

## Nitrate reductase activity: A solution to nitrate problems tested in free and Immobilized algal cells in presence of heavy metals

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### Abstract

Every organism has different potential to accumulate  $\text{NO}_3^-$  from the environment. Nitrate reduction processes are perhaps most significant in maintaining water quality by alteration of nitrate to nitrite. A comparative study between the nitrate reductase NR activity of green and blue green algae in presence of heavy metals is being conducted to present a situation where nitrate reductase process may be affected in presence of heavy metals. Metals interacted negatively with the nitrate reductase activity of a blue green alga, *Anacystis nidulans* and green algae, *Chlorella vulgaris* in both free and immobilized state. The activity was more repressed in *C. vulgaris* in presence of Ni compared to Zn and Cd. However, Cd was more toxic to NR activity in *A. nidulans* (free state). Metal dependent variation between free and immobilized cells were found to be significant ( $P < 0.01$ ) however, the concentration dependent pattern in the activity between free and immobilized state was non significant in both the test organisms. *C. vulgaris* is more efficient in conversion of nitrate to nitrite compared to *A. nidulans* in presence of heavy metals.

**Key Words:** *Chlorella. vulgaris*, heavy metals, *anacystis nidulans*, immobilization, nitrate reductase

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### Introduction

Nitrate is currently one of the most hazardous pollutant (Awasthi and Rai; 2005, Bier, 2002; Johnson and Kross, 1990; USEPA, 1987). NR is an enzyme that is being used to help clean up the environment, by conversion of nitrate to nitrite and has great potential to be part of the solution to the global problem of excess nitrate and related nitrogen nutrients in water sources (Campbell and Campbell, 1998). Nitrate reduction processes are perhaps most significant in maintaining water quality. NR protein is responsible not only for nitrate reduction, but also for nitrate uptake in *Chlamydomonas*, *Chlorella sorokiniana* (Knobloch and Tischner, 1989; Nichols, *et al.*, 1978). Assimilatory N reduction, performed by a variety of bacterial and eukaryotic organisms (Zvyagil'skaya, *et al.*, 1996; Solomonson and Barber, 1990) includes the reduction of nitrate and nitrite compounds, often mediated by nitrate or nitrite reductase enzymes, for synthesis of amino acids. Nitrate reductase (NR) activity is the limiting factor when considering the growth and protein production of algae (Lau *et al.*, 1998). There is substantial concern about the growing levels of heavy metals in the environment and the detrimental effects of these elements on living organisms. Toxicity of metals on algae has been reviewed earlier (Awasthi and Das, 2005; Demon, *et al.*, 1988; Vyamazal, 1987) confirming the deleterious effect of metals to

biological macromolecules. Besides other toxic effects, Ni, Zn, Cd, are also toxic to nitrate reductase activity of algal cells (Tripathy, *et al.*, 2004). Immobilization of algal cells is of interest because it paves the way for the immobilization of the higher plant cells and provide excellent model for studying the effects of immobilization on cell physiology. Though cells in free suspension can produce valuable information in laboratory experiments, immobilized cells are advantageous for industrial scale studies. However, comparative studies on free and immobilized are meager regarding physiological and biochemical behavior of algae subjected to metal stress.

Since heavy metals and nitrate are both harmful pollutant and often occur together, it is necessary to know the effect of heavy metals on the enzyme activity more so when NR in immobilized algae can be used to treat the nutrient pollution. The biological treatment system will definitely be affected when heavy metals are present besides  $\text{NO}_3^-$ . Although some reports are available regarding the nitrate reductase activity of algal cells in the free state however only scant information is available about the influence of these metals on the immobilized state. A comparison between different algal organism is felt necessary since each organism has different potential to accumulate  $\text{NO}_3^-$  from the

environment due to variation in NR characteristics (Berges, 1997).

The Present study deals with the comparative account of free and immobilized green algae, *C. vulgaris* and cyanobacteria, *A. nidulans* cells regarding NR activity. It also deals with the relative toxicity of one metal compared to the others.

