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Selection of Newly Isolated Mushroom Strains for Tolerance and Biosorption of Zinc *In Vitro*

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Nine newly isolated mushroom strains were tested to assess both their zinc tolerance and potential for zinc removal from an aqueous solution. Four strains of ectomycorrhizal fungi, namely *Clavariadelphus truncatus* (T 192), *Rhizopogon roseolus* (T 21), *Lepista nuda* (T 373), and *Tricholoma equestre* (T 174), along with five strains of white rot fungi, *Lenzites betulina* (S 2), *Trametes hirsuta* (T 587), *Ganoderma* spp. (T 99), *Polyporus arcularius* (T 438), and *Ganoderma carnosum* (M 88), were investigated using zinc-amended solid and liquid media. Their biosorption properties were also determined. The colony diameter and dry weight were used as tolerance indices for fungal growth. *C. truncatus* and *T. equestre* were not strongly inhibited at the highest concentrations of (225 mg/l) zinc in solid media. The most tolerant four strains with solid media, *C. truncatus*, *G. carnosum*, *T. hirsuta*, and *T. equestre*, were then chosen for tolerance tests in liquid media. An ectomycorrhizal strain, *C. truncatus*, was also detected as the most tolerant strain in liquid media. However, the metal-tolerant strains demonstrated weak activity in the biosorption studies. In contrast, the highest biosorption activity was presented by a more sensitive strain, *G. carnosum*. In addition, seven different biosorbent types from *G. carnosum* (M 88) were compared for their Zn (II) biosorption in batch experiments.

Keywords: Basidiomycetes, biosorption, ectomycorrhizal fungi, white rot fungi, zinc tolerance

Zinc is used for a variety of industrial applications, including coating for steel and iron, galvanization, the manufacture of alloys, such as brass and bronze, and the fabrication of dry-cell batteries [5]. Zinc compounds are also widely used to make white paints and ceramics, produce rubber, preserve wood, manufacture and dye fabrics, make the smoke in smoke bombs, and in the drug industry. However, this extensive use of zinc by industry has caused the pollution of soil and freshwater habitats. Although zinc is directly and/or indirectly involved as a trace element in all aspects of growth, metabolism, and differentiation of animals, plants, and microorganisms, it is toxic to these same organisms above certain concentrations, depending on the organism, physicochemical properties of the metal, and environmental factors. Barceloux [5] reported that, when zinc is present at millimolar concentrations, it is toxic to organisms.

High zinc concentrations in the environment are as toxic to microorganisms as other organisms. Metal tolerance and accumulation in soil and water by microorganisms has already been studied for biosorption and the bioremediation of these environments [13, 14, 23–26, 36, 40, 41, 44, 51, 52, 57].

Metal-tolerant mushroom strains play a key role in remediation and affect plants *via* mycorrhizal symbiosis in metal-polluted soils [1, 11, 18, 25, 30, 37, 39]. Thus, the interaction of ectomycorrhizal fungi and toxic metals is of particular importance in the reclamation of metal-polluted areas, and the effect on plant growth and productivity in these areas. Wilkinson and Dickinson [55] suggested that the ability of trees and other perennial plants to grow in polluted habitats is only attributable to mycorrhization of their roots. The *in vitro* tolerance of ectomycorrhizal fungi against Al, Fe, Cu, Zn, Ni, Cd, Cr, Pb, and Hg is vital for determining their ectomycorrhizal establishment potential in metal-contaminated sites [50].

Another major mushroom group that is known to possess tolerance to metal is white rot fungi [3, 21]. The binding activity and tolerance of these fungi to metals is also well known. White rot fungi growing on wood have been found to accumulate Cd, Fe, Zn, and Cu from the wood into their fruit bodies [3]. Furthermore, the biomass of a variety of microorganisms, including bacteria, fungi, and algae, has already been used for the removal of metals from industrial

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and municipal water. A limited number of reports have also appeared in which macrofungi have been used in remediation studies against polluted water [16, 49, 56].

