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A Correlation Matrix of Alkaline Phosphatase and Sporulation in Diazotrophic Cyanobacteria and its Thermo-tolerant Mutant

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Abstract

Alkaline phosphatase (phosphomonoesterase *i.e.* PMEase) activity in heterocystous cyanobacteria *Anabaena flos-aquae*, *Nostoc calcicola*, *Calothrix brevissima*, *Scytonema javanicum* and *Hapalosiphon intricatus* is known to be temperature and pH dependent. Maximum level of enzyme activity was recorded at either 35°C or 37.5°C. Also, the cell bound phosphomonoesterase enzyme was shown to exhibit pH optima of 10.0 or 10.2. A thermo-tolerant (*tr*) mutant isolated after MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) mutagenesis in *Calothrix brevissima* exhibited 10°C higher temperature optima and comparatively high pH optima (pH 10.4) for phosphomonoesterase enzyme. The mutant grew with a maximum growth rate (k) at 50°C. Activation energy (Ea) for cyanobacterial strains was in a narrow range between 45 to 52 kJ mol^{-1}. A little variation in temperature and pH optima was also observed in phosphomonoesterase activity of *Calothrix brevissima* and its thermo-tolerant mutant while utilizing various organic phosphates as substrate what indicated the substrate dependence temperature and pH optima. Cyanobacterial strains grown at their respective temperature and pH optima differentiated spores less frequently though, coupled with early initiation of spore.

K e y w o r d s: PMEase, organic-P utilization, sporulation, thermo-tolerant mutant (*tr*), temperature kinetics

Introduction

Cyanobacteria occur virtually in all terrestrial niches and can be found in locations which exhibit widely fluctuating chemical and physical parameters including nutrient availability, light intensity, light wavelength, temperature, and water activity (Grossmen *et al.*, 1994). While growing in tropical rice-fields cyanobacteria are often exposed to higher temperatures (*ca*. 35–40°C) during the rice cultivation cycle, surprisingly, this range of temperature supports luxuriant growth of N_2-fixing cyanobacteria both in the terms of bio-diversity and bio-mass production. Being oxyphotoautotrophic, they are ubiquitous in distribution and require solar energy, water and CO_2 for its nutrition (Whitton and Potts, 2000). Diazotrophic strains are used as a potent bio-fertiliser in the rice-agriculture. Besides fixing atmospheric nitrogen, some strains also solubilise complex organic phosphate molecules efficiently. Phosphate solubilisation is facilitated through enzyme alkaline phosphatases (APases). The enzyme, synthesized inside the cells in response to P-starvation, remains in the periplasmic space and is released extracellularly in the external medium to cleave P_i moiety of organic molecules. The APases with broad substrate specificity provides a mechanism to circumvent the process to increase the overall efficiency of phosphate recycling (Bhaya *et al.*, 2000). Phosphate depletion derepresses alkaline phosphatase activity and stimulates sporulation in several cyanobacteria. Role of PMEase is well established in *Bacillus subtilis* (Warren, 1968) however, a little work has been done on this aspect of cyanobacteria so far.

Among the environmental factors, temperature and pH play a significant role in the native propagation of cyanobacteria in agro- and natural ecosystems. They have their own temperature and pH optima which

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determine their distribution and functions in the ecosystem (Fogg *et al.*, 1973; Carr and Whitton, 1982; Stewart, 1980; Whitton and Potts, 2000). The rate of enzymatic reaction when plotted against the reciprocal of absolute temperatures (^{0}K) in an Arrhenius Plot shows its maximum activity at an optimal temperature and pH, respectively. Nakatsubo and Ino (1986) and Pandey in his various studies (Kashyap *et al.*, 1991; Pandey *et. al.*, 1992; Shukla *et al.*, 1997) observed temperature optima of 20°C for nitrogenase activity in various Antarctic cyanobacterial strains and suggested that adaptation for several thousand generations have changed the temperature response of Antarctic cyanobacteria. The temperature optima for nitrogenase enzyme in various tropical and temperate strains range between 30 to 35°C (Stewart, 1980). Some of the cyanobacteria belonging to the section IV and V (Rippka *et al.*, 1979) differentiate spores (akinetes) under stress which serves as perennating bodies.

