

## **An *in vitro* Study on the Adsorption, Absorption and Uptake Capacity of Zn by the Bioremediator *Trichoderma atroviride***

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### **Abstract**

The concentrations of Zn in the sediment of a polluted river at the Serdang Industrial Area were determined. These polluted sediment samples revealed high level of Zn (219.27 µg/g). Isolation of fungi from this polluted sediment was also carried out using Rose Bengal Agar (RBA). The isolated fungi were exposed to different concentrations of Zn (0-6000 mg/L) on Potato Dextrose Agar (PDA) to find the most tolerant isolate. *Trichoderma atroviride* was found to have the highest tolerance and it was studied for growth rate, Zn uptake capacity, its tolerance to Zn and also localization of Zn by using Potato Dextrose Broth (PDB) as the liquid culture medium. In the present study the results found out that the uptake capacity of *T. atroviride* ranged from 18.1-26.7 mg/g in liquid media at Zn concentrations from 500 to 1000 mg/L. The isolate showed 47.6-64% adsorption and 30.4-45.1% absorption for Zn. Based on the present study, 5.7-7.4% of Zn removal was observed due to biomass washing. The high adsorption, relatively low absorption and high uptake capacity of Zn suggest that *T. atroviride* is a potential bioremediator of Zn. However, further studies are needed to confirm its practical use as a bioremediating agent for Zn under field conditions.

**Keywords:** *Trichoderma atroviride*; bioremediation; adsorption; absorption; Zn.

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### **1. Introduction**

Zn is known as an environmentally ubiquitous heavy metal. This metal is an essential trace element that is needed by the normal metabolism of living organisms. However, anthropogenic inputs could cause elevated Zn concentrations in the environment due to man-induced activities (Yap *et al.*, 2005). High exposure to Zn in humans can cause nephritis, anuria and extensive lesion in the kidney. It is dissolved in aquatic ecosystems and transported by water and taken up by aquatic organisms or can be stored and transported in sediments. In Malaysia, heavy metals studies including Zn in the sediment showed that elevated levels of contamination were recorded in the aquatic areas adjacent to the industrial estates (Yap *et al.*, 2005). Since Zn is persistent and non biodegradable in the aquatic ecosystem, there is a need to remove elevated Zn before it enters the complex aquatic ecosystem.

Conventional methods for removing dissolved heavy metals from aqueous solution have been studied in detail, such as 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). However, other techniques are extremely expensive, especially for treating large amounts of water and wastewater containing heavy metals in low concentrations (Wang and Chen, 2006). On the other hand these techniques may not always be feasible as their metal-binding properties are non-specific (Price *et al.*, 2001), so they cannot be used at large scale. These are the reasons why alternative processing methods, such as biosorptions (bioremediations), are now being considered more seriously (Volesky, 1994). Biosorption technology is an alternative which utilizes various natural materials of biological origins, including bacteria, fungi, yeast, algae, etc for the treatment of heavy metal contaminated wastewaters (Bayramoglu *et al.*, 2003). Particularly fungi are considered to be best alternatives for water purification (Savvaidis, 1998). Fungi are a versatile group as they can adapt and grow under various extreme conditions of pH, temperature and nutrient availability as well as at high metal concentrations (Anand *et al.*, 2006). Some microorganisms have evolved Zn-tolerant ecotypes that can survive in Zn-toxic places, presumably by adapting mechanisms that

are in the general homeostasis of Zn (Colpaert *et al.*, 2005). Therefore, it would be useful to isolate fungi growing in polluted sediment.

*Trichoderma atroviride* is a potential candidate in this area of research with due to its frequent presence in highly polluted areas as had been reported by a few investigations (Lopez and Vazquez, 2003). However, there is no report of bioremediation by this fungus in this region. Thus, the objective of this study was to determine the adsorption, absorption and uptake capacity of Zn by *T. atroviride* under *in vitro* conditions.

## 2. Materials and Methods

### 2.1. Metal analytical procedure in sediment samples

The top 3-5 cm of the surface sediment was collected at each sampling site. Each sediment sample was placed in an acid-washed polyethylene bag and frozen (-10°C) prior to analysis. The sediment samples were later dried at 105°C for at least 16 h until constant dry weights. Afterwards, the samples were passed through a 36 µm stainless steel sieve and vigorously shaken to produce homogeneity. The dried samples were then weighted 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 4:1, first at low temperature (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 and filtrated through Whatman No. 1 filter paper into acid-washed polyethylene bottles in which they were stored until Zn determination by using a flame Atomic Absorption Spectrophotometer (Perkin-Elmer AAnalyst 800).

