International Symposium of Research Students on Materials Science and Engineering
December 20-22, 2004, Chennai, India
Department of Metallurgical and Materials Engineering, Indian Institute of Technology Madras

CHARACTERIZATION OF COBALT NANOPARTICLE FROM A COBALT RESISTANT STRAIN OF *NEUROSPORA CRASSA*

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ABSTRACT

A cobalt-resistant strain of *Neurospora crassa* (*cor*) was found to be over 150-fold more resistant to cobalt ions than wild type. DEAE-cellulose and metal-chelate affinity chromatography of cell-free extracts separated cobalt into protein-bound and free ionic fractions. In *cor* about 73% cobalt of cell-free extracts was protein bound while the same in wild type was only 25%. Cobalt content of the protein bound fraction increased with time and cobalt concentration in the growth medium, and was not influenced by related metal ions. This cobaltoprotein (CBP) which is over produced in *cor* constitutes up to 12% of total protein extract. CBP has 70 µg cobalt/mg of protein which is non-extractable by EDTA or acid washes. The CBP has characteristic absorption peaks at 280 nm, 350 nm and shoulder at 440 nm. XRD pattern of CBP showed extremely broad reflections indicating its fine particle nature. The crystalline size of cobalt nanoparticle is 143 nm based on XRD line broadening using scherrer relationship. After annealing at 300^0C, the nanoparticle is found to be 64 nm. The properties of cobalt nanoparticle and its physiological relationship to the development of metal resistance in *N.crassa* is discussed.

1. INTRODUCTION

Currently, there is a growing need to develop environmentally benign nanoparticle synthesis processes that do not use toxic chemicals in the synthesis protocol. Understanding of biological processes on the nanoscale level is one of the strong driving force behind development of nanotechnology (Whitesides, 2003). Many organisms both unicellular and multicellular are known to produce inorganic materials either intra- or extracellularly (Simkiss, 1989; Mann, 1996). Some well-known examples of bio-organisms synthesizing inorganic materials include magnetotactic bacteria synthesize magnetite nanoparticles (Lovley, 1987), diatoms produce siliceous materials (Oliver, 1995) and S-layer bacteria produce gypsum and calcium carbonate layers (Pum, 1999). Silver resistance in *Pseudomanas stutzeri* AG259 was shown to be due to the accumulation of silver based single crystals of 200 nm in size with well defined compositions and shapes (Klaus et al, 1999). These types of secrets gleaned from nature have led to the development of biomimetic approaches for the growth of advanced nanomaterials.

Metal-resistance is the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species concerned (Gadd, 1993). Metal toxicities were first investigated in *N.crassa* by Healy et al (1955). On exposure to metal ions, fungi are known to elaborate a variety of proteins, which sequester metal ions. These include metallothioneins (MTs) and other high molecular weight proteins. MT' s are involved mainly in metal ion detoxification, storage of metal ions and protection of organisms against ionising radiations (Karin, 1985). Metallothioneins are ubiquitous low molecular weight, heat stable proteins (Hamer, 1986). High cysteine content and the absence of aromatic amino acids characterize MTs. In *N.crassa* a copper metallothionein (Cu-MT) has been characterized by Lerch (Lerch 1980). Cobalt-resistance strain of *N.crassa* (*cor*) was isolated and characterized in our laboratory (Sajani, 1997; Sajani, 1998). *Cor* which is 150-fold more resistant than the parent wild type on nitrate-N medium (RamaRao, 2000). In the present study, we show that the

tolerance of *N.crassa* to cobalt ions is due to efficient conversion of toxic ionic cobalt to metallic nanoparticle form.

2. EXPERIMENTAL

2.1 Strains, Media and Growth conditions

Neurospora crassa wild type (FGSC#4200) was obtained from Fungal Genetics Stock Center, Kansas University Medical Center, USA. Cobalt-resistant strain (FGSC#7290) was isolated in our laboratory by repeated sub culturing on increasing concentrations of cobalt. Both the above mentioned strains were grown in 10 ml basal medium in 50 ml conical flasks for 72 h at 28 ± 1^0C. The general growth procedures and medium composition were similar to those described in earlier studies (Venkateswarlu and Sastry, 1970; Maruthi and Sastry, 1983). Cobalt resistance was determined by I_{50} values (50% growth inhibitory concentration) for cobalt ions from graphical plots of growth versus metal ion concentration.

