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Relevance of Alkaline Phosphatase activity of immobilized *green algae and cyanobacteria* for heavy metal toxicity monitoring

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Abstract

Harmful effects of contaminants on the ecosystem and humans cannot be assessed by standard chemical analyses of environmental samples, therefore toxicity tests using live organisms or cells represent a vital part of environmental monitoring. Biological methods based on microorganisms as test-species, have already successfully been applied to environmental toxicity/genotoxicity assessment. The enzyme activity is examined in presence of essential and non-essential heavy metals (Ni, Zn, Cd) in green algae and cyanobacteria. The activity is based on total p-nitrophenol formed in a known volume of culture suspension. The activity was deleteriously affected in a concentration dependent manner. The maximum Apase activity observed in immobilized state of *A.nidulans* was 14.6 n moles pNP µg protein^{-1} per 30 minutes in presence of non-inhibitory level of Ni and Cd. The effect due to interaction between different variables (cell state type, metal type and metal dose) depicted a significant variation ($P< 0.01$, ANOVA) in the Apase activity of free and immobilized cells of *S. quadricauda* and *A. nidulans*. This study describes the alkaline phosphatase activity which may differ with metal type, cell type or metal dose of the solution. Immobilization may protect the cell enzyme activity against the toxicity of heavy metals. The study may help in designing biosensor when estimating heavy metals quantitatively.

Key words: Immobilization, *S.quadricauda*, *Anacystis nidulans*, Alkaline Phosphatase, Heavy metals

Introduction

The unique ability to convert complex phosphorus compounds into orthophosphate is due to the possession of alkaline phosphatase enzyme (Rhee 1973; Healey and Hendzel 1980). Alkaline phosphatase is believed to have an important function in the nutrient dynamics of the aquatic environment. Alkaline phosphatase is located on the external membrane of the whole cell of many microalgae. Inhibition of this enzyme reflects the natural deletorious effect of toxicants on microalgae and represents a real ecological interest considering that algae are involved in the primary step of the food chain.

Heavy metals are usually determined by sophisticated techniques like ionic chromatography, mass spectrometry, inductively coupled plasma, and polarography, which need to be carried out in laboratories after sampling. These methods are not suitable for continuous monitoring in the environment (Rogers and Gerlach, 1999). Moreover these methods are uneconomical. Immobilized systems are designed as biomonitoring system for detecting heavy metals quantitatively. To a certain extent immobilized systems resemble natural environmental conditions as many microorganisms grow in a biotype, where they are also immobilized by encapsulation in slimes or as a partner of symbiotic systems.

However in nature, the toxic elements are present in combination presenting the synergistic or antagonistic effect on the enzyme activity and the immobilized condition may enhance/decrease the activity or the response towards heavy metals may be species specific. An attempt has been made to study the enzymatic activity under heavy metal stress and compare two organisms (*Scenedesmus quadricauda*, *Anacystis nidulans*) in free and immobilized conditions. The organisms studied presently are unique in the sense that they thrive well in polluted water. In nature the toxic elements are present in combination and may present the synergistic or antagonistic effect on the enzyme activity of different algal species. This study describes the alkaline phosphatase activity which may differ with metal type, cell type or metal dose of the solution. It may further help to design the biosensor qualitatively and help to understand the effect of immobilization on species specific cell activity.

Materials and methods

Test System

The microorganisms used were green algae (*Scenedesmus quadricauda* and *Chlorella vulgaris*) and cyanobacteria *Anacystis nidulans* (Local strain, Banaras Hindu University). The growth medium for green algae (Chu-10, Gerloff et al. 1950, pH 6.8) and *Anacystis nidulans* (Hughes medium, Hughes et al.1958, pH 6.8) was prepared using deionized double-distilled water and subsequently filter sterilized. Experiments were conducted with 100 ml cultures gently shaken at a temperature of 25 ± 1^0C with a light flux of 14.4 Wm^{-2} (18/6 h light per dark cycle). The cultures were aerated with 2% CO_2 in air at a flow rate of approximately 150 ml/min. When the cell concentration was sufficiently high, the cultures were spiked with nickel, cadmium or zinc to produce the required initial metal ion concentration. Stock solutions of $NiCl_2.6H_2O$, $ZnSO_4.7H_2O$ and $CdSO_4.H_2O$ were sterilized by passing through millipore membrane filters (0.45 μM) before adding to the culture medium. Biochemicals used were obtained from Sigma chemical company, MO, USA and BDH, UK.

