

Full Length Research Paper

Phosphate uptake kinetics and its regulation in N₂-fixing cyanobacterium *Anabaena oryzae* Fritsch under salt stress

Santosh Kumar Singh^{1*}, Vandana Pandey², and Kapil Deo Pandey³

¹Centre of Experimental Medicine and Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi - 221 005, India.

²Department of Botany, Agrasen Kanya Autonomous Post Graduate College, Maidagin, Varanasi - 221 001, India.

³Algal Research Laboratory, Department of Botany, Banaras Hindu University, Varanasi-221 005, India.

Accepted 13 August, 2007

Phosphorous (P) starved cells of the cyanobacterium *Anabaena oryzae* showed higher phosphate uptake rates than P-sufficient cells. The P-uptake obeyed saturation kinetics. The Km value for P-deficient cells was lower (54.34 μM) than P-sufficient cells (82.64 μM) while V_{max} was higher in P-deficient and lower in P-sufficient cells. Salinity (NaCl) stimulated phosphate uptake significantly in the cyanobacterium which is followed by greater amount of P-accumulation in the form of polyphosphate bodies. Inhibition of P-uptake in P-deficient cells was 45% in dark grown compared to light grown cells. P-uptake was inhibited 52 and 85% in culture treated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 10 μM) and carbonyl cyanide m-chloro phenylhydrazone (CCCP; 100 μM), respectively, suggesting that energy for uptake could be derived from oxidative phosphorylation and photophosphorelation.

Key words: *Anabaena oryzae*, metabolic inhibitors, phosphorus-uptake, salinity.

INTRODUCTION

Phosphorous (P) in municipal, agricultural and industrial effluents is an important contributory factor to eutro- fication and its recycling for further utilization is a matter of concern. It is widely accepted that phosphorus is the main nutrient that controls the development of natural populations of cyanobacteria in many terrestrial fresh water and marine environments (Healey, 1982; Mann, 1994; Whitton and Pott, 2000; Singh et al., 2006).

Although a number of dissolved organic phosphorus compounds can be utilized for growth by various algal species (Cembella et al., 1984; Mahasneh et al., 1990; Berman et al., 1969; Kumar et al., 1992), orthophosphate is the only form of phosphorus commonly taken up by

autotrophs (Healey, 1982). This is facilitated through the activity of alkaline phosphatase which appears in phos- phate deficient condition and hydrolyses phosphate from a variety of organic phosphorus compounds (Healey, 1982; Torriani-Gorini, 1994; Whitton et al., 1991; Shukla et al., 1997; Singh and Tiwari, 2000).

Phosphorus storage in cyanobacteria appears to be much larger than in other species and this capacity was reported to give them a competitive advantage over diatoms and chlorophytes when P was supplied in pulses (Sommer, 1985; Reinertsen et al., 1986; Sakshaug and Olsen, 1986). Phosphate uptake in cyanobacteria follows saturation type kinetics with kinetics parameters ($K_{1/2}$ and V_{max}) comparable to those of eukaryotic algae (Healey, 1982). The uptake process is energy-dependent (Healey, 1982; Budd and Kerson, 1987; Fernandez Valiente and Avendano, 1993) but its mechanism is unclear.

Phosphate uptake in cyanobacteria also requires speci-

*Corresponding author: E-mail: singhsk71@yahoo.com. Tel: 091-5422307549 (O) 091-9415389046 (Mobile).

fic cations. A stimulation of phosphate uptake by divalent cations Mg^{2+} , Ca^{2+} and Sr^{2+} has been reported in several strains of N_2 -fixing cyanobacteria (Falkner et al., 1980; Budd and Kerson, 1987). The cation requirement for the increased phosphate uptake and alkaline phosphatase activity suggest for their close functional relations and can be interpreted as adaptive responses to phosphorus limitation. Na^+ stimulation of phosphate uptake has been reported in cyanobacteria (Fernandez et al., 1993; Avendano and Fernandez Valiente, 1994).

A. oryzae is a dominant cyanobacterium strain of rice-field throughout the crop season where it can be used as biofertilizer. The potential of *A. oryzae* to take up phosphorus by phosphate starved cells was investigated under the influence of light and dark, metabolic inhibitors and salinity.