Accordingly, this study reports on the tolerance of different white rot and ectomycorrhizal fungi strains to elevated zinc concentrations in both solid and liquid media, and on the zinc biosorption capacities of different biosorbent types of a selected strain.

Materials and Methods

Fungus Strains and Culture Conditions

The mushrooms used in this study were originally isolated from different sites in Eskişehir, Turkey. Following field and laboratory studies, 9 species were identified after referring to relevant literature [8, 9, 20, 43], and given strain numbers. The cultures were stored on modified Melin-Norkrans (MMN) medium slants at 4°C until ready for use. Prior to each experiment, the cultures were transferred from the storage slants to the center of petri dishes (90 mm diameter) containing the MMN medium, and allowed to grow to obtain new hyphal growth at 27°C, 70% relative humidity, in the dark.

Tolerance Tests on Solid Media

The response of the mushroom strains to various concentrations of zinc was assayed by growing pure mycelial cultures on the MMN medium, pH 5.5. Zinc solutions that had been sterilized by filtration on a 0.45-μm membrane (Millipore) were added to the media after autoclaving to final concentrations of 25, 75, and 225 mg/l as $ZnSO_4$ (Merck). Agar discs (5 mm) from the actively growing edge of 10-d-old colonies were used to inoculate the center of MMN medium dishes. Growth was determined by measuring the diameters of the colonies 10 d after inoculation. The fungal colonies were then harvested following 1-min microwave exposure to melt the agar. The mycelium was blotted dry with 3 changes of Whatman paper until no further liquid was released with a moderate application of hand pressure [7]. The colonies were dried to a constant weight at 60°C and then weighed. At least three replicates were used for each strain. The results were expressed in terms of two tolerance indices (TI) for the colony diameter and dry weight [51].

$TI_{\text{colony diameter}}$=(Diameter of treated mycelium /Mean diameter of control mycelium)×100

$TI_{\text{dry weight}}$=(Dry weight of treated mycelium /Mean dry weight of control mycelium)×100

Tolerance Tests in Liquid Media

Four fungal strains were chosen for the tolerance tests in liquid media according to their tolerance indices on solid media. The cultures were grown in liquid media containing (mg/l): NaCl (200), $MgSO_4 \cdot 7H_2O$ (250), KH_2PO_4 (500), $CaCl_2 \cdot 2H_2O$ (100), KCl (100), $FeSO_4 \cdot 7H_2O$ (5), and $NaHCO_3$ (53). The glucose and peptone concentrations were 20 and 10 g/l, respectively. The $ZnSO_4$ was sterilized separately and added to the basic media after its sterilization. The final zinc concentrations were 0, 25, 75, and 150 mg/l.

Mycelium discs taken from the margin of 10-d-old colonies on MMN plates were homogenized in a sterile saline solution. These mycelia suspensions were then used as the inoculants for the liquid media. All the flasks were incubated at 27°C, 150 rpm. Following the incubation period, the mushroom mycelia were harvested by filtration. The pellets were then washed twice with distilled water, dried at 60°C until a constant weight was achieved, and weighed. Each treatment was performed using three replicates. The mycelial dry weight values were used to calculate the tolerance indices for the dry weight, as with the solid media.

Biosorption Studies

All the mushroom strains were screened for the biosorption of Zn(II) from an aqueous solution in a batch system. The batch biosorption experiments were carried out with dry and wet biosorbents. In order to prepare dried and wet biosorbents, the fungi strains were cultivated in a liquid medium. After growth, the pellets were separated by filtration. To prepare the dried biosorbent, the biomass was washed with distilled water, dried at 60°C, and powdered.

