Short Communication

Studies on phosphate uptake, accumulation and activity of alkaline phosphatase of *Nostoc muscorum* under Cd stress

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Phosphorus is an essential constituent of all living cells. The element is a part of the fundamental building blocks that constitute nucleic acids, phospholipids and complex carbohydrates. Phosphorus compounds play a central role in anabolic and catabolic pathways and in the energy conversion of the cell via transfer of energy-rich phosphoanhydride bonds, a reaction which is also involved in post translational regulation of enzyme activities (Wagner and Falkner, 2001).

In most temperate lakes, phosphate deficiency is a common phenomenon and earlier studies confirmed that phosphate-deficient organisms are capable of accumulating this nutrient in several-fold excess of their immediate demand when exposed to considerable amounts of phosphate. This tendency to store phosphate, predominantly in the form of polyphosphates, is referred to as the polyphosphate over plus phenomenon (Kulaev and Vagabov, 1983). Esterified phosphates may be released from organic phosphates through the action of cellular and extracellular alkaline phosphatases (APases) by catalyzing the hydrolysis of phosphate ester bonds. Organisms ranging from bacteria to higher plants, including algae and cyanobacteria, synthesize phosphatase in response to low availability of free phosphates to access otherwise non-metabolizable organic phosphate resources (Carr and Mann, 1994; Hallmann, 1999; Singh and Tiwari, 2000; Tadano et al., 1993).

The APase activity appears to be widespread among cyanobacteria under P-limited conditions (Healey, 1982). Ultrastructural analysis of APase in *Plectonema boryanum* showed that the enzyme is localized in layer 3 (periplasmic space) of the cell wall. During recent decades, a growing interest in the phosphate regeneration of those aquatic agroecosystems (waterlogged rice-fields) which have plenty of organic phosphate compounds, has led a number of workers to study the algal, including cyanobacterial phosphatase, activity and its regulation, since these organisms constitute the dominant flora in such ecosystems. Keeping this fact in view and considering the worldwide metal pollution problem, a study of phosphate uptake and its internal accumulation, as well as the properties of alkaline phosphatase of a *N*$_2$-fixing cyanobacterium *Nostoc muscorum* under Cd stress, may help in understanding the phosphorus metabolism and the potential of enzymatic regeneration of inorganic phosphate from polyphosphate compounds in a metal-stressed ecosystem.

The cyanobacterium *Nostoc muscorum* was used as the experimental organism. The clonal and axenic populations were obtained by employing standard microbiological techniques. The cultures were main-
tained in Allen and Arnon’s medium (Allen and Arnon, 1955) under photoautotrophic growth conditions (75 μmol photon m⁻² s⁻¹ PAR) at 24 ± 2°C. The basal medium free of combined nitrogen was used for cultivation. The pH of the growth medium was adjusted to 7.5 with HEPES/NaOH (4 mM) buffer. Cd was selected as the test metal because soils in general are found contaminated with Cd to a certain extent where PO₄ is the test metal because soils in general are found contaminated with Cd to a certain extent where PO₄ fertilizers are applied (phosphate fertilizers contain 70–150 mg Cd per kg P; Cseh, 2002). Doses of Cd selected for this study include (i) LC50, (ii) three concentrations below LC50, (iii) three concentrations below LC50, and (iii) three concentrations above LC50.

The protein content of cultures was measured following the methods of Lowry et al. (1951) as modified by Herbert et al. (1971). Phosphate uptake and intracellular accumulation (internal pool) were studied as per APHA (1998) and Husaini et al. (1996), respectively. Alkaline phosphatase activity was assayed by estimating the formation of p-nitrophenol from p-nitrophenyl phosphate (Ihlenfeldt and Gibson, 1975). Inhibition kinetics for phosphate uptake and alkaline phosphatase activity were studied at different substrate concentrations and the values of K_m and V_max were obtained by drawing the double reciprocal plots. Characterization of alkaline phosphatase was done in the presence/absence of Zn and Mg. All experiments were performed in liquid medium and repeated at least 3 times. The results were analyzed statistically by Student’s t test.

It is evident from Table 1 that not only the uptake but also the internal pool of phosphate of N. muscorum declined significantly under Cd stress (p<0.05, Student’s t test). The LC50 of Cd (0.75 μg ml⁻¹) was found to inhibit phosphate uptake by 58%, whereas intracellular accumulation was affected only by 24%. Higher concentrations of Cd were found more inhibitory to both the processes. Studies on kinetics of inhibition showed a competitive inhibition pattern for the uptake process (Fig. 1).

Figure 2A presents the induction curve for alkaline phosphatase of Nostoc muscorum under P-deficient conditions. Maximum activity (0.169 ±0.03 nmol PNP μg protein⁻¹ min⁻¹) was observed after 48 h of incubation in the control cells, whereas in Cd-treated cells the activity reached its maxima at 72 h (0.075 ±0.02 nmol PNP μg protein⁻¹ min⁻¹). Kinetics of inhibition also depicted a competitive inhibition pattern for this enzyme (Fig. 2B). It is interesting to note here that induction of this enzyme was also observed in P-rich conditions following Cd (0.15 and 0.2 μg ml⁻¹) treatment (Table 2). Figure 3 depicts the impact of Zn ions on the activity of alkaline phosphatase of N. muscorum cells grown for two generations in Zn-deficient medium. The presence of Zn was found essential for induction of this enzyme, maximum activity being observed in 0.1 μg ml⁻¹ of Zn-supplemented cultures. No such behavior was, however, noticed for Mg (data not shown).

