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RESEARCH ARTICLE

IMMOBILIZATION RESULTED SUSTAINED COPPER TRANSPORT IN *Nostoc calcicola* BREB

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ABSTRACT

The uptake pattern of copper by a cyanobacterium, *Nostoc calcicola* in its freely suspended and immobilized form is comprised of adsorption of Cu^{2+} followed by subsequent metabolism dependent uptake. Immobilized cyanobacterial cells maintained three times more metal profile (300.82 n mol Cu mg^{-1} protein) over freely suspended cells (96.89 n mol Cu mg^{-1} protein) at saturated Cu^{2+} concentration (60µM Cu). Darkness resulted in drastic reduction of Cu^{2+} uptake (90%) in freely suspended cells and least 10% in immobilized cells. Exogenously added ATP (10µM) on the other hand enhanced Cu^{2+} uptake in dark incubated free cells. However, the same ATP concentration fails to bring out any sufficient enhancement in terms of Cu^{2+} uptake in immobilized cells facing dark incubation, thus indicating that immobilized cells were able to maintain its ATP reserve even in the dark. Metabolic inhibitors such as mercaptoehanol, azide, N N' Dicyclohexycarbodimide and p-chloromercuribenzoate inhibit the metal uptake at different level. Immobilized cells exhibits remarkable Cu^{2+} transport rate even at the age of 20 and 30 days at which free living counter part took up insignificant Cu^{2+}. These findings suggest the improved metabolic efficiency of immobilized cells over freely suspended cells in term of Cu^{2+} accumulation and its use as bioreactor for metal removal in repetitive cycles without any measurable loss.

INTRODUCTION

The excess copper intake causes stomach upset, nausea, and diarrhea and can lead to tissue injury and disease. At high concentrations copper is known to produce oxidative damage to biological systems, including peroxidation of lipids or other macromolecules and several rare genetic diseases (Alzheimer disease, Huntington disease, Menkes disease, Parkinson disease, Wilson disease) are associated with the improper utilization of copper in the body (Nolan 2009). Copper is a well known plant micronutrient, an algaecide as well as fungicide, metal component of thylakoidal plastocyanin, and the enzyme superoxidase dismutase (Cavet *et al.*, 2006).

Cyanobacteria have been used to remove heavy metals from aqueous system since they have capacity to accumulate dissolve metals (Prajapati and Pandey 2007, 2008, Taranum *et al.*, 2011). Immobilized cyanobacterial cells have high capacities for metal uptake (Prajapati *et al.*, 2012). These immobilized cells were more effective and suitable than free cells for metal removal and recovery. This report is an attempt to investigate the Cu^{2+} uptake in free and immobilized cyanobacterium, *Nostoc calcicola* and its regulation by culture age, light-dark, addition of exogenous ATP and metabolic inhibitors.

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MATERIALS AND METHODS

Experimental organism and Growth 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 (Allen and Arnon, 1955) medium (pH 8.0) with A_6 trace element devoid of copper. The cultures were incubated phototrophically in culture room at 26±1°C with a light intensity of 50µ$Em^{-2}s^{-1}$ on the surface of culture vessels with 18/6h light/ dark cycle. Protein content of the cyanobacterial culture was estimated by the method of Lowry *et al.*, (1951) as modified by Herbert *et al.*, (1971).

Cell Immobilization

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.

Experimenting the Cu^{2+} uptake in free and immobilized cells of N. calcicola

Log phase cells of *N. calcicola* were centrifuged, washed and suspended in phosphate buffer (0.01M; pH 8.0) to a final density of 400µg protein ml^{-1} culture. Copper in the form of copper sulphate ($CuSO_4.7H_2O$) was supplemented to the

growth medium. The metal uptake was observed at saturated Cu^{2+} concentration (60µM) in free and immobilized cyanobacterial cells. The uptake experiments in free and immobilized cells of *N. calcicola* were conducted in light (14.4Watt m^{-2}) at 24±1°C. Samples recovered at different time intervals of Cu^{2+} exposures were suspended in aqueous EDTA (10µM disodium salt, BDH, UK) to account for EDTA-washable metal fraction. Cu^{2+} depleted from the uptake medium for both sets, were estimated by Atomic absorption spectrophotometer at 324.7nm. Cellular intake is expressed as the difference between Cu^{2+} adsorption and the total Cu^{2+} depleted from the medium in which the free cells or beads remained suspended during Cu^{2+} exposure.

