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Effect of Nutrition Factors on the Synthesis of Superoxide Dismutase, Catalase, and Membrane Lipid Peroxide Levels in *Cordyceps militaris* Mycelium

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Abstract. Effect of carbon, nitrogen, and metal ion sources on superoxide dismutase (SOD), catalase (CAT) activities, and lipid perioxide (LPO) levels in *Cordyceps militaris* mycelium were investigated at stationary growth phase by step supplementing with these nutrition factors in shake-flask cultures. Mycelium was cultivated in several growth media containing different carbon sources. The observed highest SOD and CAT activities were 44.3 U/mg protein in the presence of 20% potato broth plus 2% glucose medium and 93.7 U/mg protein in presence of 20% potato broth plus 1% glucose medium, respectively. By supplementing with either yeast extract or tryptone in 0.1–0.5% concentration range, the highest SOD and CAT activities were 21.1 U/mg protein in medium supplemented with 0.1% yeast extract and 20.7 U/mg protein in medium supplemented with 0.1% tryptone, respectively. Supplementing with Cu^{2+}, Zn^{2+}, and Mn^{2+} caused a stimulation of SOD synthesis. The minimum LPO level was observed at media presented Zn^{2+}. The time course of SOD and CAT biosynthesis showed two maxima, which correspond to the maximum of biomass. High SOD levels and low LPO levels in the medium described above indicated that the appropriate metal ions could provide a suitable protection for cells against oxygen radical damage.

Cordyceps militaris has been used extensively as a crude drug and a folk tonic food in China. This herbal medicine is very scarce in nature or takes too long a time to get a fruiting body by artificial culture. The artificial mycelial biomass shows a close chemical composition [7, 17, 29] and has a similar pharmacological efficacy to natural products of C*ordyceps* sp. [1, 20, 25]. Therefore, substantial literature has accumulated on the relationship of cellular material production to the utilization of major nutrients such as carbon and nitrogen. Fungi are themselves able to produces reactive oxygen species (ROS) depending to a large extent on the nutritional statue [23]. The ROS can damage all types of biomolecules including DNA, protein, and membrane lipids and can result in mutagenesis, inhitition of growth, and cell death [11, 12, 26]. Of course, organisms have developed defense mechanisms to provent and repair oxidative damage [12]. Enzymatic defense, including superoxide dismutase (SOD) and catalase (CAT) activities, performs an important part of the cell's defense repertoire aganist ROS [26].

Cordyceps militaris belongs to entomopathogenic fungal species, which are potential agents in bio-control [2]. There is still a lack of knowledge about the stimulatory effect of nutrition on the antioxidant enzyme, which plays a critical role in an organism's ability to infect, colonize, and thrive inside the host [8, 23, 24]. In this study, we reported the effects of carbon, nitrogen sources, and metal ions on mycelial growth rate, SOD, CAT activities, and lipid perioxide (LPO) levels, which indicate membrane defects of *C. militaris* by step supplementing with these nutrition factors in a shake flask culture. In this way, the adaptive response of *C. militaris*

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Table 1. Effect of carbon sources on growth of mycelium, SOD, CAT activities, and the LPO levels in *C. militaris* mycelium*

Carbon source (%)	Mycelial dry weight (g/100mL)	Protein content (mg/mL)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (nM/g mycelial wet weight)
Potato broth: 20	0.33[a]	1.2[a]	37.9[a]	86.7[a]	1.9[a]
Glucose: 2	0.97[c]	1.1[a]	17.4[b]	69.3[b]	6.4[c]
Sucrose: 1	1.04[c]	1.0[a]	15.4[b]	73.7[b]	5.3[b]
Maltose 1	0.68[b]	1.2[a]	12.1[c]	59.3[c]	4.9[b]

*Mean values (n = 3) with different letters in the same arrange are significantly different ($p < 0.05$).

mycelium to prevent oxidative stress was investigated depending on the environmental changes.