MATERIAL AND METHODS

Organism and growth conditions

The heterocystous cyanobacterium *A. oryzae*, a local isolates from rice field of Banaras Hindu University agriculture farm was cultivated and maintained in eight fold diluted Allen-Arnon medium (AA/8) with A5 trace element (Allen and Arnon, 1955). The clonal and axenic population of cyanobacteria was raised and their purity was tested following the standard microbiological techniques. The strain was identified using the classical and recent literatures (Desikachary, 1959; Rippka et al., 1979). The cultures were incubated for photoautotrophic growth in a culture cabinet under cool fluorescent illumination ($25 \mu \text{Em}^{-2}\text{s}^{-1}$) for 14 h a day and at a temperature of $30 \pm 2^\circ\text{C}$. The experiments were performed under diazotrophic growth condition (N_2 -medium) and repeated 3 to 4 times. The pH of the growth medium was maintained 7.6 with HEPES buffer at a final concentration of 2 mM.

Cellular protein content

The total protein contents of cultures were measured following the methods of Lowry et al. (1951) as modified by Herbert et al. (1971).

Phosphate uptake

Five days old cultures of the cyanobacterium *A. oryzae* were harvested, washed twice with P-deficient Allen-Arnon medium (AA/8) and suspended in P-sufficient or P-deficient medium for 12 h under standard growth conditions and termed as P-sufficient and P-deficient cells. The cells were collected, washed thoroughly, resuspended in P-deficient Allen-Arnon medium (AA/8) containing different concentrations of NaCl, and incubated under standard growth conditions. After an hour of preincubation, assay was started by adding K_2HPO_4 to a final concentration of 400 μM . In another set of experiment NaCl concentration remain constant (8 mM) while K_2HPO_4 concentration varied 50 - 400 μM . At different time intervals, aliquots were withdrawn and filtered to remove the cells. The filtrates were used to determine the phosphate concentration according to Healey (1978). P uptake was calculated from its depletion in the external medium.

Cellular phosphate

Cyanobacterial cells for determination of cellular phosphorus were collected by centrifugation and washed twice with sterile double dis-

tilled water. Samples were dried at 85°C , till constant weight, followed by digestion in boiling concentrated HNO_3 and H_2SO_4 (American Public Health Association (APHA), 1995). The phosphorus of the digest was determined by the ascorbic acid method (APHA, 1995).

Polyphosphate content

The total polyphosphate granules of the cells were extracted following the method of Poindexter and Fay (1983), and analyzed the Pi following the method of Healey (1978).

RESULTS

Phosphate uptake

The rate of P-uptake by P-starved cell increased with increase in phosphate concentrations up to 400 μM in a concentration-dependent manner and further increase in P-concentration in assay medium has no effect on uptake process. Rates of P-uptake determined for P-sufficient cells also showed the similar trend of uptake and the rate did not increase above the 200 μM . P-uptake in *A. oryzae* follows saturation type kinetics (Figure 1A). The 2.3 fold increase in P-uptake by P-deficient cells indicates the role of cellular P-level in controlling the process of P-uptake.

The Km value was lower (54.34 μMP) in P-deficient than P-sufficient cells (82.64 μMP) while V_{max} value was high (0.11 $\mu\text{mol P mg protein}^{-1} \text{min}^{-1}$) for P-deficient and low (0.06 $\mu\text{mol P mg protein}^{-1} \text{min}^{-1}$) for P-sufficient cells (Figure 1B). This shows that P-starvation of cells increases the rate of phosphate transport, which is much higher than the rate observed for P-sufficient cells. The P-level of cells under these conditions is attributed to be a factor controlling the phosphate transport function.

