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Bioaccumulation of copper by *Trichoderma viride*

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Abstract

Studies were carried out on interaction of *Trichoderma viride* with copper and reports bioaccumulation as a mechanism of copper tolerance during growth. There was a marked increase in the lag phase of the growth, which was concentration dependent. At a concentration of 100 mg/L of $CuCl_2 \cdot 2H_2O$, 81% of Cu(II) were removed by 3.4 g/L of the biomass in 72 h. The process was temperature and pH dependent. The maximum copper bioaccumulation occurred at 30 °C, pH 5.0. Metabolic inhibitors such as sodium azide (NaN_3) and 2,4-dinitrophenol (2,4-DNP) drastically reduced the extent of Cu(II) bioaccumulation. Electron microscopy and cell fractionation studies revealed that 70–80% of copper was present as a layer on the cell wall surface.

Keywords: Bioaccumulation; Heavy metal; Copper; *Trichoderma viride*

1. Introduction

Rapid urbanization, industrialization and technological innovations in various walks of life have lead to the problem of environmental pollution. The introduction of metal pollutants in various forms in the environment can pose a severe threat to the ecological system due to their negative impact on most life forms (Jaiswal and Malik, 2000; Gavrilesea, 2004). Although, some amount of heavy metals are required by all life forms, however, there is a threshold limit to this requirement (Cervantes and Corona, 1994). At high concentrations, heavy metal ions react to form toxic compounds in cells (Nies, 1999; Choudhury and Srivastava, 2001; Spain, 2003). Another major problem with metals is their persistence as they tend to persist indefinitely in the food chain (Gupta et al., 2000; Aleem et al., 2003).

The conventional treatment procedures used for removal of metals are uneconomical (Volesky, 1987; Say et al., 2001). Therefore, there is a need to develop rapid, economical and environmentally benign technology for the removal of metals from industrial effluents. There are certain microorganisms, which can survive in high concentrations of metals and have the potential to accumulate different metals. This is achieved by the virtue of covalent interaction of metal at cell surface or within the cell by different processes (Gadd and White, 1993; Bhanoori and Venkateswerlu, 2000). These microbes can be of immense significance in the clean up of heavy metals from the environment. In this regard, fungi are a versatile group as these can adapt and grow under various extreme conditions of pH, temperature and nutrient availability as well as high metal concentrations.

Copper is a ubiquitous metal present in the environment and is the most common contaminant of industrial effluents such as those produced by mining and metal processing (Aksu and Donmez, 2000; Savvaidis et al., 2003). It is an essential micronutrient for most living organisms since it is an important constituent of many

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metalloenzymes and proteins (Lontie, 1984). However, at high concentrations in its free-ionic form, copper is toxic to microbial cell (Domek, 1984).

Compared to other metals, copper sequestration by *Trichoderma* spp. remains little explored (Kredics et al., 2003). Therefore, the present investigation was undertaken to study the interaction of *Trichoderma viride* with copper. Here, we report copper accumulation on the surface of the cell wall of *T. viride*, as a mechanism of copper tolerance.

2. Methods

2.1. Growth and maintenance of the organism

T. viride was isolated from the soil collected from a metal polluted site in New Delhi, India. For experimentation, the organism was grown in Modified Czapex Dox Minimal Medium (Saxena and Sinha, 1973) at pH 5.0.

2.2. Bioaccumulation of copper on agar media and in broth

Appropriate amount of stock solution of copper was individually added to the agar medium to get the final concentration of 1000, 2000, 3000, 4000 and 5000 mg/L. The plates were point inoculated with *T. viride* and incubated for 168 h at 30 °C. The radius of the colony (mm) was measured against the control (medium without metal).

For experimentation in broth, appropriate amount of metal solution was added to get the desired metal concentration (0–500 mg/L). Flasks were inoculated with 5×10^7 conidia per 50 mL of the medium and flasks were incubated at 30 °C for 72 h at 200 rpm in an incubator shaker (New Brunswick, Edison, NJ, USA).

2.2.1. Preparation of biomass

After the desired incubation period, the growth was estimated as dry weight (g/L of the medium). Mycelia were washed thoroughly with de-ionized distilled water and dried in predried and preweighed Whatman filter paper no. 1 at 80 °C till a constant weight was achieved.

2.2.2. Estimation of copper in the biomass and culture filtrate

Estimation of copper in the biomass was carried out according to the procedure described by us (Ahuja et al., 2001). The residual metal concentration in the culture filtrate after the growth was also estimated. The percentage removal of the copper was calculated on the basis of total copper available in the culture filtrate before and after the growth. In all the subsequent experiments this procedure was followed with appropriate controls.

2.3. Growth of T. viride at different concentrations of copper

Cultures were raised for seven days at 200 rpm and 30 ± 1 °C in an incubator shaker in the flask containing different concentrations of copper (0–300 mg/L). The biomass was harvested at regular intervals of 24 h. The growth, copper accumulated and percentage of copper removed was estimated.

2.4. Effect of temperature and pH on copper accumulation

Accumulation of copper in a temperature range of 20–40 °C and in the pH range of 2–5 was examined for desired incubation period.

2.5. Effect of metabolic inhibitors on growth and Cu(II) accumulation

Effect of metabolic inhibitors like sodium azide (NaN_3) and 2,4-dinitrophenol (2,4-DNP) was examined to determine the metabolic nature of copper accumulation. The growth of the organism and copper accumulation was analyzed in the medium containing different concentrations of metabolic inhibitors, i.e., NaN_3 (0.01, 0.03, 0.09 mM) or 2,4-DNP (0.05, 0.10, 0.15 mM) after determining their respective inhibitory doses.

2.6. Localization of Cu(II) in T. viride

2.6.1. Electron microscope studies

Electron microscope studies were carried out according to the procedure described by us (Ahuja et al., 2001).

2.6.2. Estimation of copper removed by ethylenediamine tetraacetic acid (EDTA)

The biomass (0.10 g) was kept in 1 mL of 10 mM ethylenediamine tetraacetic acid (EDTA) for 30 min. It was then washed twice with distilled water, dried and acid digested to determine residual copper. The biomass, which was grown in the absence of copper, was also treated in a similar way which served as control. The percentage of copper desorbed by EDTA was calculated as

$$\%\text{Cu(II) removed by EDTA} = \frac{\text{Total Cu(II) accumulated} - \text{Cu(II) not removed by EDTA} \times 100}{\text{Total Cu(II) accumulated}}.$$

2.6.3. Cell fraction analysis

T. viride grown in 100 mg/L of $CuCl_2 \cdot 2H_2O$ for 72 h was harvested and homogenized. 0.50 mL of 1% sodium dodecyl sulphate (SDS) was added to 4.50 mL of homogenate. The suspension was kept for 16 h and was centrifuged (Remi RC30) at 8000*g* for 20 min at