The dried and wet biosorbents (50 mg dry wt) were added to 50 ml of a Zn(II) solution (100 mg/l, pH 6.0) in 250-ml Erlenmeyer flasks. The flasks were shaken on an orbital shaker (150 rev/min) at 30°C. Following the incubation period, the biosorbents were separated from the aqueous phases. Experimental control tests were also carried out without any biosorbents to investigate the removal of dissolved Zn(II) that might occur *via* chemical precipitation and sorption to the vessel walls.

Biosorbent Preparation

To discover the effect of the biosorbent type, seven different biosorbent types were prepared from the fungus. Dead biomass, resting, and live cells were prepared as described by Yetis *et al.* [56], whereas immobilized microorganisms in alginate and agar were prepared as described by Lebeau *et al.* [38] and Kierstan and Coughlan [34], respectively. Alginate and agar beads were also used for comparison. As a different biosorbent type, dried, ground, and sieved fruit bodies of the fungus were used according to the methods described by Muraleedharan *et al.* [45]. Lastly, the exopolysaccharide of *G. carnosum* (M 88) was also used as a biosorbent for zinc. This exopolysaccharide was prepared as suggested by Kim *et al.* [35].

In total, seven different biosorbent types of *G. carnosum* were added to 100 ml of a zinc solution (100 mg/l, pH 6.0) in 250-ml Erlenmeyer flasks. The biosorption studies were performed on a rotary shaker (150 rev/min) at a constant temperature (30°C). Samples of the liquid were taken at the beginning (0), and then after 15, 30, 60, 120, 240, 480, and 720 min.

Analytical Methods

The Zn(II) analyses were performed using an atomic absorption spectrophotometer (Perkin Elmer 3110). All the experiments were repeated three times, with the arithmetic averages of the results given. For each strain, the growth data were curve-fitted to calculate the EC_{50} (effective concentration inhibiting growth by 50%) in solid and liquid media. The amount of adsorbed Zn(II) ions per g of biosorbent was obtained using the following general equation:

$$Q=[(C_i-C_e)\cdot V]/M \quad (1)$$

where Q is the amount of Zn(II) ion adsorbed on the biosorbent (mg/g), C_i is the initial Zn(II) ion concentration in the solution (mg/l), C_e is the final Zn(II) ion concentration in the solution (mg/l), V is the volume of the medium (l), and M is the amount of the biosorbent used in the reaction mixture (g).

The results were subjected to a one-way analysis of variance (ANOVA) and LSD comparison of the means, $P<0.05$, using SPSS.

RESULTS AND DISCUSSION

In Vitro Tolerance Studies

To determine the toxicity of zinc, mushroom strains were cultivated in the presence of elevated zinc concentrations in solid and then liquid media. Table 1 shows the findings concerning the tolerance indices for the solid media. The tested mushroom strains, with the exception of *Polyporus arcularius*, were not significantly inhibited at the lowest Zn(II) concentration (25 mg/l) in an agar culture. However, *Polyporus arcularius* was inhibited 57% at this concentration and almost completely inhibited at higher Zn(II) concentrations. According to the dry weight and colony diameter tolerance indices, the highest concentration (225 mg/l) of zinc in the solid MMN medium completely or very strongly inhibited most of the mushroom strains, with the exception of *Clavariadelphus truncatus* and *Tricholoma equestre* (Table 1), where the growth was only slightly inhibited and the growth ratios were 88% and 47% of the controls, respectively. In addition, the calculated EC_{50} values were relatively high at 185 mg/l for *C. truncatus* and 85 mg/l for *T. equestre*.

Baldrian and Gabriel [4] reported that, in the presence of metals (Cd, Co, Cr, Cu, Hg, Mn, and Pb) in the growth medium, some white rot basidiomycetes mycelium turned orange and were very dense and flat. They also lacked the formation of aerial hyphae. In contrast, for the present study criteria, none of the mushrooms was significantly affected by any dose of Zn(II).