Effect of light/dark and metabolic inhibitors on phosphate uptake

The energy-dependence of phosphate uptake process was observed in light and dark and in the presence of different photosynthesis and electron-transport chain inhibitors. The cells were phosphate starved for 24 h and incubated with phosphate (400 μM) in combination with or without inhibitors. The uptake rates were determined after 4 hours of incubation The P-uptake in the presence of light (control condition) was 5.15 $\mu\text{mol P mg protein}^{-1}$ and in dark, the uptake activity was reduced to about 55% indicating the light energy depend uptake process (Table 1). DCMU (10 μM), in the photosynthetic growth condition inhibited 52% P-uptake. Arsenate, a potent inhibitor of respiration and analogue of phosphate had no effect on uptake at low concentration (25 μM). Even at higher concentrations (50, 100 μM), the percent inhibition of phosphate uptake was relatively less. CCCP (100 μM),

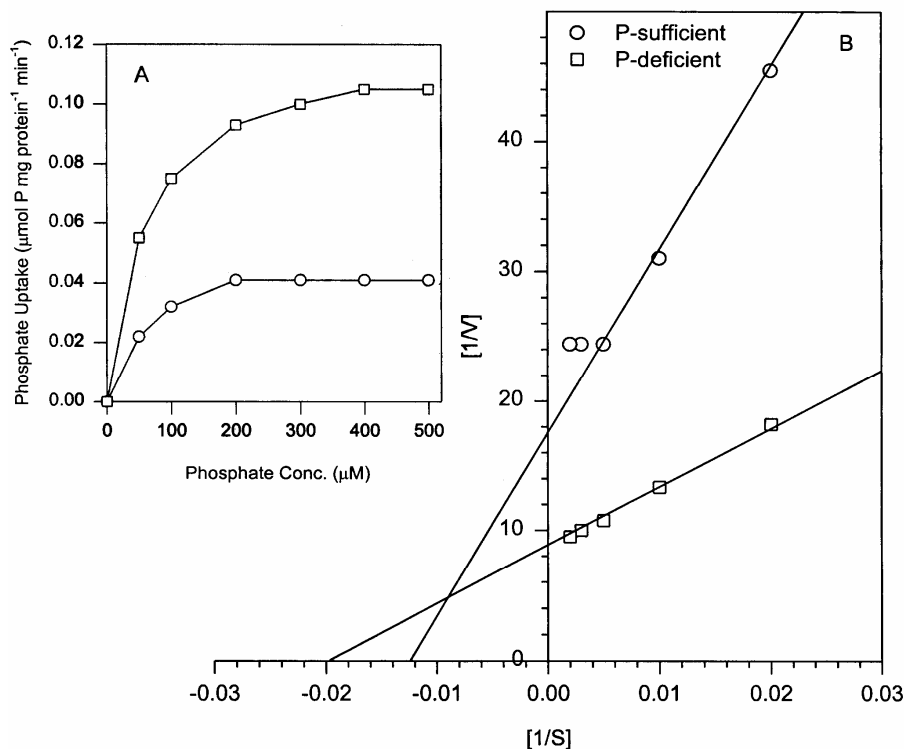


Figure 1. Phosphate uptake rate (A), Lineweaver-Burk plot (B) in P-deficient and P-sufficient cells of *Anabaena oryzae*.

Table 1. Phosphate uptake by *Anabaena oryzae* under different experimental conditions.

Experimental Condition	Phosphate uptake* ($\mu\text{mol P mg protein}^{-1}$)	Inhibition (%)
Light (L)	5.2 ± 0.21	0.0
Dark (D)	2.3 ± 0.05	55.2
DCMU (10 μM) + L	2.5 ± 0.06	52.1
Arsenate (25 μM) + L	5.2 ± 0.21	0.0
Arsenate (50 μM) + L	4.6 ± 0.14	10.1
Arsenate (100 μM) + L	3.9 ± 0.06	25.1
CCCP (25 μM) + L	4.1 ± 0.12	20.0
CCCP (100 μM) + L	0.8 ± 0.02	85.1

*After 4 h incubation.

an inhibitor of electron transport chain, severely inhibited P-uptake (85% activity) in cyanobacterium.

Effect of NaCl

The requirement of Na^+ in phosphate uptake was examined in P-starved *Anabaena* cells in the presence of 400 μM phosphate supplemented with different concentration of NaCl (0.5 - 10 mM). Phosphate uptake was periodically measured with respect to time as usual. In this short term P-uptake experiments, the data presented

(Figure 2a) illustrate the response of phosphate uptake to increasing concentration of Na^+ . The level of phosphate uptake by *Anabaena* cells increased with the increase in concentration of Na^+ upto a saturation level (8 mM), above which no further increase or decrease occurred, atleast in the range of Na^+ concentrations tested. The results presented here indicate the stimulatory effect of Na^+ on phosphate uptake.