## Materials and Methods

The organisms used in the present study are the unicellular green alga *Chlorella vulgaris* and blue green algae, *Anacystis nidulans* (Both local strain, BHU). The growth medium for *C. vulgaris* (Chu-10, Gerloff, *et al.*, 1950, pH 6.8) and *A. nidulans* was prepared using double-distilled water, filtered and sterilized. The standard microbial techniques were employed for selection, isolation and cloning of algae in pure culture. The percent survival of the organisms was scored by the plate colony count method to obtain  $LC_{50}$  dose of Ni, Zn and Cd. The concentrations of test metals used for *C. vulgaris* were Ni: 3.20  $\mu\text{mol/l}$ , 4.20  $\mu\text{mol/l}$  ( $LC_{50}$ ), and 5.20  $\mu\text{mol/l}$ , Zn: 0.75  $\mu\text{mol/l}$ , 1.75  $\mu\text{mol/l}$  ( $LC_{50}$ ), and 2.75  $\mu\text{mol/l}$ , Cd: 0.16  $\mu\text{mol/l}$ , 1.16  $\mu\text{mol/l}$  ( $LC_{50}$ ), and 2.16  $\mu\text{mol/l}$ , And for *A. nidulans* the concentrations were, Ni: 0.26  $\mu\text{mol/l}$ , 1.26  $\mu\text{mol/l}$  ( $LC_{50}$ ), and 2.26  $\mu\text{mol/l}$ , Zn: 3.16  $\mu\text{mol/l}$ , 4.16  $\mu\text{mol/l}$  ( $LC_{50}$ ), and 5.16  $\mu\text{mol/l}$ , Cd: 4.84  $\mu\text{mol/l}$ , 5.84  $\mu\text{mol/l}$  ( $LC_{50}$ ), and 6.84  $\mu\text{mol/l}$ .

Experiments were conducted with 200 ml. cultures gently shaken at a temperature of  $25 \pm 1$  °C with a light flux of  $14.4 \text{ Wm}^{-2}$  (18/6 h. light per dark cycle). The cultures were aerated with 2 %  $\text{CO}_2$  in air at a flow rate of approximately 1.5 ml/ml culture/min. Exponential phase free and immobilized cells were fixed to a final density of 500  $\mu\text{g}$  protein per ml. Stock solutions of  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and  $\text{CdSO}_4 \cdot \text{H}_2\text{O}$  were sterilized by passing through Millipore membrane filters (0.45  $\mu\text{m}$ .) before adding to the culture medium. Biochemical's used were obtained from Sigma chemical company, MO, USA and BDH, UK.

### Cell immobilization

Exponentially grown algal cells obtained by centrifugation and repeated washings, were suspended in 5% (w/v) solution of sodium alginate (Sigma). The mixture was pumped drop-wise into  $\text{CaCl}_2$  (0.2 mol/l) solution, and the beads thus formed (with an average diameter of 5 mm. and allowed to harden for 2 h.) were washed several times with sterile double-distilled water and re-suspended in a 200 ml growth medium for autotrophic growth under

culture-room conditions along with the free cells. Percent inhibition was calculated (in both free and immobilized cells) by considering the untreated cells in the free state as control. Protein was estimated following the method of Herbert *et al.*, (1971).

### Nitrate reductase activity

The estimation of NR activity was done following the method of Camm and Stein (1974). The activity is based on the total nitrite formed. For the estimation of enzyme activity known amount of algal suspension was centrifuged and washed 3-4 times with sterile double distilled water and suspended in culture medium containing 5 mM  $\text{NaNO}_3$ . For different intervals, different sets of culture tubes containing 1 ml of culture (final) were incubated. Standard curve was plotted with varying concentrations of  $\text{NaNO}_3$  dissolved in test medium. The metal treatment experiments involved in the supplementation of Ni, Zn and Cd at graded concentration. The absorbance of pink color was measured at 540 nm.

### 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).

### Statistical treatment

Values presented are the means of five independent experiments with the bar showing standard deviation in each case. The data for various metal treatments were verified for their significance at a particular probability level, and the variance ratio (F) calculated as  $F=T/R$ , where T is treatment mean square, and R residual mean square. The components for ANOVA (3-way) were metal type (Ni, Zn, Cd); cell state type (free and immobilized) and metal treatment (non-inhibitory, 50% inhibitory and more than 50 % inhibitory).