Most of the temperature sensitive mutants isolated in cyanobacteria have been characterized on the basis of their growth in two different temperatures (Ladha and Kumar, 1978). Temperature sensitive mutants of *Synechococcus cedrorum* selected by their high fluorescence at 40°C showed abnormal fluorescent kinetics, slow growth rate, decreased O_2-evolution and altered photosynthetic membrane protein patterns when grown at higher temperature (Sherman and Cunningham, 1979). The heat shock genes for cyanobacterial GroEL, GroES and DnaK homologs from *Synechococcus* PCC 7942 and *Synechococcus* PCC 6803 have been cloned and sequenced. Gene (s) GroES and GroEL constitute an operon in *Synechococcus* PCC 7942 (Webb *et al.*, 1990). Conversely, one DnaK gene has been identified in *Synechococcus* PCC 6803 (Chitnis and Nelson, 1991). However, a detailed physiological and biochemical characterization of temperature sensitive/resistant strains is lacking in adequate literature.

The temperature and pH optima of diazotrophic cyanobacteria do play a significant role during its inoculation as a bio-fertilizer in rice cultivation. This attribute is of further significance in habitats like India and South-east Asia, where temperature and pH value of the soil varies strikingly. Although a wide-spread distribution of APases in natural ecosystem and its vital role in P-recycling has been established, the role of ecological factors on this enzyme still requires great research attention as current literature contributes to only a little fraction. That enforced us to study the temperature kinetics and pH optima for APase activity in some diazotrophic cyanobacteria. Further a thermo-tolerant (*tr*) mutant of *C. brevissima* isolated after induction with MNNG was characterized along with its wild type in respect to temperature kinetics and pH optima while utilizing organic phosphates.

Experimental

Materials and Methods

Organisms and growth. The cyanobacterial strains (listed in Table I) either obtained from Indian Agricultural Research Institute (IARI), New Delhi or isolated locally, were of rice-field origin. The bacteria-free clones were raised by repeated cloning and inoculation on nutrient agar. They were identified on the basis of their morphological characteristics and cell dimensions using suitable literatures (Desikachary, 1959; Rippka *et al.*, 1979). The strains were routinely cultivated and maintained at pH 8.0 in BG-11 medium (Rippka *et al.*, 1979). The cultures were incubated under photoautotrophic growth conditions in a culture cabinet at 28 ± 2°C and illuminated with cool fluorescent light (intensity 25 μE m^{-2} s^{-1} photon flux). Resultant growth was determined in the terms of protein content.

The specific growth rate (k, $\log_{10} h^{-1}$) was calculated using the formula of Myers and Kratz (1955).

$$k = 2.303 \quad \frac{\log N_1 - \log N_0}{t_2 - t_1}$$

where N_0 = initial growth at time t_1 and N_1 = final growth at time t_2 ; t_1 and t_2 are two time intervals in the growth curve during the exponential growth phase.

Protein. Protein content of the cyanobacterial cells was determined following the method of Lowry *et al.* (1951) using bovine serum albumin (BDH) as standard.

Temperature incubation. To observe the effect of a range of temperature (10–60°C) on cellular PMEase activity of *Anabaena flos-aquae*, *Nostoc calcicola*, *Calothrix brevissima*, *Scytonema javanicum* and *Hapalosiphon intricatus*, the experiment was conducted in a temperature controlled incubator (Yarco, India) fitted with cool fluorescent tube (25 μE m^{-2} s^{-1}) and moisture regulator. The P-deficient cultures of cyanobacteria were pre-incubated at different temperatures for 60 min before the assay of enzyme. The activity of PMEase was determined by performing the enzyme assay at desired temperatures in a water bath.

pH maintenance: In order to determine PMEase activity of cyanobacterial strains at pH ranging from 7–11, the P-starved cells were incubated at different pH values for 60 min before enzyme assay. HEPES buffer was used to maintain pH at 7–8.5 whereas higher range of pH (8.5–11) was kept up with the help of glycine-NaOH (0.2 M) buffer. The enzyme assay was performed in triplicates at desired pH at 37°C temperature.

Table I
Activation energy (E_a), temperature and pH optima of PMEase enzyme in diazotrophic cyanobacterial strains

Cyanobacterial strains	Activation Energy (Ea) (kJ mol^{-1})	Temperature optima (°C)	pH optima
Anabaena flos-aquae (Lyngb.) Breb. ex Born. et Flah.	51	35	10.0
Nostoc calcicola Breb. ex Born. et Flah.	52	37.5	10.2
Scytonema javanicum (Kütz.) Bornet ex Born. et Flah.	45	35	10.2
Hapalosiphon intricatus W. et G.S. West	47	37.5	10.2
Calothrix brevissima (Wild) West, G.S.	46	37.5	10.0
Thermo-tolerant (*tr*) mutant	50	47.5	10.4

E_a (Activation energy), temperature and pH optima were determined as described in Materials and Methods

Activation energy. The value of the kinetic constant V_T was determined for every temperature value and activation energy (Ea) was calculated from the Arrhenius Plot of log V_T against 1/T as given below:

$$Ea = 2.303\ R \quad \frac{\Delta \log V_T}{\Delta 1/T}$$

where E_a = activation energy, R = gas constant, V = rate of enzyme activity and T = absolute temperature.

