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RESEARCH ARTICLE

EFFECT OF COPPER ON NITROGEN METABOLISM IN FREE AND IMMOBILIZED CELLS OF *Nostoc calcicola*

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ABSTRACT

The *Nitrogenase* and *Glutamine synthetase* activities 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 faster rate of *nitrogenase* activity than free cells. Immobilization was also associated with increase in *glutamine synthetase*, suggesting that the immobilized cells maintain sufficient ATP pool to drive energy expensive process of nitrogen metabolism. Both the enzymes in immobilized cells were less sensitive to Cu and degree of inhibition was less marked as compared to the free living cells. The tolerance of immobilized cells in terms of all the activities studied over free cells concluded that immobilized cells are more resistant to Cu in respect to nitrogen metabolism and it can be used as an effective biofertilizer.

INTRODUCTION

Copper is a well known micronutrient, metal component of plastocyanin and superoxidase dismutase (Cavet *et al.,* 2006). Exposure to Cu as a heavy metal alter the transport of nutrients and the resultant intracellular nutrient levels minimize the fidelity of nucleic acid, protein synthesis that eventually leads to viability loss. The utilization of any form of inorganic nitrogen in cyanobacteria has to depend on the availability of ATP. The ammonium taken up from the outer medium or internally generated by either nitrate reduction or dinitrogen fixation, is assimilated though its combination with carbon skeleton *via* the energy-dependent GS-GOGAT pathway (Guerrero and Lara, 1987). *Nitrogenase* is the enzyme used by the cyanobacteria to fix atmospheric nitrogen gas (N_2). Dinitrogen is quite inert because of the strength of its N-N triple bond. To break one nitrogen atom away from another requires breaking all three of these chemical bonds. *Nitrogenase* requires energy, released from the hydrolysis of ATP. ATP supplies the reducing power. Each electron transferred supplies enough energy to break one of dinitrogen's chemical bonds to convert one molecule of N_2 to ammonia. *Nitrogenase* ultimately bonds each atom of nitrogen to three hydrogen atoms to form ammonia (NH_3), which is in turn bonded to glutamate to form glutamine. *Nitrogenase* is able to reduce acetylene to produce ethylene which is estimated as *nitrogenase* activity. *Glutamine synthetase* plays an essential role in nitrogen metabolism by catalyzing ATP depending condensation of glutamate and ammonia to form glutamine.

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Glutamine is formed if an ammonium ion attacks the acyl-phosphate intermediate, while glutamate is remade if water attacks the intermediate. Ammonium ion binds more strongly than water to *glutamine synthetase* due to electrostatic forces. Another possible reaction is upon NH_2OH binding to *glutamine synthetase*, rather than NH_4^+, yields γ-glutamylhydroxamate which is used to estimate the level of *glutamine synthetase*. There are numerous reports that heavy metals also inhibit *nitrogenase* activity (Henriksson and Da Silva, 1978; Stratton and Corke, 1979; Singh and Pandey, 1981; Singh *et al.*1987; Pettersson *et al.,* 1996) and *glutamine synthetase* (Ip *et al.,* 1983; Singh *et al.,* 1987). The degree of inhibition was less marked for gel entrapped cells, the observed tolerance of immobilized cells against Cu inhibition of *nitrogenase* and *glutamine synthetase,* suggested sufficient cellular ATP pool for nitrogen metabolism than free *N. calcicola* (Potts and Morrison, 1986).

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 was

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 (0 - 60µM Cu^{2+}) and *nitrogenase* and *glutamine synthetase* activities 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.

Nitrogenase activity

The 6 days old N_2-grown free and immobilized cyanobacterial cells (400µg protein ml^{-1}) were inoculated into fresh combined nitrogen-free growth medium containing graded concentrations of Cu (0 to 60µM). 2.0 ml volume of such metal dosed free cells and a definite number of beads (having equal protein value) were taken into rubber-stoppered glass vials containing an atmosphere of 10% acetylene. Such vials were continuously agitated in light and the reaction terminated at a regular interval of 30 min up to 3 h by injecting 0.2 ml 1.0 N HCl. The amount of ethylene in a reaction vessel was determined in a gas chromatograph (Shimadzu, Japan), fitted with Porapak R column. *Nitrogenase* activity is expressed as n mol C_2H_4 produced mg^{-1} protein min^{-1} as described by Stewart *et al.,* (1968).

