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ACCUMULATION OF ZINC BY THE *LENTINUS EDODES (BERK.)* MYCELIUM CULTIVATED IN SUBMERGED CULTURE

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Abstract: To obtain rich in organic forms of zinc extracts of *Lentinus edodes*, possessing putative higher immunostimulating properties than actually used, mycelia were cultivated in zinc(II) enriched media. Culture media were enriched in zinc in concentration ranging from 0 to 90 μg mL^{-1}, added to the medium before inoculation. Total zinc concentration in submerged cultivated mycelial biomass has been determined by the use of atomic absorption spectroscopy method (AAS). Zinc concentration expressed in mg% of mycelial dry mass rose from 0,33 μg g^{-1}, estimated for mycelium cultivated in not enriched in Zn(II) medium, to 4,28 μg g^{-1} for mycelium cultured in medium containing 50 μg mL^{-1} of zinc. Higher than 50 μg mL^{-1} concentration of Zn(II) in medium caused a decrease of zinc content in mycelial dry mass. Zinc concentration in the medium strongly affected the mycelial growth. Productivity of the mycelium rose proportionally to the increase of Zn(II) concentration in the medium. The highest mycelial growth was recorded for media containing Zn(II) in concentration of 50 μg mL^{-1}. Concentration of Zn(II) in the medium upper than 50 μg mL^{-1} acted depressing on the mycelial growth. An optimal pH of the medium for zinc accumulation was estimated by cultivation of *Lentinus edodes* mycelia in media of pH ranging from 3,5 to 7, containing 50 μg mL^{-1} of zinc(II). The optimal pH of the medium for zinc accumulation was 7. Proportionally to the increasing concentration of zinc(II) in the medium rose the percentage of this metal adsorbed on the cell surface, easy to remove by washing of the mycelium with the 0,05 molar EDTA solution. The value of the percentage of zinc adsorbed on the cell surface changed in the range from 30% to 70% for concentrations of Zn(II) in the medium rising from 20 to 110 μg mL^{-1}.

Keywords: *Lentinus edodes*, zinc accumulation, submerged cultivation, zinc supplements.

Zinc is one of trace elements of fundamental importance to human health. It plays a key role in human metabolism. Inadequate intake of this metal can effect any of over 100 enzymes. The structural function of zinc is its role in the structure of proteins and membranes. Zinc finger proteins have been found to regulate gene expression by acting as transcription factors. Zinc also plays a role in cell signaling, has been found to influence nerve impulse transmission and hormone release (1-4). Adequate zinc intake is essential in maintaining the integrity of the immune system (5, 6). HIV infected individuals are particularly susceptible to zinc deficiency. The requirement for dietary zinc is 50% greater for strict vegetarians, because of high level in grains and legumes of reducing the absorption of zinc phytic acid (7).

Bioavailability and tissue distribution of zinc depends on the form ingested. Organic forms of zinc have a higher bioavailability than inorganic species (8-11).

For higher mushroom the heavy metal accumulation factors are relatively high (12, 13). Whilst it is known that the fungi take up great quantities of zinc, the mechanism of uptake and conversion and precise forms in which it is retained are little known. It is known, that zinc may stimulate organic acid production by basidiomycetes. Zinc increases excretion of organic acids with strong metal-chelating properties (oxalic and citric acid) by fungi, what suggests that a ligand-promoting mechanism is the main mechanism of zinc accumulation (14, 15). Cell cultures of mushrooms, cultivated in zinc enriched media are therefore a potentially good source of highly bioactive and safe organic zinc species.

Lentinus edodes (Berk.) is one of medicinal mushrooms found to contain highly potent immune system stimulators (16). Extracted from *L. edodes* fruit bodies highly purified polysaccharide fraction (lentinan) is an approved drug, used in cancer treatment as well as in AIDS research (17, 18). Hot water extracts from *L. edodes* mushroom mycelium

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and culture media (LEM, LAP) both demonstrate strong immunostimulating and antitumor activity (19, 20). Efficient growth of mushroom mycelia in liquid culture is an alternative and low-cost method to produce fungal biomass (21-22). Submerged cultivated mycelium of examined strain *of L. edodes* was found an efficient source of lentinan – the immunostimulating β-D-glucan (23). The objective of this study was to optimize submerged culture conditions for good growth of *L. edodes* mycelium and high zinc content in cultivated mycelial biomass, probably in form of the high bioavailable organic compounds. High zinc content in mycelial biomass would enhance immunostimulating activity of mushroom extracts. Reach in zinc mycelial extracts would be therefore used as zinc supplements for individuals at risk of zinc deficiency with additional stimulating effect on immune system.