2.2 Purification of cobaltoprotein

Step 1. Growth and extraction: Mycelia of *N.crassa* c*or* grown for 72 h in presence of 8 mM $CoSO_4$ in nitrate-N medium were washed with distilled water and homogenized with acid-washed sand in 20 volumes (W/V) of Tris buffer (50 mM) pH 6.5. The homogenate was centrifuged at 600 g for 5 min to pellet out cell debris and sand. The supernatant was further centrifuged at 15,000 g for 20 min. The pellet was re-homogenized in 10-15 ml buffer and centrifugation was repeated. The supernatants were pooled.
Step 2. Heat treatment: The clear supernatant from step 1 was gently stirred for 10 min at 60^0C. The resulting precipitate was then removed by centrifugation for 20 min at 10,000 g.
Step 3. DEAE cellulose: The supernatant from step 2 was applied on to a DEAE cellulose column (30 ml bed volume) preequilibriated with 50 mM Tris buffer, pH 6.5. The column was washed with 90 ml of this buffer and the bound proteins were eluted with a linear gradient of NaCl (0-1 M) in the same buffer. Fractions (3 ml) were collected and cobalt-containing fractions were distinguished by Atomic Absorotion Spectrophotometer and pooled.
Step 4. Sephadex G-50: The cobalt containing fraction from above step was dialyzed and lyophilized to concentrate (20-30 fold), and applied to a column of sephadex G-50 (1× 90 cm) preequilibriated with 50 mM Tris-HCl, pH 6.5 containing 0.1 M NaCl. The column was developed at a flow rate of 30 ml h^{-1} and fractions (2 ml) were collected and monitored at 280 nm. The cobalt-containing peak fractions were pooled, dialyzed and lyophilized. Metal content of mycelia and cell free extracts was determined following wet acid digestion (Venkateswarlu and Sastry, 1970) by atomic absorption spectrophotometry (AAS Perkin Elmer 2380). Cobalt content was determined by atomic absorption spectrophotometry (AAS Perkin Elmer 2380).

Absorption spectra of cobaltoprotein were recorded on Beckmann DU-6 spectrophotometer. The cobaltoprotein was further characterized by powder X-ray diffraction (Philips, PW 1710) using Cu-Kα radiation before and after annealing (300^0C) in the muffle furnace.

3. RESULTS

3.1 Cobalt toxicity

Cobalt toxicity in nitrate-N medium under stationary conditions was examined comparatively in wild and cobalt resistant strain (*cor*) of *N.crassa.*The results of Fig 1 and 2 indicate that *cor* is 150-fold more resistant when compared to wild type. The 50% growth inhibitory concentration of cobalt is 0.1 mM for wild and 15 mM for *cor* strains respectively.

3.2 Fractionation of CBP in nitrate N-medium

DEAE-cellulose chromatography of cell free extracts of the wild type and the *cor* strain grown at their respective I_{50} cobalt concentrations (0.1 mM and 15 mM). In wild type most of the cobalt (75%) is in the flow through fraction and the rest in the DEAE-cellulose bound protein fraction (table 1). In case of *cor* most of the cobalt (73%) was located in the protein bound fraction bound to DEAE cellulose and little (27%) in flow through fraction. Fractionation of metal chelate affinity matrix showed 75% of cobalt in wild type is in ionic form (bound to column), while only <25% was observed in *cor* strain.

3.3 Absorption spectrum of cobaltoprotein

Cobaltoprotein is brown coloured with characteristic absorption peaks at 275 nm and 350 nm and a shoulder at 440 nm as shown in Fig. 3.

3.4 XRD studies

Powder XRD pattern of CBP as in Fig 4 showed broad reflections indicating fine particle nature of the material obtained. The crystallite size was calculated from the XRD line broadening Scherrer relationship, $d=0.9\ \lambda\ /\beta\ \cos\ \theta$, where d is the diameter in A^0, β is the full width half maxima (FWHM) and λ is the wavelength of X-rays. The crystallite as calculated size was found to be 143 nm. The XRD pattern of annealed sample in fig 5 indicates extremely broad reflection. The crystallite size was calculated from the XRD line broadening Scherrer relationship, was found to be 64 nm of the annealed CBP. The cubic lattice parameter was calculated to be a=8.36 A^0.

4. DISCUSSION

The ability of microorganisms to grow in the presence of high metal concentrations might result from specific mechanisms of resistance amplified by the organisms. Such mechanisms include: efflux systems; alteration of solubility and toxicity by changes in the redox state of the metal ions; extracellular complexation or precipitation of metals and the lack of specific metal transport systems (Beveridge et al., 1997). Here, we describe the biological synthesis of cobalt-based nanoparticle of 143 nm bound firmly to cobaltoprotein. This forms the basis for resistance mechanism in *N.crassa,* wherein the toxic ionic form of cobalt is converted to relatively non-toxic metallic form.

