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RESEARCH ARTICLE

EFFECT OF COPPER ON PHOSPHATE METABOLISM AND $^{14}CO_2$-INCORPORATION IN FREE AND IMMOBILIZED CELLS OF *NOSTOC CALCICOLA*

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ABSTRACT

The phosphate uptake, ATPase activities and $^{14}CO_2$-incorporation were investigated in free living and immobilized cells of *Nostoc calcicola* under copper stress conditions. The maximum Cu concentration in free and immobilized *N. calcicola* cells was 60µM at which the immobilized cells were characterized by a faster rate of phosphate uptake than free cells. Immobilization was associated with decrease in vivo activities of ATPase(s), suggesting that the immobilized cells maintain sufficient ATP pool. $^{14}CO_2$-incorporation in immobilized cells was less sensitive to Cu and degree of inhibition was less marked compared to free living cells. The tolerance of immobilized cells in terms of all the activities studied over free cells suggested that such a system could be successfully applied to remove heavy metals from polluted water through repeated cycles with no loss of cells in bioremediation.

INTRODUCTION

Copper is a well known micronutrient, an algaecide as well as fungicide, metal component of thylakoidal plastocyanin and superoxidase dismutase (Cavet *et al.,* 2006). Beneficial range of Cu is extremely narrow so that even the slightly elevated concentration in the part of the ocean is toxic to the cyanobacteria. Cyanobacteria are capable to accumulating toxic heavy metals to the concentration several order of magnitude higher than the surrounding media (Taneja and Fatima, 2002; Yoshida *et al.,* 2005). Cyanobacteria have been used to remove Cu from aqueous system (Awasthi *et al.,* 2006; Banerjee *et al.,* 2004; Hameed, 2006). Phosphate is an important macronutrient for cyanobacteria. It is generally extracted from the surrounding medium by uptake process (Betterson and Baleen, 1968). There are several reports of energy-dependent uptake and accumulation of phosphorus-phosphate in cyanobacteria (Falkner *et al.,* 1980), where light plays a crucial regulatory role (Healey and Whitton, 1982). The activities of polyphosphate synthatase results in the formation of polyphosphate bodies in the cyanobacteria exposed to phosphate-excess medium (Grillo and Gibson, 1979). Several reports reveal the involvement of heavy metals in the inhibition of phosphate uptake in cyanobacteria (Bilal *et al.,* 2010). There are two types of ATPase(s) in cyanobacteria, Ca^{2+} dependent ATPase is a coupling factor of the thylakoid mediating photosynthetic production of ATP. Mg^{2+} dependent ATPase predominately located in plasmamembrane, plays a vital role in cation transport. Lockau and Pfeffer (1982) observed sensitivity of these ATPase(s) towards vanadate. Stimulations in membrane ATPase by heavy metals has been attributed to alterations in sterol and phosholipid composition of the plasmamembrane (Cooke *et al.,* 1989). The present observations clearly establish that Cu affect both the enzymes (Ca^{2+}- and Mg^{2+} dependent ATPases), results more ATP hydrolysis in free *N. calcicola* than immobilized cells. Cyanobacteria depend largely on photosynthesis for the generation of ATP and reductants. Since Cu inhibited $^{14}CO_2$-assimilation in free and immobilized cells and the degree of inhibition was less marked for gel entrapped cells, the observed tolerance of immobilized cells against Cu inhibition of $^{14}CO_2$-incorporation, suggested higher photosynthetic O_2 evolution in the immobilized state and maintenance of sufficient cellular ATP pool in the cyanobacteria to drive the various physiological reactions (Potts and Morrison, 1986).