MATERIALS AND METHODS

Fermentation

Microorganism and media

Used in this study *Lentinus edodes* strain was taken from the medicinal mushroom strain collection of Department of Drug Technology of Medical University of Warsaw. It originates in Korea. In nature it grew in Pobwang Peak area, Mt. Myohyang.

The seed culture was grown in 250 mL flasks containing 150 mL of basal medium (glucose 5% w/v, yeast extract 1%, casein hydrolysate 1%, KH_2PO_4 0,1%) at 26°C, on a rotary shaker (New Brunswick Scientific, USA), at 110 rev min^{-1} for 7 days.

Fermentation medium used for the submerged cultivation of *L. edodes* in zinc enriched media was composed of waste products of the food industry (beet molasses 10%, corn steep liquor 0,5%, grain worth 5%, KH_2PO_4 0,1%) in daylight.

Growth conditions of mycelium in shake flask culture of zinc enriched media

In the first series of experiments culture medium was enriched in zinc(II) by the addition of zinc sulfate. The concentration range of zinc(II) added to the medium was 0 to 90 μg mL^{-1}, (0, 10, 30, 50, 70, 90, μg mL^{-1}). Zinc-containing compound ($ZnSO_4$) was added to the medium before inoculation. Initial pH of medium was 6.5.

Mycelia were grown in shake flask culture in 500 mL flasks containing 200 mL of medium. The fermentation medium was inoculated with 5% (v/v) of the seed culture. Cultures were incubated at 26°C in a rotary shaker (New Brunswick Scientific, Edison, NY), at 110 rev min^{-1} for 10 days. Mycelia were harvested by filtration under vacuum and washed three times either with 0,9% solution of NaCl (approximately half of filtered biomass), or with 3 mmol/L solution of EDTA (the second part of filtered biomass). Both parts of filtered mycelium were dried at 60°C. Total volume of filtrates (medium, NaCl solution and EDTA solution) were measured, and 5 mL samples were prepared for determination of Zn(II). All experiments were conducted three times to ensure reproducibility. Total zinc content was determined for each mycelium by use of the atomic absorption spectrometry method (AAS).

The specific growth rate (μ_x, day^{-1}) was obtained by the regression of the exponential logarithmic growth phase, while productivity (P_x) was calcula-

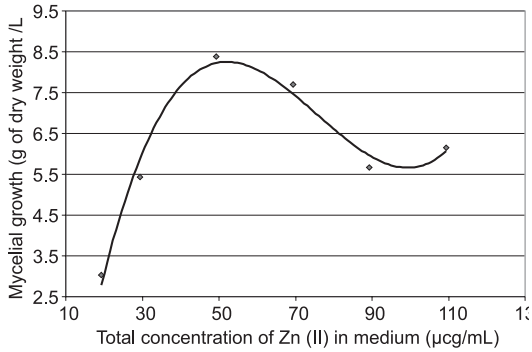

Figure 1. Relationship between concentration of Zn(II) in medium and mycelial growth.

Table 1. Furnace programme for zinc determination in *Lentinus edodes* mycelium samples.

Stage	Temperature (°C)	Hold time (s)	Air flow (L min^{-1})
Drying	150	30	1,5
Ashing	300	20	1,5
Atomizing	1300	3	0.0
Cleaning	1500	1	1,5

