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Adsorption and Absorption of Cu in *Trichoderma atroviride*

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ABSTRACT

Conventional methods for removing heavy metals from polluted waters, using chemical precipitation, sludge separation, chemical oxidation or reduction, and ion exchange, have been uneconomical and are weak processes. An alternative technique is the use of fungi as bioremediating agents. A strain of *Trichoderma atroviride*, isolated from a river passing through the metal polluted Serdang industrial area, was studied for its uptake and tolerance to Cu. This study found that the uptake capacity of *T. atroviride* for Cu ranged from 0.77 to 11.20 mg/g in Potato Dextrose Broth in liquid media over the Cu concentration range of 25 to 300 mg/L. The isolate showed 50.3 to 85.4% adsorption and 9.6 to 47.1% absorption. These adsorption and absorption values are comparable to any good bioremediators for Cu found in the literature. This study suggests that *T. atroviride* is a potential bioremediator of Cu. However, further studies are still needed to confirm its practical use as a bioremediating agent under field conditions.

Keywords: *Trichoderma atroviride,* fungus bioremediation, adsorption and absorption of Cu

ABBREVIATIONS

C	:	Centigrade
Cu	:	Copper
DDW	:	Double distilled water
EDTA	:	Ethylenediamine tetraacetic acid
g	:	Gram
mg	:	Milligram
µg/g	:	Microgram per gram
mg/l	:	Milligram per litre
PDB	:	Potato Dextrose Broth
RBA	:	Rose Bengal Agar

INTRODUCTION

Rapid industrial development has led to an increased discharge of industrial effluents, which usually contain heavy metals in concentrations well beyond the permissible limits, into the environment (Ahuja *et al*., 2001). Cu 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 (Anand,

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2006) or those used in vineyards, ranging from the application of fertilizers, dumping of agricultural, and municipal wastes. Cu is also an element essential for all living organisms as a co-factor for a variety of enzymes; however, an excess of this element can be mutagenic and can cause the appearance of highly reactive oxygen radicals (Zapotoczny *et al.,* 2006). An excess of Cu can disrupt the ecological status of biota, whereas the occurrences of heavy-metal resistant micro-organisms in the soil and water of industrial regions have been reported (Aleem, 2003). In order to survive in such an adverse stressful situation, the micro-organisms develop mechanisms which confer upon them resistance against Cu by accumulating high amounts of heavy metals, either intracellulary or extracellulary or by a combination of both mechanisms (Ahuja *et al*., 2001).

Fungi are a versatile group as they can adapt and grow under various extreme conditions of pH, temperature and nutrient availability, as well as high metal concentrations (Anand, 2006); recently, they are considered as the best bioremediators for polluted waters purification (Savvaidis, 1998) in comparison to conventional methods for removing dissolved heavy metals from aqueous solution, such as by chemical precipitation and sludge separation, chemical oxidation or reduction, ion exchange, electrochemical treatment, membrane technologies, reverse osmosis, filtration, adsorption on activated carbon, and evaporative recovery (Lopez and Vazquez, 2003).

Trichoderma atroviride (family: Hypocreaceae) is a potential bioremediator in this area of research with regard to its frequent presence in high polluted areas, and for which just a few investigations have been carried out on. On the other hands, there has been no report of bioremediation by this particular isolate in this region. Thus, the objective of this study was to determine the adsorption and absorption of Cu by *T. atroviride* under laboratory conditions.

MATERIALS AND METHODS

Metal Analytical Procedure in the Sediment Samples

The top 3-5 cm of surface sediments were collected from metal polluted site at Kuyoh River Industrial Area and were placed in an acid-wash polyethylene bag and frozen (-10 °C) prior to analysis. They were later dried at 105 °C for at least 16 h until a constant dry weight was obtained (Tanner *et al.,* 2000). The samples were then passed through a stainless steel sieve of 63 µm size and vigorously shaken to produce homogeneity. The dried samples were then weighed and digested in a combination of concentrated nitric acid (Anala R grade, BDH 69 %) and perchloric acid (Anala R grade, BDH 60 %) in the ratio of 4:1; first at a low temperature of 40 °C for 1 hour and then at 140 °C for at least 3 hours (Yap *et al*., 2002). The digested samples were then diluted to 40 ml with double distilled water (DDW) and filtered through Whatman No. 1 filter paper into acid-washed polyethylene bottles, where they were stored until ready for Cu determination using a flame Atomic Absorption Spectrophotometer (AAS) (Perkin-Elmer Model AAnalyst 800).

