Development and characterization of nickel accumulating mutants of *Aspergillus nidulans*

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Abstract Stable mutants of *Aspergillus nidulans*, resistant to 1 mM Ni were developed by step-by-step repeated culturing of the fungus on the medium containing increasing concentrations of nickel chloride. Characterization of mutants could differentiate them into two categories Ni^R I and Ni^R II. Each category of mutants exhibited alterations in growth, conidial germination and melanin secretion both in Ni-free and Ni-containing media. Ni^R II mutants were little slow in growth with sparse mycelia and conidiation but showed high melanin secretion and higher Ni-uptake in comparison to Ni^R I mutant. Studies involving metabolic and translational inhibitors could prove that Ni-accumulation was biphasic. The initial energy independent surface accumulation was found to be followed by energy dependent intracellular uptake. Increase in the concentration of the metal in the medium or the time of exposure did not proportionately increase the metal uptake by the mutants. Ni-uptake followed Michaelis-Menton saturation kinetics, which was enhanced under optimum pH of 6.5–7.5 and reduced complexity of the medium due to free availability of ions. Resistance to Ni was found to be constitutive in Ni^R I mutant, and could be induced in Ni^R II mutant.

Keywords *Aspergillus nidulans* · metabolic · inhibitors · kinetics · mutants · nickel · resistance · uptake

Introduction

The heavy metal nickel (Ni) is one of the essential micronutrients, required by various organisms for a range of structural organizations and metabolic activities. Nickel is a cofactor for enzymes like hydrogenases, urease and methyl-S-coenzyme M-reductase, and some key enzymes in the metabolism of strictly anaerobic bacteria¹. Microorganisms and plants acquire and accumulate micronutrients from their surrounding environment and in turn become the major source of metals for humans and animals. However, in excess amounts, even the essential metal-micronutrients become toxic to most of the organisms. Toxic levels of nickel inhibit growth and sporulation of various filamentous fungi and yeasts² and cause reduction of RNA and protein synthesis and photosynthesis³.

Nickel naturally occurs in soil, sea-salts, volcanic ash, plant-exudates and particles in smoke from forest fires and volcanoes. Nickel is extensively used in electroplating and production of pigments, steel and other alloys formed with different metals⁴. These natural and man made processes including tobacco smoking and burning of coal, petroleum waste, etc. lead to the accumulation of Ni to a toxic level in the environment, particularly in soil and water. Increase of heavy metals in the environment leads to the selection of resistant organisms via molecular mechanisms^{5,6}. Metal resistant microbes have a repertoire of mechanisms by which they can accumulate heavy metals and radionuclides from industrial effluents, contaminated sewage, sludge, coal wastes, low grade ores and urban waste; and thereby

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prove beneficial both in environmental decontamination as well as recovery of valuable metals^{7,8}. To exploit the microorganisms for such benefits, it is essential to have microorganisms possessing high resistance against the metal. Microorganisms, tolerant to metals, have been isolated from the environment contaminated with metals. However, many of the indigenous microbes show resistance to low levels of metal⁹. Nevertheless, training the microorganisms to resistance by stepwise exposure to higher and higher levels of toxic element or by mutagenesis using a mutagen can lead to the development of microbial strains having resistance to high levels of metals. We developed strains of *Aspergillus nidulans*, having very high tolerance to Ni and characterised them for various aspects including Ni accumulation. The findings are reported in this communication.

Materials and Methods

Organism and the growth conditions: A metal sensitive strain *riboA_p*, *biA₁* of *Aspergillus nidulans* was used as the original wild type strain (Ni^S). This strain was obtained from Culture Collection Centre, Glasgow, UK and maintained in the Department of Genetics, University of Delhi, South Campus, New Delhi, India.

The wild type as well as the mutant strains, selected in this study were normally grown at 37°C for 120 h on either supplemented Czapeck-Dox medium (CDM)¹⁰ or complete medium (CM). Both these media were made in deionised glass distilled water. CDM having pH 6.6 contained (g L⁻¹ medium): glucose, 10; NaNO₃, 6; KH₂PO₄, 1.52; KCl, 0.52; MgSO₄·7H₂O, 0.52; Cu(NO₃)₂·3H₂O, 0.001; FeSO₄·7H₂O, 0.001; ZnSO₄·7H₂O, 0.001 and supplemented with vitamins (μg L⁻¹): riboflavin, 111 and biotin 22. To make CM, the CDM was supplemented with (g L⁻¹ medium): peptone, 2; yeast extract, 1; casein hydrolysate, 1.5 and vitamins (mg L⁻¹ medium): riboflavin, 1; biotin, 0.02; nicotinamide, 1; p-aminobenzoic acid, 0.5; pyridoxine – HCl, 0.5 and thiamine-HCl, 0.5. The pH was adjusted to 6.1. All the chemicals were of AR-grade. Molar stock solution of nickel chloride (NiCl₂·6H₂O) was used for obtaining Ni-resistant mutants.