Protein was estimated following the method of Herbert et al. (1971).

Cell immobilization

Exponentially grown algal cells (500 μg protein per ml) obtained by centrifugation and repeated washings, were suspended in 5% (w/v) solution of sodium alginate (Sigma). The mixture was pumped drop-wise into $CaCl_2$ (0.2M) solution, and the beads thus formed, were washed several times with sterile deionized double-distilled water and re-suspended in a 200 ml growth medium for autotrophic growth under culture-room conditions along with the free cells.

Release of immobilized cells

The assays were determined by dissolving the solid matrix (alginate beads). Matrices of calcium alginate can be readily dissolved by exposure to hexametaphosphate. (Bozeman et al.1989)

Enzyme activities

For studying the effect of metals on Apase activity, cultures were incubated in medium lacking PO_4^{-3}. A bubble column reactor was used for Apase activity. The column was aerated at 250-300 cc/min. with an air bubbler. Beads were placed into a bubble column reactor in 500 ml growth medium for 24 h.

Alkaline phosphatase activity was assayed by the method of Ihlenfeldt and Gibson (1975). The activity is based on total p-nitrophenol formed in a known volume of culture suspension.

Percent inhibition was calculated (in both free and immobilized cells) by considering the untreated cells in the free suspension as control.

The actual metal concentrations used were the LC_{50} values determined previously by the plate/colony method.

Statistical analysis was done through't' test and (3-way) ANOVA. [Components: metal type (Ni, Zn, Cd); cell state type (free and immobilized cells) and metal treatment (non-inhibitory, 50% inhibitory and more than 50% inhibitory concentration)]

Results and discussion

Alkaline phosphatase activity (n moles pNP µg protein^{-1} per 30 min.) in *Scenedesmus quadricauda* (Table 1) demonstrates that at LC_{50} dose of Ni, Zn and Cd, the percent inhibition of Apase activity in the free state of cells was found to be 64.1, 50.9, 83.0 in *S.quadricauda*. In immobilized cells, it was 52.8, 51.5, and 75.5. The Apase activity of *A.nidulans* is presented in fig.1. The percent inhibition was 60.0, 60.0, 58.6 and 52.0, 49.3, 46.6 in free and immobilized state respectively.

Nickel toxicity to APase was higher in *S. quadricauda* followed by *A. nidulans* in both free and immobilized state. In contrast to this zinc was more toxic to *A. nidulans* followed by *S. quadricauda.* In free state, Cd was more toxic to *S. quaduicauda* followed by *A. nidulans* while in immobilized state the hierarchy for Cd toxicity was: *S. quadricauda* > *A. nidulans*. A significant inhibition ($P< 0.05$, students "t" test) in APase activity following metal treatment has been observed in both free and immobilized state.

Immobilized state of cells without any metal treatment showed 1.30 and 1.09 fold higher APase activity than free state of *S. quadricauda and A. nidulans* respectively. Alterations in the Apase activity with changing the cell type, metal type and metal concentration were highly significant ($P< 0.001$, ANOVA). The effect due to interaction between different variables (cell state type, metal type and metal dose) depicted a significant variation ($P< 0.01$, ANOVA) in the APase activity of free and immobilized cells of *S. quadricauda* and *A. nidulans*. A significant variation ($P< 0.01$) was also observed for metal types vs metal doses type of interaction in both the test organisms.

