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RESEARCH ARTICLE

CHARACTERIZATION OF COPPER TOLERANT (Cu^r) STRAIN OF *Nostoc calcicola* Breb

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ABSTRACT

Characterization of Copper tolerant (Cu^r) strain of *Nostoc calcicola* Breb revealed its ability to grow at 70µM Cu being 60µM Cu is saturating concentration for sensitive (Cu^s) strain. The overall pattern indicated the acquisition of metal tolerance in the former. In Cu uptake comparisons, the Cu^r strain showed only 26% less cellular metal buildup (72.15 n mol Cu mg^{-1} protein) at 60 min compared to Cu^s (96.89 n mol Cu mg^{-1} protein). The Cu^r strain exhibited the lower level of Phosphate uptake, Ca^{2+} and Mg^{2+} dependent ATPase activities, $^{14}CO_2$-incorporation, *Nitrogenase* and *Glutamine synthetase* activities.

INTRODUCTION

Copper has a number of important functions in human body. Cu is required to fix calcium in the bones and to build and repair all connective tissues including tendons, ligaments, skin, hair, nails, arteries and veins. Imbalance of Cu can contribute to osteoporosis and cardiovascular problems. Copper is closely related to estrogen metabolism, and required for women's fertility to maintain pregnancy. Imbalance can cause every conceivable female organ-related difficulty such as premenstrual syndrome, ovarian cysts, infertility, miscarriages and sexual dysfunctions. It affects men less than women in this area, but it may affect men's potency and sexual drive as well as that of women. Cu stimulates production of neurotransmitters epinephrine, norepinephrine and dopamine. It is also required for *monoamine oxidase*, an enzyme related to serotonin production resulting Cu is involved deeply with all aspects of the central nervous system. Copper imbalances are highly associated with most psychological, emotional and often neurological conditions. These include memory loss, especially in young people, depression, anxiety and schizophrenia (Nolan 2009). Cyanobacteria have been used to remove Cu from aqueous system since they have capacity to accumulate dissolve metals (Prajapati *et al.*, 2011, 2012; Taranum *et al.*, 2011). Cu-tolerant (Cu^r) strain of *Nostoc calcicola* Breb has been isolated in Department of Biotechnology, Bundelkhand University, Jhansi revealed its ability to grow at 70µM Cu. The 60µM Cu was saturating concentration for Cu-sensitive (Cu^r) strain that cannot be grown at 70µM Cu (Prajapati and Pandey 2011). Thus the experiments in present study for comparing Phosphate uptake, Ca^{2+} and Mg^{2+} dependent ATPase activities, $^{14}CO_2$-incorporation, *Nitrogenase* and *Glutamine synthetase* activities in Cu^s and Cu^r strains were carried out at 60µM Cu.

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MATERIALS AND METHODS

Isolation of Cu^r strain from Cu^s cells of *N. calcicola* Breb

Log phase population of *N. calcicola* Breb with a cell density of approximately $1x10^5$ cells ml^{-1} were seeded on nutrient agar prepared in Allen-Arnon's (Allen and Arnon, 1955) medium (pH 8.0) containing Cu concentrations (60-100µM). The cultures were incubated phototrophically in culture room at 25±2°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). As cyanobacterial cells could not grow at 60µM Cu (saturating metal concentration for Cu^s strain) form the basis of isolation of colonies, growing in the presence of 70µM Cu under diazotrophic condition. The Cu^r clones were grown as nutrient plates arose and its stability was checked though replating on nutrient plates containing 70µM Cu. This population was grown in bulk. All the experiments for comparing various metabolisms in Cu^s and Cu^r strains were carried out at 60µM Cu because Cu^s strain cannot be gown at 70µM Cu.

Effect of Cu on various metabolisms in Cu^s and Cu^r strains

Phosphate uptake estimation

For phosphate uptake, 6 days old Cu^s and Cu^r strains of *N. calcicola* Breb were starved for 4h in phosphate free medium.

Phosphate uptake experiment proceeded by using 2.5mM K_2HPO_4. To 1ml of standard K_2HPO_4 solution of culture supernatant 5N H_2SO_4 (1ml), ammonium molybdate (1ml) and reducing agent (0.1ml) [1.2gm sodium metabisulphate, 1.2gm sodium sulphite, 0.2gm ANSA (1-amino-2-nepthol-4-sulphonic acid)] were added and appropriate dilution made by sterilized distilled water (final 10ml). The mixture was incubated at 25±2°C. The optical density of the blue color developed was measured at 660nm spectrophotometrically as per the method of Fiske and Subba Row, (1925).