In this study, a strong interspecific variation in zinc tolerance was determined, as *C. truncatus* (T 192), *G. carnosum* (M 88), *T. hirsuta* (T 587), and *T. equestre* (T 174) were more tolerant to zinc than the other species on solid media. A similar variation has also been detected in some other studies [4, 7, 11, 17, 39, 51]. Gast *et al.* [27] concluded that species differences were a more important factor for metal levels in mushrooms than soil factors.

Vodnik *et al.* [51] suggested that a tolerance index based on the diameter of the fungal culture on solid media was not an appropriate parameter for estimating fungal metal tolerance. However, the current findings pertaining to the dry weight and colony diameter were correlated for all the examined fungi. *C. truncatus* and *T. equestre* were the most tolerant strains for both indices, whereas *G. carnosum* and *T. hirsuta* were the most tolerant strains at a 75 mg/l concentration based on the dry weight of the mycelia. Therefore, these four strains, *C. truncatus*, *T. equestre*, *G. carnosum*, and *T. hirsuta*, were all chosen for further tolerance studies in liquid media owing to their different tolerance abilities.

The zinc tolerances of the four selected mushroom strains in liquid media were lower than those for solid media, with the exception of *T. equestre* (Tables 1 and 2). The growth of this strain in a liquid culture with 25 and 75 mg/l Zn(II) concentrations was reduced to only 4% and 23% of the controls, respectively, whereas according to the dry weight data, the inhibition values for solid media were 18.5% and 36%, respectively. *C. truncatus* was also the most tolerant strain in liquid media.

Contrary to the results reported by Blaudez *et al.* [7], the present results revealed that the metal toxicity increased when growing the fungi in liquid media compared with solid media. Some other researchers have also reported similar results [28, 50, 51]. In this study, the EC_{50} values were generally considerably lower for the liquid cultures compared with the solid cultures. For example, the EC_{50} values for *C. truncatus* decreased from 185 (in solid culture) to 115 mg/l (in liquid culture), plus the EC_{50} values for all

Table 1. Tolerance indices based on dry weight (mg) and colony diameter (mm) of mycelium to elevated zinc concentrations in solid media.

Mushroom strains	Zn(II) (mg/l)[a]						EC_{50} (mg/l)
	25		75		225		
	DW[b]	CD	DW	CD	DW	CD	
Lenzites betulina S 2	53.0±2.5ab	100.0±0.0a	64.0±2.7ac	75.0± 0.0a	2.0±0.6ab	0.0± 0.0a	70
Trametes hirsuta T 587	97.0±3.8acd	100.0±0.0a	117.0±2.0b	62.0±0.0b	0.0±0.0a	0.0±0.0a	78
Tricholoma equestre T 174	81.5±3.0abd	100.0±0.0a	64.0±6.0ac	80.0±0.0c	47.0±3.2c	30.0±0.0b	85
Ganoderma spp. T 99	107.0±7.0cd	90.0±0.0ab	68.0±2.6ac	76.0±5.8ac	0.0±0.0a	0.0±0.0a	72
Lepista nuda T 373	142.0±7.2cef	92.0±15.5b	97.0±4.1ab	80.0±0.0c	9.0±3.2b	0.0±0.0b	74
Polyporus arcularius T 438	43.0±2.3b	77.0±8.0ab	4.5±0.9d	0.0±0.0d	0.0±0.0a	0.0±0.0a	43
Clavariadelphus truncatus T 192	124.0±6.6de	100.0±4.8a	107.0±4.3b	92.0±4.2e	88.0±2.1d	72.0±2.1c	185
Rhizopogon roseolus T 21	125.0±7.4de	100.0±0.0a	50.0±2.3c	50.0±0.0f	7.0±0.3ab	0.0±0.0a	77
Ganoderma carnosum M 88	171.0±12.4f	100.0±0.0a	117.0±7.1b	90.0±0.0e	7.0±0.4ab	0.0±0.0a	82

[a]Mean±SE, n=3, one-way ANOVA. Values in each column followed by the same letter do not differ significantly.
[b]DW, Dry weight; CD, Colony diameter.