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MATERIALS AND METHODS

Experimental organism and culture conditions

Nostoc calcicola, an isolate of rice field obtained from Algal Research Laboratory, BHU, Varanasi, was cultured in 250 ml Erlen-Mayer flask containing 100 ml Allen-Arnon's combined nitrogen free medium (pH 8.0) with A_6 trace element devoid of copper The cultures were incubated phototrophically in culture room at 25±1°C with a light intensity of 50µ$Em^{-2}s^{-1}$ on the surface of culture vessels with 18/6 light/ dark cycle. Cell immobilization was carried out by the method of Singh *et al.,* (1989). The beads thus prepared subsequently suspended in 200 ml basal medium and incubated phototrophically under culture room conditions along with free cells. The culture were starved from copper by growing free and immobilized cells in a medium devoid of copper for 72 hr. the copper in the form of copper sulphate ($CuSO_4.7H_2O$) was supplemented

to the growth medium in varying concentration (10, 20, 40 and 60µM Cu^{2+}) and phosphate uptake, ATPase(s) activities and $^{14}CO_2$-incorporation estimated after 6 days growth. All the experiments were carried out in a completely randomized design and treatment replicated four times. The experiments were repeated to reconfirm the results. The data obtained was statistically analyzed using standard statistical procedures.

Phosphate uptake estimation

For phosphate uptake, 6 days old free and immobilized cyanobacterial cells were starved for 72 hr in a phosphate free medium. Phosphate uptake experiment proceeded as stated by using K_2HPO_4 concentrations (0.2-2.5mM for free cells and 0.5-3.0 mM for immobilized cells) To 1 ml of standard K_2HPO_4 solution or culture supernatant 5N H_2SO_4 (1ml), ammonium molybdate (1ml) and reducing agent (0.1 ml) [1.2 g sodium metabisulphate, 1.2 g sodium sulphite, 0.2 g ANSA (1-amino-2-nepthol-4-sulphonic acid)] were added and appropriate dilution made by sterilized distilled water (final 10 ml). The mixture was incubated for 10 min at 25±1°C. the optical density of the blue color developed was measured at 660 nm spectrophotometrically as per the method of Fiske and Subba Row, (1925).

Measurement of ATPase

The extraction of ATPase from cells was done as per the method of Lockau and Pfeffer (1983). Cu starved and supplemented (0-60µM) free and immobilized cyanobacterial cells were incubated at 25±1°C and then centrifuged, washed and re-suspended in extraction buffer (300 mM Tris-HCl, pH 8.1). The cells were ruptured in liquid nitrogen and centrifuged (10,000 g). The supernatant obtained was dialyzed for 3 hr against 10 mM preparation used as crude extract.

Mg^{2+} dependent ATPase

The Mg^{2+} dependent ATPase was assayed by determining the amount of inorganic phosphate liberated as described by Ohnishi *et al.,* (1975). The assay mixture (2.0 ml) contained 6 mm $MgCl_2$, 6 mM ATP in 30 Mm Tris-HCl buffer (pH 8.1). The reaction was initiated by adding above enzyme preparation and stopped at 1 h by adding 0.25 ml Tricloroacetic acid (40%).

Ca^{2+} dependent ATPase

The enzyme was activated prior to assay. The crude enzyme was treated with trypsin (Sigma USA, 0.75mg ml^{-1}) for 10 min followed by the addition of 0.75 mg ml^{-1} of trypsin inhibitor (Sigma USA). The Ca^{2+} dependent ATPase assay was performed as per the method of Owers-Norhi *et al.,* (1979) except that $MgCl_2$ was replaced by 60 mm $CaCl_2$.

