reports about the phosphate regulation of alkaline phosphatase and the effect of macro ($Ca^{2+}$ and $Mg^{2+}$) and micro ($Zn^{2+}$, $Cu^{2+}$, $Fe^{3+}$ and $Mn^{2+}$) nutrients on the enzyme activity of diazotrophic cyanobacteria. *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus*. Further, the role of phosphate metabolism on sporulation of cyanobacterial strains is discussed.

## Experimental

### Materials and Methods

**Organisms and growth.** The diazotrophic cyanobacteria *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus* were isolated from local rice-fields of Banaras Hindu University while the *Anabaena torulosa* was obtained from Indian Agricultural Research Institute (IARI), New Delhi. All the strains were initially cultivated in BG-11 nutrient medium with $A_5$ trace elements (Rippka *et al.*, 1979). The cultures were incubated photo-autotrophically in a culture cabinet maintained at $25 \pm 2°C$ and illuminated with cool fluorescence tubes with a photon flux of 25 $\mu E\ m^{-2}\ s^{-1}$ on the surface of culture vessel for 14 h a day. The clones were isolated in broth and the strains were raised to axenic population. The lack of bacteria was tested using standard microbiological techniques (Norris and Ribbons, 1968) and identified with the help of standard literatures (Desikachary, 1959; Rippka *et al.*, 1979).

**Protein.** Growth of cyanobacterial strains was measured following changes in protein content of the cells determined by phenol reagent (Lowry *et al.*, 1951 as modified by Herbert *et al.*, 1971) using lysozyme (Sigma, USA) as the standard.

**Phosphate uptake.** The experiment to determine phosphate uptake in cyanobacteria strains was conducted in presence of 10 and 40 $mg\ l^{-1}\ P_i$ taking the phosphate starved cells as inoculum. The uptake of phosphate was estimated on the basis of phosphate depletion from the medium and the inorganic phosphate (Pi) present in the medium was determined following the method of Fiske and Subbarow (1925).

**Enzyme (alkaline phosphatase/PMEase) assay.** Alkaline phosphatase (phosphomonoesterase, PMEase) activity was determined using cyanobacterial cells grown in phosphate supplemented or phosphate depleted growth medium depending upon the requirement of experiment. Enzyme activity was assayed following the method described by Ihlenfeldt and Gibson, (1975) using *p*-nitrophenyl phosphate (*p*-NPP) as substrate and reagents (SIGMA chemicals, USA). The extracellular PMEase activity was determined in the spent medium after filtration through Millipore filter (0.43 μm; Millipore, India).

The reaction mixture of enzyme assay contains culture suspension (0.2 ml, protein *ca.* 150 μg ), glycine-NaOH buffer (1.6 ml; 0.2 M) and *p*-NPP (0.2 ml; 0.75 mM concentration). The test mixture was incubated at 37°C for 20 min in a temperature controlled water bath and the activity was terminated by the addition of 8.0 ml NaOH (0.2 M). The *p*-nitrophenol liberated during the enzymatic reaction was quantified spectrophotometrically using itself as standard and the enzyme activity was expressed as nmol *p*-NPP hydrolysed $mg^{-1}$ protein $h^{-1}$.

**Sporulation and spore frequency.** Culture of cyanobacterial strains grown in P-sufficient condition was harvested through centrifugation (10,000 × g; 15 min) washed and inoculated in BG-11 nutrient agar medium. The Petri dish with inoculated cyanobacteria was incubated in light grown conditions as described earlier. The growth on nutrient agar was examined microscopically everyday to observe initiation of sporulation. After the sporulation completed, the spore frequency was determined following the method of Pandey and Kashyap (1987).

$$\text{Frequency of spores formation} = \frac{\text{n (S)}}{\text{n (T)}} \times 100$$

where, n = number, S = spores and T = (V + H + S); V = vegetative cell and H = heterocyst.

Size of spores was measured microscopically using stage and ocular micrometers.

**Statistical analysis.** Each experiment was repeated thrice with 3–4 replicates and mean data of an experiment have been presented here. The three replicates of an experiment were analysed using analysis of variance (ANOVA) as described by Underwood (1997).

**Enzyme nomenclature.** Alkaline phosphatases (ALPs; orthophosphoric-monoester phosphohydrolase – 1 PMEase; EC 3.1.3.1) are a group of membrane-bound phosphomono-esterases (Seargeant and Stinson, 1979).