### 2.2. Fungi isolation and culture conditions

The dilution method was carried out based on Lopez and Vazquez (Lopez and Vazquez, 2003) in order to avoid overlapping colonies. Isolation of fungi was performed on Rose Bengal Agar (1.0 g of KH<sub>2</sub>PO<sub>4</sub> +0.5 g of MgSO<sub>4</sub>.7H<sub>2</sub>O+5.0 g of peptone+10.0 g of dextrose per 1 liter of sterile double distilled water) in 1 liter conical flasks. Then, PDA (Potato Dextrose Agar) was used for subculturing. Afterwards, the petri dishes were observed for seven days for the emergence of fungal colonies.

### 2.3. Isolation of the most tolerant fungus on solid medium

PDA was used as a solid medium for fungal isolation despite the fact that agar may affect metal

speciation and solubility, because a solid medium was more appropriate for growth observations of all fungi. Stock solutions of the metal salt were prepared in double distilled water and were added to PDA media at various concentrations. The medium was inoculated with one disk of young mycelium from the edge of the stock solid PDA culture which was cut using a sterile cork borer of 0.5 cm in diameter (Zapotoczny *et al.*, 2006).

### 2.4. Toxicity testing of selected species on liquid medium

PDB as a liquid medium was prepared with filtered river water obtained by using ultra filtration (micro pore filter paper 0.45 µm). An appropriate amount of a stock solution of Zn was added to the liquid medium (vol/vol) of each conical flask (250 ml) to reach the desired nominal concentration and volume of 100 ml. The medium was inoculated with three disks of young mycelium from the edge of the solid stock PDA culture which was cut using a sterile cork borer, each 0.5 cm in diameter (Zapotoczny *et al.*, 2006).

### 2.5. Determination of growth rate

The cultures were raised for seven days at 200 rpm and 27±1°C (the fungus habitat temperature) in darkness on an incubator shaker in conical flasks (100 ml) containing different concentrations of Zn. After the desired incubation period (seven days), the growth was determined as dry weight (g/l of the medium). The biomass was washed thoroughly with DDW and dried on a predried and preweighed filter paper Whatman No. 1 at 80°C till a constant weight was achieved (Anand *et al.*, 2006).

### 2.6. Estimation of Zn in the culture filtrate

Estimation of Zn in the biomass was performed according to the procedure described by Ahuja *et al.* (2001). The concentrations of Zn in the liquid cultures were measured with an atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800) before inoculation with fungus. The residual Zn concentrations in the liquid culture filtrates after fungus growth were also estimated. Zn uptake was estimated as the amount of Zn (mg) per unit of mycelium dry weight (g) (Lopez and Vazquez, 2003).

$$Q = [(C_i - C_f) / m] v$$

Where Q= Zn uptake (mg metal/mg biomass), C<sub>i</sub>= initial Zn concentration (mg/L), C<sub>f</sub>= final Zn concentration (mg/L), m= quantity of dry biomass (mg), v= suspension volume (ml).

Table 1. *In vitro* response of *T. atroviride* at elevated Zn concentrations in liquid medium of PDB.

Zn nominal concentration in liquid medium (mg/L)	Zn measured concentration in liquid medium (mg/L)	Growth (dry weight) g/L of the medium	Zn uptake (mg Zn/g dry biomass)
500	507.73	3.14	18.1
600	585.80	2.96	18.4
700	676.76	1.99	21.9
800	791.93	1.73	22.7
900	903.41	1.60	23.0
1000	1016.37	1.14	26.7