Glutamine synthetase activity

Cell-free extracts of Cu starved and supplemented (0-60µM) free and immobilized cyanobacterial cells (having equal protein value) were prepared by sonication at 4°C in 50 mM Tris-HCl buffer (pH 7.5) containing 2mM Na_2EDTA, 3mM DDT and 5mM $MgCl_2$ followed by centrifugation at 10,000g and the supernatant was used as crude enzyme extract. 0.5ml of enzyme extract was mixed with 1ml of reaction mixture consisting of Tris-HCl (pH 7.0), $MnCl_2$, 40mM; potassium arsenate, 3mM; sodium-ADP, 20 mM; Hydroxylamine, 0.4mM; neutralized with 2N NaOH 60mM glutamate, and 30 mM; was allowed to proceed in dark for 10 min at 30°C. The reaction was terminated after 10min by adding 2 ml of stop mixture consisting of 10% $FeCl_3$, 4ml; 24% Trichloroacetic acid, 1ml; 6N HCl, 0.5ml and 6.5ml distilled water. The turbid debris was removed by centrifugation from the resultant solution and the intensity of the coffee colour solution was taken at 540 nm against the reagent blank prepared by eliminating glutamine and hydroxylamine. *Glutamine synthetase* activity was expressed as µmol γ-glutamyl hydroxamate mg^{-1} protein min^{-1} as quantified by a reference to a standard curve obtained with γ-glutamyl hydroxamate as described by Sampaio *et al*, (1979).

RESULTS AND DISCUSSION

Nitrogense is the key enzyme in biological nitrogen fixation by cyanobacteria (Stewart and Lex, 1970; Stewart, 1973, 1980; Stewart *et al.,* 1975). The Cu-less free cells showed increasing trends in the enzyme activity attaining a maximum of 6.12 n mol C_2H_4 produced mg^{-1} protein min^{-1} (Fig.1a). The present observation on deleterious effect of Cu on *nitrogenase* activity reveal that the process was sensitive to all the metal concentrations used (0-60µM). The Cu concentration (10µM) brought about 26% declines in *nitrogenase* activity (at 3 h) although lowering could be seen even after 30 min of metal exposure of cells. The cyanobacterial cells retained at least some level of *nitrogenase* activity (0.72 n mol C_2H_4 produced mg^{-1} protein min^{-1}; 3 h) at the highest metal concentration (60µM Cu). Immobilization resulted resistance of cells against Cu inhibition because 10µM Cu causing 27% decline in *nitrogenase* activity of free cells, could do so only by 7.15%, and the Cu-resistance of immobilized cells was also reflected by a comparison of 50% inhibitory concentration as it came to 60µM Cu compared to 20µM Cu for free cells (Fig.1b).

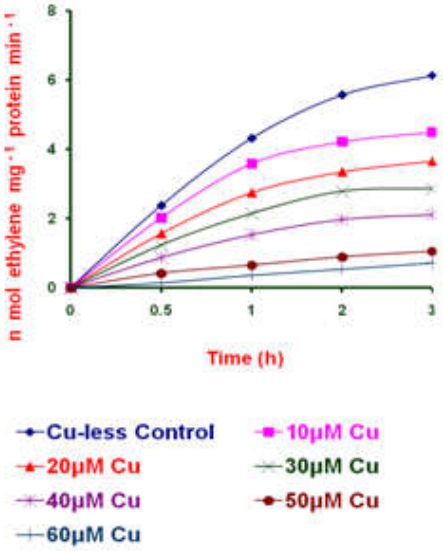

Fig.1a. *Nitrogenase* activity in free *N. calcicola* cells exposed to graded Cu concentration.

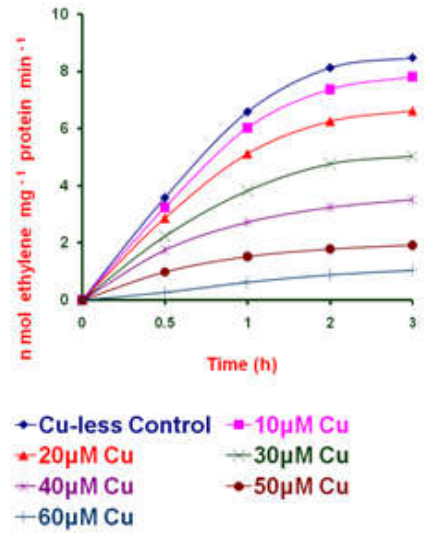

Fig.1b. *Nitrogenase* activity in immobilized *N. calcicola* cells exposed to graded Cu concentration.