## Results

**Phosphate regulation of PMEase activity.** Cyanobacterial strains *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus* grown in the presence of 40 mg $l^{-1}$ phosphate for 8 days with negligible amount of PMEase activity were transferred to phosphate depleted medium. The switch-on time (onset of first appearance of PMEase activity) differed for the expression of cellular PMEase and extracellular enzyme activity among the strains studied. The switch-on time for cellular PMEase activity was minimum in *C. brevissima* (16 h) and maximum for *H. intricatus* (26 h), while it was found to be 18 and 21 h in *A. variabilis* and *S. javanicum*, respectively. The expression of extracellular PMEase activity took longer time and the first sign of enzyme's activity was noticed in *C. brevissima* (24 h) among the strains followed by *A. variabilis* (30 h), *S. javanicum* (35 h) and *H. intricatus* (36 h).

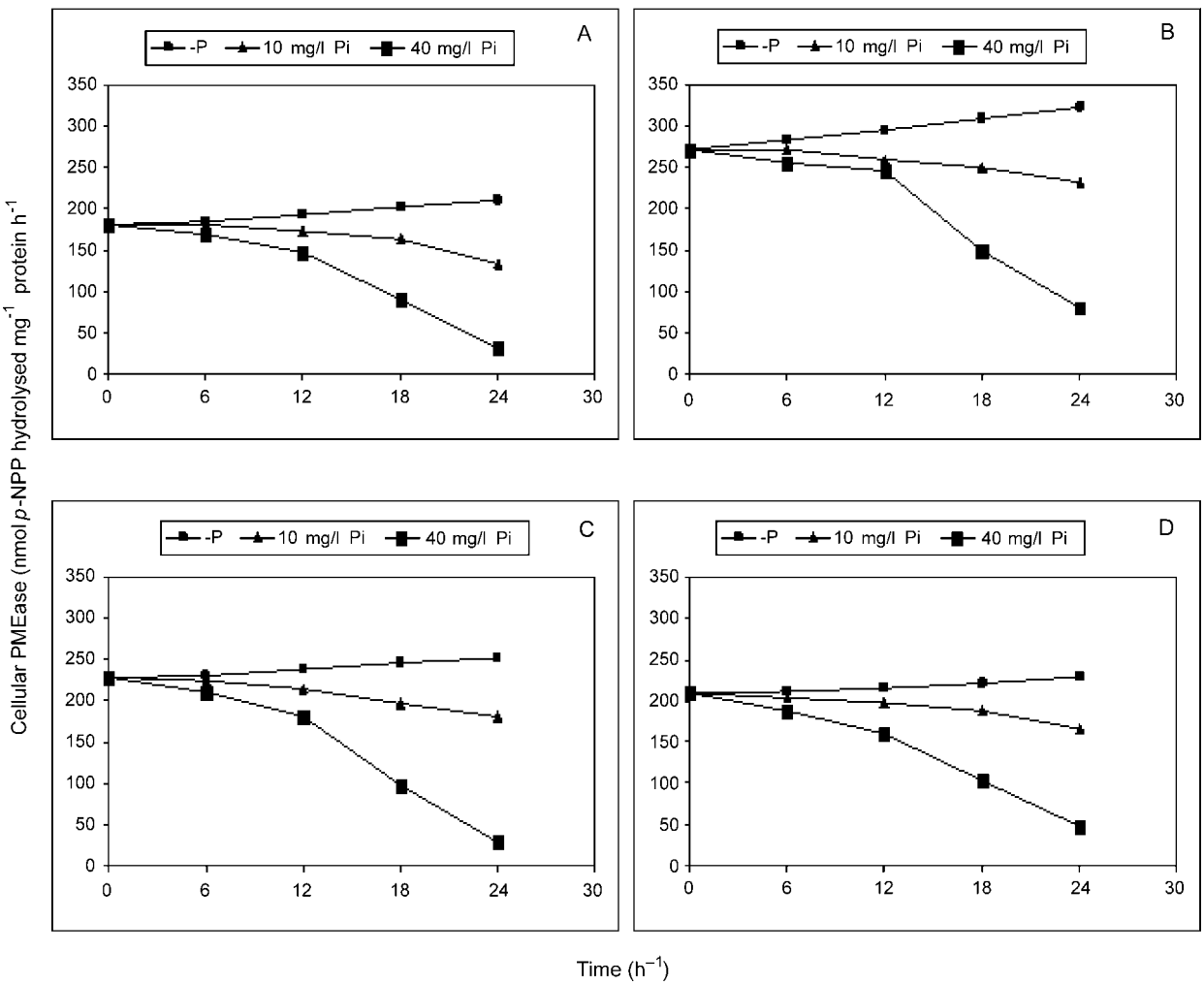


Fig. 1. Inorganic phosphate ($K_2HPO_4$) dependent cellular PMEase activity in cyanobacterial strains: *Anabaena variabilis* (A), *Calothrix brevissima* (B), *Scytonema javanicum* (C) and *Hapalosiphon intricatus* (D).