The observed competitive inhibition of phosphate

### Table 1. Effect of Cd on phosphate uptake and internal phosphate pool of Nostoc muscorum after 72 h of treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Phosphate uptake²</th>
<th>Internal phosphate pool²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd (μg ml⁻¹)</td>
<td>(μg PO₄⁻³ / μg protein⁻¹)</td>
<td>(μg PO₄⁻³ / μg protein⁻¹)</td>
</tr>
<tr>
<td>Control</td>
<td>5.93 ±0.11 (00)</td>
<td>0.208 ±0.01 (00)</td>
</tr>
<tr>
<td>0.01</td>
<td>5.55 ±0.13 (06)*</td>
<td>0.208 ±0.01 (00)*</td>
</tr>
<tr>
<td>0.03</td>
<td>5.03 ±0.21 (15)</td>
<td>0.208 ±0.01 (00)*</td>
</tr>
<tr>
<td>0.05</td>
<td>3.89 ±0.15 (35)</td>
<td>0.176 ±0.03 (15)</td>
</tr>
<tr>
<td>0.075 (LC50)</td>
<td>2.49 ±0.11 (58)</td>
<td>0.159 ±0.01 (24)</td>
</tr>
<tr>
<td>0.10</td>
<td>1.68 ±0.099 (72)</td>
<td>0.101 ±0.01 (52)</td>
</tr>
<tr>
<td>0.15</td>
<td>0.53 ±0.099 (91)</td>
<td>0.041 ±0.01 (80)</td>
</tr>
<tr>
<td>0.20</td>
<td>0.30 ±0.09 (95)</td>
<td>0.022 ±0.01 (89)</td>
</tr>
</tbody>
</table>

*Values in parentheses denote % inhibition.
All values are mean ±SE.
* ‘*’ significant at p<0.05, * not significant.
uptake (Fig. 1) under Cd stress indicates a direct competition between Cd and PO₄³⁻ ions for a common entry site. This was further confirmed when the toxic effect was counteracted at increasing substrate concentrations. As in many algae and cyanobacteria, phosphate deficiency induced N. muscorum cells to synthesize alkaline phosphatase, which is otherwise repressed in P-rich conditions (Fig. 2A). The P-rich Nostoc cells with no detectable alkaline phosphatase activity required a period of 12 h in P-free medium to express the initiation of alkaline phosphatase activity. This is quite earlier as compared to other cyanobacteria where the switch-on times reported under P-free medium vary from 54 to 148 h (Mahasneh et al., 1992). The medium containing LC₅₀ of Cd required a longer switch-on time (48 h) to express the respective enzyme activity (Fig. 2A). Interestingly, induction of alkaline phosphatase in N. muscorum cells was observed even in P-rich medium when the test cyanobacterium was exposed to higher doses of Cd (Table 2). It can also be visualized from Table 1 that higher concentrations, i.e. 0.15 and 0.2 μg ml⁻¹ of Cd, resulted in severe reduction (91% and 95%, respectively) in phosphate uptake. Therefore, such a low uptake potential under higher doses of Cd could account for the switch-on of the enzyme, alkaline phosphatase, for breakdown of the polyphosphates for cellular metabolism.

Alkaline phosphatase is also notable in being a metalloenzyme (Vincent et al., 1992). An extensive study on the enzymology of APase of the enteric bacterium Escherichia coli demonstrated that it is a homodimeric complex (Mᵣ ∼94 kDa) with two Zn (II) ions and a single Mg (II) ion within the active site of each subunit. The Zn (II) ions most likely possess a catalytic role in rendering phosphate monoesters susceptible to hy-
drolysis (Wyckoff, 1987).

Though studies conducted with algae and cyanobacteria depict similarity with bacterial and metazoan APase with regard to their phosphate repressible characteristics, no consensus for Zn (II) ion requirement appeared. For example, the best characterized APase included in this group is that purified from the freshwater cyanobacterium, *Synechococcus* sp. PCC 7942, which is a periplasmic enzyme having a single subunit of \( M_r \sim 145 \text{kDa} \) (Block and Grossman, 1988; Ray et al., 1991). The gene encoding this APase (*pho A*) displays no homology to the previously characterized APase and the deduced amino acids provide no basis for conventional Zn (II) ion requirement. Moreover, the APase of chlorophytes *Chlamydomonas reinhardtii* (Quisel et al., 1996) and *Volvox carteri* (Hallmann, 1999) appeared to be Ca (II) ion dependent. Wagner et al. (1995), however, reported a Zn (II)-requiring, constitutively expressed APase from *Synechococcus* sp. PCC 7942. Encoded by *pho V*, the deduced amino acid sequence displays 34% identity to bacterial APase and predicts a protein of \( M_r \sim 61.3 \text{kDa} \). In this study, the cyanobacterium, *Nostoc muscorum* (Fig. 3) also depicted the requirement for Zn (II) ions for induction of this phosphate-repressible APase. This apparent divergence of some algal and cyanobacterial forms with respect to their metal ion requirement could be ascribed to a mechanism such as metal replacement, as proposed for carbonic anhydrase (McKay et al., 2001).

These results imply that cadmium pollution has a severe impact on the uptake as well as the internal pool of phosphate, and also on the APase activity of the N\(_2\)-fixing cyanobacterium, *Nostoc muscorum*. Cadmium, depending on its concentration, may activate the Zn-requiring, phosphate-repressible APase of *N. muscorum* even under P-rich conditions. Moreover, the early inducible APase of *N. muscorum* demonstrates its superiority over other cyanobacteria for phosphate regeneration of agroecosystems.

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**References**