Isolation of a thermo-tolerant (*tr*) mutant of *Calothrix brevissima.* The exponentially growing trichomes of *C. brevissima* were homogenized using sterile glass beads (2 mm. dia.) and washed in phosphate buffer of pH 7.0. The small fragments (3–4 cells) were treated with 100 µg MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) ml^{-1} at pH 7.0 (phosphate buffer 0.01 M), temperature 28°C and light intensity of 25 µE $m^{-2}s^{-1}$. Following the 60 min of treatment, permitting 5–7% survival of cells, the treated population after centrifugation (10,000×g; 15 min) was allowed to grow and segregate for 10 days in basal medium (BG-11 medium) at 50°C and maintained henceforth in an incubator (light intensity of 25 µE m^{-2} s^{-1}). The mutagenised population was then transferred to liquid medium and allowed to grow at 28°C. The population was treated with penicillin-G (Sigma Chemicals) to kill actively growing population at this temperature. The remaining population after 24 h of penicillin-treatment was washed and spread on nutrient agar plates and incubated at 60°C. *Calothrix brevissima* does not form colonies however small pin-head visible patches were picked up by micro capillaries and grown in 5 ml broth. Several such clones were isolated, checked for its reversible character and finally a stable actively growing clone at 50°C was selected for the further study.

Enzyme (alkaline phosphatase/PMEase) assay. Alkaline phosphatase activity (PMEase; cellular and extra cellular) was determined in glycine-NaOH buffer (pH 10.2; 0.2 M) using *p*-nitrophenyl phosphate (*p*-NPP) as substrate and follows the method descried by Ihlenfeldt and Gibson (1975). The reaction mixtures containing culture suspension or supernatant (0.2 ml), buffer (1.6 ml) and substrate (0.2 ml) at final concentration of 0.75 mM were incubated for 20 min. at 37°C or at required temperature in a thermal controlled water bath. The reaction was terminated by NaOH (0.2 M) and the yellow colour of *p*-nitrophenol was quantified using itself as standard. The enzyme activity was expressed as nmol *p*-NPP hydrolyzed mg^{-1} protein h^{-1}.

PMEase assay and organic phosphates at various pH and temperatures. The millipore (0.22 µm) filter (Millipore, India), sterilized organic phosphates (Sigma Chemicals) such as *p*-nitro phenylphosphate (*p*-NPP), glucose 6-phosphate (G-6-P), fructose 6-phosphate (F-6-P), β-glycerophosphate and adenosine triphosphate (ATP) were used at 40 mg l^{-1} concentration.

PMEase derepressed cells of *C. brevissima* and its thermo-tolerant mutant (*tr*) cultivated for 7 days under the phosphate-depleted condition were harvested through centrifugation (10,000 × g; 15 min), washed and incubated in presence of *p*-NPP, G-6-P, F-6-P, β-glycophosphate, ATP and in P_i-stress (as control) under photoautotrophic growth conditions. Depending on the experimental requirements, the cells in the organic-P were incubated at different pH and temperature in the range of 9.0–10.5 and 30–60°C respectively. After 60 min of incubation, the cells were harvested and extra cellular release of PMEase activity was measured.

Sporulation. Cyanobacterial strains grown in phosphate sufficient medium were harvested, washed with sterile distilled water and inoculated on BG-11 nutrient agar. The inoculated Petri dish was incubated in light under growth conditions as described above. Growth on nutrient agar was examined periodically under a binocular microscope without disturbing its aseptic condition to observe initiation and spore formation. At the end of sporulation, the spore frequency was determined as described by Pandey and Kashyap (1987).

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

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

Statistical analysis. The mean data of three replicates of an experiment were analyzed 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 phosphomonoesterases (Seargeant and Stinson, 1979).

Results

Temperature kinetics of PMEase enzyme. In order to understand whether temperature kinetics of PMEase enzyme differ among the various species of diazotrophic cyanobacteria, five strains – *Anabaena flos-aquae*, *Nostoc calcicola* (an isolate of a rice field in reclamation stage of "Usar land" with a pH as high as 9.5), *Scytonema javanicum*, *Hapalosiphon intricatus* and *Calothrix brevissima* and its thermo-tolerant mutant (*tr*) were taken as test organisms. The results of temperature dependent alkaline phosphatase activity in cyanobacterial strains have been presented in Figures 1 and 2A. The strains incubated at 5°C did not show PMEase activity, however gradually increased after 10°C and reached its optimal level at a distinct temperature *i.e.* temperature optima. The PMEase activity among the cyanobacterial strains incubated at various temperatures differed significantly ($F_{3,35}$ strains-temperature × PMEase = 93.06; $p = < 0.001$) as evident in statistical analysis (Two way ANOVA).