Measurement of ATPase

The extraction of ATPase from Cu^s and Cu^r strains was done as per the method of Lockau and Pfeffer (1983). Both the strains were incubated at 25±2°C, then centrifuged (10,000 g), washed and re-suspended in extraction buffer (300 mM Tris-HCl, pH 8.1). The supernatant obtained was dialyzed for 20 min against 10mM 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 (2ml) contained 6mM $MgCl_2$, 6mM ATP in 30mM Tris-HCl buffer (pH 8.1). The reaction was initiated by adding above enzyme preparation and stopped at 20 min by adding 0.25ml Tricloroacetic acid (40%).

Ca^{2+} dependent ATPase

The enzyme was activated prior to assay. The crude enzyme was treated with trypsin (0.75mg ml^{-1}) for 20 min followed by the addition of 0.75mg 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 Cu^s and Cu^r strains after transfer to fresh growth medium were pre-incubated in dark for 3h. The 1ml volume of the dark incubated Cu^s and Cu^r strains were transferred to glass scintillation vials, containing 60µM Cu and 0.05µCi ml^{-1} $NaH^{14}CO_3$ (BARC, India). The Cu^s and Cu^r strains in the scintillation vials, were light-incubated at 25±2°C and $^{14}CO_2$-incorporation stopped by adding 0.1ml 2N HCl, followed by the addition of 5ml 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.

***Nitrogenase* activity**

Cu^s and Cu^r strains (400µg protein ml^{-1}), inoculated into fresh combined nitrogen-free growth medium containing 60µM Cu. 2ml of such cells were taken into rubber-stoppered glass vials containing an atmosphere of 10% acetylene and the vials were continuously agitated in light and reaction terminated by injecting 0.2 ml 1N 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 nmol C_2H_4 produced mg^{-1} protein as described by Stewart *et al.,* (1968).

***Glutamine synthetase* activity**

Extracts of Cu^s and Cu^r strains (400µg protein ml^{-1}) were prepared by sonication at 4°C in 50mM Tris-HCl buffer (pH 7.5) containing 2mM Na_2-EDTA, 3mM DDT and 5mM $MgCl_2$ followed by centrifugation at 10,000g and the supernatant was used as crude enzyme extract. To 0.5ml of enzyme extract was mixed with 1ml of reaction mixture consisting of Tris-HCl (pH 7), $MnCl_2$, 40mM; potassium arsenate, 3mM; Na_2-ADP, 20mM; Hydroxylamine, 0.4mM; neutralized with 2N NaOH 60mM glutamate, and 30 mM; was allowed to proceed in dark at 30°C. The reaction was terminated by adding 2ml 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 540nm against the reagent blank prepared by eliminating glutamine and hydroxylamine. *Glutamine synthetase* activity was expressed as µmol γ-glutamyl hydroxamate mg^{-1} protein as quantified by a reference to a standard curve obtained with γ-glutamyl hydroxamate as described by Sampaio *et al*, (1979).

RESULTS AND DISCUSSION

The Cu^r strain showed a slightly lower pace for growth compared to its parent in the normal growth medium lacking metal although it tolerated 70µM Cu. The overall pattern of Cu-uptake event in Cu^r strain was characterized by lowered uptake rate (1.2 n mol Cu mg^{-1} protein min^{-1}). The Cu uptake pattern for Cu^s and Cu^r strains taking 60µM Cu showed time-dependent metal uptake (Fig.1). Cu^r strain accumulated 72.15 n mol Cu mg^{-1} protein, during 60 min as compared to higher values for the Cu^s strain (96.89n mol Cu mg^{-1} protein) or in other words, accumulating to the 26% reduction in the total Cu-intake. The acquisition of Cu tolerance in *N. calcicola* Breb was associated with lowered Cu^{2+} uptake in Cu^r strain as evident from observations in Cu-tolerant *N. calcicola* (Prajapati and Pandey, 2007, 2008). Fig.2 reflected the phosphate uptake in Cu^s and Cu^r strains of *N. calcicola* Breb. There was a lower phosphate uptake (0.018µmol PO_4^{3-}µg^{-1} protein) observed in Cu^r strain as compared to Cu^s strain (0.02µmol PO_4^{3-}µg^{-1} protein) at 4h. A similar trend of lower profile for phosphate uptake was recorded in Cu^r strain of *Scenedesmus* (Mierle and Stokes, 1976). Table-1 showed ATPase (Ca^{2+} and Mg^{2+} dependent) activities in Cu^s and Cu^r strains. Ca^{2+} and Mg^{2+} dependent ATPase activities in Cu^r were lesser as compared to the Cu^s strain at 20 min. These finding are similar as reported in Ni-tolerant *Synechococcus* (Gleason and Wood, 1988). Fig.3 showed $^{14}CO_2$-incorporatin in Cu^s and Cu^r strains and no significant counts of β-particles were observed in both the strains at 3h directing the deleterious effect of Cu as also reported in Cd-tolerant *Anacystis nidulans* (Singh and Pandey, 1982; Singh and Yadava, 1986). Table-2 exhibited the sensitivity towards the