In another experiment, the effect of Na^+ on P-uptake was also examined in the presence of different concentrations of phosphate (50 - 400 μM). As shown in Figure 2b the P-uptake level in the cyanobacterium was

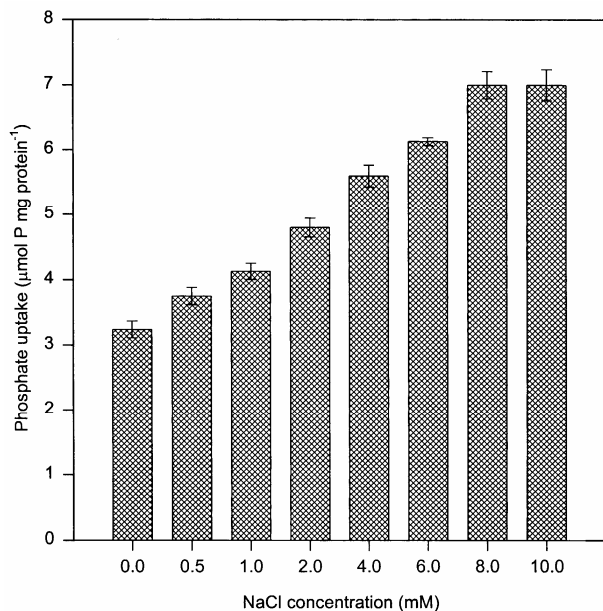


Figure 2a. Phosphate uptake by P-deficient cells of *Anabaena oryzae* in the presence of different concentrations of NaCl (after 30 min incubation).

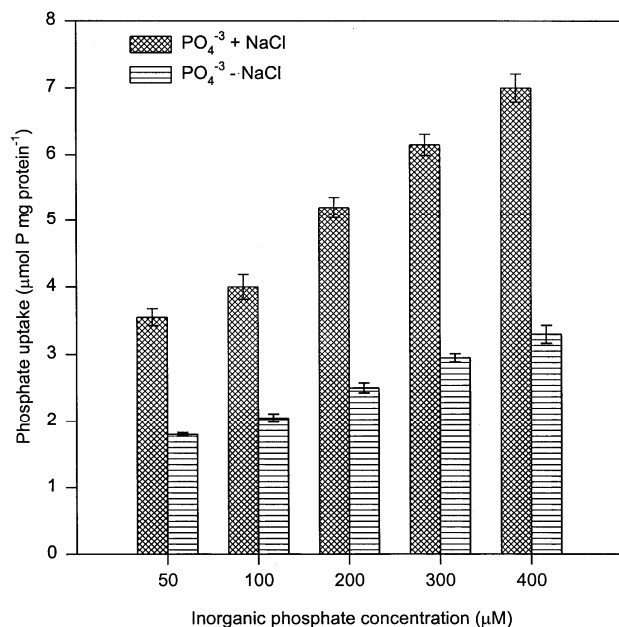


Figure 2b. Phosphate uptake by P-deficient cells of *Anabaena oryzae* in the presence and absence of NaCl under different phosphate concentrations (after 30 min incubation).

significantly reduced when cells were incubated in the absence of Na⁺. A two-fold decrease in P-uptake was noted in the absence of Na⁺. However, the uptake activity was found to be dependent on phosphate concentrations irrespective of the presence or absence of NaCl (8 mM)