Phosphate ($P_i$) 10 mg and 40 mg $l^{-1}$ was added in the PMEase induced cells and the phosphate less condition served as control. The enzyme activity was assayed at indicated times as described in Materials and Methods.

The APase activity is maximally expressed when phosphate of the medium is consumed or depleted to a level, which is not inhibitory for its activity. The P-deprived cells of cyanobacterial strains (*C. brevissima*, *A. variabilis*, *S. javanicum* and *H. intricatus*) for 48 h were grown with 40 mg $P_i l^{-1}$ (the default level of $P_i$ used in BG-11 medium), 10 mg $P_i l^{-1}$ and without phosphate (P-stress) and the results of PMEase repression has been presented in Figure 1. APase activity in all the four strains under P-stress remained either stable or slightly elevated during the incubation period from zero to 24 h. In contrast, cells transferred to 10 mg $P_i l^{-1}$ showed decrease in PMEase activity from 6 h onwards and the degree of inhibition increased along with the incubation period. The incubation of cells in 40 mg $P_i l^{-1}$ showed greater degree of inhibition in enzyme activity which was sharp between 12–24 h. The phosphate mediated inhibition was maximum (78%) in *C. brevissima* and minimum (70%) in *S. javanicum*.

It is evident from the results and statistical analysis of the data (Three way ANOVA) that phosphate regulated PMEase activity in cyanobacterial strains is $P_i$ – concentration dependent and also the degree of inhibition was strain-specific. ($F_{3, 179}$ $P_i$ concentrations x cyanobacterial strains at different time = 92.48; $p<0.001$).

## Macro and micronutrient modulation of PMEase activity

**Effect of macronutrients.** To observe the effect of macronutrients – $Ca^{2+}$ and $Mg^{2+}$ ions (divalent cations) – on PMEase activity, the cells grown in P-supplemented medium till early exponential phase were inoculated in P-deprived condition containing $10^{-3}$ mM (1 µM) to 10 mM (10,000 µM) ionic strength of $Ca^{2+}$. The $CaCl_2$ present in the medium was replaced with NaCl and $MgSO_4$ with $Na_2SO_4$ at their equimolar concentrations, respectively.

Both of the ions as a nutrient enhanced the PMEase activity in all the four strains examined and $Ca^{2+}$ stimulated modulation of PMEase activity was more pronounced than the $Mg^{2+}$ ions. The increase in ionic