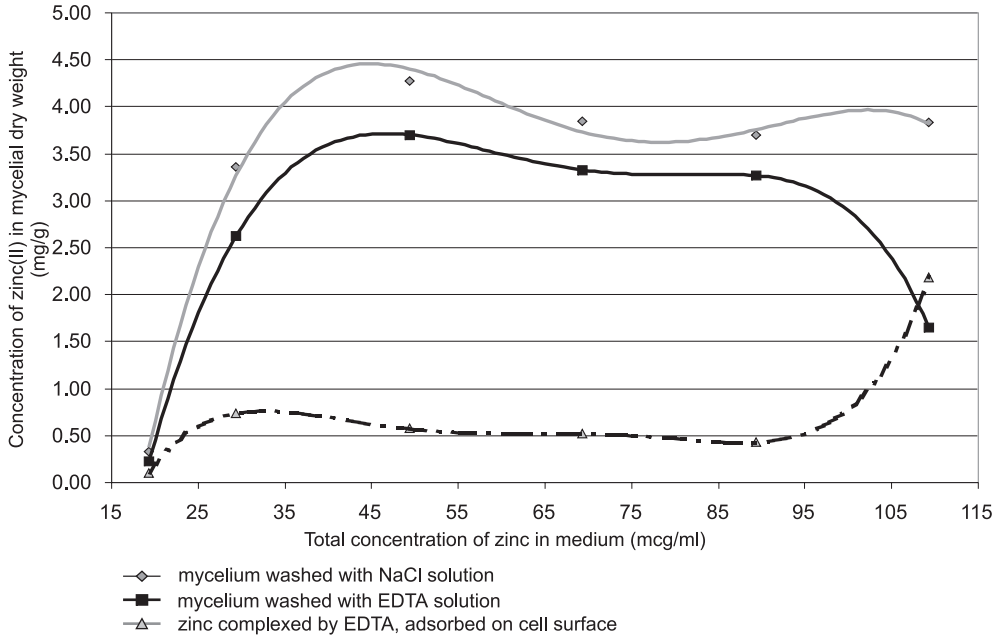

Figure 2. Relationship between concentration of zinc(II) in medium and in mycelial dry weight.

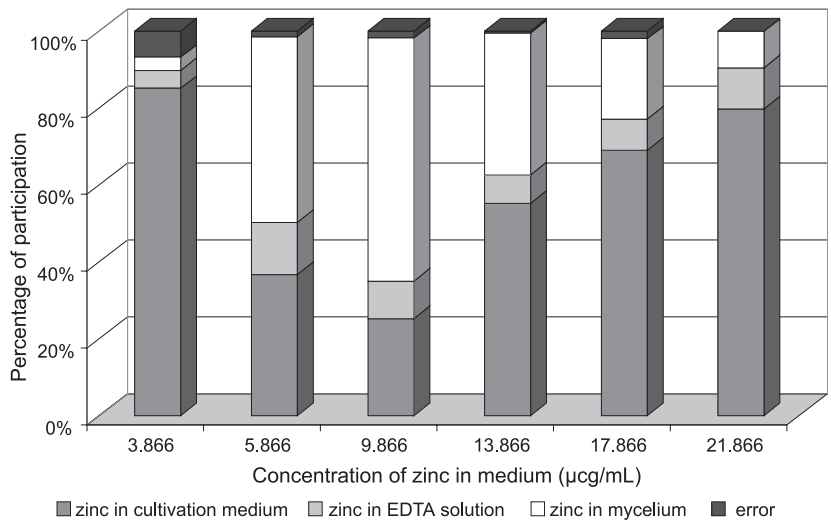

Figure 3. Recovery of zinc added to the 200 mL of cultivation medium (mycelia washed with solution of EDTA).

ted from the quantity of *L. edodes* mycelial biomass grown per liter of medium per day (g L^{-1} day^{-1}, dry weight basis).

Determination of the optimal pH for accumulation of zinc by the *Lentinus edodes* mycelium

In this experiment culture medium was enriched in zinc(II) by the addition of zinc sulfate in

concentration of 50 µg mL^{-1}. $ZnSO_4$ was added to the medium before inoculation. The pH range of tested cultures was from 3,5 to 7,0 (3,5, 4,0, 4,5, 5,0, 5,5, 6,0, 6,5 and 7,0).

The growth conditions and method of filtration of mycelia was the same as described above.

ANALYTICAL METHODS

Atomic absorption spectrometry (AAS) method of determination of zinc in mycelium and culture media

Digestion procedure

Wet digestion procedure was performed. Amount of 0.2 g of sample (mycelium dried in 100°C) was placed in a quartz crucible and mineralized for 4 h at 450°C. When cooled, the sample was dissolved in 5 mL of 25% HNO_3 solution. Liquid samples before mineralization were evaporated to dryness. 2 mL samples were evaporated, mineralized at 450°C for 4 h and dissolved in 5 mL of 25% HNO_3 solution. Afterwards, samples were diluted to 25 mL with deionized water. If necessary, the solutions were diluted several times in a few steps with deionized water. A blank digest was conducted the same way. Zinc standard solutions of concentrations 10, 20, 30 µg/mL were prepared under the same conditions.