Table 2. Tolerance indices based on dry weight (mg) of mycelium to elevated zinc concentrations in liquid media.

Mushroom strains	Zn(II) (mg/l)[a]			EC_{50} (mg/l)
	25	75	150	
C. truncatus T 192	98.3±7.0a	88.7±4.3a	84.5±4.4a	115
T. hirsuta T 587	101.7±3.4b	65.0±5.6a	45.9±3.6b	105
T. equestre T 174	96.0±2.6b	77.1±6.9ab	31.2±2.1c	90
G. carnosum M 88	115.3±6.4b	80.4±4.5ac	11.0±1.6d	74

[a]Mean±SE, n=3, one-way ANOVA. Values in each column followed by the same letter do not differ significantly.

the strains were lower in a liquid culture, with the exception of *T. equestre*. Therefore, as previously recognized by Rapp and Jentscke [46], it may be possible to expect that liquid cultures give a better estimation of the toxicity values of metals. However, mycelial growth in liquid media does not reflect mycelial growth in soil [7, 28]. Furthermore, fungal mycelia differentiation in the absence of a solid substrate may affect the toxicity of metals to mycelia [29]. Therefore, the high zinc tolerance of mycorrhizal strains, such as *C. truncatus* and *T. equestre*, on solid media could be accepted as expected results for subsequent mycorrhizal seedling production studies.

It is well documented that metal phytotoxicity is decreased by ectomycorrhizal fungi [11, 15, 19, 32, 39, 54]. Leyval *et al.* [39] suggested that mycorrhizae reduce metal concentrations in plant tissue through a variety of different mechanisms, such as binding to the cell walls of extrametrical hyphae, protection by the fungal mantle, reduction of metal availability by the whole fungal biomass, and modification of the rhizosphere. The metal-ion binding mechanism in biosorption involves different processes, such as complexation, coordination, electrostatic attraction, microprecipitation, and ion exchange [31]. Bellion *et al.* [6] recently reported evidence of potential extracellular and intracellular mechanisms that may be involved in the tolerance of ectomycorrhizal fungi to excess metals in their environment. According to Bellion *et al.* [6], Zn(II) tolerance in *Suillus bovinus* may be primarily due to a reduced Zn(II) accumulation within the cells. Meanwhile, another study ascertained that *S. bovinus* reduced the needle Zn(II) amount of a high external metal concentration, and this effect increased when the fungus was pretreated with Zn(II) for 6 months [12]. It has been suggested that tolerance to one metal does not imply tolerance to all metals. However, zinc tolerance has been detected as being positively correlated with Cu and Cd tolerance [7]. In study by Denny and Wilkins [18], Zn(II) toxicity in mycorrhizal *Betula* sp. was reduced by the adsorption of Zn(II) to the hyphal surface.

Nonmycorrhizal trees are inhibited by metal concentrations that are generally far lower than those that inhibit the growth of ectomycorrhizal fungi in pure cultures [7]. Therefore, axenic studies into the metal tolerance of ectomycorrhizal fungi may help in the selection of fungi likely to increase host tolerance. Thus, four of the fungi strains used in this study were ectomycorrhizal (*T. equestre*, *L. nuda*, *C. truncatus*, and *R. roseolus*). The high zinc tolerance of these strains on solid media may lead to studies about metal tolerance under symbiotic conditions.