Materials and Methods

Strain and seed culture. The culture method was according to Wang and Schellhorn [27]. *Cordyceps militaris* SH strain (stored in our lab) was maintained on potato dextrose agar (PDA) (Difco Laboratories) slants at 4°C, and was grown on PDA at 23°C for 15 days before inoculation with pieces of culture (5 × 5 mm) into 250-mL conical flasks containing 100 mL 30% potato dextrose solution. The cultures were incubated on a rotary shaker (160 rpm) at 23°C for 4 days as seeds.

Effect of carbon sources. The basal medium used, which contained 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$ (all subsequent media contained both salts), 1% glycine, supplemented separately with 2% glucose, 1% saccharose, 1% maltose, or 20% potato broth (peeled, 200g), was cut into pieces (1 × 1 cm) and boiled in 1000 mL water for 30 min. Then the potato broth was obtained by filtering the boiled solution through gauze and supplemented with water to the final volume of 1000 ml.). Seeds were inoculated at the final concentration of 5% into 250-mL conical flasks containing 100 mL of the medium described above. The cultures were incubated on a rotary shaker (160 rpm) at 23°C for 5 days. After cultivation, biomass was filtered through gauze and washed with 50 mM PBS buffer, pH 7.4, two times. The cultured filtrates were stored at −20°C. All subsequent incubation and harvest conditions were the same as above, except for length of culture conditions at the time course of the experiment.

Effect of glucose concentration. Basal medium (20% potato broth) supplemented with glucose (1–6%) was used for determination of the effect of glucose concentration.

Effect of supplemental nitrogen sources. The basal medium containing 20% potato broth and 2% glucose was supplemented separately with either yeast extract or tryptone in 0.1–0.5% concentration ranges as a nitrogen sources.

Effect of supplemental metal ions. The basal medium (20% potato broth, 2% glucose, and 0.3% yeast extracts) was supplemented separately with different metal ions ($CuSO_4$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 2H_2O$, or $FeSO_4 \cdot 7H2O$). Metal ion in above basal medium was substituted separately with metal ions ($CuSO_4$ + $ZnSO_4 \cdot 7H_2O$ + $MnSO_4 \cdot 2H_2O$) in 4 ppm of each ion at the time course of experiments. Seeds (final concentration is 5%) were inoculated into 36 conical flasks (500 mL) containing 200 mL of the above medium. Flasks were then agitated (160 rpm) at 23°C for different length of periods (up to 144 hours) and harvested. There were no additional metal ions in control medium.

Preparation of cell-free extracts. Wet mycelia were frozen at −20°C, thawed overnight at 4°C, and then suspended in 4 volumes 50 mM PBS buffer (pH 7.4). The suspended material was homogenized with a sonic power. Cell debris was removed by centrifugation at 15,000*g*, for 15 min at 4°C. Supernatants were used as samples for following analysis.

Analysis methods. SOD activity assay system was based on the inhibitory effect of SOD on the spontaneous autoxidation of pyrogallol [22]. CAT activity was determined in cell-free extracts by the method of Bergmeyer [4]. The extinction coefficient at 240 nm of 43 M^{-1} cm^{-1} for the chromophore was used to calculate the amount of CAT. Protein was determined according to the Bradford method [5]. Lipid peroxidation was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBA. The extinction coefficient at 532 nm of 155 M^{-1} cm^{-1} for the chromophore was used to calculate the MDA-like TBA produced [6]. The Tukey test, for multiple comparisons, was used for statistical significance analyses at $p < 0.05$. The values are the means of three separate experiments.

Results

Effect of carbon sources. The biomass of mycelia, SOD, CAT activities, and the LPO levels in mycelium were investigated with respect to the carbon sources. As shown in Table 1, the maximum SOD activity (37.9 U/mg protein) was observed in medium containing 20% potato broth and was about 3.14-, 2.47-, and 2.18-fold higher than those in the medium containing 1% maltose, 1% sucrose, and 2% glucose, respectively ($p < 0.05$). The maximum CAT activity (86.7 U/mg protein) was also observed in the medium containing 20% potato broth. The minimum LPO level was obtained in the medium containing 20% potato broth. Potato broth caused stimulation of SOD and CAT synthesis, but the quantity of biomass was about 3-fold less than those in the medium containing both glucose and sucrose.