## Results

A decrease in the activity of nitrate reductase was noticed with increase in metal concentration in both *C. vulgaris* (Table 1) and *A. nidulans* (Fig. 1).  $LC_{50}$  concentrations of Ni, Zn and Cd, inhibited the nitrate reductase activity by 87.2, 50.0, 72.1 and 53.6, 34.6, 38.0 % respectively in free and immobilized *C. vulgaris*. 100% activity (0% inhibition) in *Anacystis nidulans* as shown in Fig. 2 is equal to 116 ng  $\text{NO}_2^- \mu\text{g/protein}$  (free cells). Fig. 2 also reveals more pronounced activity by immobilized cells.

Table 1: Impact of Ni, Zn and Cd on nitrate reductase activity (after 72 h.) of free and immobilized *Chlorella vulgaris*

Metal concentration ( $\mu\text{mol/l}$ )		Nitrate-reductase activity ( $\text{ng NO}_2^- \mu\text{g/protein}$ )	
		Free living	Immobilized
Ni	Control	98.84 $\pm$ 0.03	120.00 $\pm$ 0.04 (21.4)*
	3.20	40.52 $\pm$ 0.02 (59.0)	56.80 $\pm$ 0.04 (42.5)
	4.20	12.65 $\pm$ 0.03 (87.2)	45.80 $\pm$ 0.09 (53.6)
	5.20	9.88 $\pm$ 0.04 (90.0)	31.40 $\pm$ 0.06 (68.2)
Zn	0.75	59.30 $\pm$ 0.04 (40.0)	64.60 $\pm$ 0.03 (34.6)
	1.75	49.42 $\pm$ 0.04 (50.0)	60.90 $\pm$ 0.04 (38.3)
	2.75	24.71 $\pm$ 0.03 (75.0)	35.40 $\pm$ 0.05 (64.1)
Cd	0.16	35.00 $\pm$ 0.02 (64.0)	65.00 $\pm$ 0.07 (35.1)
	1.16	27.50 $\pm$ 0.05 (72.1)	61.20 $\pm$ 0.07 (38.0)
	2.16	15.00 $\pm$ 0.05 (84.8)	40.20 $\pm$ 0.08 (59.3)

All the values are mean  $\pm$  SE

\* Sign shows stimulation over control (free cells)

Data in parentheses denote % inhibition. All the treatments are significantly different ( $P < 0.005$ ) from their respective control according to students 't' test

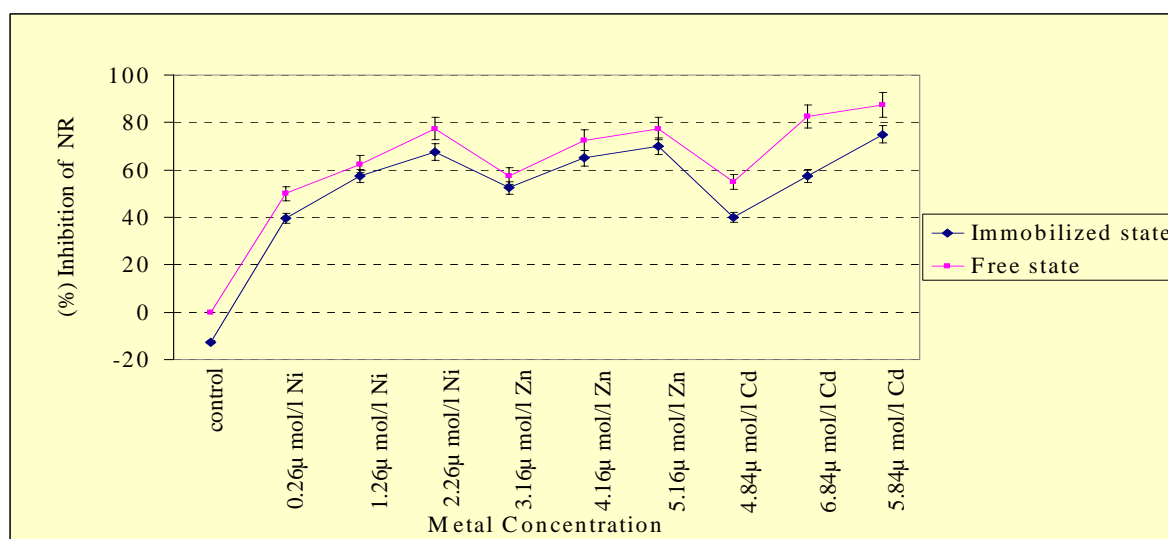


Fig. 1: Pattern of metal concentration dependent inhibition of nitrate reductase activity in anacystis nidulans in free and immobilized states