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

The present observations on *nitrogenase* inhibition by Cu are analogous to the previous reports on inhibition of *nitrogenase* by heavy metals like Cu, Cd, Hg, Ni, Cr, Ag, Pb, Zn As and Al (Horne and Goldman, 1974; Henriksson and DaSilva, 1978; Stratton and Corke, 1979; Stratton *et al.,* 1979; Pettersson *et al.,* 1985; Rai and Raizada, 1985, 1986; Raizada and Rai, 1985; Dubey and Rai, 1987; Singh *et al.,* 1987). A decline in nitrogen fixation by the Cu treated *Aphanizomenon*

flos-aquae cells have been attributed to the breakdown of photosynthesis in vegetative cells that ultimately channelize energy to heterocyst (Wurtsbaugh and Horne, 1982; Scherer *et at.,* 1988) and irreversible inactivation of *nitrogenase,* because of insufficient supply of energy to remove oxygen within heterocyst (Foy and Cox, 1967; Murray and Horne, 1980). The increased resistance of immobilized cells indicated their superiority over free cells. The enhanced *nitrogenase* activity in immobilized cells is agreement with other reports (Musgrave *et al.,* 1982; Brouers and Hall, 1986; Kerby *et al.,* 1986; Shi *et al.,* 1987; Karpunina *et al.,* 1989). Also, the enhanced heterocyst frequency of immobilized *N. calcicola* cells over free cells is agreement with the reports of Musgrave *et al.,* (1982) for *Anabaena* sp. with the heterocyst frequency up to 9.8 over 7.5% in free cells.

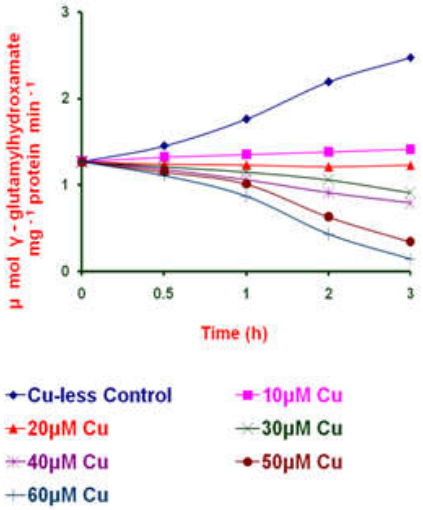

Fig.1a. *Glutamine synthetase* activity in free *N. calcicola* cells exposed to graded Cu concentration

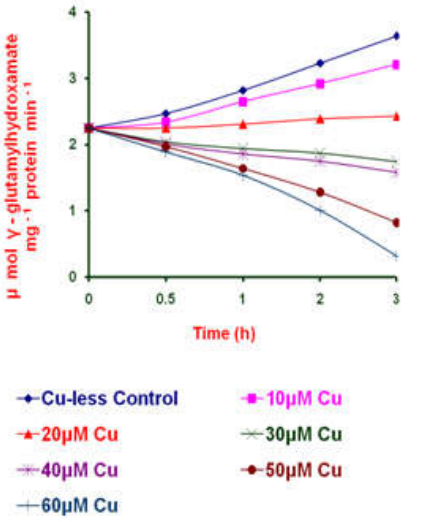

Fig.1b. *Glutamine synthetase* activity in immobilized *N. calcicola* exposed to graded Cu concentration

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

Glutamine synthetase, main enzyme involved in cyanobacterial NH_4^+ assimilation (Stewart, 1980; Chapman and Meeks, 1983) and may be regarded as the first step in biosynthesis of vital metabolic compounds. *Glutamine synthetase* activity in N_2 grown, *N. calcicola* free cells (basal level 1.25µ mol γ-glutamylhydroxamate mg^{-1} protein min^{-1}) attained a maximum of 2.4µ mol γ-glutamylhydroxamate mg^{-1} protein min^{-1} within 3 h of photoincubation (Fig.2a). Based on comparisons made for *nitrogenase* inhibition, the *glutamine synthetase* activity was slightly more sensitive to Cu (10µM) as reflected by a 24% inhibition within 1 h of Cu exposure. Similarly, immobilized cells showed 05% inhibition of enzyme activity within 1 h exposure to Cu concentration (10µM; Fig. 2b). Inhibition could possibly arise from the disrupt energy supply resulting from the Cu-membrane interaction and such ions could occupy the action site first then move to the regulatory site(s) of enzyme following saturation as reported by Singh *et al.,* 1987; Ip *et al.,* 1983) on *glutamine synthetase* sensitivity towards Hg and Cu. The superiority of immobilized cells over their free cell counterpart could also be seen that *nitrogenase* and *glutamine synthetase* activities were less inhibited in immobilized cells against Cu.

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