The optimal level of PMEase activity (283 nmol *p*-NPP hydrolyzed mg^{-1} protein h^{-1}) was observed in *C. brevissima* at 37.5°C (Figure 2A). *Anabaena flos-aquae* and *S. javanicum* showed their maximum PMEase activity at 35°C whereas other strains have their temperature optima of the enzyme at 37.5°C. The activation

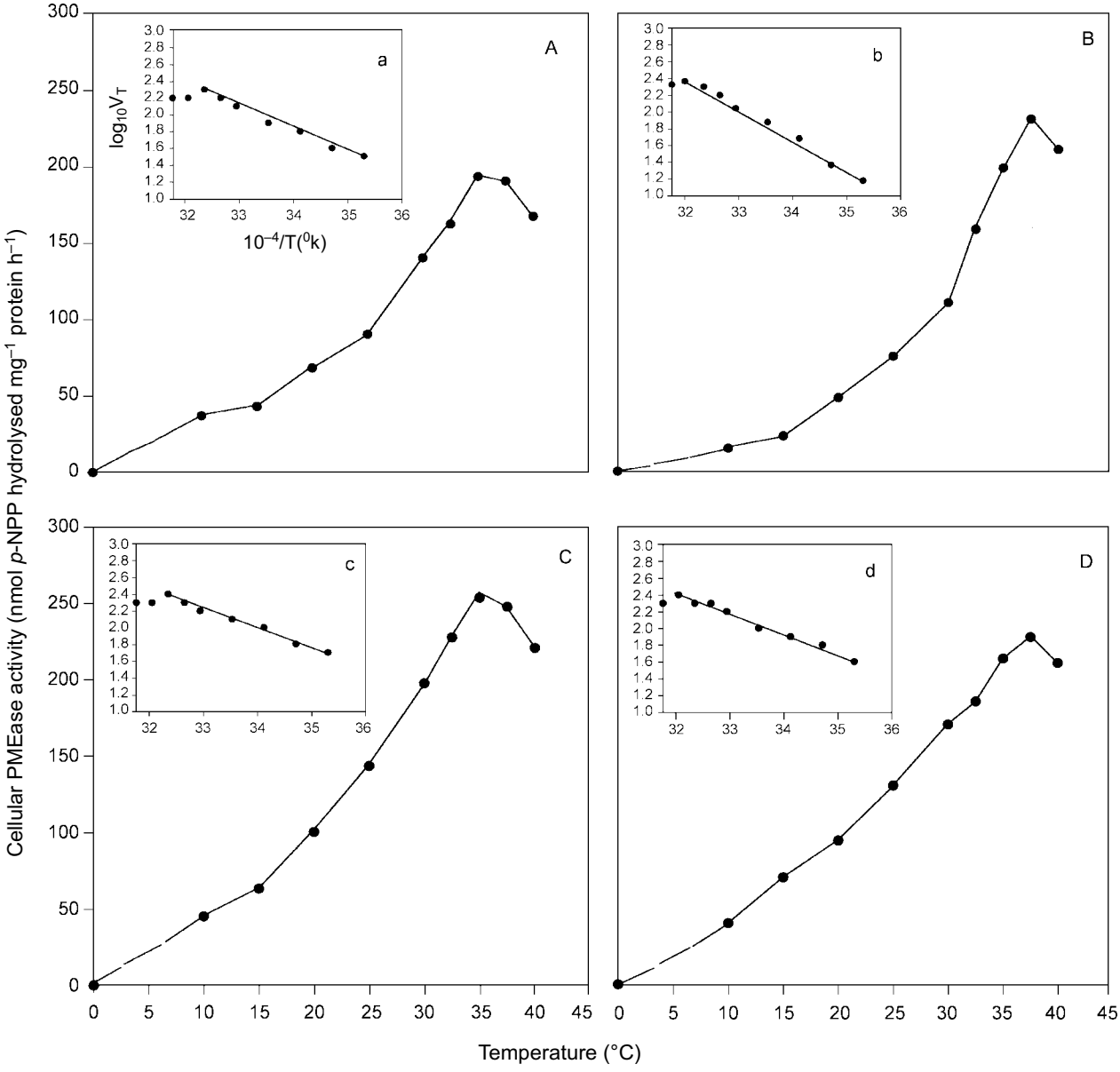

Fig. 1. Cellular phosphomonoesterase activities of the cyanobacteria *Anabaena flos-aquae* (A), *Nostoc calcicola* (B), *Scytonema javanicum* (C) and *Hapalosiphon intricatus* (D) at different temperatures (in degree centigrade, °C) with their corresponding Arrhenius plots (a, b, c and d) having reciprocal absolute temperatures (in Kelvin, K^{-1})

