# Effect of Dissolved Oxygen Concentration on Superoxide Dismutase Production by *Humicola lutea* Cells

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Cultures of the fungal strain *Humicola lutea* 110 were grown in a 3-l bioreactor. Effects of dissolved oxygen concentration (DO) on cell growth, intracellular protein content and antioxidant enzyme activities (SOD and catalase) were investigated. Controlling DO from 20 to 60% lead to: (I). The lethal phase of growth was reached faster; (ii) strong reduction of the intracellular protein content, and (iii) increase of antioxidant enzyme activities. The most efficient SOD biosynthesis was achieved at the 1st maximum of activity in the culture grown under DO uncontrolled conditions.

## Introduction

Metabolism of oxygen produces reactive and potentially toxic species such as the superoxide anion radical ($O_2^-$), $H_2O_2$ and the hydroxyl radical (Sies, 1993; Crockford *et al.*, 1996). The aerobic organisms maintain a strong defense against activated oxygen. Superoxide dismutases (SODs), which catalyze the conversion of superoxide radicals into $H_2O_2$ and molecular oxygen, are part of the cellular defense system (Hassan, 1989). Bacteria generally contain a manganese type of SOD (MnSOD), an iron type (FeSOD), or both in the cytoplasm (Hassan, 1989). Copper-zinc SOD (Cu/ZnSOD), the third type, is generally found only in eukaryotes (Beyer *et al.*, 1991).

SOD expression is markedly enhanced by increasing intracellular production of $O_2^-$ under the following conditions: (i) increasing dissolved oxygen concentration (DO) in the growth medium; (ii) increasing the specific growth rate; and (iii) addition of redox-active compounds (Hassan, 1983). The effect of DO on the SOD biosynthesis has been documented for prokaryotes (Hassan, 1983; Gregory and Fridovich, 1973 and Vercellone *et al.*, 1990) and some lower eukaryotes including yeasts (Shilova *et al.*, 1989), but it is much less clear in the case of filamentous fungi.

In a previous work (Angelova *et al.*, 1993), a strain *Humicola lutea* 110 was selected for its ability to release high level of SOD activity in the cells. *Humicola lutea* belongs to filamentous fungi, multicellular lower eukaryotes. These microorganisms are suitable for a SOD investigation because of their potential advantages, abundant mycelium, intensive respiration and high level of cyanide-resistant respiration (Sakajo *et al.*, 1993; Moore and Siedow, 1991), which are prerequisites for the generation of reactive oxygen species (Hassan and Fridovich, 1979). We have also reported the optimal culture conditions for the production of both SOD and biomass in shake-flask culture (Angelova *et al.*, 1996a). Evidences from this strain have shown an association between increase $O_2^-$ generation and the enhanced intracellular SOD activity by induction of *de novo* protein synthesis (Angelova *et al.*, 1995; Angelova *et al.*, 1996b).

The objective of the present study was to investigate the effect of DO on SOD biosynthesis by the strain *H. lutea* 110 grown in a bioreactor.

## Materials and Methods

### *Microorganism*

The fungal strain, *H. lutea* 110 from the Mycological Collection of the Institute of Microbiology, Sofia was used throughout and maintained at 4 °C

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on beer agar pH 6.3 (half-diluted brewer's wort and 2% agar, Fassatiova, 1986).

*Cultivation, equipment and conditions*

Cultivation was performed in a 3-l bioreactor ABR-09 developed and realized by the former Central Laboratory for Bioinstrumentation and Automatisation. (CLBA) of the Bulgarian Academy of Sciences. The bioreactor was equipped with a pH monitoring system, an automatic DO monitoring and controlling system.

The compositions of the culture media were as described earlier (Angelova *et al.*, 1996a). For the inoculum, 80 ml of seed medium was inoculated with 5 ml of spore suspension at a concentration of $2x10^8$ spores/ml in 500-ml Erlenmeyer flasks. The cultivation was performed on a shaker (220 rpm) at 30°C for 24 h. For bioreactor cultures, 150 ml (8% v/v) of the seed culture were inoculated into 3-l bioreactor containing 1850 ml of the production medium. The cultures were grown at 30°C for 120 h. The fermentation parameters under DO uncontrolled conditions were an impeller speed 500 rpm and an air flow of 1 vvm. In this case, the changes in DO levels during fermentation were only measured. For DO controlled culture system aeration and impeller speed were regulated to produce 20, 35, 50 or 60% oxygen saturation in the liquid.

*Analytical methods*

The cell-free extract was prepared as described earlier (Angelova *et al.*, 1995). The SOD activity was measured by the nitro blue tetrazolium (NBT) reduction method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of SOD required for inhibition of the reduction of NBT by 50%, and was expressed as units per mg protein (U/mg protein). Catalase was assayed by the method of Beers and Sizer (1952). Protein was estimated by the Lowry procedure (Lowry *et al.*, 1951), with crystalline bovine albumin as standard. The dry weight of the mycelium was determined after washing of the biomass and subsequently drying it to a constant weight at 105 °C.

The results obtained in this investigation were evaluated from repeated experiments using three or five parallel samples.