Effect of initial glucose concentration. The biomass of mycelia, SOD, CAT activities, and the LPO levels in mycelium were investigated with respect to the glucose concentrations in 20% potato broth. The results were summarized in Table 2. The maximum SOD activity (44.3 U/mg protein) was obtained in medium (20% potato broth) supplemented with 2% glucose. CAT activity was decreased from 93.7 U/mg to 46.3 U/mg as

Table 2. Effect of glucose concentrations on growth of mycelium, SOD, CAT activities, and, the LPO levels in *C. militaris* mycelium*

Glucose (%)	Mycelial dry weight (g/100mL)	Protein content (mg/mL)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (nM/g mycelial wet weight)
1	0.91^{a}	0.9^{a}	21.7^{a}	93.7^{a}	4.5^{a}
2	1.12^{b}	1.0^{a}	44.3^{c}	67.9^{b}	4.6^{a}
3	1.29^{c}	1.1^{a}	39.1^{c}	53.7^{b}	3.8^{a}
4	1.37^{c}	1.1^{a}	32.7^{b}	46.3^{c}	4.0^{a}

*Mean values (n = 3) with different letters in the same arrange are significantly different ($p < 0.05$).

Table 3. Effect of nitrogen concentrations on growth of mycelium, SOD, CAT activities, and LPO levels in *C. militaris* mycelium*

Nitrogen source (%)	Mycelial dry weight (g/100 ml)	Protein content (mg/ml)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (nM/g mycelial wet weight)
Yeast extract					
0.1	1.10^{a}	2.5^{a}	21.1^{a}	19.6^{a}	4.1^{a}
0.3	1.21^{a}	3.0^{b}	19.6^{a}	18.4^{a}	3.8^{a}
0.5	1.20^{a}	3.6^{c}	16.1^{b}	10.7^{b}	3.6^{a}
Tryptone					
0.1	1.18^{a}	3.3^{b}	17.7^{b}	20.7^{a}	4.9^{b}
0.3	1.15^{a}	3.5^{c}	13.9^{c}	13.4^{b}	4.7^{b}
0.5	1.11^{a}	3.5^{c}	15.4^{c}	12.4^{b}	3.9^{a}

*Mean values (n = 3) with different letters in the same arrange are significantly different ($p < 0.05$).

the glucose concentration increased from 1% to 6% $p < 0.05$. The glucose concentrations did not significantly affect the LPO levels and protein content ($p > 0.05$), but biomass tended to increase as the glucose concentration increased from 1% to 6% ($p < 0.05$).

Effect of initial nitrogen concentration. The biomass, SOD, CAT activities, and the LPO levels were investigated depending on initial nitrogen concentrations. As shown in Table 3, the maximum SOD activity was 21.1 U/mg protein in medium with 0.1% yeast extract and then decreasing slowly with an increase of yeast extract concentration up to 0.5%. The same trend was observed in the media containing 0.1–0.5% tryptone ($p < 0.05$). The maximum CAT activity was 20.7 U/mg protein in the medium containing 0.1% tryptone. CAT activity declined significantly depending on the increase of both tryptone and yeast extract concentrations from 0.1% to 0.5% ($p < 0.05$). A low LPO level was observed in the media containing yeast extract in 0.1–0.5% concentration range and containing 0.5% tryptone (Table 3). The biomass was not affected by either yeast extract or tryptone concentrations ($p > 0.05$). But the protein content was increased when both yeast extract and tryptone concentration increased from 0.1 to 0.5% ($p < 0.05$).