Factors Regulating Cu^{2+} uptake

Cu^{2+} uptake and Culture Age

This experiment was aimed to compare the metabolic longevity of free and immobilized cells. Cu^{2+} uptake was monitored every 6^{th} day lasting 30 days in free as well immobilized cells maintained initially the same level of protein.

Light and Exogenous ATP

Free cells and beads prepared the same age free cell stock were dark incubated for 72 h at 24±1°C. Since, 10µM ATP was non-growth inhibitory to the organism as reported (Singh, 1987). The same concentration was applied to dark incubated free and immobilized cells during the subsequent monitoring of Cu^{2+} uptake.

Inhibitors / Uncouplers

DCCD (N N' Dicyclohexycarbodimide), pCMB (p-chloromercuribenzoate) were obtained from Sigma, USA and 2-mercaptoethanol and NaN_3 from BDH, UK. DCCD was solublized in ethanol and added to the assay medium in a way that the final ethanol concentration never exceeds 0.1% (v/v non-inhibitory to cell growth). Likewise, pCMB was dissolved in 0.1N NaOH initially and azide in sterile water. All such chemicals were added separately to the free and immobilized *N. calcicola* containing 60µM Cu.

RESULTS AND DISCUSSUION

The saturated Copper concentration for maximum uptake was estimated at 60µM for free and immobilized cells in which the free cells showed metal buildup of 96.89 n mol Cu mg^{-1} protein as compared to immobilized cells showed 3 fold (300.82 n mol Cu mg^{-1} protein) at 1 h. Fig. 1 reflects the higher efficiency of immobilized cells in term of Cu^{2+} accumulation. The enhanced metal buildup in immobilized cells has proved the superiority of immobilization. Similarly, the immobilized cells of *Aulosira fertilissima* have been reported to accumulate more Cr and Ni (Banerjee *et al.*, 2004). Also, the immobilized *Scenedesmus quadricauda* accumulate more Zn than the free cells (Awasthi and Rai, 2006). The pattern of Cu^{2+} uptake by aging free *N. calcicola* cells remained saturation within 6 days thus suggesting that the active transport of Cu^{2+} is characteristic of exponentially growing cells. After 6 days the free cells started declining. A 24 days, free cells did not show Cu^{2+} buildup whereas the immobilized cells showed 50% buildup at the similar age thus suggesting the higher efficiency of immobilized cells in term of Cu^{2+} accumulation (Fig. 2). A sharp contrast representing Cu^{2+} uptake by immobilized cells suggests their longevity as sufficient Cu^{2+} uptake could be seen even in one month old beads. The immobilized cells were still able to sustain sufficient ATP reserve, as reported in *Anabaena cylindrica* (Lambert *et al.*, 1979). Cu^{2+} uptake by *N. calcicola* under light and dark conditions showed a wide difference.

The light grown cells maintained a 14 fold difference in free and 1.5 fold in immobilized cells over the dark incubated cells in each state, suggesting for the photosynthetically generated energy dependent Cu^{2+} uptake (Fig. 3a and b). Similar results were reported for Ni and Cd uptake in *A. cylindrica* (Pattersson *et al.*, 1986). The exogenous supply of ATP (10µM) to dark cells in free state showed favorable response in terms of improvement in Cu^{2+} uptake over the non supplemented free cells. The immobilized cells, however, did not show much reduction in Cu^{2+} uptake even after 72h dark incubation. Exogenously added ATP could not raise Cu^{2+} uptake in such dark cells, thus suggesting that immobilized cells were still able to sustain ATP reserve optimum drive to active transport of Cu^{2+}. Similarly, Potts and Morrisson (1986) reported that immobilized cells of N. commune maintain their ATP pool size when undergoing various shift in metabolism. The respective contribution of selected metabolic inhibtitors / uncouplers towards Cu^{2+} uptake in free and immobilized cells is shown in Table 1. Thioles (mono and di) are known to reduce metal toxicity (Prajapati and Pandey, 2011). Non-inhibitory concentration of mercaptoethanol (30µM) brought about an almost 25% inhibition in metal uptake over the control (free as well as immobilized) cells.