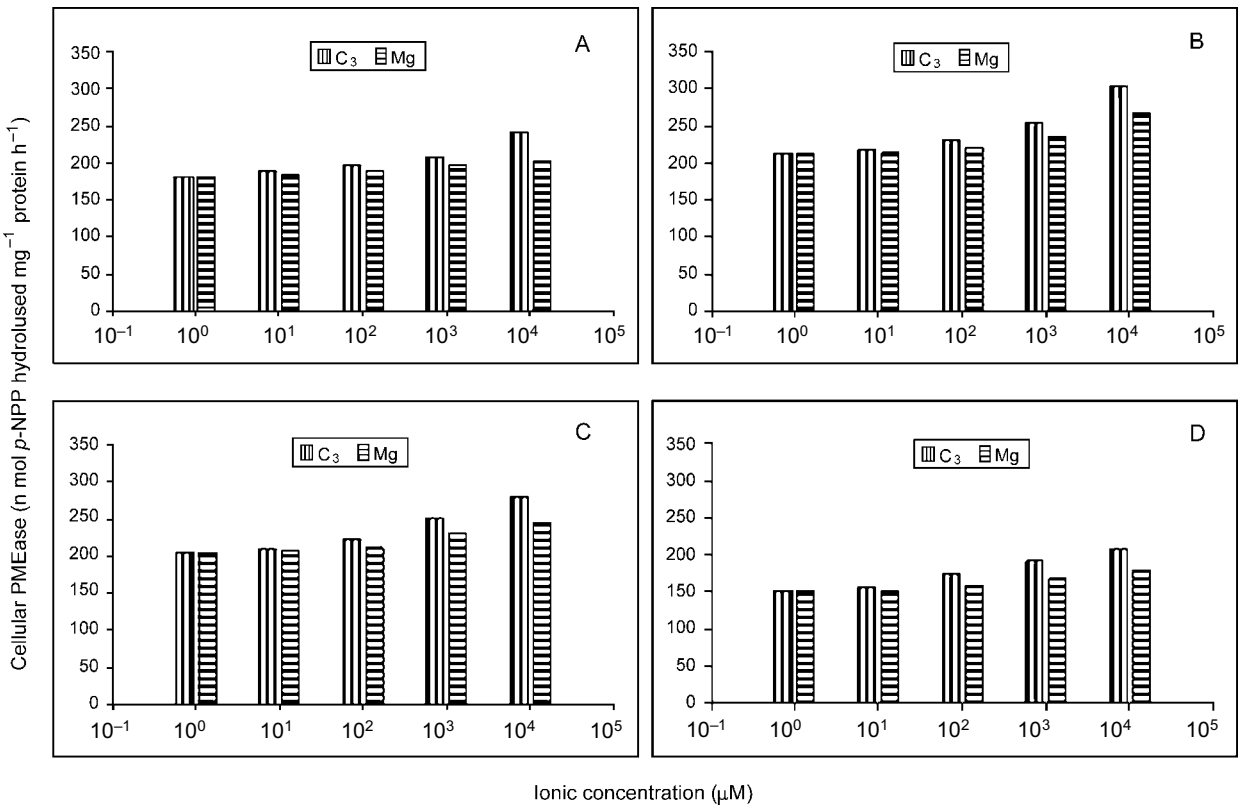


Fig. 2. A comparative analysis of cellular PMEase activity in different cyanobacterial strains: *Anabaena variabilis* (A), *Calothrix brevissima* (B), *Scytonema javanicum* (C) and *Hapalosiphon intricatus* (D) as influenced by macronutrients $Ca^{2+}$ and $Mg^{2+}$. The PMEase derepressed cells were incubated in the presence of 1 µM to 10,000 µM (l e + 0 to le + 4) ionic concentrations of calcium and magnesium salts. To observe the effect of these two macronutrients, calcium chloride ($CaCl_2 \times 2H_2O$) present in the BG-11 medium was replaced with sodium chloride (NaCl) and magnesium sulphate ($MgSO_4 \times 7H_2O$) with sodium sulphate ($Na_2SO_4$) at their equimolar concentrations, respectively. BG-11 medium served as control (C, 1 µM on X-axis). PMEase activity was detected after 24 h of incubation as described in Materials and Methods.

concentration of both the macronutrients gradually promoted the PMEase activity in all the strains upto 10 mM concentrations. The stimulation of PMEase activity was about 33–45% by $Ca^{2+}$ ions whereas $Mg^{2+}$ ions could enhance it approximately by 11–27% only in the studied cyanobacterial strains. The maximum increase in enzyme activity was observed in *H. intricatus* whereas the *A. variabilis* was found to have shown the minimum response. The differences in PMEase activity of various cyanobacterial strains in response to $Ca^{2+}$ and $Mg^{2+}$ ions was statistically (two way ANOVA) significant. ($F_{3, 12}$ ionic concentration × cyanobacterial strains = 18.44; $p<0.001$). The results indicated that the $Ca^{2+}$ ion requirement for the optimal expression of PMEase activity may have some regulatory role on APase enzyme in diazotrophic cyanobacteria (Figure 2) however, the significance of other macronutrients like $Mg^{2+}$ can not be ruled out.

**Role of micronutrients.** The experiment similar to the above was performed to observe the effect of micronutrients on PMEase activity. The desired micronutrient was deleted from the trace elements of the culture media and the same micronutrient was supplemented at an ionic concentration of $10^{-4}$ mM (0.1 μM) – 1 mM (1,000 μM). The $Mn^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and $Fe^{3+}$ were selected to observe their effects on the development of PMEase activity in the studied cyanobacterial strains.

The results showed that the relatively low level (1 μM) of all the micronutrients either enhanced the PMEase activity or kept it stable. The stimulatory effect by $Zn^{2+}$ was more prolific as it enhanced the PMEase activity about 30%, 47%, 35% and 32% at 10 μM concentration in *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus,* respectively. $Cu^{2+}$ upto 1 μM gradually enhanced (15–18%) the enzyme activity whereas $Fe^{3+}$ and $Mn^{2+}$ at that concentration have no significant effect. Further increase in ionic concentration of micronutrients suppressed the enzyme activity in a concentration dependent manner. The effect of micronutrients on PMEase activity of the cyanobacterial strains differs significantly (three way ANOVA) among the strains and concentration of micro elements ($F_{3, 239}$ ionic concentration × micronutrients × cyanobacterial strains = 16.56; $p<0.001$).