The maximum Apase activity observed following 72 h incubation of immobilized state of *A.nidulans* was 14.6 n moles pNP µg protein^{-1} per 30 minutes in presence of non-inhibitory level of Ni and Cd (Fig. 1) while in case of *S. quadricauda*, it was only 9.12 n moles pNP µg protein^{-1} at 1.4 µM Zn respectively (Tables 1).

Two independent mechanisms of regulation of alkaline phosphatase activity are reported in plankton (Waldemar et al. 1982). The interaction of heavy metal with the activity indicates that heavy metal may displace any essential metal ion which forms the central and functional part of the enzyme protein and secondly, interference with sulphydryl (-SH) groups which often determine the secondary and tertiary structure of the proteins may be the reason for the inhibition of APase activities after metal supplementation in both free and immobilized state (Van Assche and Clijsters, 1990). Besides, a reduced energy supply due to the inhibition of $^{14}CO_2$ incorporation (Awasthi and Das, 2005) or the interaction of metal with phosphate binding sites (Husaini and Rai 1991) might be other reasons for inhibition.

Conversely, a less pronounced inhibition of enzymatic activities of immobilized state as compared to free suspension could be ascribed to the operation of an efficient energy generation process in the immobilized state (Lau et al. 1998). Immobilization stabilizes the photosynthetic apparatus and thus protects the cells against the metal toxicity. Alginate gels have been successfully used to minimise the toxic effects of heavy metals in entrapped algal cells (Bozeman et al., 1989). Any stimulation in the ATP pool and availability of NADPH will stimulate ATP dependent process like APase activity. Enhanced photosynthetic activity also indicates towards increase of the enzymatic activities (Awasthi and Das, 2005).

The comparative study does suggest the selection of suitable algae for any biosensor specifically used for qualitative operation. Yet, to confirm the hypothesis, further techniques are required.

Several studies on toxicity of metals on algae confirmed the deleterious effect of metals to biological macromolecules (Tripathi *et al*., 2004; Awasthi, 2004; Awasthi & Das, 2005). Bozeman *et al*. (1989) used free and immobilized *Selenastrum capricornutum* in algal toxicity assays.

Table 1 Influence of Ni, Zn and Cd to alkaline phosphatase activity (after 72 h) of free and immobilized *Scenedesmus quadricauda*

Metal concentration (µM)		**Alkaline Phosphatase (n moles pNP µg protein^{-1} per 30 minutes) x 10 $^{-1}$**	
		Free living	**Immobilized**
Control		127.20 ± 0.04	163.20 ± 0.05 (28.30) *
Ni	1.10	57.60 ± 0.04 (54.70)	74.40 ± 0.04 (41.50)
	2.10	45.60 ± 0.03 (64.10)	60.00 ± 0.03 (52.80)
	3.10	21.60 ± 0.04 (82.00)	43.20 ± 0.04 (66.04)
Zn	1.40	74.40 ± 0.05 (41.50)	91.20 ± 0.04 (28.30)
	2.40	62.40 ± 0.06 (50.94)	74.40 ± 0.03 (41.50)
	3.40	45.60 ± 0.04 (64.15)	57.60 ± 0.02 (54.71)
Cd	1.30	36.00 ± 0.02 (71.69)	50.40 ± 0.03 (60.37)
	2.30	21.60 ± 0.04 (83.00)	31.20 ± 0.03 (75.47)
	3.30	9.60 ± 0.03 (92.40)	24.00 ± 0.04 (81.13)

All the values are mean ± SE
∗Shows stimulation over control (free cells)
Data in parentheses denote % inhibition.
All the treatments are significantly different ($p<0.001$) from their respective control according to students 't' test
No. of analysis, n = 3 (for each treatment)