The cobalt-resistant strain of *N.crassa* (*cor*) was found to be 150-fold more resistant to cobalt ions than wild type. This high cobalt resistance observed in *cor* is due to overexpression of cobalt binding protein that binds to cobalt ions and thereby helps in detoxification. It is a well-known fact that metal ions exert toxicity more in ionic form than when complexed (Gadd and Griffith, 1978). Fractionation of cell-free extracts by DEAE-cellulose separated cobalt into protein-bound and ionic fractions. Most of the cobalt of *N.crassa cor* was located in the protein-bound fraction, in sharp contrast to that of parental wild type. Related metal ions (Fe, Zn, Cu and Ni) did not induce this protein nor were they bound to this protein. Hence, cobaltoprotein is a cobalt specifc protein, overexpressed only in the *cor* strain, where it accounts for about 12% of the total protein; this clearly points to a detoxification function. Compositional analysis(data not shown) indicated the presence of a high content of cysteine and glycine as in metallothioneins, but with an excess of acidic amino acids (glutamic and asparatic acids) making CBP a highly acidic protein. This property is in fact responsible for the binding of CBP to DEAE-cellulose even at pH 6.5. The advantageous feature of CBP is the characteristic brown colour with absorption peaks accounting for aromatic aminoacids at 275 nm unlike other metallothioneins

and chromophore at 350 nm and a shoulder at 440 nm. The most interesting feature of CBP is the broad reflections observed in the powder XRD pattern is 143 nm, which upon annealing at 300^0C stabilizes to 64 nm particle.

From the applications point of view, it would be important to harvest the metal nanoparticles formed within the fungal biomass. In *Verticillium sp*, intracellular silver and gold nano particles formed are released by ultrasound treatment (Murali, 2003). The most advantageous feature with CBP is it gets secreted out after 72 h of growth there by harvesting becomes easier. This study opens new avenues towards the biological mechanisms of metal nanoparticles and possible applications of the novel materials.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge grant from the Department of Science and Technology (SP/S0/D55/99), New Delhi and University Grants Commission (UGC-SAP (DRS-1)), Government of India.

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TABLES

Table 1: Fractionation of CBP in nitrate-N medium

Fraction	Wild-Co^{2+} (µg)	*Cor*-Co^{2+} (µg)
Total soluble	138 (100 %)	1490 (100%)
DEAE cellulose bound	34 (25%)	1040 (70%
Free cobalt	102 (75%)	450 (30%)

Both wild type and c*or* strains were grown for 72 h in nitrate-N medium at their I_{50}. Mycelia were harvested, homogenized and the soluble fraction (20 mg protein) was passed through DEAE cellulose column. The DEAE bound protein fraction was eluted with 0.3 M NaCl in Tris buffer. Cobalt content of soluble fraction, DEAE cellulose bound protein fraction and flowthrough fractions were estimated. Data shown is average value of three experiments with duplicates.