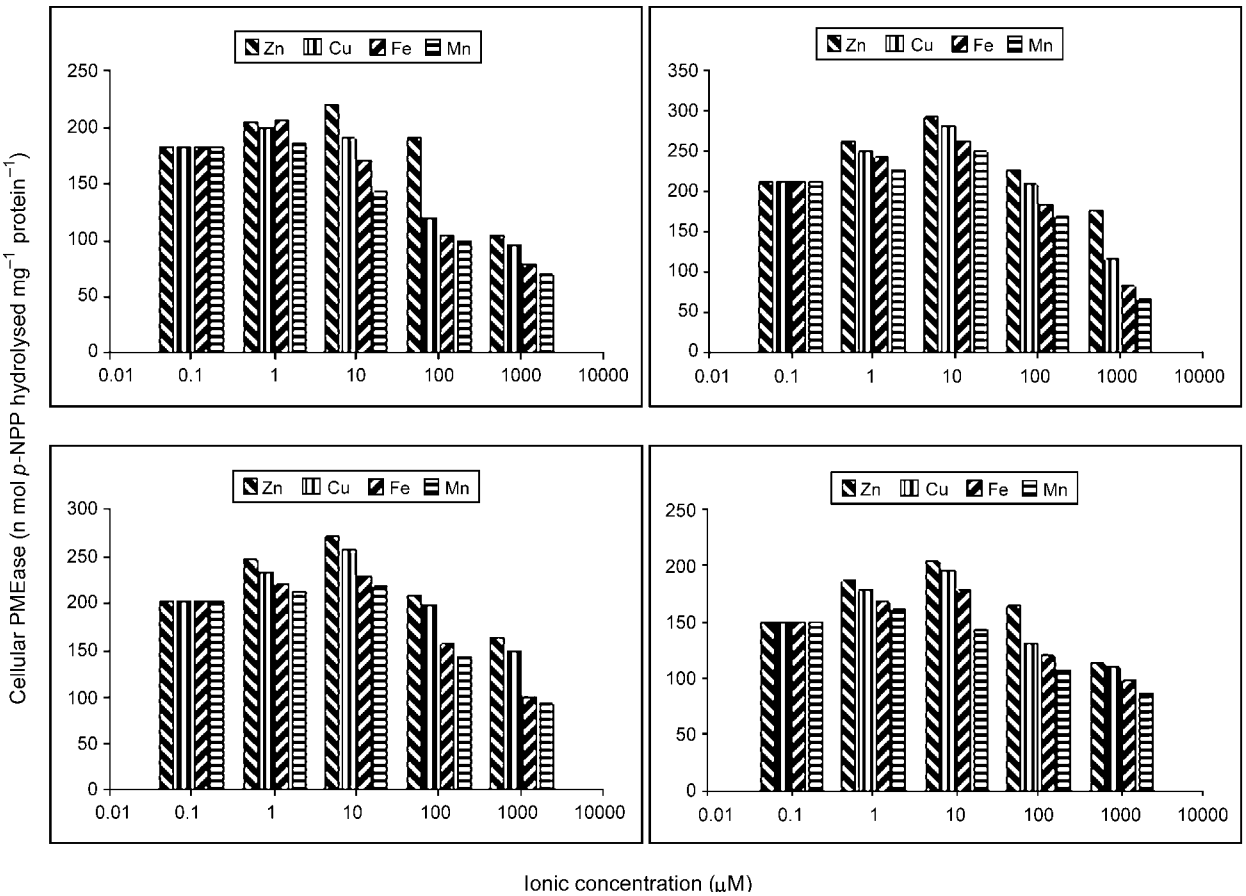


Fig. 3. Effect of micronutrients $Zn^{2+}$, $Cu^{2+}$, $Fe^{3+}$ and $Mn^{2+}$ on cellular PMEase activity of cyanobacterial strains: *Anabaena variabilis* (A), *Calothrix brevissima* (B), *Scytonema javanicum* (C) and *Hapalosiphon intricatus* (D).