T. Atroviride *Isolation*

For isolation, the collected sediments were mixed with a little double distilled water (DDW) in sampling polyethylene bottles (50 ml) and kept in a refrigerator at 7 ºC in the Mycology laboratory. The dilution was then carried out based on Lopez and Vazquez (2003). Rose Bengal Agar (RBA) was used for the direct isolation of fungal colonies from the sediment solution, where they were counted as Colony Forms Unite (CFU), while Potato Dextrose Agar (PDA) was used for sub-culturing and storage media.

Estimation of Cu in the Culture Filtrate

Estimation of Cu in the biomass was performed according to the procedure described by Ahuja *et al*. (2001). The concentrations of Cu in the

liquid cultures were measured with a flame AAS before inoculation by the fungus. After the growth, the residual Cu concentration in the liquid cultures filtrate was also estimated. Metal uptake was estimated as the amount of Cu (mg) per unit of mycelium dry weight (g) (Lopez and Vazquez, 2003).

$$Q = \frac{C_i - C_f}{M} \times V$$

Where Q= Cu uptake (mg metal/mg biomass), Ci= initial Cu concentration (mg/l), Cf= final Cu concentration (mg/L), M= quantity of dry biomass (mg), V= suspension volume (ml).

Localization of Cu in T. Atroviride

The amount of Cu associated with the cell biomass was fractionated as the ethylenediamine tetraacetic acid (EDTA) washable fraction was present on the cell surface and the EDTA non-washable fraction was also present intracellularly. The biomasses were washed first with DDW and next with 10 ml of 0.5 mM EDTA for 30 minutes. It was then centrifuged, while the supernatant was collected and the pellet was dried and used for the Cu estimation through wet digestion. Based on this method (wet digestion), the entire dried and weighed biomass from each conical flask was taken in a digestion tube. The biomass was digested in 3 ml of acid mixture of nitric acid (AnalaR grade, BDH 69%) and perchloric acid (AnalaR grade, BDH 60%) in the ratio of 6:1 at 100 °C for 1 hour. These samples were subsequently made up to 5 ml with DDW. Digested samples were diluted with DDW to a certain volume for Cu determination using a flame AAS (Ahuja, 2001).

RESULTS AND DISCUSSION

Cu Concentrations in the Surface Sediment of Sg. Kuyoh River

Based on the findings by Yap *et al*. (2002), there are advantages in the use of sediment samples to assess human impacts on the aquatic environment. Firstly, sediment plays a main role in the transport of metals. Secondly, it is frequently used to identify sources of pollution spatially and temporally. Thirdly, sediment can be used to locate the major sink for heavy metals and these elements are persistent in the marine environment.

The mean of total Cu concentration, based on the aqua-regia method in the sediment of Kuyoh River Industrial Area (the micro-organism's habitat), was 347.64 µg/g.

Growth of T. Atroviride *in Presence of Cu in Liquid Medium*

Results showed that the increase in the concentrations of Cu (i.e. from 0 to 300 mg/L) decreased the levels of fungal biomass (*Fig. 1*). There is an almost constant note of decrease of biomass between 0 and 200 mg/L concentrations of Cu, but there is a remarkable decrease of fungal biomass at 300 mg/L. Lopez and Vazquez (2003) reported a similar finding for *T. atroviride*, except that the dramatic decrease was observed at 350 mg/L and a complete absence of growth at 400 mg/L, as compared to this study which still observed a little biomass at 400 mg/L. This could be due to the different media used. PDB was used as the liquid medium in the present study, while Lopez and Vazquez (2003) used Sabouraud liquid medium (Scharlau) supplemented with 1% peptone and 2% dextrose. They used Peptone, rather than other nitrogen-containing substrates, because of its comparatively low metal binding capacity. Thus, the metal availability of the liquid medium increased and the lethal concentration of their experiment was limited to 350 mg/L compared to the final result of 400 mg/L found in the present study.

