Copper (II) Accumulation and Superoxide Dismutase Activity during Growth of *Aspergillus niger B-77*

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The influence of copper (II) ions on the growth, accumulation properties and superoxide dismutase (SOD) activity of a growing culture of *Aspergillus niger B-77* were studied. Microbial growth, the level of copper (II) accumulation and SOD activity depended on the initial copper (II) concentration. *Aspergillus niger* is able to accumulate large amounts of copper (II) from the nutrient medium with 200 mg.l^{-1} copper (II) ions without loosing its biological activities. Addition of copper (II) ions increased the SOD activity in the growing cell cultures. The changes in enzyme activity induced by heavy metal ions might be used as an indicator of intracellular oxy-intermediate generation in a cell culture growing under stress conditions.

Introduction

Superoxide dismutases (SODs) are antioxidant enzymes that disproportionate superoxide ($O_2^{\cdot}$) to hydrogen peroxide (H_2O_2) and dioxygen (Srinivasan *et al.,* 2000; Niven *et al.,* 1999; Gort *et al.,* 1999). This class of enzyme is found in almost all aerobic organisms (Greco *et al.*, 1990; Angelova *et al.*, 2000; Hamilton and Holdom, 1999). Eukaryotes contain Mn-SOD in the matrix of the mitochondria and CuZn-SOD elsewhere in the cell, most notably in the cytoplasm, nucleus and lysosomes. Exposure of aerobic microorganism to variety of stresses, such as heat shock, oxidative stress, treatment with drugs, X- and UV-irradiation, stress caused by immobilization, has been shown to induce the synthesis of the antioxidant enzymes (Angelova *et al.*, 2000; Fink-Boots *et al.*, 1999; Farr and Kogoma, 1991).

There are a few reports of superoxide dismutase and copper (II) accumulation by growing fungal cells. The purpose of the present study was to investigate the copper (II) uptake capacity and the activities of SOD in cells of *Aspergillus niger B-77*, growing in the presence of high copper (II) concentration.

Materials and Methods

Microorganism, medium and cultivation

The mutant strain *Aspergillus niger B-77*, an industrial producer of glucoamylase (No. 65/1980, National Bank of Industrial Microorganisms and Cell Cultures, Bulgaria), was used in this study. Spores from an established culture (6–7 days old) incubated on potato/glucose agar slants at 30 °C were used for inoculation. The growth medium contained the following (per litre): 30 g starch, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ and 1 g peptone. The copper (II) ions were added as $CuSO_4.5H_2O$ in four concentrations: 50, 100, 200 and 300 mg per litre and pH was adjusted to 4.8–5.0 before sterilization.

Cultivation of *Aspergillus niger* was carried out in 500-ml Erlenmeyer flasks with 100 ml growth medium on a rotary shaker at 30 °C (220 rpm, 72 hours). The medium was inoculated with 0.1 ml diluted spore suspension (about 10^6 spores per ml).

During the experiments while the fungus growth, the pH values were measured continuously and it was observed that although the initial pH decreased a bit, the difference was always less than 1.0–1.5 pH degree so pH change did not cause copper (II) precipitation. Samples were taken at predetermined time intervals (0 min, 12, 24, 36, 48, 60 and 72 h) for the microorganism growth and residual copper (II) concentrations in the culture media. Before analysis the samples were centrifuged at 5000 × g for 10 min and the supernatant fraction was analyzed for copper (II) ions. Uptake values were determined as the difference between the initial copper (II) concentration and the one in the supernatant. All experiments

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were carried out at least twice. The values used in calculations were mostly the arithmetic average of the experimental data.

Analytical methods

Cell growth was measured by drying of washed mycelia at 85 °C. The residual copper (II) concentration in the broth was determined using an atomic absorption spectrophotometer (model 2380; Perkin Elmer, Überlingen, Germany).

Standard literature procedures were used for enzyme and protein assays: SOD (Beauchamp and Fridovich, 1971) and soluble protein in cell extracts (Lowry *et al.*, 1951). The levels of superoxide dismutase were measured by incubating protein fractions in 56 μM potassium cyanide, 10 mM methionine, 1.17 μM riboflavin and 50 mM K_2HPO_4 (pH 7.8) in a light chamber with a 25 Watt bulb for 6 min.

Under these conditions, riboflavin is excited by a photon, and is able to oxidise an electron donor molecule – in this case methionine. This donation of an electron results in the production of a superoxide molecule ($O_2^{\cdot}$). The $O_2^{\cdot}$ molecule is able to reduce the nitro blue tetrazolium (NBT), giving an insoluble purple formazan. This colour change is measured spectrophotometrically at A_{560}. The presence of SOD leads to a reduction in the level of formazan being produced. One unit of SOD is defined as the amount of enzyme required for inhibition of the reduction of NBT by 50%.

Results and Discussion

Microbial growth and copper (II) consumption kinetics

Maximum growth expressed as dried biomass concentration was found in the absence of copper (II) ions. The presence and increasing of copper (II) concentration in the growth medium caused inhibition on the growth of the microorganism. Copper (II) accumulation was highest at the 12th h of growth. The rapid uptake observed in the initial stage of kinetics would be correlated to copper (II) adsorption to the membrane of fungus involving surface phenomena (Dönmez and Aksu, 1999). The lag period is the same with increasing metal level in the medium indicates that metal accumulation is not depended on metabolic activity.