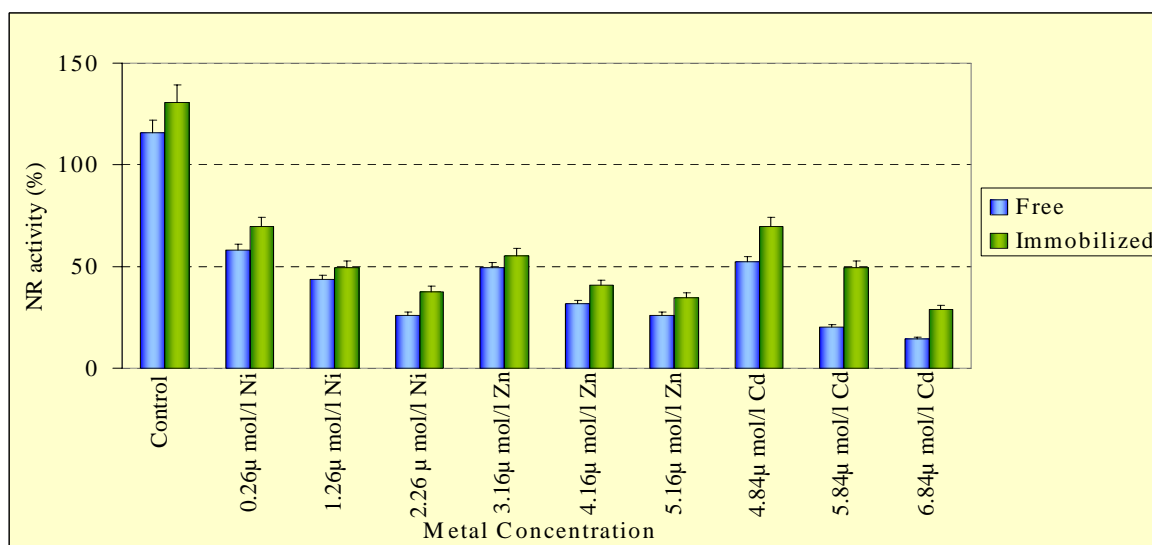


Fig. 2: Effect of different concentrations of heavy metals on nitrate reductase activity of anacystis nidulans in free and immobilized states

Table 2: Analysis of variance (3-way) table for nitrate reductase activity of *Chlorella vulgaris*

Source of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significance level
A	1	2620.87	2620.87	288.28	P < 0.001
Metal	3	21947.97	7315.99	804.73	P < 0.001
Conc.	3	1722.67	861.33	94.74	P < 0.001
A x metal	3	332.70	110.90	12.19	P < 0.01
A x conc.	3	49.46	24.73	2.72	P > 0.05
Metal x conc.	9	773.63	128.93	14.18	P < 0.01
Error	9	34.54	9.90		
Total	31			27501.86	

A = Cell type (free and immobilized cells)

Metals = Ni, Zn, Cd

Conc.= Concentration of metals (Non-inhibitory, 50% inhibitory level, inhibitory level)

Table 3: Analysis of variance (3-way) table for nitrate reductase activity of *Anacystis nidulans*

Source of variation	Degree of freedom	Sum of squares	Mean square	F-value	Significance level
A	1	853.53	853.53	116.15	P < 0.001
Metal	3	31178.76	10392.92	1415.95	P < 0.001
Conc.	3	2394.93	1197.46	163.14	P < 0.001
A x Metal	3	113.67	37.89	5.16	P < 0.05
A x Conc.	3	15.09	7.54	1.02	P > 0.05
Metal x Conc.	9	1039.31	173.21	23.59	P < 0.01
Error	9	44.03	7.33		
Total	31			35638.35	