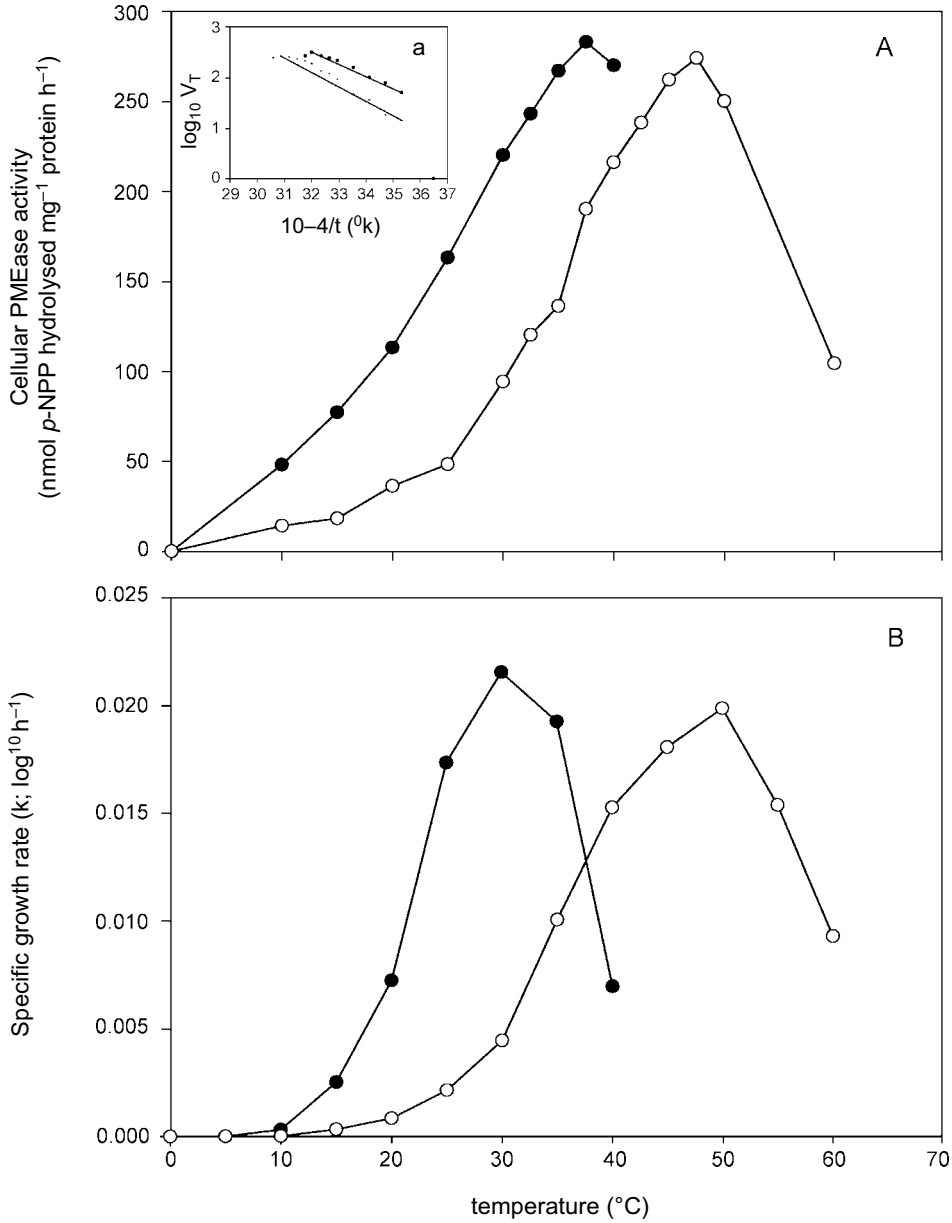

Fig. 2. Phosphomonoesterase activity (A) with their respective Arrhenius plot (a) and growth in terms of specific growth rate, k (B) of the cyanobacterium *Calothrix brevissima* (l) and its MNNG induced thermo-tolerant (*tr*) mutant (O) in response to different temperature.