To observe the effect of a particular micronutrient, its salt present in the trace element was omitted. Various metal ions (micronutrients) of different ionic concentrations (0.1 μM–1000 μM) were added to the PMEase derepressed cells and APase activity was measured after 24 h of incubation in respective ionic concentrations. Nutrient medium with $A_5$ trace elements served as control (C, 0.1 μM on X-axis).

**Effect of metabolic inhibitors.** To observe the role of photo-synthetically and respiratory generated energy on the regulation of PMEase synthesis, the cyanobacterial strains were exposed to three different conditions: (i) cells incubated in dark (ii) cells incubated in light in the presence of DCMU and (iii) cells incubated in dark in the presence of sodium azide.

The cells grown in light served as control. The enzyme activity measured in dark grown cells showed greatly reduced enzyme activity in all the strains and the inhibition was reflected maximum (*ca.* 80%) in *A. variabilis*. The enzyme activity in *S. javanicum*, *H. intricatus* and *C. brevissima* was higher than the *A. variabilis* in the dark grown cells. The light grown cells with DCMU (an inhibitor of PS-II) showed a sharp decline in enzyme activity in all the above four strains investigated which constituted about 35–40% of the light grown cells. However, the degree of inhibition was less prolific than that of in the dark grown cells. The cyanobacterial cells incubated in dark in the presence of sodium azide (a respiratory electron transport chain inhibitor) expressed a little enzyme activity which was nearly 10–13% of their respective light grown cells (Table I). The PMEase activity of cyanobacterial strains in the presence of inhibitors and in darkness showed significant contrasts (two way ANOVA: $F_{3, 47}$ inhibitors x cyanobacterial strains = 6.86;

Table I
Effect of photosynthetic and respiratory inhibitors on the alkaline phosphatase activity of cyanobacteria

| Cyanobacterial Strains | Enzyme (PMEase) activity (n mol *p*-NPP hydrolysed $mg^{-1}$ protein $h^{-1}$) | | | |
|---|---|---|---|---|
| | Light | Dark | Light + DCMU [1] | Dark + Sodium azide[2] |
| *Anabaena variabilis* | 210 | 44 | 73 | 21 |
| *Calothrix brevissima* | 315 | 82 | 118 | 49 |
| *Scytonema javanicum* | 253 | 74 | 96 | 34 |
| *Hapalosiphon intricatus* | 228 | 65 | 84 | 29 |

[1] DCMU – 10 µM, [2]Sodium azide – 1 µM
The P-rich cells incubated in P-deprived medium with or without inhibitors were transferred under different conditions and APase activity was determined after 48 h of incubation.

Table II
Phosphate uptake, alkaline phosphate activity and sporulation in diazotrophic cyanobacteria under P-deprived and P-conditions

| Cyanobacterial strains | Phosphate concentration | Phosphate[1] uptake | APase[2] activity | Sporulation | | | | |
|---|---|---|---|---|---|---|---|---|
| | ($mg\ l^{-1}$) | (µmol P $mg^{-1}$ protein) | (nmol *p*-NPP hydrolysed $mg^{-1}$ protein $h^{-1}$) | Position | Shape | Initiation days | Frequency | Size |
| *Anabaena variabilis* Kutzing ex Born. et Flah. | -P<br>10<br>40 | ND<br>2.2<br>4.3 | 215<br>113<br>32 | Centrifugal | Barral | 17<br>19<br>23 | 67 ± 4<br>51 ± 2<br>27 ± 2 | 6.7–8.9 µm broad and 8–12 µm long |
| *Anabaena torulosa* (Carm.) Lagerh. ex Born. et Flah. | -P<br>10<br>40 | ND<br>1.8<br>3.7 | 266<br>147<br>41 | Centripetal | Cylindrical | 20<br>18<br>17 | 12 ± 0.5<br>25 ± 2<br>39 ± 3 | 6.7–11.5 µm broad and 14.5–27.6 µm long |
| *Calothrix brevissima* West, G.S. | -P<br>10<br>40 | ND<br>1.9<br>3.9 | 325<br>197<br>84 | ND | ND | ND | ND | ND |
| *Scytonema javanicum* (Kutz.) Bornet ex Born. et Flah. | -P<br>10<br>40 | ND<br>2.3<br>4.5 | 258<br>174<br>51 | ND | ND | ND | ND | ND |
| *Hapalosiphon intricatus* W. et G.S. West | -P<br>10<br>40 | ND<br>1.7<br>3.8 | 232<br>161<br>45 | Random | Rounded | 35 | 18 ± 1 | 14–16 µm broad |

[1] Phosphate uptake determined at 150 min incubation period. [2] APase activity measured at 24 h incubation period.