The comparative growth and copper (II) accumulation properties of *Aspergillus niger B-77* were investigated as a function of initial copper (II) concentration. The results are given as units of dried biomass per 1 l, specific copper (II) uptake determined as the amount of copper (II) per unit of dry weight of cells and the uptake yield were calculated (Table I).

At copper (II) concentrations of 50, 100 and 200 mg.l^{-1} growing *Aspergillus niger* cells took up 74.8, 68.5 and 60.4% of total amount of copper (II) within 24 h, respectively. The metal uptake (mg copper (II) ions per g biomass) increased with increasing of copper (II) concentrations. At 50, 100 and 200 mg.l^{-1} copper (II) ions for 24 h of cultivation, the uptake was 3.7, 7.2 and 19.2 mg/g respectively. At 300 mg.l^{-1} of initial copper (II) concentration, neither microbial growth nor copper (II) uptake was observed. The maximum cop-

Table I. The effect of initial copper (II) concentrations on the microbial growth and copper (II) uptake per g of dried biomass.

Copper (II) ions [mg·l^{-1}]	Dried biomnass [g·l^{-1}]			Copper (II) uptake [mg·g^{-1}]			Uptake yield (%)		
	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
0	3.6	10.5	17.5	–	–	–	–	–	–
50	3.4	10.1	16.7	9.5	3.7	2.2	65.0	62.8	57.0
100	3.2	9.4	15.6	19.6	7.3	4.4	74.8	68.5	60.4
200	2.2	6.3	10.5	51.9	19.2	11.5	74.8	68.5	60.4
300	0.3	0.6	0.8	–	–	–	–	–	–

The uptake yield (%) is defined as the ratio of accumulated concentration of copper (II) at 12, 24 and 48 h to the initial copper (II) concentration.

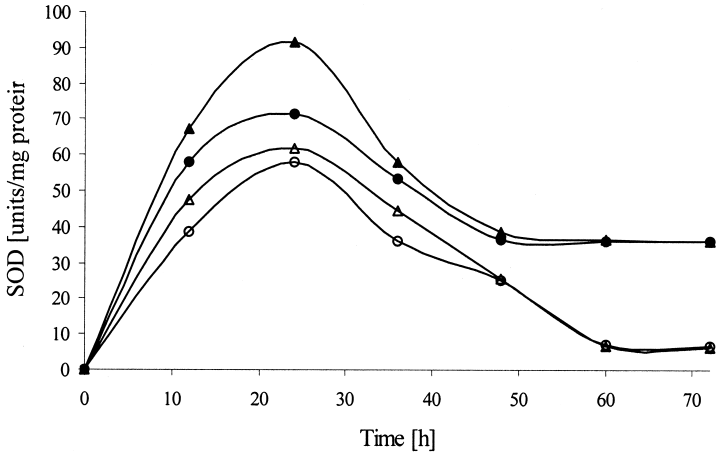

Fig. 1. Effect of copper (II) addition on SOD activity during the growth of *Aspergillus niger B77* at different copper (II) concentrations; (○, △, ● and ▲ show the variations of SOD with time at 0, 50, 100 and 200 mg.l^{-1} initial copper (II) concentrations, respectively). The data shown are mean values of three experiments. Individual values deviate less than ±10% of the mean values.

per (II) uptake capacity of the fungus 19,2 and 51,9 mg/g was observed at 200 mg.l^{-1} initial copper (II) concentration at 12th h and 24th h of cultivation respectively.

Effect of copper (II) ions on SOD formation of growing Aspergillus niger B-77 cells

To determine the effect of copper (II) ions on SOD biosynthesis growing mycelia systems at different heavy metal concentrations were used. For the experiments fungal cells were grown in the medium with the addition of $CuSO_4.5H_2O$ at 50, 100 and 200 mg.l^{-1} copper (II) ions or without heavy metal. Fig. 1 presents the courses of SOD activities in cultures during the growth. SOD formation was significantly affected by heavy metal concentrations. During the logarithmic growth phase, mycelium dry weight at 50 and 100 mg.l^{-1} copper (II) ions were found to be slightly lower in comparison with these of control culture (Fig. 1). In contrast, SOD activities were found to be visible higher in the presence of heavy metal. SOD production was positively influenced by copper (II) ions and increased during first 24 h when maximum enzyme levels were achieved. Thereafter, a steady decrease were noted. After 48 h inoculation, the SOD activity showed no further decrease and remained constant values. We suppose that cellular physiology correlating with the enhance of an SOD activity under these conditions, can indicate the role(s), which such enzyme activity plays in the cellular adaptation to environmental stress. Greco *et al.* (1990) reported that Cu, Zn SOD in yeasts has the characteristics of a Cu-stress protein, suggesting that it has a critical function in the aerotolerance of this organism under conditions of oxidative stress. SOD, an enzyme responsible for the dismutation reaction (Gralla and Kosman, 1992) and superoxide radicals, is suggested to be involved in initiating the fungal cell response to temperature stress (Fink-Boots *et al.*, 1999).

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