energy (Ea) for cyanobacterial strains further confirms the narrow range of temperature kinetics of enzyme (PMEase) as the Ea values range between 45–52 kJ mol^{-1}. The *N. calcicola* and *A. flos-aquae* both have highest as well as identical values of Ea while the lowest Ea for PMEase was observed in *S. javanicum* (Table I).

pH optima. The cyanobacterial strains have their peculiar optimal level of PMEase activity either at pH 10.0 or 10.2. Only two pH optima for the enzyme were obtained for various strains, which corroborate its uniformity and stability with respect to pH and strain specificity (Table I). Statistical analysis (Kruscal-Wallis one way ANOVA) of the data on activation energy, temperature and pH optima of various cyanobacterial strains revealed that these characteristics of PMEase activity differed significantly ($p = < 0.001$) among the strains ($H = 26.6$ with one degree of freedom).

Characterization of a thermo-tolerant mutant (*tr*) of *Calothrix brevissima*. An attempt to isolate a thermo-tolerant mutant spontaneously through successive increase in temperature was a failure since population adapted at higher temperature reverted back easily. After the induction of MNNG, the thermo-tolerant mutant appeared at a frequency of 5.9×10^{-4} and they were stably inherited in cultures for several generations. During the routine cultivation, mutants were found to revert back to the parental type with a reversion frequency of 1.6×10^{-6}.

A comparative account of specific growth rate (k) of wild type *C. brevissima* and its thermo-tolerant (*tr*) mutant has been presented in Figure 2B. Unlike the wild type, mutant grew poorly until 30°C reaching its peak (k) at 50°C, while the wild type has its highest rate (k) at 30°C. The further increase in temperature drastically reduced the growth. The mutant, however, tolerated 60°C with just half of the value of specific growth rate (k).

The PMEase activity of wild type *C. brevissima* and its thermo-tolerant mutant (*tr*) plotted against different temperatures (Figure 2B) ranging from 0–60°C indicated a distinct temperature optima for parent and its mutant. The difference in temperature optima was about 10°C. At the lower temperatures mutant behaved in conformity with its growth pattern and no appreciable enzyme activity was observed until 30°C.

Organic phosphate, PMEase, temperature and pH optima: A correlation matrix. The extra cellular PMEase activity *i.e.* phosphate solubilisation of various organic phosphates supplemented with the medium by *C. brevissima* and its thermo-tolerant (*tr*) mutant at their optimal pH and temperature has been presented in Table II. The wild type cyanobacterium *C. brevissima* under phosphate stress (as control) showed extra cellular PMEase activity 75 and 72 nmol *p*-NPP hydrolyzed mg^{-1} protein h^{-1} at pH 10 and temperature 37.5°C, respectively which indicated its potential to utilize organic-P. The enzyme released was able to solubilise the organic P substrates which in turn curtailed the P_i-starvation up to some extent. This has resulted in reduced level of cellular PMEase and its release in the medium. The experiment shows that ATP and glycerophosphate are the least utilized organic phosphates as transpired from the higher level of enzyme activity (although lesser than the control). Among the other organic-P sources, *p*-NPP was found to be the best organic-P leveraged by PMEase and is followed by G-6-P and F-6-P. The pH required for their optimal utilization ranged between 9.6 and 10. Similarly the optimal temperature of the organic-P utilization was obtained either at 32.5°C or 37.5°C.

Table II
Phosphate solubilisation (extra cellular phosphomonoesterase activity) of different organic phosphates by *Calothrix brevissima* and its thermo-tolerant mutant (*tr*)

Factors	Characteristics	Various organic P-sources						
		Strains	Control (-P)	*p*-NPP	G-6-P	F-6-P	β-glycero-phosphate	ATP
pH	pH optima	Wild	10	10	9.8	9.8	9.6	9.6
		Mutant	10.4	10.4	10.2	10.3	10.2	10.0
	Extra cellular PMEase activity	Wild	75	39	44	49	57	63
		Mutant	68	35	40	44	50	57
Temp.	Temp. optima (°C)	Wild	37.5	37.5	37.5	35	35	32.5
		Mutant	47.5	47.5	45	45	45	45
	Extra cellular PMEase activity	Wild	72	43	48	49	60	65
		Mutant	65	39	42	42	58	60

APase derepressed cells of wild type and its thermo tolerant mutant (*tr*) were incubated in various organic P-sources (40 mg l^{-1}) such as *p*-nitrophenyl phosphate (*p*-NPP), glucose 6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), β-glycero-phosphate and adenosine triphosphate (ATP) for 24 hours under photoautotrophic growth conditions. The pH optima were determined by growing the cells at different pH (for 2 h) in the range of 9.00–10.5, while keeping the temperature (28 ± 2°C for wild type and 50 ± 2°C for mutant) constant. Similarly the cells grown in different organic phosphates at a constant pH of 8.0 (Default pH of BG-11 medium) were incubated (1 h) at different temperatures ranging from 30–60°C to determine the temperature optima. The phosphate depleted condition served as control medium.