Effect of metal ions. The effect of metal ions on the biomass, SOD, CAT activities, and the LPO levels was investigated. The results are summarized in Table 4. The SOD activities were much higher in the media containing Cu^{2+}, Zn^{2+}, and Mn^{2+} in comparison with that in the control culture ($p < 0.05$). The maximum SOD activity (42.0 U/mg protein) was obtained in the medium containing Cu^{2+}+ Zn^{2+}. In contrast, the maximum CAT activity (25.7 U/mg protein) was obtained in the medium containing Cu^{2+}, and no more differences were observed between the medium containing Fe^{2+}, Zn^{2+}, Mn^{2+}, and control culture. Supplemental metal ions did not cause a change of biomass and protein content, but Zn^{2+} caused the lowest LPO levels, compared with the other metal ions and control ($p < 0.05$) (Table 4). In addition, metal ions (Cu^{2+}+ Zn^{2+}+ Mn^{2+}) in the 4 ppm–40 μM concentration range imsignificantly affected SOD, CAT activities, protein content, biomass production, and LPO levels ($p > 0.05$) (data not shown).

Variations of SOD, CAT activities, mycelial growth rate, and LPO levels in the shaking flask culture. Time course of SOD, CAT production, mycelium growth rate, and LPO level during the cultivation in the shaking flask culture are shown in Figure 1. Figure 1A demonstrated that the time course of SOD activities shows two maxima in both media at early exponential phase (the 48th h) and the late stationary phase (the 144th hour), respectively. SOD

Table 4. Effect of metal ions on growth of mycelium, SOD, CAT activities, and LPO levels in *C. militaris* mycelium*

Elements (20 μM)	Myceiial dry weight (g/100ml)	Protein content (mg/ml)	SOD (U/mg protein)	CAT (U/mg protein)	MDA (nM/g mycelial wet weight)
Con^b	1.19^a	2.7^a	19.5^a	17.8^a	4.0^a
Cu^{2+}	1.10^a	2.3^a	40.3^c	25.7^b	3.5^b
Zn^{2+}	1.21^a	2.5^a	35.3^c	17.7^a	1.2^c
Mn^{2+}	1.26^a	3.4^b	23.2^b	19.5^a	4.9^a
Fe^{2+}	1.15^a	2.5^a	18.5^a	16.9^a	5.0^a
$Cu^{3+} + Zn^{2+}$	1.17^a	2.4^a	42.0^c	18.9^a	1.1^c
$Cu^{2+} + Zn^{2+} + Fe^{2+}$	1.18^a	2.5^a	41.2^c	19.8^a	2.7^b
$Cu^{2+} + Zn^{2+} + Mn^{2+}$	1.14^a	2.7^a	30.7^b	22.1^b	2.2^b

Con^b has no additional metal ions.
*Mean values (n = 3) with different letters in the same arrange are significantly different ($p < 0.05$).

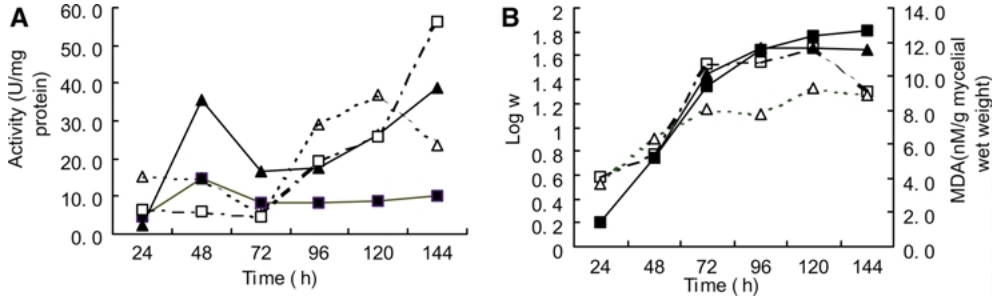

Fig. 1. (A) Variations of SOD and CAT activities in cordyceps militaris mycelium depending on the incubation period. (■) SOD in mycelium in control medium, (▲) SOD in mycelium in medium with metal ions, (Δ) CAT in mycelium in control medium, (□) CAT in mycelium in medium with metal ions. (B) Variations of mycelium growth (log w) and MDA in *cordyceps militaris* mycelium. (■) log W of mycelium in control medium, (▲) log W of mycelium in medium with metal ions, (□) MDA of mycelium in control medium, (Δ) MDA of mycelium in medium with metal ions.