FIGURES

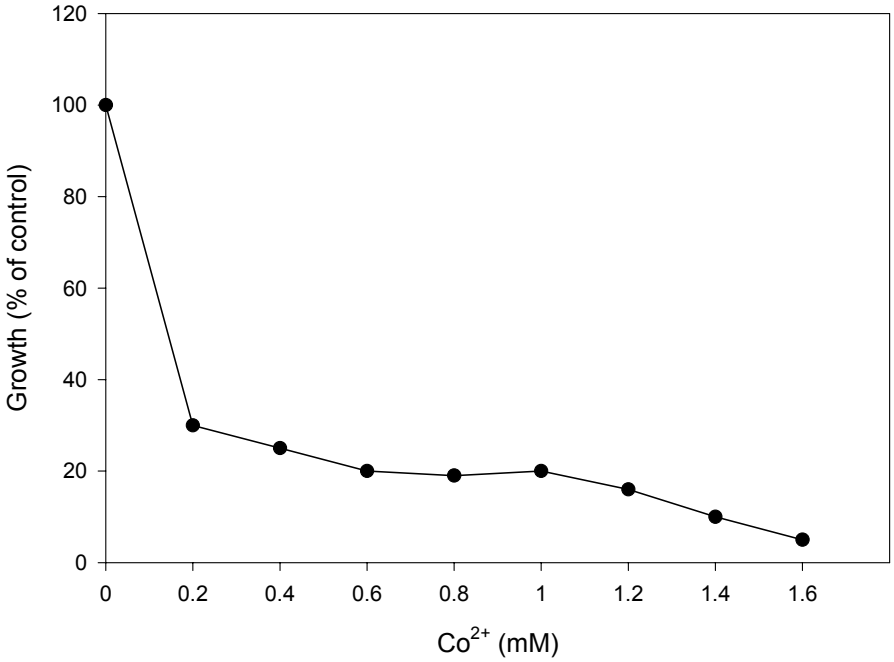

Fig. 1 Cobalt toxicity in *N.crassa* (wild): *N.crassa* was grown in 20 ml nitrate-N medium in 50 ml conical flasks at 28±1^{0}C. Cobalt was included to the required concentrations as indicated. After incubation the mycelia were washed, dried and mycelial weights of controls are taken as 100% (mg dry wt).

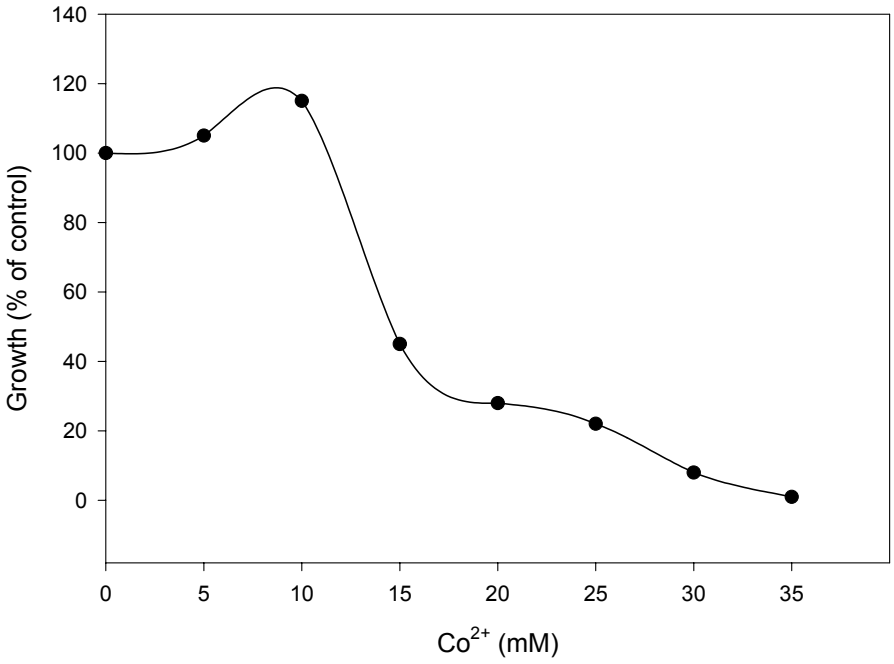

Figure 2 Cobalt toxicity in *N.crassa* (*cor*)**:** *N.crassa* was grown in 20 ml nitrate-N medium in 50 ml conical flasks at 28±1^{0}C. Cobalt was included to the required concentrations as indicated. After incubation the mycelia were washed, dried and mycelial weights of controls are taken as 100% (mg dry wt).

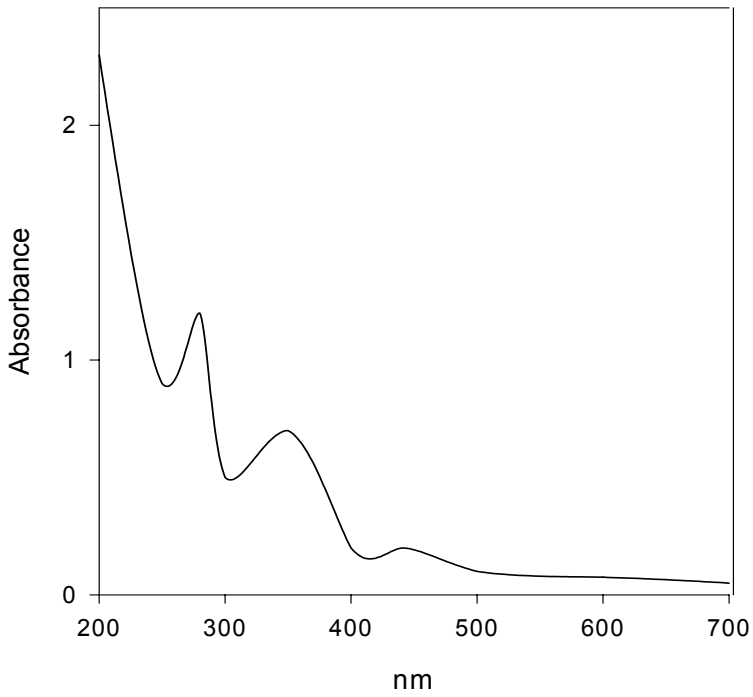

Figure 3 Absorption spectrum of cobaltoprotein: Cell free extracts from *cor* mycelia grown on nitrate-N media were loaded on DEAE cellulose column and cobalt binding protein was eluted with 0.3 M NaCl in Tris buffer (50 mM). The spectral features of this DEAE bound fraction was recorded on Beckman DU6 spectrophotometer.