The thermo-tolerant mutant (*tr*) of the cyanobacterium behaved similarly with respect to the various organic P utilization at their optimal pH and temperature values. The mutant with a higher temperature optima (47.5°C) for PMEase enzyme also showed pH tolerant behaviour as its pH optima was found to be the highest (pH 10.4) among the strains studied (Table II). The utilization of organic phosphates by wild type (*C. brevissima*) and its thermo-tolerant (*tr*) mutant reflected that the temperature and pH optima may change to a little extent while solubilising the organic-P. In other words the substrate dependent pH and temperature optima exist among the diazotrophic strains of cyanobacteria. A significant difference in temperature and pH optima against PMEase activity is also evident in the statistical analysis (Two way ANOVA) of the results obtained for organic phosphates utilization by *C. brevissima* and its thermo-tolerant (*tr*) mutant ($F_{1.47}$ pH-temp.optima, PMEase in P sources = 67.12; $p = < 0.001$).

Table III
Sporulation in diazotrophic cyanobacteria as influenced by temperature and pH optima of PMEase

Cyanobacterial strains	Effect of PMEase on Sporulation					
	At normal pH and temperature (Control)		At temperature optimum and pH normal		At pH optimum and temperature normal	
	Spore frequency (%)	Days of initiation	Spore frequency (%)	Days of initiation	Spore frequency (%)	Days of initiation
Anabaena flos-aquae	9.2	16	5.4	14	4.7	14
Nostoc calcicola	57	18	43	15	65	16
Scytonema javanicum	ND	ND	ND	ND	ND	ND
Hapalosiphon intricatus	23	29	14	21	15	23
Calothrix brevissima	ND	ND	ND	ND	ND	ND

Cyanobacterial strains are routinely cultivated at 28 ± 2°C and pH 8.0. Their respective temperature and pH optima for enzyme (PMEase) activity along with a normal pH (pH 8.0) and temperature (28°C) were selected to observe their effect on sporulation.
ND = not detectable

Sporulation and alkaline phosphatase. Since the phosphate starvation signalled the synthesis of PMEase as well as sporulation initiation in several cyanobacteria (Adams and Carr, 1981), an attempt was made to investigate the sporulation in cyanobacterial strains mentioned above at their respective temperature and pH optima of alkaline phosphatase. The results presented in Table III indicated that only three cyanobacteria among the five selected strains differentiated spores (under phosphate stress condition). The frequency of sporulation in *N. calcicola* and *H. intricatus* was high because in *N. calcicola*, the initiation started off between two heterocysts and the cyanobacteria form strings of spores. The *H. intricatus* differentiated spores randomly both in the prostrate and erect systems. The *A. flos-aquae* with the lowest spore frequency differentiated spores only on both the sides of heterocysts. The initiation time of sporulation decreased when the cyanobacterial strains were grown at their optimal temperature and pH of PMEase. That indicated a reciprocal relationship between initiation day and PMEase activity at higher temperature and pH (optima), which acted inversely towards the good growth. However, reduction in spore frequency in the strains at their respective temperature and pH optima may be due to lyses of the cells. The *N. calcicola*, an isolate of the higher pH, unlike the others, differentiated spores at higher frequency coupled with reduction in initiation days (Table III). The relationship between sporulation characteristics and PMEase activity were analysed by using controlled and optimal parameters (pH and temperature). The result was found to differ (Two Way ANOVA) significantly ($F_{2.17}$ PMEase activity vs. sporulation = 4.12; p = 0.05).

Discussion

Temperature as an environmental factor significantly affects the biodiversity and biomass of cyanobacteria in the rice-field of tropical countries. At the same time, the pH of the rice-growing regions of the tropics ranges from slightly acidic to as high as 9.5 (Singh, 1961). Cyanobacteria in rice-fields are often used as a potent bio-fertilizer to fulfil the demand of nitrogen in eco-friendly way. They also play another economic role by solubilising the complex organic phosphate molecules with the help of alkaline phosphatases (APases). Our studies substantiate further the environmental adaptive role of diazotrophic cyanobacteria in the rice cultivation by fixing nitrogen and solubilising complex organic phosphate.