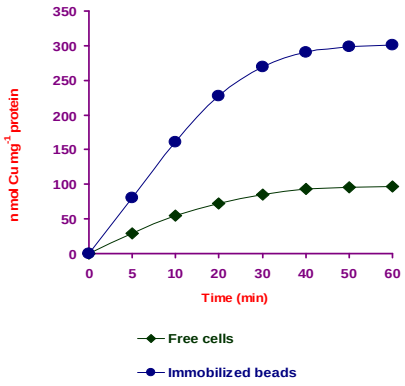


Fig.1. Cu uptake in free and immobilized *N. calcicola* at 60µM Cu

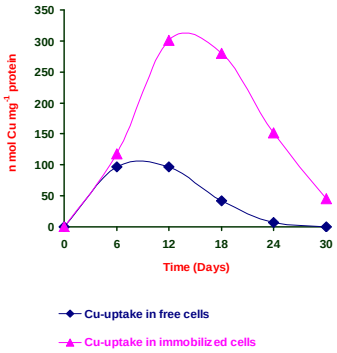
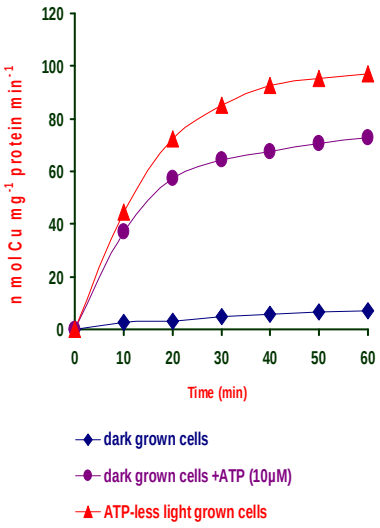
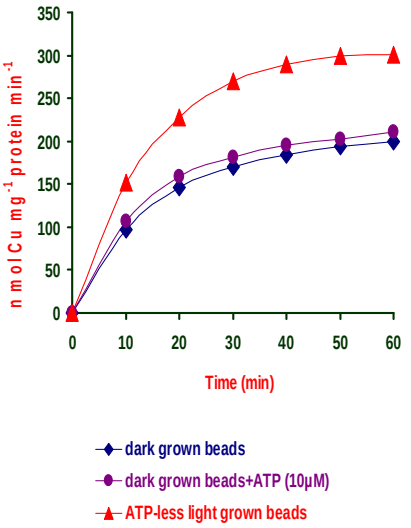


Fig.2. Cell age vs. Cu^{2+} uptake



(a)



(b)

Fig.3. Effect of light and exogenous ATP on Cu^{2+} uptake by (a) free and (b) immobilized *N. calcicola* cells

(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 different metabolic inhibitors on Cu^{2+} uptake in free and immobilized cells of *N. calcicola*

(n mol Cu mg^{-1} protein)

Metabolic inhibitor / uncoupler	Inhibition at 20 min
Free cells of N. calcicola	
Control	72.2
pCMB (1µM)	0.0
Mercaptoethanol (30µM)	54.17
Azide (10µM)	36.88
DCCD (10µM)	18.53
Immobilized cells of N. calcicola	
Control	227.31
pCMB (1µM)	0.0
Mercaptoethanol (30µM)	154.18
Azide (10µM)	114.53
DCCD (10µM)	54.74

(The data are mean of two independents experiments with four replicates each. The maximum variation from mean value was less than 5%) Azide (10µM), the well known inhibitor of respiratory electron transport and uncoupler of oxidative phosphorylation (Hewitt and Nicholas, 1963), is even a substrate for nitrogenase (Rubinson *et al.*, 1985) inhibited Cu^{2+} uptake more than 50%, since the treatment medium did not contain any combined nitrogen. It is presumed that azide could be used as a nitrogenase substrate in a major way while lesser amount could be available. A strong inhibition of Cu^{2+} uptake by DCCD (10µM) was due to alteration in the energy transfer processes via inhibition of ATP synthase dependent ATP synthesis. The strongest inhibition (100%) by pCMB (1µM) within Cu^{2+} uptake can be explained on the basis of the interaction of pCMB with –SH groups present on the cell membrane (Brachet, 1975) are always signifying the integrity of the membrane to maintain active ion transport. The cell membrane as well as –SH group containing enzyme receptor might be effected as pCMB in term through a damaged plasma membrane resulting in the complete abolition of metal uptake in both the states. The apparent superiority of immobilized cells in long term Cu^{2+} uptake experiments thus suggest that the such a system could be successfully applied to remove heavy metals from polluted water through repeated cycles and with no loss of cells in bioremediation.

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