Cu Uptake by T. Atroviride *in Liquid Media*

Based on the data presented in *Fig. 2*, it can be seen that by increasing the concentrations of Cu from 0 to 300 mg/L, the Cu uptake also increased, whereas the levels of biomass were found to decrease based on *Fig. 1*. Moreover, the maximum biosorption (11.20 mg/g) from the

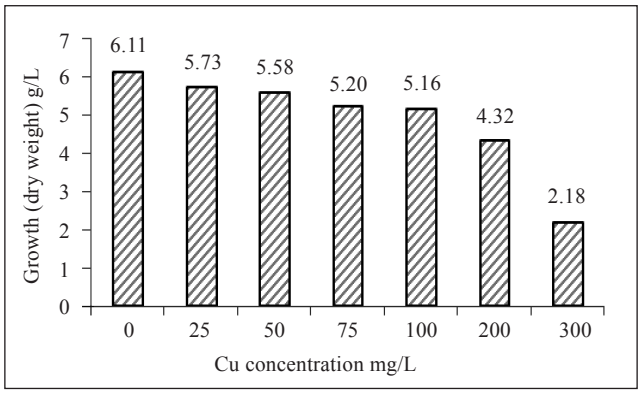

Fig. 1: Biomass weight of T. atroviride *in the presence of different Cu concentrations in PDB*

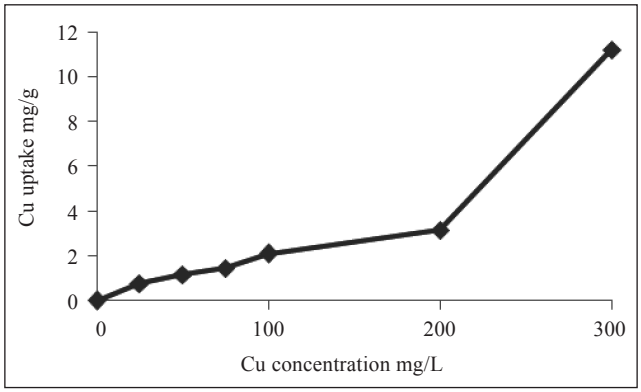

Fig. 2: Cu uptake of T. atroviride *at elevated Cu concentrations (mg/L) in liquid medium*

metal solution occurred at a concentration of 300 mg/L. This concentration is close to the Cu level in sediment of the sampling site at Kuyoh River (346.91 mg/L), while the minimum biosorption occurred at the concentration of 25 mg/L (0.77 mg/g). There are significant differences between the concentrations below and above 100 mg/L. From 0 to 100 mg/L, the uptake increased with a slow constant speed as similarly found by Wang and Chen (2006). They found that the uptake rate of the metal had increased along with increasing the initial concentration, if the amount of biomass was kept unchanged. From 100 to 300 mg/L, on the other hand, *T. atroviride* showed a sudden increase up to the maximum uptake (300 mg/L). The increase in the uptake might often be associated with toxicity and/or increased permeabilization of cell membrane on the account of further binding of the metal to exposed intracellular sites (Gadd, 1990) in relation to the decreased biomass with elevated Cu concentrations.

There are a few studies on biosorption of heavy metals by species of the genus of *Trichoderma*. Anand *et al*. (2006) reported 17 mg/L Cu removal from the liquid medium with 100 mg/L initial Cu concentration by *T. viride* at 30 ºC over a 72 hours incubation period. This finding is different from the result gathered in the present study (10.93 mg/L Cu removal by *T. atroviride* at 100 mg/L of Cu in liquid medium with 7 days incubation). Some researchers have demonstrated factors such as contact time, biomass dosage, temperature, and

pH influence biosorption of metals (Yan and Viraraghavan, 2003). Variations in the tolerance of metals for different or the same species of a genus might be due to the presence of one or more types of tolerance strategies or resistance mechanisms exhibited by different fungi (Zafar *et al.,* 2006).

Localization of Cu in T. Atroviride

EDTA could remove 50.3 to 72.9% of Cu from the cell surface of *T. atroviride,* when this metal was present in the range of 25 to 75 mg/L and 81.2 to 85.4% in the range of 100 to 300 mg/L (*Fig. 3*). The mentioned percentage of the Cu removal in *T. atroviride* indicated that most of the Cu was surface bound. However, as clearly depicted in *Fig. 3*, only 50.3% could be removed at 25 mg/L of Cu concentration. This could be due to the fact that a good amount of mycelia was formed at this concentration, and that metal was adsorbed and entrapped in the deeper layers of the hyphal network. Therefore, it was difficult to extract the metal with EDTA (Anand *et al*., 2006). Using a scanning electron microscope, Zapotoczny *et al*. (2006) also observed a gradual increase in the thickness of the cell wall due to toxicity of Cu on *Acremonium pinkertoniae.*

According to Anand *et al*. (2006), 48.15% and 80 to 85% of Cu could be removed using *T. viride* in the media of 50 mg/L and 100 to 200 mg/L, respectively. It seems that both species of the same genus show a similar response to Cu concentrations but the Cu percentage removal by *T. atroviride* demonstrated more adsorption at low concentration and less adsorption at higher concentration of Cu in comparison with *T. viride*.