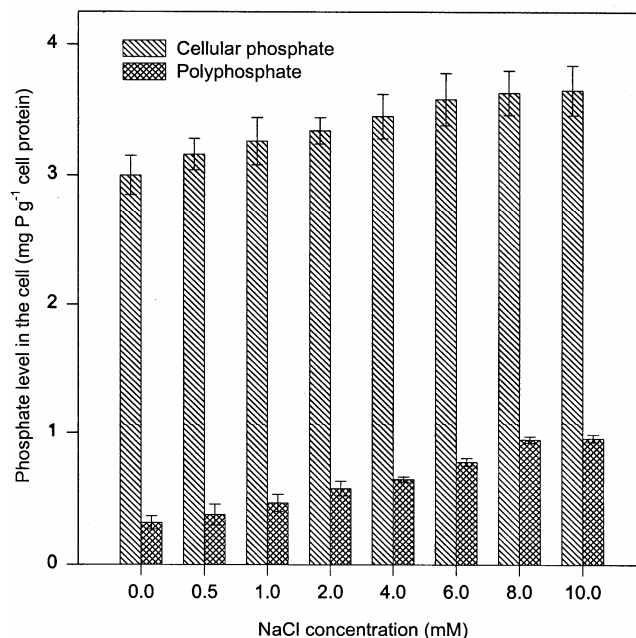


Figure 3. Effect of different concentrations of NaCl on cellular and polyphosphate of *Anabaena oryzae*.

in the medium and the activity increased with respect to phosphate concentration. The considerably enhanced P-uptake in the presence of NaCl indicates the requirement of Na⁺ in the uptake process in *A. oryzae*.

Effect of salinity on cellular and polyphosphate of *Anabaena oryzae*

Cyanobacteria, under phosphate sufficient condition accumulate polyphosphate in the form of granules which is comparatively more under diazotrophically grown cells. The effect of sodium chloride on cellular P-uptake and phosphate accumulation in the form of polyphosphate showed that the cell-P increased with increment in NaCl (0 - 10 mM) level. Cellular-P was (3.0 mg P g⁻¹ cell protein) minimum in absence of NaCl and sodium chloride stimulated which was maximum (3.63 mg P g⁻¹ cell protein) in NaCl (8 mM) supplemented medium. Similar trend was also observed in poly P-accumulation in cell.

There seems to exist a positive correlation between poly-P-accumulation and cellular P-content. Poly-P contributed about 10% to cellular P-content and sodium chloride enhanced accumulation up to 26% of the total cell P (Figure 3).

DISCUSSION

Microbes, including cyanobacteria, have evolved the potential to respond to environmental changes by changing themselves. This potential for rapid adaptation is uni-

quely associated with the life style of microbes and resides in their genome which carries all the information necessary for organism to confer upon them the ability to respond to changes in their environment. Thus an organism of a given genotype is a very much product of its environment (Tempest and Neigssel, 1978). One of the environmental parameters in natural ecosystems is the frequent depletion of one or more essential nutrients which commonly influence the properties of microbial cells in nature and cultural conditions, and for that microbes, it possess a wide variety of mechanisms for adapting them self both structurally and functionality with the objective to ensure their prolonged survival in the environment.

Phosphate uptake by P-sufficient cells was saturated at 200 μM phosphate concentration while P-deficient cells had a saturation point at 400 μM concentration. The 2-3 fold increase in P-uptake by P-deficient cells over P-sufficient cells also indicates the role of cellular P-level in regulating the uptake process. Like other microbial systems, the P-uptake in *A. oryzae* also follows saturation kinetics. The K_m for P-deficient cells was lower than the P-sufficient cells while V_{\max} was higher in P-deficient and lower in P-sufficient cells. This provides evidence to suggest that P-starvation of cells increases the phosphate transport rate. This important function was also found to be energy regulated as the activity was greatly reduced in dark or in cells treated with inhibitors of photosynthesis and respiration. The requirement of energy for P-uptake varied under various P_i concentrations in the medium. The very low concentration of available phosphate (P_i) in the soil (Ragothama, 1999) and a high demand for P_i in cell poses a unique problem. *A. oryzae* adopted strategies to overcome this low P_i concentration problem by increasing P_i affinity through decrease in K_m value (NaCl mediated) or energy provided by ATP. It was of interest to note that this process also required Na^+ for optimal activity which increased with increasing concentration of phosphate. The uptake rate was three fold higher in the presence of NaCl than its absence. The kinetic parameter of P-uptake was also affected by NaCl that is, the presence of NaCl significantly increased V_{\max} and decreased K_m value, compared to the control. This confirms the previous report on Na^+ -stimulation of P-uptake in unicellular and filamentous cyanobacteria (Apte and Thomas, 1985; Avendano and Fernandez Valiente, 1994). Energy required for P-uptake can be provided either by ATP or by electro-chemical gradient. The possibility of Na^+ /phosphate cotransport has been envisaged as has been reported for Na^+ -dependent nitrate transport in *Anacystis nidulans* (Rodriguez et al., 1992) and for phosphate uptake in the heterotrophic bacterium *Streptococcus faecalis* (Rabaste et al., 1991) and in some non-nitrogen fixing cyanobacteria (Avendano and Valiente, 1994).