A = Cell type (free and immobilized cells)

Metals = Ni, Zn, Cd

Conc. = Concentration of metals (Non-inhibitory, 50% inhibitory level, inhibitory level)

62.5, 72.5, 82.5 and 57.5, 65.0 and 57.5 % inhibition in NR activity of free and immobilized cells of *A. nidulans* as obvious from Fig. 1. The nickel toxicity to the activity was higher in *C. vulgaris* compared to *A. nidulans* which is in contrast to immobilized cells where the Ni toxicity was more to *A. nidulans*. However, zinc and cadmium were more toxic to *A. nidulans* than *C. vulgaris*, both in free and immobilized conditions.

Tables 2 and 3 represent the statistical analysis of the results found in *C. vulgaris* and *A. nidulans* respectively. A significant decrease ('t' significant at P < 0.005) in the NR activity following metal treatment has been observed in both free and immobilized state. The efficiency of nitrite production in the immobilized (untreated) cells was 1.21 and 1.15 fold higher (ANOVA significant at P < 0.05) compared to free suspension of *C. vulgaris* and *A. nidulans* respectively. Although, on treatment with LC<sub>50</sub> dose of Ni, Zn and Cd, the immobilized cells depicted 3.6, 1.2 and 2.2 fold increase (P < 0.005) for *C. vulgaris* and 1.04, 1.18, 1.44 fold increase (P < 0.005) for *A. nidulans* in nitrite production. Metal dependent variation between free and immobilized cells were found to be significant (P < 0.01) and *A.*

*nidulans* (P < 0.005) however, the concentration dependent pattern in the activity between free and immobilized state was non significant (P < 0.05, ANOVA).

## Discussion and Conclusion

Uptake of metals and metalloids can be harmless (i.e., no effects), toxic or even beneficial. Experiments on NR activity revealed that Ni, Zn and Cd inhibit the synthesis of NR. The displacement of an essential metal ion forming the central and functional part of the enzyme protein may be one of the reasons for inhibition of nitrate reductase by heavy metals and secondly, interference with sulphhydryl (-SH) groups which often determine the secondary and tertiary structure of proteins. Besides, a reduced energy supply due to the inhibition of <sup>14</sup>CO<sub>2</sub> incorporation, photosynthetic electron transport and an indirect inhibition of uptake of substrate (NO<sub>3</sub><sup>-</sup>) of enzyme may be other important reason. Yet, a less pronounced inhibition of enzymatic activities of immobilized cells as compared to free suspension could be ascribed to the operation of an efficient energy generation process in the immobilized state. Immobilization allows the cells to

grow in close physical association with each other, and together with the slow growth, it results in chemical and physical communication between cells. Besides, any stimulation in the ATP pool and availability of NADPH may stimulate ATP dependent processes like, NR activity. Thus enhanced photosynthetic activity indicates towards increase of the enzymatic activities. *C. vulgaris* is more efficient in nitrite production compared to *A. nidulans* and thus recommends the use of green algae compared to cyanobacteria in vitro condition until any other blue green algae is found which is equally/more efficient in removing the nitrate from the environment especially in presence of heavy metals like Ni and Cd.

As a result, the following inferences is concluded from the present study:

- Ni toxicity was higher in *C. vulgaris* compared to *A. nidulans* which is in contrast to immobilized cells where the Ni toxicity was more to *A.*
- Zn and Cd were more toxic to *A. nidulans* than *C. vulgaris*, both in free and immobilized conditions.
- The efficiency of nitrite production in the immobilized (untreated) cells was 1.21 and 1.15 fold higher compared to free suspension of *C. vulgaris* and *A. nidulans* respectively.
- On treatment with LC<sub>50</sub> dose of Ni, Zn and Cd, the immobilized cells depicted 3.6, 1.2 and 2.2 fold increase ( $P < 0.005$ ) for *C. vulgaris* and 1.04, 1.18, 1.44 fold increase ( $P < 0.005$ ) for *A. nidulans* in nitrite production.

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