$p<0.001$). The results inferred that the energy for the enzyme synthesis is primarily fulfilled by photosynthetic reactions but to some extent through respiratory processes also.

**Phosphate metabolism and sporulation.** To define the role of phosphate nutrition on the spore formation, the P-influenced sporulation characteristics was studied in the selected strains (Table II). Phosphate depletion derepressed the PMEase enzyme while the phosphate supplementation repressed the enzyme activity in a concentration dependent manner. A substrate concentration dependent phosphate uptake was also evident in all the five cyanobacteria studied, which showed an increasing trend with higher level of phosphate ions. The phosphate uptake saturated at about 100 mg $l^{-1}$ phosphate in the cyanobacterial strains (data not presented). *A. variabilis* and *A. torulosa* differ markedly in response to phosphate level and the sporulation characteristics. In *A. variabilis* the barrel shaped spore initiation was centrifugal, with a decrease in spore frequency and delay in spore formation in response to fluctuating phosphate levels. In contrast, the cylindrical spores formed in *A. torulosa* was phosphate dependent as the presence of phosphate stimulated spore frequency and also the initiation of sporulation stage was shortened. In this species, spores are formed centripetally adjacent to the heterocyst.

*C. brevissima* and *S. javanicum* although did not form spores either under natural conditions or under culture. Both behaved similarly with respect to phosphate concentration dependent phosphate uptake and inhibition of PMEase enzyme.

In cyanobacterium *H. intricatus* where the rounded spores are formed randomly both in erect as well as prostrate system, the pattern of phosphate uptake and APase activity was the same as in above strains. The phosphate stress induced the spore formation both in terms of time required for the initiation and spore frequency in the strain. However, the role of phosphate on the sporulation is strain specific as phosphate stimulated spore formation in *A. torulosa*.

## Discussion

The phosphatases are quite stable enzymes and they are synthesised within the cells in response to availability of inorganic phosphate outside the cell medium. During the early phase of enzyme synthesis, phosphatases synthesised in the cells are transported to periplasmic space and at later stage they are released outside the cell either in the medium or in the external environment probably in search for organic phosphate. The presence of high level of phosphate represses the synthesis of phosphatases (Block and Grossman, 1988) however, the time required for the expression of APase varies with the concentration of phosphate initially supplied in the medium and with the cellular P-level (Kumar *et al.*, 1992). Several studies have shown that an internal phosphate pool regulates the synthesis of repressible phosphatases in cyanobacteria (Fitzgerald and Nelson, 1996). When the pool is filled, synthesis of enzyme is shut down and orthophosphate may act as an inhibitor which competes with the monoesters for the active sites on the phosphatases (Jansson *et al.*, 1988). The APase activity and phosphate uptake functions are phosphate repressible in *Anabaena* PCC7120 and enzyme synthesis is under transcriptional control (Pandey and Tiwari, 2003).

The results on the four diazotrophic cyanobacterial strains also confirm the above findings since low level of added phosphate (10 mg $l^{-1}$ $P_i$) is less effective in repressing the enzyme activity compared to high level (40 mg $l^{-1}$ $P_i$) of inorganic phosphate addition. Further, the activity decreases along with the time of incubation, which indicates the gradual increase of internal $P_i$ pool. The phosphate induced inhibition of cyanobacterial strains of alkaline phosphatase activity is strain specific as well as concentration dependent. Phosphorus limitation is also known to cause drastic decrease in photosynthetic $O_2$ evolution, ATPase, nitrogenase ($N_2$ase) and other enzyme activity associated with nitrogen metabolism (Grossman *et al.*, 1994).

Among the various factors inducing spore differentiation in phosphate level seems to be most important as the phosphate stress stimulates sporulation in several cyanobacteria (Adams and Carr, 1981). The induction of APase activity during P-stress may be considered as an early biochemical event preceding sporulation. The assumption is strengthened by the observation that: (a) addition of phosphate inhibits sporulation (Adams and Carr, 1981) as well as alkaline phosphatase activity (Ihlenfeldt and Gibson, 1975) and (b) spores do not contain polyphosphate bodies. The phosphate starvation might be leading to the energy limitation which appears to be the only major factor involved in the induction of spore differentiation, though phosphate deprivation did not induce spore differentiation in *Nostoc* 7524 (Sutherland *et al.*, 1975) and also in *A. torulosa* during the present investigation in contrast to the rest of the strains.