Liquid inoculum, adjusted to ~1×10⁶ conidia mL⁻¹ normal saline was used. The diameter of colonies and dry weight of biomass were measured to determine the growth of the organisms grown on solid and liquid media, respectively.

Development of Ni-resistant mutants: Ni resistant mutants (Ni^R) of *A. nidulans* were obtained by stepwise selection against increasing concentrations of Ni. The conidia from the fungal colonies were repeatedly subcultured in plates containing increasing concentrations of Ni in CDM agar

until the growth of colonies was found stable and comparable with those on Ni-free medium. The process led to the selection of Ni^R strains that exhibited good growth and conidiation at 1mM concentration of Ni, beyond which the growth of the mutant strains was inhibited. Therefore, 1mM Ni was considered as the maximum tolerable concentration (MTC) of the metal for the fungus under study.

To understand whether the mechanism of Ni-resistance was inducible, the conidia of mutant strains were allowed to germinate for first 8 h in CDM containing 0, 0.1 and 0.25 mM Ni and there after transferred to the medium having Ni concentration raised to 1mM. Fungal growth and accumulation of Ni in the fungal biomass was monitored for next 5 d at 24 h intervals.

Ni-accumulation study: The fungal biomass raised in Ni containing liquid CDM was washed with normal saline, dried, weighed and incinerated at 600°C for 6h in a muffle furnace. The ash was dissolved in 2 mL of 1N HCl. In case of cytosol samples (as described below), 1 mL of the sample was mixed with 2 mL of 1N HCl. Suitable dilutions were made with deionised distilled water and subjected to Ni estimations at 232 nm in an atomic absorption spectrophotometer (AAS- Shimadzu: AA-640-13).

To estimate the accumulation of metal in the cell wall, the mycelial biomass from 72 h old culture was harvested, washed extensively in normal saline and blotted to dry. The mycelial mass was homogenized in 1% sodium dodecyl sulphate (SDS), shaken at 200 rpm for overnight and centrifuged at 10,000 × g for 20 min at 4°C. The pellet containing cell walls was washed extensively with distilled water and then sequentially with 80% ethanol and ether¹¹. The cell wall preparation was dried at 80°C, weighed and incinerated at 600°C. The supernatant contained the cytosolic fraction. Both the cell wall ash and the cytosolic fraction were subjected to Ni estimation by AAS.

Effect of metabolic inhibitors on Ni accumulation: Studies on the effect of metabolic inhibitors on Ni uptake were conducted to elucidate energy dependence of Ni uptake by the mutants. Metabolic inhibitors: sodium azide (NaN₃) and 2,4-dinitrophenol (DNP) and the translational inhibitor cycloheximide were used for this study. The concentrations of inhibitors that inhibited 50 percent growth (IC₅₀ values) of the sensitive and the mutant strains in 72h was determined; and the effect of inhibitors on the growth of the strains and the metal accumulation was examined by culturing the strains in liquid CDM containing 1mM Ni along with IC₅₀ concentration of the respective inhibitor.

Kinetics of Ni uptake: The Ni resistant mutant strain was grown for 60h in metal-free CDM and the mycelial biomass was harvested, washed extensively with normal saline

and blotted to dry. One hundred mg of wet biomass was transferred to 10 mL of the liquid CDM and subjected to different treatments, which included varying concentrations of Ni, metabolic inhibitors, absence of carbon source, H^+ concentration, and the nature of the medium. Ni uptake was estimated in the biomass at 1 h intervals for 5 h. For determining the role of C-source, the pre-grown mycelia of the mutants were starved for 5 h by incubating in liquid medium without glucose, before subjecting to metal uptake assay.

Uptake of Ni at external concentrations of 0.5–2.5 mM Ni was recorded after 3 h. The K_m and V_{max} for the uptake of Ni were calculated for the Ni^R mutants. The Lineweaver-Burk plot was prepared using $1/S$ and $1/V$ values.