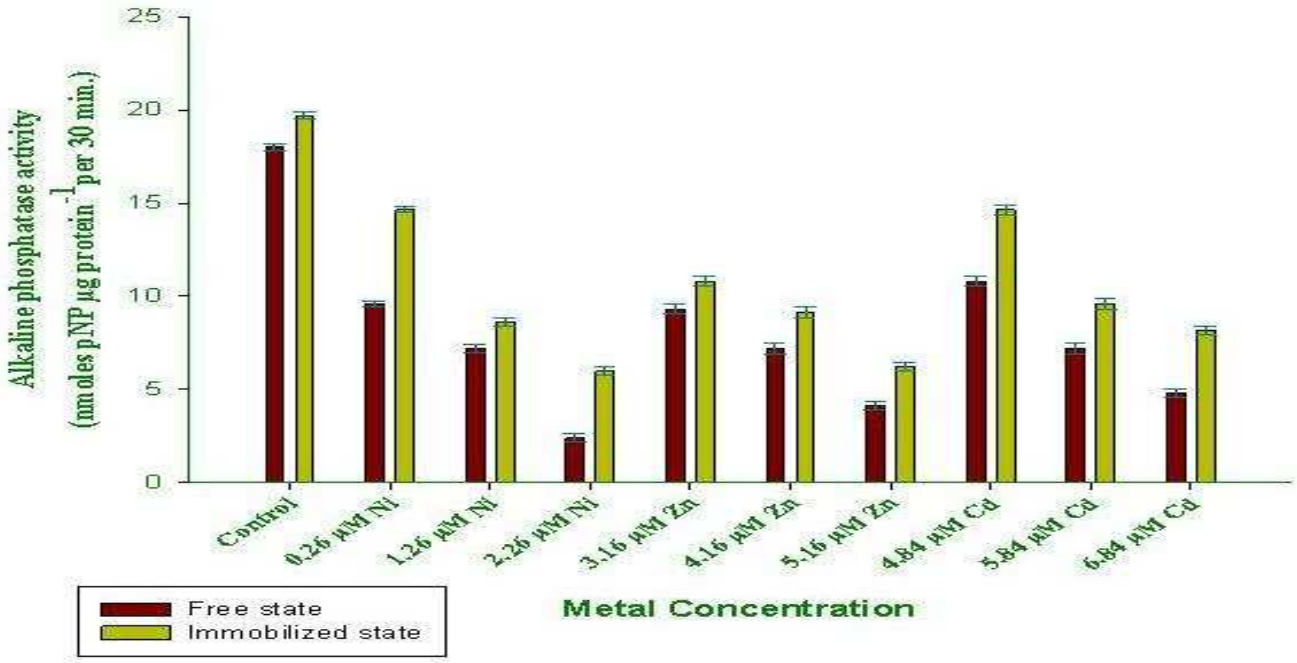

Figure 1 Effect of different concentration of heavy metals on alkaline phosphatase activity in free and immobilized *Anacystis nidulans*

Many studies are reported regarding the use of immobilized algae as biosensor. Frense *et al*. (1998) developed an optical biosensor for the determination of environmental impurities of water based on immobilized living algal cells. Naessens *et al*. (2000) constructed a new biosensor for the detection of some herbicides based on kinetic measurements of chlorophyll-a fluorescence in immobilized *Chlorella vulgaris* cells. Naessens and Tran-Minh (2000) made a biosensor using *Chlorella* microalgae immobilized on the membrane of an oxygen electrode to determine volatile organic compounds in form of aerosols by measuring the oxygen production during the algae photosynthetic process. A toxicity biosensor based on immobilized *Anabaena torulosa* for the determination of copper toxicity was developed by Chia *et al.* (2005). Chouteau *et al*. (2005) used a conductometric biosensor using immobilized *Chlorella vulgaris* microalgae as bio-receptors as a bi-enzymatic biosensor for heavy metal ions and pesticides detection in water samples.
Alkaline phosphatase activity is also affected by heavy metals which have been reported by a number of workers. Durrieu and Tran-Minh (2002) constructed a biosensor to detect heavy metals from inhibition of alkaline phosphatase present on external membrane of *Chlorella vulgaris* microalgae. The immobilized condition may inhibit/enhance the activity of the enzyme. Moreover the presence of different metals in combination may act antagonistically or synergistically towards any cell activity. Therefore the biosensor may be designed for qualitative estimation of the metals and species specific for better result.

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