### 2.7. Localization of Zn in selected species

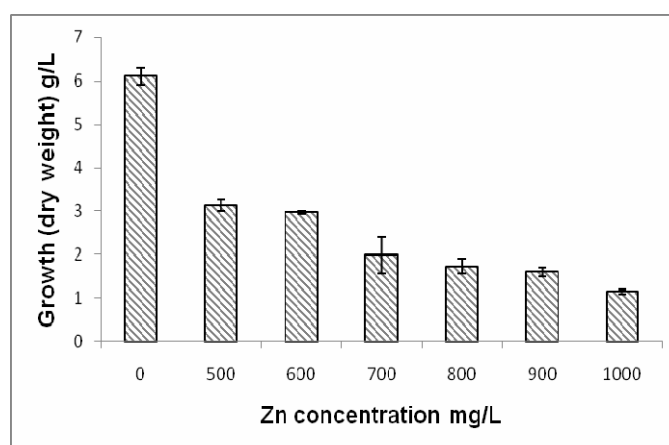
The amount of Zn associated with the cell biomass was fractionated as the ethylenediamine tetraacetic acid (EDTA) washable fraction present on the cell surface and the EDTA non-washable fraction present intracellularly. Each biomass was washed first with DDW and next with 10 ml of 0.5 mM EDTA for 30 minutes. Afterwards, it was centrifuged, and the supernatant was collected and the pellet dried and used for Zn estimation by wet digestion method. In this method (wet digestion) the entire dried and weighed biomass from each conical flask was placed into a digestion tubes. The biomass was digested in 3 ml of an acidic 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. The digested samples were diluted with DDW to a certain volume to be able to be read by the AAS machine, and the amount of Zn was determined with an atomic absorption spectrophotometer (Perkin-Elmer AAnalyst 800) (Ahuja *et al.*, 2001).

### 3. Results

Among thirty isolated fungi from the sediment samples, only one isolate, later identified by Centraalbureau voor Schimmelcultures (CBS), as *T. atroviride*, was found to be the only species which was able to grow at 6000 mg/L of Zn concentration on PDA. Already at 1000-6000 mg/L of Zn on agar medium, milky mycelia were clearly visible which was indicative of the presence of Zn.

*T. atroviride* was isolated from the Zn polluted sediment of the Kuyoh River at the Serdang Industrial Area. A high Zn concentration was found (219.75 µg/g) in the habitat of this fungus. *In vitro* assays on *T. atroviride* grown in the liquid media confirmed a high tolerance to Zn. In the present study as is shown in Table 1, we observed that *T. atroviride* survived at high concentrations of Zn.

As is shown in Fig. 1, with increasing concentrations of Zn from 0 to 1000 mg/L the level of biomass decreased. A remarkable decreasing of biomass was observed for media between 600 to 700 mg/L and also in media between 900 to 1000 mg/L of

Figure 1. Biomass weight of *T. atroviride* in the presence of different Zn concentrations in liquid medium of PDB.

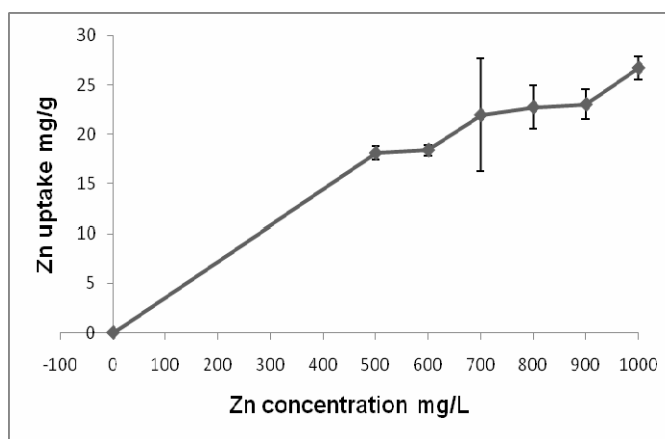


Figure 2. Zn uptake of *T. atroviride* at elevated Zn concentrations in liquid medium.

Zn concentrations. Milky colored mycelia were clearly visible at all concentrations of Zn.

The concentrations of Zn uptake are given in Fig. 2. With increasing concentrations of Zn from 500 to 1000 mg/L, the Zn uptake increased. Furthermore, the maximum and minimum biosorption from metal solution occurred at initial concentrations of 1000 mg/L (26.7 mg/g) and 500 mg/L (18.1 mg/g), respectively. Noticeable decrease in the biomass could be seen at Zn concentrations between 600 to 700 mg/L and also at 900 to 1000 mg/L of the media used in this study.

In the study on the localization of Zn in *T. atroviride*, 47 to 64% of the Zn was removed by EDTA from the cell surface (adsorption) of *T. atroviride* when Zn was in the range of 500 to 1000 mg/L (Fig. 3). In this study the percentage of Zn adsorption decreased with increasing Zn concentrations from 500 to 1000 mg/L. On the other hand, 30.4 to 45.1% of Zn was accumulated (absorption) by *T. atroviride* in the range of 500 to 1000 mg/L. The higher percentages of adsorption than those at absorption indicated that the *T. atroviride* is a potential bioremediator of Zn.