Protein estimation: Proteins were isolated and partially purified¹² to determine their role in conferring Ni-resistance. Seventy two h old mycelia from the cultures, grown under different experimental conditions, were harvested and washed in physiological saline and Tris-HCl buffer (10 mM, pH 8). One g of wet mycelium from each set was homogenized in 3 mL of Tris-HCl and the homogenate was centrifuged at 20,000 $\times$ g for 30 min in Beckman 70 refrigerated centrifuge. The supernatant was heated at 60°C in the heating block for 10 min and then centrifuged at 50,000 $\times$ g for 10 min. The supernatant, referred to as partially purified fraction, was evaporated *in vacuo*. The residue was dissolved in 0.5 mL of Tris-HCl buffer for protein estimation and subjected to electrophoresis¹³.

Results and Discussion

Development of Ni resistant mutants: Most fungi accumulate metals by employing physico-chemical mechanisms and transport systems of varying specificity^{14–16}. However, both essential and non-essential metals in concentrations, higher than the optimal level, prove toxic to organisms. Under such conditions, these organisms may activate and adapt a mechanism of detoxification to ensure survival^{17,18}. We, in this study were successful in developing Ni-resistant (Ni^R) mutants from *Aspergillus nidulans* strain *riboA₁*, *biA₁* by step-by-step repeated culture and selection on the medium containing increasing concentrations of Ni. This process has successfully been employed for such purpose by many other workers⁵. The selected mutants remained resistant to 1mM Ni, even after several subculturing on metal free medium and thus confirmed them to be genetically stable Ni^R mutants.

Characterization of Ni^R mutants: The selection procedure provided two types of Ni^R mutants of *A. nidulans*. Mutants that formed relatively large colonies, dense bright green conidiation and secreted very little or no melanin were designated as Ni^R I (Fig. 1A). The mutants that formed medium size colonies, sparse mycelial growth and conidiation and secreted melanin profusely were designated as Ni^R II (Fig. 1B). The growth studies on solid as well as in liquid CDM showed that both Ni^R I and Ni^R II mutants, in spite of their tolerance to Ni, showed extended lag phase, though

Fig. 1 Colonies of Ni^R mutants on supplemented Czapeck-Dox agar containing 1 mM Ni at 37°C after 120 h. A: Ni^R I, B: Ni^R II. Growth of wild type control strain (Ni^S) is highly inhibited (marked by $\rightarrow$).

Fig. 2 Growth of Ni^S and Ni^R strains on supplemented Czapeck-Dox agar at 37°C. A: Ni-free medium, ○ = Ni^S, △ = Ni^RI, □ = Ni^RII. B: Ni (1mM) containing medium, ● = Ni^S, ▲ = Ni^RI, ■ = Ni^RII.

later picked up the growth and became comparable to that of sensitive strain, grown on Ni free medium (Fig. 2A & B). In the medium containing even 0.5mM Ni, the hyphal growth of Ni^S strain after 120 h, was found to be inhibited by about 90 percent and Ni^RI and Ni^RII mutants by about 12 and 45 percent, respectively. At 1mM Ni concentration, the inhibition increased to 98 percent for Ni^S, 32 and 65 percent for Ni^RI, Ni^RII mutants respectively. The Ni^S strain showed almost no growth except for very title hyphal extension and no conidiation. In Ni^RI mutant, conidiation showed up by 72 h, while in Ni^RII was seen by 48 h and reached to maximum by 72 h. Such alterations in morphology, growth and morphogenesis of many other fungi due to exposure to heavy metals including Ni in the medium are well known^{19,20} and have been considered as a strategy by the organism to evade the toxicity for survival.

Alterations in cell wall composition and accelerated production of melanin can be induced by toxic metals in the medium. Melanin in the cell wall is used as a defence mechanism as such structures are impermeable to heavy metals and other toxicants. The addition of melanin to the Cu-containing culture of albino strain of *A. pullulans* reduced the metal toxicity to a certain extent²¹.