Nitrogenase and *Glutamine synthetase* respectively at 20 min. The lower activities of both the enzymes were observed in Zn-tolerant strain of *A. nidulans* (Shehata and Whitton, 1982).

Table1. ATPase (Ca^{2+} and Mg^{2+} dependent) activities in Cu^s and Cu^r strains

Cu^s strain of *N. calcicola* at 20 min	
Ca^{2+} dependent ATPase	28.14 nmol Pi liberated
mg^{-1} protein	
Mg^{2+} dependent ATPase	19.79 nmol Pi liberated
mg^{-1} protein	
Cu^r strain of *N. calcicola* at 20 min	
Ca^{2+} dependent ATPase	27.12 nmol Pi liberated
mg^{-1} protein	
Mg^{2+} dependent ATPase	18.16 nmol Pi liberated
mg^{-1} protein	

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

Table2. *Nitrogenase* and *Glutamine synthetase* activities in Cu^s and Cu^r strains

Cu^s strain of *N. calcicola* at 20 min	
Nitrogenase	0.071 nmol C_2H_4
produced mg^{-1} protein	
Glutamine synthetase	0.141 µmol γ-
glutamylhydroxamate mg^{-1} protein	
Cu^r strain of *N. calcicola* at 20 min	
Nitrogenase	0.069 nmol C_2H_4
produced mg^{-1} protein	
Glutamine synthetase	0.137 µmol γ-
glutamylhydroxamate mg^{-1} protein	

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

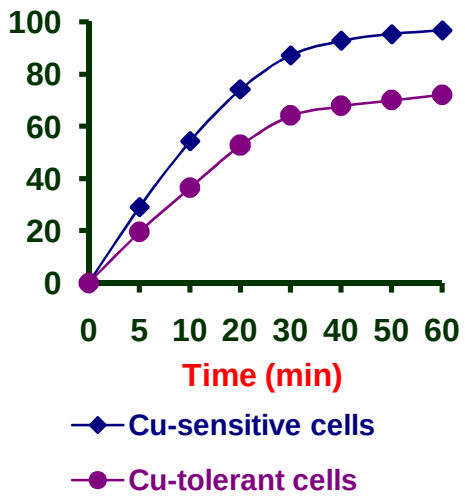

Fig. 1. Cu influx in Cu^s and Cu^r strains of *N. calcicola* at 60µM Cu

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

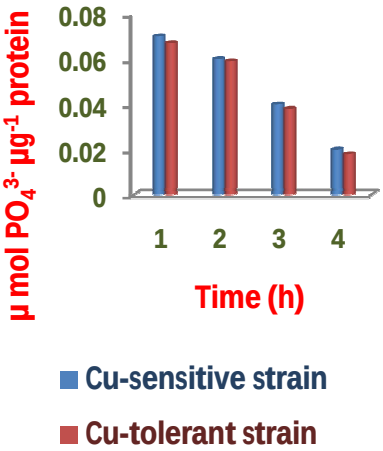

Fig. 2. Phosphate uptake in Cu^s and Cu^r strains of *N. calcicola*

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

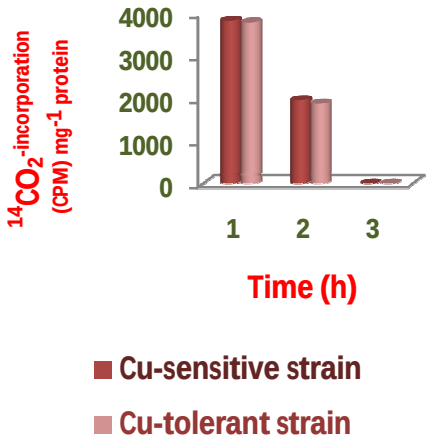

Fig. 3. $^{14}CO_2$-incorporation in Cu^s and Cu^r strains of *N. calcicola*

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

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