Evidences have been presented against the involvement of proton motive force in the process of phosphate

uptake (Budd and Kerson, 1987). The possibility that Na^+ -electrochemical gradient can act as energy source for this process must be however taken into consideration. Illuminated cyanobacteria maintain a downhill Na^+ electrochemical gradient from outside to inside which can provide energy for secondary active transport (Ritchie, 1992).

Cyanobacteria growing under P-stress evolve several strategies to cope up in the P-deficiency. Mechanisms involved include the synthesis of phosphates, high efficiency for P-uptake and utilization of its stored polyphosphate. The gene(s) involved in the regulation of these process are located on different operons (Bhaya et al., 2000) however, it seems to belong to the same gene family having different regulon under similar physiological condition of P-stress. Another physiological stress created by NaCl acts synergistically to the above mentioned functions which are of great practical significance. The findings are important and can be exploited biotechnologically in the production of APases and removal of P_i through uptake process and its accumulation in the form of poly-P body.

ACKNOWLEDGEMENTS

The authors thank Head, Department of Botany, B.H.U. for providing the facilities. SKS is grateful to Director IMS for encouragements.

REFERENCES

- Allen MB, Arnon DI (1955). Studies on nitrogen-fixing blue-green algae. I. growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. 30: 366-372.
- American Public Health Association (APHA) (1995). Phosphorus. In Eaton AD, Clesceri LS, Greenberg AE (eds) Standard Methods for the Examination of Water and Waste Water, Am. Public Health Assoc., Washington, pp. 107-115.
- Apte SK, Thomas J (1985). Effect of sodium on membrane potential, uptake of phosphate, nucleoside phosphate pool and synthesis and expression of nitrogenase in *Anabaena torulosa*. Ind. J. Exp. Biol. 23: 518-522.
- Avendano MDC, Fernandez Valiente E (1994). Effect of sodium on phosphate uptake in unicellular and filamentous cyanobacteria. Plant Cell Physiol. 35(7): 1097-1101.
- Bhaya D, Schwarz R, Grossman R (2000). Molecular responses to environmental stress. In Whitton BA, Potts M (eds) Ecology of Cyanobacteria, Kluwer Academic Publishers, The Netherlands, pp. 397-442.
- Berman T (1969). Phosphate of inorganic phosphorus in lake Kinneret. Nature 224: 1231-1232.
- Budd K, Kerson GW (1987). Uptake of phosphate by two cyanophytes: Cation effects and energetics. Can. J. Bot. 65: 1901-1907.
- Cembella AD, Antia NJ, Harrison PJ (1984). The utilization of inorganic and organic phosphorus compounds as nutrients by eukaryotic microalgae-A multidisciplinary perspective. Part I. CRC. Crit. Rev. Microbiol. 10: 317-391.
- Desikachary TV (1959). Cyanophyta, Indian Council of Agricultural Research, New Delhi, pp. 623-660.
- Falkner G, Horner F, Simonis W (1980). The regulation of energy dependent phosphate uptake by the blue-green alga *Anacystis nidulans*. Planta 149: 138.
- Fernandez Valiente E, Avendano MDC (1993). Sodium stimulation of