The Ni^R mutants, despite their tolerance to 1mM Ni, exhibited delayed and differential rates of conidial germination and developmental process irrespective of the presence of the metal in the medium. In first 8 h, conidia of Ni^RI mutant appeared in different stages of distension with only very few conidia showed the beginning of germ tube formation. Subsequently, the rate of further development of conidia into formations of germ tube, mycelial mats and balls continued to vary. The pattern of growth

and development of Ni^RII mutant was similar to that of Ni^RI mutant except that comparatively high percentage of Ni^RII conidia showed faster rate of germination and growth. Mycelial mats of Ni^R mutants were much thinner irrespective of presence or absence of Ni in the medium but more so in Ni-containing medium. Mycelial balls of Ni^R mutants were fragmented at all the phases of their growth. The developmental aberrations brought about by Ni were irreversible as the conidia from a single mycelial ball, when transferred to Ni-free MM continued to produce colonies of fragmented mycelial balls of different sizes. The rate of disintegration was more rapid in Ni^S strain in Ni-free as well as in Ni-supplemented medium than in the mutants cultured in presence in Ni. The germination of conidia of Ni^S strain was completely inhibited by MTC level of Ni.

The effect of Ni on delayed conidial germination of Ni^R mutants was further evident by assessment of their biomass during the first 24 h. Ni^RI and Ni^RII mutants grown in Ni-free medium registered only about 50% dry weight of the biomass formed by Ni^S in the same medium. However, in Ni-containing medium, both Ni^R mutants registered 4 to 6 folds higher biomass. In other studies^{22,23}, the germination of conidia of the wild type strains of *A. nidulans* was found to be inhibited to 50 percent by 175μM Cu and 350μM Cd, whereas the same or higher concentrations of these metals could slightly stimulate the germination of Cu- and Cd-resistant strains.

Nickel uptake by the Ni^R mutants: Both Ni^RI and Ni^RII mutant strains showed progressive increase in Ni uptake up to 72 h of their growth, thereafter exhibiting a declining trend (Fig. 3). Ni^RII mutant accumulated two folds more Ni than

the Ni^RI mutant. By 96 h, when the exponential growth had ended and mycelial disintegration had commenced, Ni^RII mutant retained more than 5 times Ni than the Ni^RI mutant. Thus, Ni^RII was better Ni-accumulator than Ni^RI. Very low content of Ni in the mutant biomass in later phases of growth can be attributed to the changes in the biosorptive properties of the fungus due to the chemical changes in the mycelium⁷.

Fig. 3 Ni uptake by Ni^S and Ni^R strains in liquid Czapeck-Dox medium containing 1mM Ni over a period of 120 h at 37°C. ● = Ni^S, ▲ = Ni^RI, ■ = Ni^{RII}.

The metal accumulation sites can be both cell wall and the cytoplasm. The structure of mycelial network and the

Table 1 Distribution of Ni in cellular fractions of Ni^S and Ni^R strains, cultured in Czapeck-Dox broth containing 1mM Ni for 72 h at 37°C.

Cellular fraction	Ni ^S	Ni ^R	Ni ^{RII}
Cell wall (µg mg DW ⁻¹)	3.4±0.31	15±1.97*	120.7±5.52*
Cytosol (µg ml ⁻¹)	6.36±1.03	150±9.11*	526.5±14.92*

± indicates standard errors of mean.

*Significance at 1% level.

Table 2 Ni uptake (µg mg⁻¹ DW) by Ni^R mutants in Czapeck-Dox broth containing 1mM Ni and the metabolic inhibitors (NaN₃ and 2,4-DNP).

Time h	Ni ^{RI}			Ni ^{RII}		
	Ni	Ni+NaN ₃	Ni+DNP	Ni	Ni+NaN ₃	Ni+DNP
0	0.06±0.01	0.07±0.01	0.05±0.01	0.27±0.01	0.26±0.01	0.58±0.04
24	0.98±0.04	0.95±0.02	1.19±0.06	5.50±0.21	4.98±0.08	4.24±0.23
48	6.02±0.07	3.51±0.21	4.16±0.18	9.71±0.30	4.55±0.19	4.95±0.24
72	56.81±2.44	4.57±0.22	5.72±0.20	125.8±2.34	6.56±0.33	6.08±0.23
96	20.00±0.86	4.93±0.03	5.96±0.29	94.65±4.36	5.81±0.29	6.61±0.26
120	5.82±0.32	0.28±0.014	0.35±0.021	28.22±1.47	3.01±0.09	4.30±0.16

IC₅₀ values: NaN₃ (µM mg DW⁻¹) for Ni^{RI}=2.5, Ni^{RII}=7.5; 2,4-DNP (mM mg DW⁻¹) for Ni^{RI}=0.75, Ni^{RII}=1.