Biosorption Studies

The results of the biosorption studies are given in Fig. 1. All strains demonstrated a higher biosorption capacity than charcoal and clay, with the exception of two dry (*T. equestre* and *R. roseolus*) and one wet (*C. truncatus*) biosorbent. *G. carnosum* was found to be the most potent biosorbent for both the wet and dried biosorbent types. Interestingly, this strain was determined to be sensitive in the liquid culture tolerance experiments (Table 2). The Zn(II) tolerances of *G. carnosum* in solid and liquid cultures at the highest Zn(II) concentration were only 7% and 11%, respectively. In contrast, the most tolerant strain, *C. truncatus*, presented weak biosorption activity for both biosorbent types. *T. hirsuta* and *L. betulina* were determined as other significant strains. Some researchers have reported that metal biosorption by the microbial biomass is not correlated to metal tolerance [47]. Similar results were also obtained from liquid and solid media with the fungi investigated in this study. Although *C. truncatus* and *T. equestre* were determined to be the most tolerant strains against zinc, both the wet biosorbent type of *C. truncatus* and dried biosorbent type of *T. equestre* demonstrated the weakest biosorption capacity among all tested strains. In contrast, both of the biosorbent types of *G. carnosum* represented the highest biosorption capacity for Zn(II) in the present study. Although this strain was the most sensitive strain in liquid media, its biosorption capacity was higher than the more tolerant strains, such as *C. truncatus, T. equestre*, and *T. hirsuta*. Therefore, according to these results, it would seem that metal tolerance was not a factor in Zn(II) biosorption capacity.

In addition, the Zn(II) biosorption abilities of different biosorbent types prepared from *G. carnosum* were observed in batch experiments. To determine the best biosorbent type of *G. carnosum*, seven different biosorbents were compared. To prepare the dead biomass, resting, and live cells, the flasks were incubated at 30°C at an agitation rate of 150 rev/min for 192 h, corresponding to the accelerated growth phase of the biomass (Fig. 2).

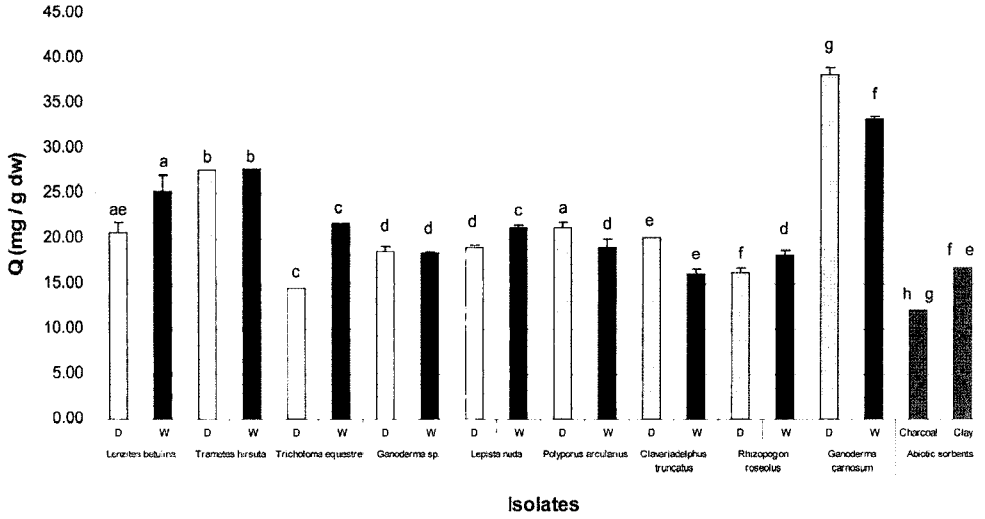

Fig. 1. Zinc biosorption to wet and dried biosorbents from several mushroom strains.
Bars with different letters indicate values with significant difference. Bars represent the means of three replicates at $P \leq 0.05$ (Biosorbent: 50 mg dry wt; Zn(II): 100 mg/l; pH 6.0). Letters above the histogram bars represent Analysis of Variance (ANOVA). D and W indicate dry and wet biosorbents, respectively.