Analytical procedure

Zinc atomic absorption standard solution in 1% HNO_3, 998 µg/mL (Buck Scientific) was used for the

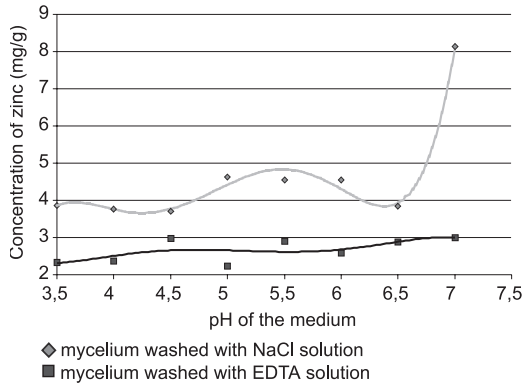

Figure 4. Relationship between pH of cultivation medium and concentration of zinc in mycelial dry weight.

Table 2. The zinc content in *Lentinus edodes* mycelia cultivated in enriched in $ZnSO_4$ media, zinc accumulation factors (AF) and factors of element mobilization (EMF).

Zn(II) added to the medium (µg mL^{-1})	Total concentration of Zn(II) in medium (µg mL^{-1})*	Mycelia washed after filtration with NaCl solution			Mycelia washed after filtration with EDTA solution		
		Concentration of Zn mg g^{-1} D.W. (S.D.)	AF**	EMF***	Concentration of Zn(II) mg g^{-1} D.W. (S.D.)	AF**	EMF***
0	19,33	0,33 (0,07)	17,01		0,22 (0,04)	11,38	
10	29,33	3,36 (0,33)	114,56	10,18	2,62 (0,73)	89,32	11,91
30	49,33	4,28 (0,43)	86,76	12,96	3,70 (0,23)	75,00	16,82
50	69,33	3,85 (0,36)	55,53	11,66	3,33 (0.58)	48,03	15,14
70	89,33	3,70 (0,09)	41,42	11,21	3,27 (0,17)	36,61	15.14
90	109,33	3,83 (0,07)	35,03	11,6	1,65 (0,58)	15,09	7,5

* Concentration of zinc in not enriched medium was equal 19,33 µg/mL

** AF – accumulation factor – ratio of the zinc concentration in mycelial dry weight and the concentration in the medium.

** EMF – factor of element mobilization – ratio of the zinc concentration in mycelial dry weight cultivated in the zinc-enriched medium and zinc concentration in the mycelial dry weight of the control culture, cultivated in the not zinc enriched medium.

Table 3. Relationship between concentration of Zn (II) in medium and the productivity (P_x) and specific growth rate (μ_x) of *Lentinus edodes* mycelia (biomass dry weight basis).

Zn(II) added to the medium (μg mL^{-1})	Total concentration of Zn(II) in medium (μg mL^{-1})	P_x (g L^{-1} day^{-1})	μ_x (day^{-1})
0	19,33	0,30	0,18
10	29,33	0,54	0,28
30	49,33	0,84	0,29
50	69,33	0,77	0,28
70	89,33	0,57	0.25
90	109,33	0,62	0,26

preparation of selenium standards. The Shimadzu AA--660 atomic absorption spectrometer, provided with the autosampler and a transversely heated graphite atomizer was used throughout. 10 mL of zinc(II) standard or sample solution were wet-injected in the graphite furnace. The time/temperature program is shown in Table 1. Slow solution uptake and slow solution injection conditions were selected. Three standard additions (four replications of each) and peak height measurements were used for quantification.

RESULTS

Mycelial growth on zinc-enriched media

Selected medium was composed of waste products of the food industry and therefore costs of the ingredients were low. The biomass accumulation was 3 g of mycelial dry weight per one liter of culture medium, recorded after 10 days of cultivation (for not enriched in zinc medium). The mycelial growth was strongly affected by the zinc concentration in the medium. When the concentration of Zn(II) in the medium rose in range from 19,33 μg mL^{-1} (concentration of zinc in not enriched medium) to 49,33 μg mL^{-1} (medium enriched in 30 μg of zinc), the biomass accumulation in medium rose proportionally. The zinc concentration in medium higher than 50 μg mL^{-1} slightly inhibited mycelial growth, however, the mycelial yield was still higher than for not enriched in zinc media (Figure1). Thus the optimal mycelial growth was recorded for the medium enriched in 30 μg of zinc(II), for which zinc concentration totaled approximately 50 μg mL^{-1}. The productivity of mycelium varied between 0,3 and 0,8 g/L/day depending on the concentration of zinc(II) in cultivation medium. The specific growth rate was the highest for cultivation in the medium containing 50 μg of zinc in 1 mL of medium and reached value of 0,29 day^{-1} (Table 3).