Based on present study there are differences between the levels of Zn removal from liquid medium by fungus and the addition of adsorption and absorption values obtained by the wet digestion method. In our study, the above differences were assumed to be due to washing during the experiment. Anand *et al.* (2006) reported 5% Cu removal from mycelia because of washing the hyphal during the experiment without any report of the initial concentration of Cu in the liquid medium while the result of washing in this study showed 5.7 to 7.4% Zn loss in the range of 500 to 1000 mg/L of Zn in the media.

#### 4. Discussion

In this study, *T. atroviride* was found to be the only species able to grow at 6000 mg/L of Zn concentration in PDA while Colpaert *et al.* (1992) reported 1000 mg/L of Zn for six strains of an ectomycorrhizal fungi on agar plate and they found that *Suillus bovinus* P2 is the only species be able to survive at maximum 1000 mg/L of Zn<sup>2+</sup>. Anand *et al.* (2006) and Zapotoczny *et al.* (2006) reported 5000 and

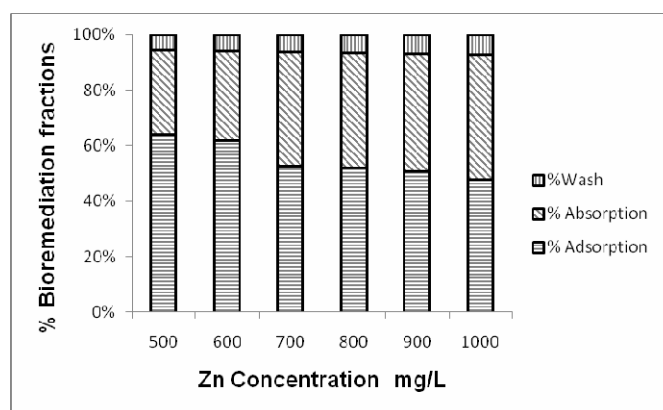


Figure 3. Zn removal percentages by *T. atroviride* from liquid medium at elevated Zn concentrations.

600 mg/L of Cu on agar medium and malt agar for *T. viride* and *Acremonium pinkertoniae*, respectively. The different reported ranges of heavy metal tolerance on solid medium may be caused by metal binding property of various agar medium or diverse ability of different fungi. Lopez and Vazquez (Lopez and Vazquez, 2003) reported that when the mycelia were transferred to medium without dextrose, *T. atroviride* was capable of uptaking more metal ions than in the presence of dextrose.

Several authors have reported the formation of colorful mycelia in the presence of heavy metals on agar media. For examples, Subramanyam and Gupta (1986), Venkateswerlu et al. (1989), Anand et al. (2006) and Zapotoczny et al. (2006) reported blue colored mycelia for Cu on solid medium in *Neurospora crassa*, *Cunninghamella blackesleeana*, *Acremonium pinkertoniae* and *Trichoderma viride*, respectively. They suggested that the blue color of the mycelia was due to the binding of Cu to the fungal cell wall protein of the mycelium. The present results of the *in vitro* assays on *T. atroviride* in liquid media of Zn are supported by a study reported by Lopez and Vazquez (Lopez and Vazquez, 2003). They found that *T. atroviride* was capable of surviving at high concentrations of Zn, i.e. up to 750 mg/L. Colpaert et al. (1992) and Sintuprapa et al. (2000) showed a maximum Zn tolerance at 1000 mg/L for ectomycorrhizal fungi and *Penicillium* sp., respectively. However, other fungi are less tolerant, for example, *Aspergillus flavipes* tolerated 200 mg/L of Zn (Lopez and Vazquez, 2003) and *Arbuscular ectomycorrhizal* mycelium tolerated 100 mg/L of Zn in the media of exposure (Joner et al., 2000).

The differences between the inhibition to growth on solid medium and liquid medium were due to the high availability of Zn in liquid medium (Anand et al., 2006). On the other hand the differences between the inhibition to the growth and levels of Zn removal in liquid medium for the same species come from the properties and components of the liquid medium.

This high tolerance to Zn observed in *T. atroviride* could be attributed to the fact that the fungus was isolated from a sediment sample containing high levels of Zn. It is known that microorganisms isolated from natural environments contaminated with heavy metals often exhibit tolerance to multiple pollutants because they have adapted well to such environments. Indeed, several authors have established *in vitro* adaptation of fungi to heavy metals (Lopez and Vazquez, 2003).

The milky colored mycelia at all concentrations of Zn may be due to the binding of Zn to the fungal cell walls (Anand et al., 2006). *T. atroviride* hyphae showed spherical shape in cultivation flasks after seven days of incubation in liquid medium (PDB).