The time course profile of the biosorbent materials showed that an equilibrium was attained in a relatively short time. Yet, a sudden increase was observed after an equilibrium period of 240 and 480 min as regards the biosorption capacity of the resting and live cells, respectively. The different biosorption tendencies of the resting and live cells of *G. carnosum* may have been due to the combined effect of biosorption and desorption during the equilibrium period, as after a long equilibrium period, the biosorption phenomenon appeared to play a more dominant role in the process rather than desorption. However, according to the results, it was possible to use each type of biomass to remove Zn(II) (Fig. 3), although the resting cells of *G. carnosum* were preferred, being easy to cultivate, readily available, and the method of preparation much easier. The biosorption capacity obtained in this study was comparable to and higher than that of many corresponding biosorbents. Whereas earlier studies have shown that this strain can be used for the removal of lead (II) ions from aqueous solutions [2], the present report constitutes the first evidence of the biosorption of Zn(II) ions by *G. carnosum*.

The Zn(II) biosorption capacity values for the examined mushroom strains, in particular those for *G. carnosum*, were comparable to those in existing literature [42, 48, 53]. For instance, the biosorption capacity of the biomass of *Volvariella volvacea* is 11.7 mg Zn(II)/g biomass [42]. The biosorption capacity of *Sargassum* sp. and *Ulva* sp. is 32.5 and 35 mg Zn(II)/g biomass, respectively [48]. The macrophyte biomass of *Alternanthera philoxeroides* has been used for the removal of Zn(II) ions and its biosorption capacity found to be 18.57 mg Zn(II)/g biomass [53]. Meanwhile, the biosorption capacity of the resting cells of *G. carnosum* was determined as 31 mg Zn(II)/g biomass.

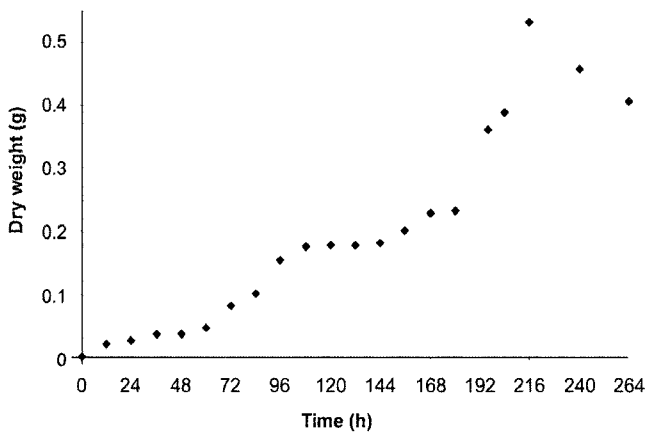

Fig. 2. Growth curve of *Ganoderma carnosum* (M 88).

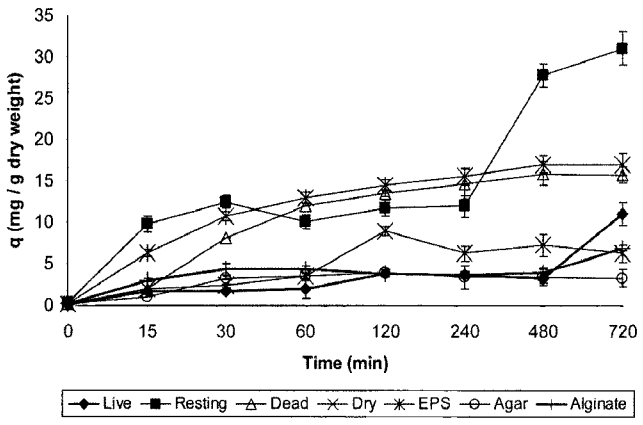

Fig. 3. Time course of Zn(II) sorption by different biosorbent types from *Ganoderma carnosum* (M 88).

In conclusion, *Clavariadelphus truncatus* (T 192) was found to be the most zinc-tolerant strain in both culture types. However, its biosorption capacity was weaker than that of the other strains, especially in wet biosorbents. Interestingly, a more sensitive strain, *Ganoderma carnosum* (M 88), was the most effective strain for biosorption capacity. Therefore, the bioremediation and/or mycorrhizal seedling production for zinc-contaminated environments should be further investigated through these strains.

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