$^{14}CO_2$-incorporation

The photoautotrophically grown 6 days old free and immobilized cyanobacterial cells after transfer to fresh growth medium, were pre-incubated in dark for 24 h. The 1 ml volume of the dark incubated cyanobacterial free cells (400µg protein ml^{-1} culture) and beads (corresponding to the same protein value), were transferred to glass scintillation vials, containing Cu (0-60µM) and 0.05 µCi ml^{-1} $NaH^{14}CO_3$ (BARC, India). The simultaneously run metal-less control and metal-treated free and immobilized cells in the scintillation vials, were light-incubated at 25 ± 1°C and $^{14}CO_2$-incorporation stopped at regular intervals of 1 hr by adding 0.1 ml 2 N HCl, followed by the addition of 5.0 ml scintillation cocktail, containing 4 parts of 0.8% PPO (2,5-diphenyloxazole) with 0.01% POPOP [1,4-bis (4-methyl-5-phenyl-2 oxazole)-benzene] in toluene and 3 parts of ethanol. Such reaction mixtures were surface blown for 5 min to remove the $^{14}CO_2$ gas, and the clear solution subjected to counting the emission of β-particles from incorporated $^{14}CO_2$ in a liquid scintillation counter (Beckman, USA). The value of counts obtained is expressed as CPM mg^{-1} protein.

RESULTS AND DISCUSSION

The concentration of Cu (0-60µM) in growth medium affected the phosphate uptake of *N. calcicola.* Figure 1a shows phosphate uptake in Cu-less free cells increased linearity up to 4 h with saturation at 5 h attaining a maximum of 0.78µ mol PO_4^{3-} μg^{-1} protein. Cu loading of such cultures caused a concentration-dependent inhibition of phosphate uptake with 50% decline by 10µM Cu. The Cu concentration 60µM causing 86% inhibition of phosphate uptake. Figure 1b shows Phosphate uptake in immobilized *N. calcicola* cells was slightly higher over free cells attaining maximum of 1.12µ mol PO_4^{3-} μg^{-1} protein after 5 h. For such cells there was a concentration-dependent inhibition of uptake between 10-60µM Cu. Immobilization resulted in the resistance of cells as 40µM Cu inhibiting 86% of phosphate uptake in free cells, caused 52% less inhibition of uptake. Also, the maximum Cu concentration for immobilized cells (60µM) allowed 12.25% phosphate uptake.

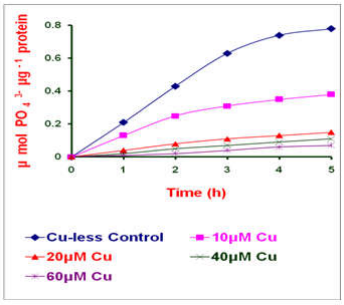

Fig. 1a. Phosphate uptake in free *N. calcicola* cells *N. calcicola* cells at graded Cu concentration

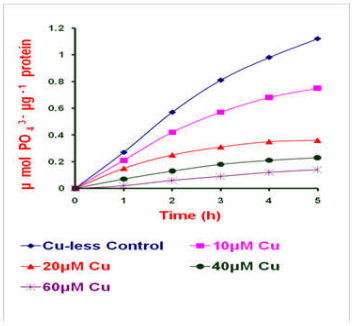

Fig. 1b. Phosphate uptake in immobilized cells at graded Cu concentration

(The data are mean of two independents experiments with four replicates each. The maximum variation from mean value was less than 5%)

Table 1. Effect of Cu on ATPase (Ca^{2+}-dependent and Mg^{2+}-dependent) activities in free and immobilized *N. calcicola* cells

	n mol Pi liberated mg^{-1} protein			
Concentration of Copper (μM)	Free cells (Ca^{2+}-dependent ATPase)	Immobilized cells (Ca^{2+}-dependent ATPase)	Free cells (Mg^{2+}-dependent ATPase)	Immobilized cells (Mg^{2+}-dependent ATPase)
Cu-less Control	38.26	31.74	29.76	23.85
20μM Cu	37.52	29.79	28.14	21.77
40μM Cu	32.14	26.12	22.52	18.69
60μM Cu	28.14	23.78	19.79	15.72

Table 2. Effect of Cu on $^{14}CO_2$-incorporation in free and immobilized *N. calcicola* cells

	CPM mg^{-1} protein	
Concentration of Cu (μM)	Free cells	Immobilized cells
Cu-less Control	13580	22368
20μM Cu	8763	18765
40μM Cu	6786	16850
60μM Cu	0.0	14267