± indicates standard errors of mean.

unique composition of the cell wall of filamentous fungi offer desired sites for metal bindings. Both Ni^{RI} and Ni^{RII} mutants accumulated appreciable levels of Ni in the cell walls (Table1) and much higher amount of Ni in their intracellular compartment. Of the two mutants, Ni^{RII} showed more metal amassing capability. Cell surface accumulation occurs due to metal complexation with charged reactive groups like hydroxyl, sulphahydryl, carboxyl, phosphate or amino groups²⁴. Other cell walls components like glycans, chitosan, polyuronides and chitin are also active in biosorption²⁵. Cell wall-deficient slime mutants of *N. crassa* accumulated much less Cu than wild type strain indicating Cu distribution both in the cell wall and the cytoplasm²⁶. In *Saccharomyces cerevisiae*, Cu has been located both in the cytoplasm as well as the cell wall²⁷. Sequestration of Ni into vacuole associated with free histidine has been reported in yeasts and other fungi¹⁴.

The effect of metabolic inhibitors sodium azide (NaN₃) and 2,4 dinitrophenol (2,4-DNP) on the uptake of Ni was studied to elucidate the energy dependence of Ni accumulation. It is known that after cell surface binding, which is an instant passive process at the cell surface, the transport of metal(s) is an energy involving active process. In the presence of inhibitors, the passive process of cell surface binding is not affected but active uptake is affected owing to less availability of energy. IC₅₀ concentration of NaN₃ for Ni^S and Ni^{RII} strains was found to be 7.5 µM and for Ni^{RI} mutant 2.5 µM. For 2,4-DNP, the IC₅₀ values were 1mM for Ni^S and Ni^{RII} and 0.75 mM for Ni^{RI} strains. Ni^{RI} mutant was thus, more sensitive to the metabolic inhibitors than Ni^S and Ni^{RII} strains. There was no change in the pattern of uptake of metal in the presence of metabolic inhibitors at their IC₅₀ values by either of the mutant strains during the initial growth phase of 24 h. However, by 72 h, Ni^{RI} and Ni^{RII} mutants showed about a 10 to 20-folds decrease, respectively (Table 2). Thus, Ni²⁺ uptake was biphasic in nature and was evident both in long-term and short-term metal uptake assay. Such biphasic uptake of metal ions

Fig. 4 Effect of pH on Ni uptake by Ni^{R} mutants in liquid Czapeck-Dox medium. Mycelia grown for 60 h in Ni-free medium were transferred to medium containing 1mM Ni. A: $\text{Ni}^{\text{R}}\text{I}$ mutant; B: $\text{Ni}^{\text{R}}\text{II}$ mutant; pH: $\triangle$ = 4.5, $\square$ = 5.5, $\circ$ = 6.5, $\blacksquare$ = 7.5, $\blacktriangle$ = 8.5

has been demonstrated in many filamentous fungi as well. Cell wall adsorption accounts for rapid, non-specific and energy-independent process resulting from ionic interactions, followed by a progressive energy-dependent phase of intracellular uptake^{28,29}.

Similarly, when the fungal biomass from 60 h old cultures, grown in Ni-free medium, was exposed to metabolic inhibitors or the absence of carbon source in the CDM, after the initial accumulation in first 1 h, the Ni^{R} mutants did not register any significant increase in Ni uptake. These results again confirmed that the initial energy-independent passive metal uptake by diffusion and surface binding, was followed by an active process requiring the availability of metabolic energy.

Fig. 5 Effect of the complexity of the medium on Ni uptake. Mycelia of Ni^{R} mutants grown in Ni-free medium for 60 h were transferred to CM containing 1mM Ni. $\triangle$ = $\text{Ni}^{\text{R}}\text{I}$ in MM+Ni, $\square$ = $\text{Ni}^{\text{R}}\text{II}$ in MM+Ni (control), $\blacktriangle$ = $\text{Ni}^{\text{R}}\text{I}$ in CM+Ni, $\blacksquare$ = $\text{Ni}^{\text{R}}\text{II}$ in CM+Ni.

Metal uptake by microorganisms is greatly influenced by pH^{30,31}. Ni uptake in both the Ni^{R} mutants was maximum at pH 6.5 (Fig. 4). The effect of pH can be accounted for the chemical behaviour of the ionic species involved. Acidic conditions though increase the metal availability; also increase H^+ ion concentration that may compete for the binding to anionic sites on cell surface or for transport in the cell. Increase in alkalinity on the other hand, can precipitate the metal as hydroxide, oxide or carbonates of varying solubility and toxicity. Such complexation of metal ions may lead to their unavailability for binding or uptake. The hydroxylated species may also associate more efficiently with the microbial cell than the corresponding metal cation³².