Phosphate depletion (or stress) probably helps generating signal(s) to start endospore formation in *Bacillus subtilis* (Warren, 1968) and may also be valid in cyanobacteria by differentiating akinetes (spores) in response to phosphate starvation. Phosphate deficiency has been shown to induce sporulation in cyanobacteria (Wolk, 1965). A temporal relationship has been observed among phosphate uptake, induction of alkaline phosphatase activity and sporulation in *Anabaena doliolum* (Pandey *et al*., 1991). The akinetes serve as a perennating structure and generally formed under stress conditions. The effect of temperature which has a strong influence on alkaline phosphatase activity, on the sporulation of cyanobacterial strains indicated that under the higher temperature and pH (optima) conditions, N_2-fixing cyanobacterial strains solubilise phosphates at their optimal level, and promote the sporulation in the strains. Among the strains studied, *N. calcicola* behaved differently with respect to pH optima in terms of sporulation and the observed higher sporulation frequency may be due to the adaptation for several generations to high pH at its origin's

place (*ca* pH 9.5). Akinete differentiation in cyanobacteria is induced at low (Wolk, 1965) or higher temperature (Pandey and Kashyap, 1987), compared to the temperature which they are routinely cultivated. It seems that both low and high temperatures act as environmental stress to promote sporulation in the previous and present study.

It is interesting to note that the diazotrophic cyanobacteria isolated from rice-fields have shown two categories of temperature and pH optima. *Nostoc calcicola* and *H. intricatus* with higher temperature optima showed comparatively more basic values of pH optima while the *A. flos-aquae* with low temperature optima also possessed low pH optima. Similarly, PMEase enzyme in a thermo-tolerant mutant (*tr*) of *C. brevissima* with a higher temperature optima (47.5°C) exhibited a higher pH optima of 10.4. However, the pattern is not always consistent with the above, as *C. brevissima* with low pH and high temperature optima and *S. javanicum* with high pH and low temperature optima led to conclude that their temperature and pH optima are strain specific. The extra cellular PMEase has shown different affinities for the utilization of various organic phosphates for its metabolism. Their utilization by *C. brevissima* (wild type) and its thermo-tolerant mutant (*tr*) was pH and temperature dependent although the optimal pH and temperature for the PMEase enzyme has shown to operate within a narrow range. The temperature kinetics of alkaline phosphatase of *Plectonema boryanum* showed the maximum activity of both cell-bound and cell-free enzymes at 40°C and 70°C, while the pH optima was 8.8 and 9.0, respectively. The enzyme could hydrolyze a number of different organic compounds such as *p*-NPP, F-6-P, β-glycerophosphate and others in decreasing order (Doonan and Jenson, 1979).

Cyanobacterial strains have the potential to utilize organic phosphate as was evident in several reports (Ihlenfeldt and Gibson, 1975; Whitton *et al.*, 1991), however, the utilization of organic phosphate was found to be strain specific as well as substrate-specific. Sarma and Mehta (1985) observed that *Anabaena torulosa* could utilize glycerophosphate whereas *Anabaena doliolum* is not being able to do so. *Oscillatoria* BA 010 cells grown with G-6-P confirms that this is a P-sufficient culture since the initial high level of APase activity of P-deficient cultures lead to P-sufficient condition, which is comparable to the control culture level. As a result, the released phosphate that acts as an inhibitor of enzyme starts accumulating in the culture medium (Doonan and Jensen, 1979).

Al-Mousauri and Whitton (1983), while using a temperature gradient plate to enrich the cultures in the range of 20–50°C observed a marked difference in the temperature at which a particular species predominate in the mixed community. Substantial growth of a mixed cyanobacterial community occurred at 45°C but none at 48°C. In cultures, the most thermo-tolerant clones of *Synechococcus* grew at a maximum rate in the range of 63–68°C, while at temperatures within the range of 68–72°C they were observed having super optimal growth (Meeks and Castenholz, 1971). All clones of *Oscillatoria* cf. *terebriformis* had respectable growth rates below 48°C, although under natural conditions in the springs of East Oregon and North Nevada, the *Oscillatoria* mat ended abruptly at about 47–48°C (Wickstrom and Castenholz, 1985). A low and high temperature adapted population of cyanobacteria in a mixed population was shifted from low to high temperature. As a result of temperature shift, 16 S rRNA of high temperature adapted cyanobacteria increased while low temperature type 16 S rRNA decreased. Thus, suggesting that they were derived from population having peculiar temperature optima (Ruff-Roberts *et al.*, 1994).

In this study, the growth of the thermo-tolerant strain (*tr*) isolated during investigation reached its peak at 50°C, though it was not noticed until at 30°C. The isolation of such a type of thermo-tolerant strain has a significant potential for its inoculation as a biofertiliser in rice cultivation, especially in tropics.

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