Biosorption of toxic metals is based on the ionic species associating with the cell surface or extra-cellular polysaccharide, proteins and chitins (Zafar *et al.,* 2006). Different species/ strains and cell types of the same fungus vary in terms of their chemical composition of the cell wall due to variation in their uptake capacities (Mowll and Gadd, 1983).

As shown in *Fig. 3*, around 2.7 to 5% of Cu in the liquid medium was removed by washing. These values were obtained by the assumption of differences between the levels of Cu removal from the liquid medium by fungus and the addition of adsorption and absorption values obtained by the wet digestion method.

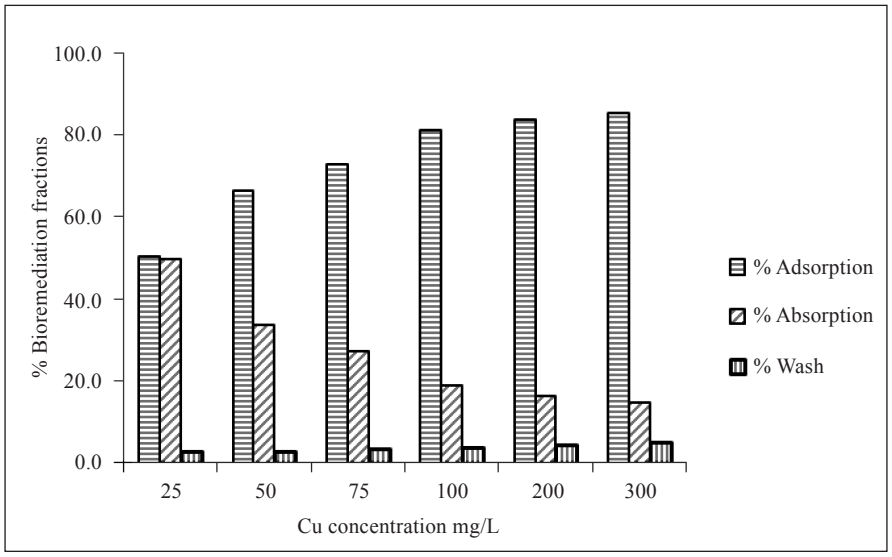

Fig. 3: Cu removal percentage by T. atroviride *from the liquid medium at elevated Cu concentrations*

Anand *et al*. (2006) reported 5% of Cu removal from mycelia form washing hyphal during the experiment, without any report of initial Cu concentration in the liquid medium, whereas the result of washing in this study showed 2.7 to 5.0% of Cu loss in the range of experiment using 25 to 300 mg/L.

This assumption seems to be more accurate than what Price *et al.* (2001) reported in their experiment. They treated the fungal mycelium with 0.1 N HCl to remove any heavy metals adsorbed into the mycelium and then all the remaining heavy metals with the biomass after the treatment was assumed to be absorbed intracellularly. However, both assumptions can not clearly present the real level of washing during the experiment. Therefore, there is a need to use more accurate methods such as cell fraction analysis in order to show the final heavy metal removal by fungi over the period of the experiment.

CONCLUSIONS

The *T. atroviride* showed the ability to bind Cu into its cell wall surface and this appeared to be the main mechanism of metal tolerance in the present study. It plays this role by showing 50.3 to 85.4% adsorption and 9.6 to 47.1% absorption. Binding of Cu onto the cell surface (or adsorption) immobilized the metal making it less available in the medium, thereby reducing its toxicity. This allowed the organism to further resume its normal growth (Anand *et al*., 2006). This study revealed 2.7 to 5.0% Cu loss in the range of 25 to 300 mg/L due to washing, based on the wet digestion method. However, further studies should be designed to elucidate the mechanisms of metal tolerance by *T. atroviride*.

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