Ni^{R} mutants accumulated lesser amount of Ni when incubated in complete medium than in CDM (Fig. 5). Several components of microbial media like agar, peptone, yeast extract, casamino acids and chelators like oxalate, citrate, glutamate, cysteine, EDTA, etc. are known to complex the metal ions affecting their bioavailability³³. Such complex formation may reduce the metal toxicity in certain microbial systems^{34,35}.

Studies on kinetics of Ni uptake: The kinetic studies with 60 h old mycelium of the mutants, grown in Ni-free medium, suggested that the amount of Ni accumulated by both $\text{Ni}^{\text{R}}\text{I}$ and $\text{Ni}^{\text{R}}\text{II}$ strains varied with the Ni concentration in the medium to a certain extent. There was a significant increase in Ni uptake in medium having concentration up to 1mM Ni during the first 3 h. Beyond this time, there was no appreciable enhancement in the metal uptake. The initial rapid Ni uptake was followed by more gradual uptake, which continued to saturation by 24 h. Further enhancement in the exposure time or the external concentration of the metal influenced the rate of uptake to a limited extent only. In these

short term Ni uptake assays, higher efficiency of Ni^RII over Ni^RI mutant was again established.

A Lineweaver-Burk plot of the medium concentration of Ni (1/S) versus the rate of uptake (1/V) followed the Michaelis-Menten kinetics in both the mutants. The K_m and V_{max} values for Ni^RI were 0.355mM and 0.098 mmoles⁻¹ mg DW⁻¹h, respectively and for Ni^RII mutant, 1.067 mM and 0.345 mmoles⁻¹ mg DW⁻¹h, respectively. A low half saturation constant (K_m) for the uptake of Ni in Ni^RI mutant indicated a higher affinity of the strain for the metal (Fig. 6). However, Ni^RII mutant showed significantly higher V_{max} value suggesting that the mutant could efficiently accumulate metal. High affinity transport system for es-

Fig. 6 Line weaver-Burk graph for uptake of Ni. ○ = Ni^RI, ● = Ni^RII.

sential ions has been reported in several fungi³⁶. One of the Ni-resistant (Ni^{R2}) mutant of *N.crassa* was found to accumulate more Ni than the other Ni-resistant mutants¹⁹. Transporters with differential affinity towards the same metal, reciprocal relationship between metal concentrations in the medium and the affinity of the transport system are a common phenomenon in metal-microbe relationship^{37,38}.

Nature of resistance: The nature of resistance in Ni^R mutants was found to be constitutive. Constitutive and inducible resistance to different metal ions have been reported in several microorganisms³⁹. The constitutive system of metal resistance indicates that the microbe has an indigenous property of regulating the inflow of heavy metals from the environment. The gene expression may be induced by the exposure to the toxicant, to mediate the physiological responses. Induction may result from increased expression of existing genes or from their amplification in the gene pool of the community.

Incubation of conidia during first 8 h in medium free of Ni or having low concentrations (0.1, 0.25mM) and then at elevated concentration (1mM Ni) showed inhibited growth and fragmented colonies of Ni^RI. In case of Ni^RII, similar effect was observed on pre-exposure to 0.0 or 0.25 mM Ni, while pre-exposure to sub toxic level of Ni (0.1mM), induced the mycelial growth to almost 4 folds. (Fig. 7). In terms of metal uptake, in both Ni^RI and Ni^RII mutants, initial growth in the absence of Ni or at low concentrations of Ni stimulated the mycelia to accumulate much higher amount of metal. This probably resulted in more toxicity causing growth inhibitions (Fig. 8).