activities in medium containing metal ions were significantly higher than those in control culture for incubation periods after the 24th h ($p < 0.05$). The maximum SOD activity was 39.6 U/mg protein at the 144th h. In contrast, CAT activities that showed also two maxima with respect to the time course were lower in the medium containing metal ions than those in the control medium before the 120th h (Fig. 1). After the 120th h, this trend was reversed. The second maximum CAT at the 120th h was 35 U/mg protein and 2.5-fold higher than that at the first maximum (at the 24th h) in the control culture. Metal ion prolonged this period to the 144th h for the obtaining of the maximum CAT production (58 U/mg protein) in the medium containing metal ions.

A sharp increase in the LPO level was observed in the control culture for the 72nd h. Afterward, LPO levels increased slowly (Fig. 1B). However, the LPO level in the medium with metal ions increased slowly during the incubation period and was much lower than that in the control culture between the 72nd and 120th h ($p < 0.05$), when the cell growth was at late log growth phase and stationary growth phase.

Discussion

The rate of electron transport in the respiratory chain is where the major source of ROS of filamentous fungus via glycolysis and citric acid cycles can be affected by the changes of carbon and nitrogen sources [14]. The highest SOD and CAT activities of *Cordyceps militaris* were observed as 37.9 and 86.7 U/mg protein in medium with 20% potato broth as carbon source, respectively (Table 1). The difference in values of SOD and CAT among the carbon sources may be due to the differences in their bioenergetics and entrance to the glycolysis pathway at different steps, which may influence production of ROS and biomass [3]. SOD activity in *C. militaris* mycelium significantly decreased with an increase glucose concentrations from 2% to 6% ($p < 0.05$) in 20% potato broth (Table 2). This result is consistent with a report by Ayar-Kayali et al. [3]. In this case, it is possible that the elevated sensitivity to an osmotic upshift results from an increase in intracellular levels of O^{-2} [16]. CAT activity decreased with glucose concentration increasing from 1% to 6%. This result also agreed with a report by other researchers [28]. This may

be due to the fact that low glucose concentrations in the culture medium of fungus induce the expression of glucose oxidase, which oxidizes glucose and reduces dioxygen to hydrogen peroxide, and then hydrogen peroxide induces biosynthesis of CAT indirectly [18, 21].

In general, edible fungi are rich in heavy metals and can build up large concentrations of some heavy metals [13, 19]. Because heavy metals cause a metal stress in the cells resulting in the formation of ROS, survival of the microorganism in these stresses is attributed to the induced biosynthesis of enzymes responsible for antioxidant defence or other bio-reactions [9, 10, 15]. Compared with control culture, Cu^{2+}, Zn^{+2}, and Mn^{2+} induced biosynthesis of SOD, and Zn^{+2} caused a decrease of LPO levels in mycelia (Table 4). The data confirmed the regulated effect of Cu^{2+}, Zn^{+2}, and Mn^{2+} on SOD biosynthesis. It was reported that the presence of Cu^{2+} or Fe^{2+} starvation leads to an increase in expression of SOD in *Aspergillus* [15]. Low LPO levels indicated high SOD protected cells from free radical damage. These effects were confirmed in the time course of experiments during the cultivation dependent on metal ions in the shaking flask culture (Fig. 1). SOD levels in media supplemented with the metal ions were higher than those in control medium after the 24th h (Fig. 1A), and caused lower LPO levels between the 72nd h and the 144th h in mycelium (Fig. 1B). These results indicated that these metal ions play an important role in increasing SOD activity during this period.

According to the above results, low glucose and the appropriate Cu^{2+}, Zn^{+2}, and Mn^{2+} were more effective in increasing the synthesis of antioxidant enzyme and, subsequently, reduced lipid peroxidation. Especially, the appropriate metal ions are critical factors in keeping relative high SOD and low LPO levels in mushroom mycelia during submerged culture.

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