Accumulation of zinc in mycelium of *Lentinus edodes*

For *Lentinus edodes* cultivated in submerged cultures in media enriched in Zn(II) concentrations of zinc were determined in mycelial dry weight, in filtered media and in solutions used for washing of filtered biomass. Accumulation factors (AF) from medium to the cultivated mycelium were calculated from the ratios of the zinc concentration in mycelial dry weight and concentration in the medium. Factors of element mobilization (EMF) were calculated as a ratio of the zinc concentration in mycelial dry weight cultivated in the medium enriched in Zn(II) and concentration in the mycelial dry weight of the control culture, cultivated in the not enriched in zinc medium.

The uptake of zinc to the submerged cultivated mycelium of *Lentinus edodes* was very effective. The accumulation factors (AF) of zinc from the medium were, depending on the concentration of zinc(II) in the medium, about 100-times higher than that for fruiting bodies of white rot fungi. Factors of element mobilization (EMF) were 30 to 300 times higher for the mycelium (depending on the zinc concentration in the medium) than these for fruiting bodies of cultivated mushrooms. The highest concentration of zinc in mycelial dry weigh was obtained for mycelium cultivated in the medium enriched in 30 μg of zinc(II) in 1 mL (total zinc content 50 μg mL^{-1}), and equals about 4,28 μg in 1 gram of dry weight. However, about 13% of determined amount of zinc mass was only weakly combined to the *L. edodes* cell surface. After washing of the enriched in zinc mycelial biomass with solution of EDTA, which forms stable complex compounds with heavy metal cations weakly combined to the mushroom cell surface, only 87% of total amount of accumulated zinc mass remained in the tested sample. The percentage of the total zinc amount remaining in the

mycelial biomass after washing with EDTA solution decreased for mycelia cultivated in media of higher zinc concentration (Figure 2). The optimal concentration of zinc(II) in medium, for which was recorded the highest efficacy of zinc accumulation inside of mushroom cell, expressed in the highest value of the AF and RMF factor was equal 30 μg mL^{-1}. Factors of element mobilization for mycelia cultivated in media containing 20-90 μg of zinc(II) in 1 mL of medium, washed with EDTA solution, were higher than these determined for media washed with NaCl solution. It suggests, that for the mentioned range of zinc(II) concentration in the medium zinc is effectively accumulated inside the cell. Higher zinc(II) concentration in the medium inhibited zinc uptake. The big amount of zinc(II) was adsorbed on the cell surface, therefore the zinc accumulation factors for mycelia washed with NaCl solution were for all cultivations higher than those for mycelia washed with EDTA solution.

The recovery of zinc(II) added to the medium estimated by the determination of the zinc amount in mushroom mycelium, in filtered medium and in washed medium for each 200 mL cultivation reached 90-100% (Figure 3).

Relationship between the pH of medium and efficacy of accumulation of zinc by the *Lentinus edodes* mycelium

The highest efficacy of zinc(II) accumulation by the cultivated mycelium was recorded for medium of pH equal 7 (neutral). The zinc(II) concentration in cultivated mycelium rose in accordance with the pH growth, however, this relationship was more distinct for total zinc content (mycelia washed with NaCl solution). For mycelia washed with solution of EDTA this relationship was also observed, nevertheless it was not so sharp (Figure 4). Very likely the pH value is more significant for the process of zinc(II) adsorption on the cell surface, than for the transport to the cell inside.

CONCLUSIONS

Efficacy of submerged cultivation of *L. edodes* mycelium in enriched in zinc(II) medium is high. Supplementation of cultivation medium with zinc(II), giving in effect concentration of 50 μg mL^{-1}, caused growth of mycelial yields by about 250%. The productivity of mycelium for this medium was equal 0,8 g/L/day whilst specific growth rate reached value of 0,29 day^{-1}.

For cultivated in this medium mycelium of *Lentinus edodes* the total concentration of zinc in mycelial dry weight was equal 4,28 μg g^{-1}. About 86% of zinc(II) determined in the mentioned above mycelium was accumulated inside the mushroom cell; only 14% were weakly adsorbed on the cell surface.

Supposing the good bioavailability of zinc accumulated in mycelium probably in organic form, enriched in zinc extracts from *L. edodes* mycelial biomass could be a good food supplements. This kind of food supplement may be even accepted by vegetarians which requirement for dietary zinc is fifty percent greater, because of high level in grains and legumes of phytic acid, reducing the absorption of zinc.

The results obtained suggest that submerged cultivation of *Lentinus edodes* mycelial biomass on low-cost media composed of waste products of food industry, enriched in zinc, may have a significant impact even on the industrial scale, to obtain food supplements.

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