In the case of the uptake capacity of Zn by *T. atroviride*, the sudden increase in the concentration from 600 to 700 mg/L and also from 900 to 1000 mg/L may often be associated with toxicity and/or increased permeability of the cell membrane due to further binding of the metal to exposed intracellular sites (Deshmogh and Rai, 2005) causing the biomass to decrease with elevated metal exposure.

Similarly to reports of difference in biosorption for the same species on solid medium, there were also differences in liquid media. For example, Colpaert and Van (1992) reported different biosorption of Zn for *Suillus bovinus* isolated from unpolluted and Zn-polluted sites in Belgium. The strain isolated from the Zn-polluted area showed 2.8, 7.9, 10.8 and 17.4 mg/g of Zn uptake in media with Zn concentrations of 10, 100, 500 and 1000 mg/L, respectively, whereas *Suillus bovinus* isolated from an unpolluted area showed 0.654, 3.6, 9.4 mg/g of Zn uptake in media with 10, 100 and 500 mg/L of Zn, respectively, but without any growth at 1000 mg/L of Zn exposure. Some researchers demonstrated that factors such as contact time, biomass dosage, temperature and pH are known to influence the biosorption of metals. The variations in the metal tolerance 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., 2007) apart from being different fungal species.

The data on biosorption of Zn varied from study to study. Sintuprapa et al. (2000), Joner et al. (2000) and Price et al. (2001) reported 16, 5.6-76 and 0.15 mg/g of Zn uptake by *Penicillium* sp, arbuscular mycorrhizal mycelium and *Aspergillus niger*, respectively. These variations were attributed to differences in the biomass concentration and treatment, biosorption conditions including pH and the methods employed (Zafar et al., 2007). 47 to 64% of Zn was removed by EDTA from the cell surface of *T. atroviride* when Zn was in the range of 500 to 1000 mg/L (Fig 3). In comparison, the data from the work of Anand et al. (2006) showed that 48.15% and 80 to 85% of Cu could be removed by *T. viride* grown at Zn concentration of 50 mg/L and 100 to 200 mg/L, respectively. In this study, the percentage of Zn adsorption decreased with increasing Zn concentrations from 500 to 1000 mg/L. A similar pattern of decreasing Zn adsorption was observed in the work of Price et al. (2001). In a 0.01 mM solution of Zn, *Aspergillus niger* removed 49% of Zn by adsorption. At 0.1 mM Zn, 34% of Zn was adsorbed by *A. niger*. These comparisons showed that a higher percentage of Zn adsorption was found in *T. atroviride*.

The biosorption of toxic metals is based on ionic species associating with the cell surface or extra cellular

polysaccharide, proteins and chitins (Zafar *et al.*, 2007). Different species/strains and cell types of the same fungus vary in the chemical composition of the cell walls, leading to variations in their uptake capacities (Zapotoczny *et al.*, 2006).

The biosorption of Zn in the present study was determined in according to the wet digestion fraction method described by Ahuja *et al.* (2001). However, this method or other methods like treatment of the fungal mycelium with 0.1 N HCl to remove any heavy metal adsorption to the mycelium (Akthar and Mohan, 1995; Price *et al.*, 2001 and Anand *et al.*, 2006) were described in the literature to determine adsorption and absorption but none of these studies could show cell wall adsorption and intercellular accumulation accurately.

However, this assumption seems to be more accurate than what Price *et al.* (2001) did in their experiment in which they treated the fungal mycelium with 0.1 N HCl to remove any heavy metal adsorbed to the mycelium and then all heavy metal remaining with the biomass after the treatment was assumed to be absorbed internally. However, both assumptions cannot clearly show the real amount of metals being washed during the experiment. Hence, the isolated *T. atroviride* from sediment samples of the Serdang Industrial Area showed a high level of tolerance in the presence of high Zn concentrations under this *in vitro* study. *T. atroviride* was able to uptake 18.1 to 26.7 mg/g of Zn in the range of 500-1000 mg/L Zn concentration in PDB as liquid medium. The uptake of Zn was followed by 47.6-64% adsorption and 30.4 to 45.1% absorption. The high tolerance to Zn observed in *T. atroviride* could be attributed to the fact that the fungus was isolated from a sediment sample containing high levels of Zn. It is known that microorganisms isolated from natural environments contaminated with heavy metals often exhibit tolerance to pollutants because they have adapted to such environments. In conclusion, this study suggests that *T. atroviride* is a potential bioremediator of Zn. However, further studies are needed to confirm its practical use as a bioremediating agent under field conditions.

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