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# Improved production by fed-batch cultivation and some properties of Cu/Zn-superoxide dismutase from the fungal strain *Humicola lutea* 103

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**Abstract**

Cu/Zn-containing superoxide dismutase (Cu/Zn-SOD) from the fungal strain *Humicola lutea* 103 is a naturally glycosylated antioxidant enzyme which has been demonstrated to have a protective effect against myeloid Graffi tumor in hamsters and experimental influenza virus infection in mice. In this work, an improvement of enzyme production was achieved by using both an improved growing strategy and a more efficient purification protocol. The optimized fed-batch cultivation, with 7.5 mg/ml glucose fed daily after 24 h, resulted in prolonged growth and abundant mycelium production, as well as in improvement in enzyme production. A 2-fold increase in the total SOD activity and a significant increase in enzyme production were achieved (from 1.5- to 3.2-fold). Fed-batch technology contributed to a 24 h stabilization period of biosynthesis in which every stop of fermentation can be acceptable, thus making this fermentation a process of industrial interest. In addition, the improved purification procedure offers a reduction of purification steps and enhanced enzyme yield (1.6-fold).

The molecular mass was proven to be 15,940 Da for the subunit and the conformational dynamics of the protein in solution was studied by electrospray ionization mass spectrometry (ESI-MS).
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*Keywords:* Filamentous fungi; Fed-batch cultivation; Superoxide dismutase; Improved production; Enzyme analysis; Electrospray ionization mass spectrometry

## 1. Introduction

Filamentous fungi are important organisms in some biotechnological industries where they provide a wide range of native products, mainly enzymes [1]. They have a number of advantages over traditional microbial cultures. Amongst others, filamentous fungi possess a wealth of enzymes that can modify a variety of substrates [2]. Moreover, they demonstrate fast growth, an abundant mycelium, intensive respiration and a high level of cyanide-resistant respiration [3]. Based on these physiological peculiarities, an effective use of fungi for the production of the intracellular antioxidant enzyme, superoxide dismutase (SOD), could be developed [4–6].

SOD, an enzyme naturally present in all aerobic cells, catalyzes the dismutation of the highly reactive superoxide radical anion to hydrogen peroxide and molecular oxygen [7]. The removal of superoxide effectively blocks secondary reactions that otherwise would lead to formation of the promiscuously reactive hydroxyl radical, which is highly damaging to all classes of biological macromolecules. The generation of ROS is an unavoidable consequence of the oxidative metabolism that can cause damages in all cellular constituents (DNA, lipids and proteins). It affects several cell functions, including replication, growth, protein synthesis, and ion transport [8,9]. Additionally, many diseases are linked to damage from ROS as a result of an imbalance between radical-generating and radical-scavenging systems in favour of the first—a condition called oxidative stress [10–13]. The involvement of oxidative stress in human diseases is the basis for antioxidant therapy [13,14]. The use of SOD as a therapeutic agent has been proposed for several diseases where an important role by ROS has been suggested [15,16].

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Limitations of SOD as a therapeutic agent are mainly due to its rapid clearance from blood by glomerular filtration [17,18]. Among the strategies developed so far to improve the therapeutic action of SOD, its conjugation with polymers, its incorporation into transport systems (liposomes), its chemical modification and the development of recombinant SOD should be mentioned [19–21]. It would therefore be advantageous to provide SOD with higher pharmacological activity. Our previous investigations have shown that the fungal strain *Humicola lutea* 103 is a high producer of naturally glycosylated Cu/Zn-SOD [6,22], which could be isolated in very few other cases. The secretory tetrameric extracellular mammalian SOD is the only glycosylated SOD, besides the *H. lutea* enzyme, described so far [23]. Glycoenzymes of this kind do not need additional processing for conjugation and modification as do non-glycosylated enzymes.

*H. lutea* Cu/Zn-SOD (HLSOD) was used in an in vivo model to demonstrate its protective effect against myeloid Graffi tumor in hamsters [6]. Moreover, the fungal enzyme has been shown to protect mice from mortality after experimental influenza A/Aichi/2/68 (H3N2) virus infection. Using the glycosylated HLSOD, the survival rate is increased and the survival time prolonged, similar to the application of ribavarin, while non-glycosylated bovine SOD conferred lower protection [22].

The present study aims at the enhancement of the yield of naturally glycosylated Cu/Zn-SOD by using both fed-batch culture techniques to increase enzyme production and a more efficient purification protocol. We also describe some properties of the purified enzyme.

## 2. Materials and methods

### 2.1. Microorganism

The fungal strain, *H. lutea* 103, from the Mycological Collection of the Institute of Microbiology, Sofia, was used throughout and maintained at 4 °C on beer agar, pH 6.3.

### 2.2. Cultivation, equipment and conditions

The compositions of the culture media (g/l) were as follows: (1) seed medium—glucose, 40.0; $NH_4NO_3$, 3.0; $KH_2PO_4$, 1.0; $MgSO_4{\cdot}7H_2O$, 0.5; KCl, 0.5; $FeSO_4{\cdot}7H_2O$, 0.001 and (2) production medium—glucose, 48.0; casein, 3.0; soybean flour, 4.0; $MgSO_4{\cdot}7H_2O$, 0.5; $CuSO_4{\cdot}5H_2O$, 0.0011; $ZnSO_4{\cdot}7H_2O$, 0.0029; $FeSO_4{\cdot}7H_2O$, 0.0043; $MnSO_4{\cdot}7H_2O$, 0.0013.