(The data are mean of two independents experiments with four replicates each. The maximum variation from mean value was less than 5%)

There are reports covering heavy metal inhibition of phosphorus-phosphate uptake in cyanobacteria (Singh and Yadava, 1984) and phosphate uptake and photosynthesis of planktonic communities in selected Precambrian shield lakes (Nalewajko and Paul, 1985). Pettersson *et al.,* (1988), observed that Al, severely affected growth of *Anabaena cylindrica* and induced symptoms of phosphorus-starvation. However, these investigators could not observe Al-inhibition of phosphate uptake, and the rapid accumulation of polyphosphate granules in cells exposed to Al in such cases, also established that the cation did not disturb phosphate incorporation although it lowered the activity of enzyme acid *phosphatase* along with the mobilization of polyphosphate. They conclude that Al acts on the intracellular metabolism of phosphate, which eventually leads to phosphorus-starvation rather than on its uptake in *A. cylindrica*. Higher phosphate uptake by immobilized *N. calcicola* cells was due to their higher energy state in comparison to free cells. There are similar observations by Chevalier and de la Noue (1985) on immobilized *Scenedesmus quaricaudata, S. obliquus* and *S. acutus* that quite efficiently removed phosphorus from urban secondary effluents. The immobilized cells as control, showed nearly 40% less *in vivo* activity of Mg^{2+}-dependent ATPase in comparison to free cells. Similarly, there was more than 20% drop in Mg^{2+}-dependent ATPase activity in such cells compared to free cells (Table 1). Such observations suggest that the immobilized cells maintain sufficient ATP pool in *N. commune* (Potts and Morrison, 1986). Inhibition by Cu of both the ATPase(s) that elevated concentrations can be attributed to the Cu-effect on plasmamembrane of the cyanobacteria. These observations are in agreement of those reported for different metal ions (Cooke *et al.,* 1989; Ros *et al.,* 1990). The present study was an attempt to ascertain the nature of Cu action with respect to such aspect in *N. calcicola* involving $^{14}CO_2$-incorporation against Cu ions for free and immobilized cells. The free cells without Cu showed 13,580 CPM mg^{-1} protein $^{14}CO_2$ incorporation and such cells dosed with Cu concentration (60μM), displayed 100% inhibition. The immobilized control cells are more efficient in $^{14}CO_2$ incorporation with 22,368 CPM mg^{-1} protein (Table 2). However, the similar Cu concentration (60μM) brought about only 36 % reduction in $^{14}CO_2$ incorporation in immobilized cells. The inhibitory effects of Cu on $^{14}CO_2$-incorporation in *N. calcicola* are in line with the report of Rai and Raizada (1985) on Ag and Ni inhibition of carbon-fixation in *N. muscorum* and Singh and Singh (1987) on Hg in *N. calcicola.*

Takamura *et al.,* (1992) observed that the cyanobacteria were sensitive to Cu, Cd and Zn. The concentration of Cu that caused 50% inhibition of photosynthesis was 3.5μM for *Phormidium ramosum* and 0.01μM for *Chamaesiphon polymorphus.* The physiological basis of Cu inhibition of photosynthesis has been in terms of inhibition of dark reaction in *Chlorella vulgaris* (Greenfield, 1942), retardation of feredoxin-dependent reaction (Babich and Stotzky, 1978; Shioi *et al.,* 1978; De Filippis *et al.,* 1981), metal action on photochemical processes generating reductants from Calvin cycle (Cedano-Maldonado *et al.,* 1972; Singh and Singh, 1987; Scherer *et al.,* 1988). Cu inhibition was less marked for immobilized cells, the observed tolerance of immobilized cells against Cu inhibition of $^{14}CO_2$-incorporation suggesting maintenance of sufficient reserve energy in the form of ATP in cyanobacteria drive the various physiological processes. The overall it can be concluded that the immobilized cyanobacterial cells are more resistant to Cu in respect to all the parameters studied and it can be effectively used in bioremediation.

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