Protein analysis: The inducibility of resistance mechanism in Ni^RII mutant was further investigated by using cyclo-

Fig. 7 Growth of mutants pre-exposed to the absence of Ni and lower concentrations of Ni for first 8 h and subsequently grown in the medium containing 1mM Ni over a period of 120 h at 37°C. A : Ni^RI, B : Ni^RII; ○ = grown directly in 1mM Ni (control); pre-exposure Ni concentration: △ = 0 mM, ▲ = 0.1mM, ■ = 0.25mM. (Time 0h in the graphs denotes 8thh i.e. the end of the pre-exposure period)

Fig. 8 Ni uptake by mutants unexposed and pre-exposed to lower concentrations of Ni for 8 h and then grown in medium containing 1 mM Ni over a period of 120 h at 37°C. A : $\text{Ni}^{\text{R}}\text{I}$; B : $\text{Ni}^{\text{R}}\text{II}$; $\circ$ = grown directly in 1 mM Ni (control); pre-exposure Ni concentration: $\triangle$ = 0 mM, $\blacktriangle$ = 0.1 mM, $\blacksquare$ = 0.25 mM. (Time 0h in the graphs denotes 8thh i.e. the end of the pre-exposure period)

Fig. 9 Ni accumulation by mutants grown in medium containing Ni (1 mM) and cycloheximide at its IC_{50} value over a period of 120h. Ni uptake in medium containing only Ni is the control. MM+Ni : $\triangle$ = $\text{Ni}^{\text{R}}\text{I}$, $\square$ = $\text{Ni}^{\text{R}}\text{II}$, MM+Ni + cycloheximide: $\blacktriangle$ = $\text{Ni}^{\text{R}}\text{I}$, $\blacksquare$ = $\text{Ni}^{\text{R}}\text{II}$.

heximide, an inhibitor of protein synthesis. IC_{50} levels of cycloheximide for Ni^{S} and $\text{Ni}^{\text{R}}\text{II}$ strains were $60 \mu\text{g mL}^{-1}$ and for $\text{Ni}^{\text{R}}\text{I}$ mutant $40 \mu\text{g mL}^{-1}$. Presence of 1 mM Ni along with IC_{50} levels of cycloheximide inhibited the growth of Ni^{S} and $\text{Ni}^{\text{R}}\text{II}$ strains to a much higher level as compared to $\text{Ni}^{\text{R}}\text{I}$ strain. However, Ni accumulation by the Ni^{R} mutants was not inhibited in the presence of IC_{50} levels of cycloheximide and the accumulated metal quantities were comparable to those accumulated in inhibitor-free medium (Fig. 9).

To understand the possible involvement of protein(s) in the regulation of metal uptake, the strains were grown under appropriate conditions. The SDS PAGE analysis

Fig. 10 SDS-PAGE analysis of partially purified protein extract of Ni^{R} strains. Lanes: M - Markers, 1 - $\text{Ni}^{\text{R}}\text{II}$ mutant pre-exposed to 0.1 mM Ni and simultaneously treated with cycloheximide, 2 - $\text{Ni}^{\text{R}}\text{II}$ mutant pre-exposed to 0.1 mM Ni, 3 - $\text{Ni}^{\text{R}}\text{II}$ mutant grown directly in 1 mM Ni, 4 - $\text{Ni}^{\text{R}}\text{I}$ mutant grown directly in 1 mM Ni, 5 - Wild-type grown in the absence of Ni.

of the partially purified protein preparation from 72 h old biomass revealed that $\text{Ni}^{\text{R}}\text{II}$ mutant after induction by 0.1 mM Ni produced a protein having MW of 125 kDa (Fig. 10). This protein could not be detected in Ni^{S} or $\text{Ni}^{\text{R}}\text{I}$ strains. Under uninduced condition or cycloheximide treatment during induction, the amount of this protein was reduced to several folds. Thus, 0.1 mM Ni appears to

stimulate production of this protein in Ni^RII mutants. These results indicated some role of this high MW protein in inducing resistance against Ni in Ni^RII mutant. It could also be inferred that *de novo* protein synthesis was not required for Ni accumulation by the Ni^R mutants of *A. nidulans* and the protein synthesised by Ni^RII mutant did not regulate the entry of metal, instead employed other mechanism to detoxify the effect of Ni. Cu-resistance in *S. cerevisiae* can be induced by Cu to produce metallothionein, which may bind other metals also. Such metal stimulated, metal binding polypeptides have also been reported from *Candida glabrata* and several *Mucor* species^{5,40}. Ni-resistant strains of *S. cerevisiae* accumulated large amounts of histidine in response to intracellular Ni indicating the role of amino acid in combating Ni-toxicity⁴¹.

Thus, this study while providing a procedure to develop metal resistant accumulating fungal strain has yielded efficient Ni accumulating strains of *A. nidulans*. The information generated regarding the characteristic of the strains and the nature of the Ni-resistance can be exploited to develop bio-inoculants of this fungus for bioremediation of Ni from contaminated environment.

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