The $Mg^{2+}$ modulated stimulation of cellular PMEase activity of all the four diazotrophic cyanobacterial strains with an increasing trend of ionic concentration indicates requirement of $Mg^{2+}$ by the alkaline

phosphatase. Block and Grossman (1988) also observed $Mg^{2+}$ ions requirement by the *A. nidulans* for the phosphatases where this metal ion may substitute another ion bound to enzyme. Stimulation in APase activity by $Mg^{2+}$ was also observed in a microaerophilic diazotrophic cyanobacterium *Plectonema boryanum* (Doonan and Jenson, 1979). The $Ca^{2+}$ ion requirement has been reported for phosphatase activity in algae (Quisel *et al.*, 1996). In cyanobacteria, Healey (1973) has reported the dependence of *A. variabilis* on $Ca^{2+}$ ions and the findings are subsequently confirmed by Grainger *et al.* (1989) in *C. parietina* APase, grown in batch cultures. The mechanism of $Ca^{2+}$ stimulation of enzyme activity is not known. It may act as an integral part of the cell bound enzyme or it increases the accessibility of the substrates to the enzyme or may be playing a role in stabilising the enzyme and thus increasing the activity. The alkaline phosphatase is known to be a $Zn^{2+}$ requiring enzyme in the procaryotes (Coleman, 1987) however, $Zn^{2+}$ severely inhibit the APase activity in *A. nidulans* (Block and Grossman, 1988), *N. commune* (Whitton *et al*., 1990), *C. parietina* (Grainger *et al.*, 1989) and *Anabaena* species (Kumar *et al.*, 1992). In contrast to above, the observation that $Zn^{2+}$ at lower concentrations stimulate APase activity in the above mentioned four diazotrophic strains are more pronounced compared to $Cu^{2+}$, $Fe^{3+}$ and $Mn^{2+}$. It is interesting and requires more information to define the role of this divalent cation in the mechanism of catalysis and the differences between the phosphatases present in different cyanobacteria. The exposure of $Zn^{2+}$ to fresh water microbial community greatly affects the phosphate retention in biomass accumulation and decreases APase activity (Adams and Carr, 1981). One of the alkaline phosphatase in *Synechococcus* (Pho V) requires $Zn^{2+}$ for its activity however it is inhibited by phosphate (Wagner *et al.*, 1995). The expression of the $Cu^{2+}$ containing plastocyanin protein increases with added copper which is regulated at the level of transcription in *A. variabilis* (Bovy *et al.,* 1992). Some cyanobacteria can exploit copper as an alternative to electron transport (Robinson *et al.,* 2000). Rueter (1983) has reported inhibition of APase activity by $Cu^{2+}$ in a marine diatom *Thalassiosira pseudonana*. The results of current investigation that the lower concentrations of iron and copper stimulated APase activity in cyanobacterial strains is a requirement as a micronutrient for cell metabolism which is affecting indirectly the PMEase activity and their higher concentration inhibition may be qualified to be metal induced. Among the micronutrients, $Mn^{2+}$ even at fairly low concentration of 10 μM, inhibited APase activity in four examined cyanobacterial strains. The second phosphate-derepressible APase, Pho V was inhibited by $Mn^{2+}$ ion. However, at this stage we are not sure about the existence of such phosphatase in diazotrophic cyanobacterial strains.

A marked decrease in enzyme activity in the cells incubated in dark compared to cells incubated in light indicates the requirement of light energy in the synthesis of the enzyme. This is further confirmed when the light incubated cells of cyanobacterial strains are treated with DCMU and to some extent sodium azide results in loss of enzyme activity compared to light grown cells alone. These observations imply that photoenergy is required for the synthesis of APases, if not for the enzyme activity in cyanobacteria, as has been reported recently in *Anabaena oryzae* (Singh and Tiwari, 2000). These observations are contradictory to the observations made in two species of red algae *Gelidium* in which the APase activity is independent of light (Hernandez *et al.,* 1995). The difference in enzymatic behaviour may explain the ecological succession within the planktonic settlements.

Under the nutrient stress, synthesis of APase enzyme is of downright importance in concurrence with nitrogenase in diazotrophic cyanobacteria. These two unique characteristics coupled with spore formation under P-stress enables them to survive the nitrogen and phosphorus limitation. In the natural environment or under the laboratory conditions the macro and micronutrients present in the surroundings and inside the cell play a significant role in the regulation of these two enzymes. The current study confirms the above view in spite that only APase has been considered for the investigation.

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