The cultivation was performed in 500 ml Erlenmeyer flasks or in 3 or 12 l bioreactor ABR-09 (working volume 2 and 7 l, respectively), developed and constructed by the former Central Laboratory for Bioinstrumentation and Automatisation (CLBA) of the Bulgarian Academy of Sciences. The bioreactor was equipped with pH monitoring, automatic DO monitoring and a control system.

For the inoculum, 80 ml of seed medium was inoculated with 5 ml of spore suspension at a concentration of $2 \times 10^8$ spores/ml in 500 ml Erlenmeyer flasks. The cultivation was performed on a shaker (220 rpm) at 30 °C for 24 h. For shake-flask cultures, 6 ml of the seed culture were inoculated into 500 ml Erlenmeyer flasks, containing 74 ml of the production medium. The cultures were grown at 30 °C for 120 h.

The bioreactors cultures were performed with 8% (v/v) 24-h-old shake-flask inoculum at 30 °C for 120 h. The fermentation parameters were: impeller speed, 600 rpm, and air flow, 1 vvm (1 volume air per 1 volume liquid per min). The results obtained in this investigation were evaluated from repeated experiments using three or five parallel runs.

### 2.3. Effect of fed-batch glucose addition

For shake-flask cultures, a sterile glucose solution (400 g/l) was added daily, starting from 24 h of culture, to bring the final glucose concentration to 2.5, 5.0, 7.5 or 10.0 mg/ml, respectively. As batch variants, cultures without glucose addition were used. For bioreactor cultures, feeding glucose solution was added to bring the fermentation vessel up to concentration of 7.5 mg/ml according to the same scheme.

### 2.4. Analytical methods

The cell-free extract was prepared as described earlier [24]. Briefly, mycelium biomass was harvested by filtration, washed in distilled water and then in cold 50 mM potassium buffer (pH 7.8), and was resuspended in the same buffer. The cell suspension was disrupted by homogenizer model ULTRA Turax T25 IKA WERK. The temperature during treatment was maintained at 4–6 °C by chilling in an ice-salt bath and during the filtration through filter paper. Cell-free extracts were clarified at 13,000 × *g* for 20 min at 4 °C.

The SOD activity was measured by the nitro blue tetrazolium (NBT) reduction method of Beauchamp and Fridovich [25]. One unit of SOD activity was defined as the amount of SOD required to inhibit the reduction of NBT by 50% of maximum ($A_{560}$) and was expressed as units per mg protein [U/mg protein]. Cyanide (2 mM) was used to distinguish between the cyanide-sensitive isoenzyme Cu/Zn-SOD and the cyanide-resistant Mn-SOD. The Cu/Zn-SOD activity was obtained as total activity minus the activity in the presence of 2 mM cyanide. Protein was estimated by the Lowry procedure [26], using crystalline bovine albumin as standard. Soluble reducing sugars were determined by the Somogyi–Nelson method [27] and total nitrogen by the micro Kjeldal method [28].

The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) No. 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105 °C.

The kinetic parameters were studied according to the procedures of Pirt [29].

### 2.5. Purification of H. lutea Cu/Zn-SOD

Cell-free extract from *H. lutea* 103 obtained as described above was saturated to 30% with ammonium sulfate, placed at 4 °C for minimum 2 h, then centrifuged for 30 min at 16,000 × *g*. The resulting supernatant was first applied to: an Octyl-Sepharose CL-4B (Pharmacia, Fine Chemicals, Uppsala, Sweden) column (40 mm × 32 mm), equilibrated with 0.02 M Tris–HCl (pH 7.8) buffer saturated to 30% with ammonium sulfate and washed with the same buffer until unbound substances, including SOD, eluted out from the column (absorbance at 276 nm dropped to about 0.08). The collected SOD containing fractions (10–12 ml each) were then applied to a Phenyl-Sepharose CL-4B (Pharmacia, Fine Chemicals, Uppsala, Sweden) column (62 mm × 35 mm), equilibrated with the same buffer as used in the previous chromatographic step and eluted under the same conditions. In the presence of 30% ammonium sulfate most of the impurities were not retained, while the SOD was adsorbed. The enzyme eluted with the same buffer containing 10% of ammonium sulfate as a narrow peak. Both steps column chromatographic steps were performed at a high flow rate of 70–80 ml/h.

Fractions containing SOD were concentrated with Amicon (10 kDa cut-off) and further purified by gel-filtration on a Sephadex G-100 column (26 mm × 540 mm), eluted with 0.02 M potassium phosphate buffer, pH 7.8 containing 0.02 M sodium chloride, at a flow rate of 12 ml/h. SOD-containing fractions in all chromatography steps were detected by measuring enzyme activity with NBT method [25].

### 2.6. Polyacrylamide gel electrophoresis

The SOD isoenzyme profile was performed on polyacrylamide gels. Forty μg total protein was applied to 10% non-denaturing PAGE and was stained for superoxide dismutase activity, as described by Beauchamp and Fridovich [25]. Cu/Zn-SOD from bovine erythrocytes and Mn-SOD from *Escherichia coli* were used as standards. Purity control of the enzyme was performed by 10%

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