- phosphate uptake in the cyanobacterium *Anabaena* PCC 7119. *Plant Cell Physiol.* 34: 201-207.
- Healey FP (1978). Phosphate uptake. In Hellebust JA, Craigie JS (eds) *Handbook of Phycological Methods. Physiological and Biochemical Methods*, Cambridge University Press, Cambridge, pp. 411-417.
- Healey FP (1982). Phosphate. In Carr NG, Whitton BA (eds) *The Biology of Cyanobacteria*, Blackwell Scientific Publishers, pp. 105-124.
- Herbert D, Phipps PJ, Strange RE (1971). Chemical analysis of microbial cells, In Norris JR, Ribbons DW (eds) *Method in Microbiology*, Academic Press London and New York, pp. 209-344.
- Kumar TA, Mahasneh IA, Tiwari DN (1992). Alkaline phosphatase activities of an *Anabaena* from deep-water rice. *World J. Microbiol. Biotechnol.* 8: 585-588.
- Lowry OH, Rosenbrough NJ, Farr, AL, Randall RJ (1951). Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Mahasneh IA, Grainer SLJ, Whitton BA (1990). Influence of salinity on hair formation and phosphatase activities of the blue-green alga (cyanobacterium) *Calothrix viguieri* D253. *Br. Phycol. J.* 25: 25-32.
- Mann NH (1994). Protein phosphorylation in cyanobacteria. *Microbiology* 140: 3207-3215.
- Poindexter JS, Fay LF (1983). Combined procedure for assays of poly- β -hydroxybutyric acid and inorganic poly phosphate. *J. Microbiol. Methods*, 1: 1.
- Rabaste F, Dauphin G, Jeminet G, Guyot J, Delort A (1991). Phosphate-dependent sodium transport in *S. faecalis* investigated by ^{23}Na and ^{31}P NMR. *Biochem. Biophys. Res. Commun.* 181: 74-79.
- Ragothama KG (1999). Phosphate acquisition. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50: 665-693.
- Reinertsen H, Jensen A, Langeland A, Olsen Y (1986). Algal competition for phosphorus: the influence of zooplankton and fish. *Can. J. Fish. Aquat. Sci.* 43: 1135-1141.
- Rippka R, Deruelles J, Waterbury JB, Hardman M, Stanier RY (1979). Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol.* 111: 1-61.
- Ritchie RJ (1992). Sodium transport and the origin of membrane potential in the cyanobacterium *Synechococcus* R-2 (*Anacystis nidulans*) PCC 7942. *J. Plant. Physiol.* 139: 320-330.
- Rodriguez R, Lara C, Guerrero MG (1992). Nitrate transport in the cyanobacterium *Anacystis nidulans* R-2: kinetic and energetic aspects. *Biochem. J.* 282: 639-643.
- Sakshaug E, Olsen Y (1986). Nutrient status of phytoplankton blooms in Norwegian waters and algal strategies for nutrient competition. *Can. J. Fish. Aquat. Sci.* 43: 389-396.
- Shukla SP, Singh SK, Mishra AK (1997). Energetics of alkaline phosphatase (monoesterase) activity in Antarctic and tropical isolates of a cyanobacterium, *Anabaena*. *Cytobios.* 9: 29-33.
- Singh SK, Singh SS, Pandey VD, Mishra AK (2006). Factors modulating alkaline phosphatase activity in the diazotrophic rice-field cyanobacterium, *Anabaena oryzae*. *World J. Microbiol. Biotech.* DOI 10.1007/s11274-006-9137-1 Online.
- Singh SK, Tiwari DN (2000). Control of alkaline phosphatase activity in *Anabaena oryzae*. *J. Plant Physiol.* 157: 467-472.
- Sommer U (1985). Comparison between steady state and non steady state competition: Experiments with natural phytoplankton. *Limnol. Oceanogr.* 30: 335-346.
- Tempest DW, Neigssel OM (1978). Ecophysiological aspects of microbial growth in aerobic nutrient limited environment. *Adv. Microb. Ecol.* 2: 105-153.
- Torriani-Gorini A (1994). Introduction: the pho regulon of *Escherichia coli*. In Torriani-Gorini A, Yagil E, Silver S (eds) *Phosphate in microorganism: Cellular and molecular biology*. Am. Soc. Microbiol., Washington DC, pp. 1-5.
- Whitton BA (2000). Soils and rice-fields. In Whitton BA, Potts M (eds) *Ecology of Cyanobacteria*, Kluwer Academic Publishers, The Netherlands, pp. 233-255.
- Whitton BA, Grainger SLJ, Hawley GRW, Simon JW (1991). Cell-bound and extracellular phosphatase activities of cyanobacterial isolates. *Microb. Ecol.* 21: 85-98.