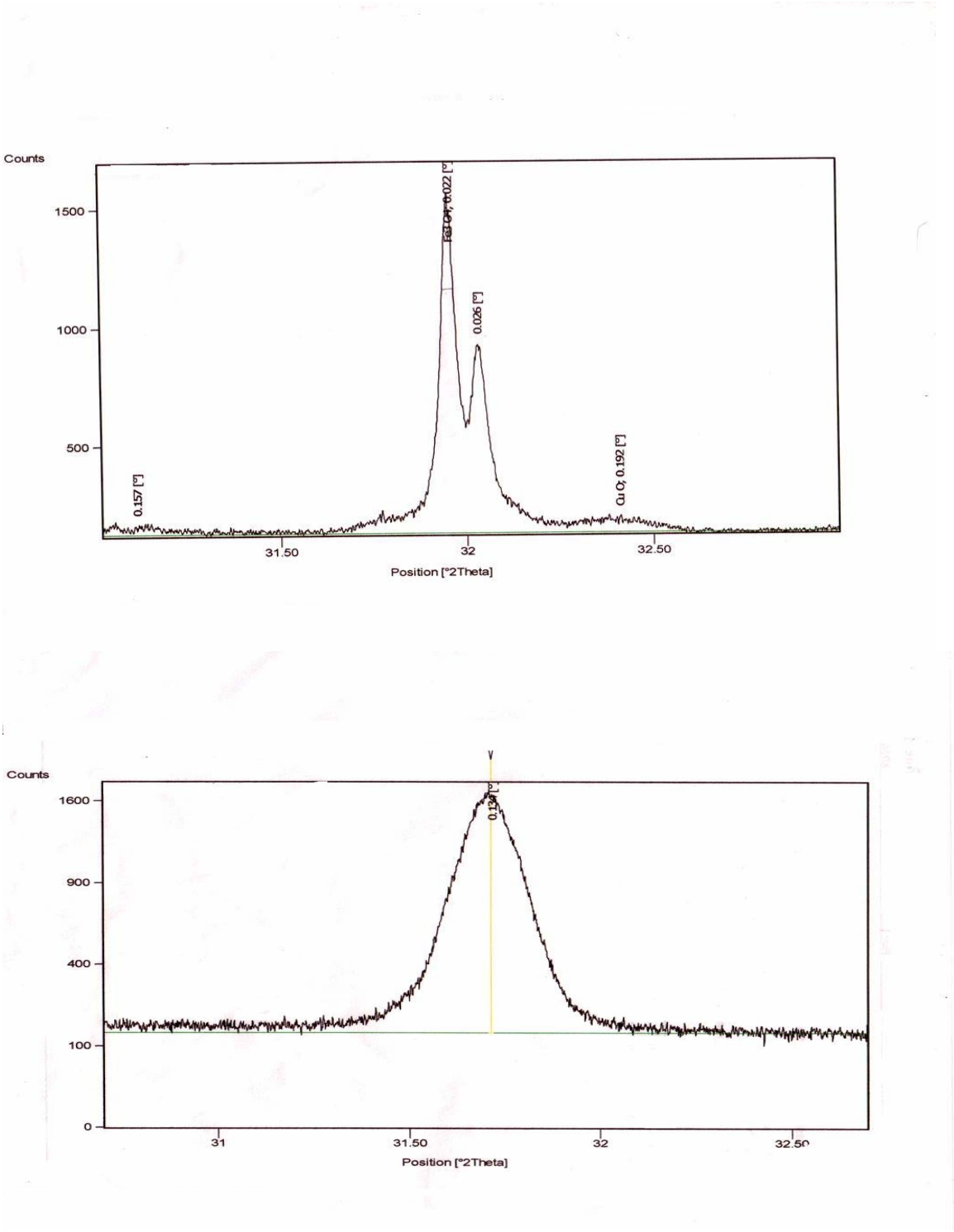

Figure 4 (a and b): XRD pattern of CBP before and after annealing.