## Results and Discussion

*Growth and enzyme production in DO uncontrolled system*

We have previously reported that maximum SOD activity in shake-flask culture of *Humicola lutea* 110 is estimated to be about 120 U/mg protein (Angelova *et al.*, 1996a). To produce SOD in large quantities, we examined the cultivation of submerged culture in a bioreactor. The initial experiments were carried out under DO uncontrolled conditions. Time courses of dry mycelium weight, SOD and catalase production, DO and intracellular protein content during the cultivation are shown in Fig. 1 for a typical fungal growth experiment. Biomass amount increased rapidly without lag period. The stationary phase was reached after 36–42 h of growth, when the glucose and

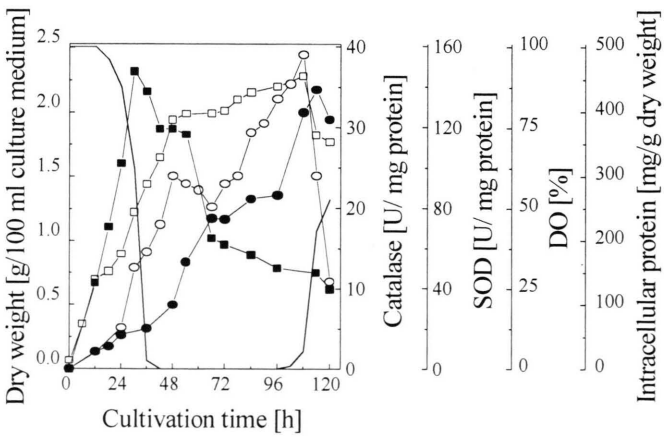


Fig. 1. Time courses of mycelia growth (□), SOD (○) and catalase (●) production, dissolved oxigen concentration (DO) (—) and intracellular protein content (■) in *H. lutea* culture grown under DO uncontrolled conditions.

nitrogen content (data not shown) were exhausted.

Because of the pronounced consumption of dissolved oxygen in the exponential phase, the DO level decreased sharply up to 36 h. For the period among 42 and 102 h, the DO level oscillated around zero and then displayed an increasing tendency. The sudden drop of oxygen concentration might be attributed to the require oxygen for cell metabolism (Petruccioli *et al.*, 1995). On the other hand, high oxygen consumption took place in relation to growth also. The most intensive consumption was detected during the exponential and early stationary phases. In fact, when the culture entered on a later half of the stationary phase and the lethal phase, the DO concentration rose again. The same observation was made by Petrucchioli *et al.* (1995) and Friedrich *et al.* (1994) during the growth of *Penicillium variabile* and *Aspergillus niger*, respectively.

SOD and catalase synthesis started while the cells were still growing and DO level was high. Catalase activity increased up to 114 h. SOD was produced in two steps; first during the exponential phase, with a maximum at 54 h (96.6 U/mg protein), followed by a slight decrease, and second during the late stationary phase, reaching a maximum at 108 h – 156.6 U/mg protein. Therefore, SOD activity of the bioreactor culture was about 25% higher in comparison with that of the shake-flask culture.

It is interesting to note that the active SOD synthesis was carried out during a period of intensive oxygen consumption. Furthermore, a rising tendency in DO level during the later period of cell cultivation coincided with a sharp decrease in SOD activity. As is known, the respiration accounts for a substantial portion of $O_2^-$ production in eukaryotic organisms (Guidot *et al.*, 1993). *H. lutea* minimizes the toxicity of $O_2^-$ by inducing SOD production to scavenge these radicals. In this case, a maximum of catalase activity was also established, because $O_2^-$ generated intracellularly will, by dismutation, give rise to a higher level of $H_2O_2$ (Hassan and Fridovich, 1979).

The amount of intracellular protein increased sharply in the early exponential phase when nutrient consumption (data not shown) was high and cell growth took place. The intracellular protein content showed a maximum at 30–36 h (457 mg/g dry weight), followed by a significant reduction, when the fermentation reached the stationary phase and biomass was relatively constant.

### *Effect of DO control on the growth and enzyme production*

As mentioned above, growth of the fungal strain *H. lutea* 110 and active SOD biosynthesis correlated with a significant drop in the DO level implying that oxygen demand and consumption was raised, and thus, the need to investigate the effect of controlled DO. Experiments have been conducted at various DO level ranging from 20–60% to evaluate their effect on the growth and biosynthesis of the antioxidant enzymes.

The effects of increasing DO levels on the biomass production and intracellular protein content are shown in the Fig. 2. The results showed that the growth of the fungal strain was significantly influenced by DO concentrations. The controlling DO from 20 to 60% lead to strongly reduction in duration of the stationary phase as well as to decrease of the biomass amount in the late metabolite stage (lethal phase of growth). In the same time, maximum dry weight showed negligible difference among four DO levels.

The changes in the growth of *H. lutea* at high DO level correspond to the similar tendency observed in the culture of *Saccharomyces cerevisiae* (Guidot *et al.*, 1993). On the other hand, the contradictory data about the effect of high DO level on microbial growth have been demonstrated. Roshkova *et al.* (1992) reported that the growth of *Kluyveromyces lactis* MP-11 increased with the increasing aeration and stirring. In contrast, under similar conditions, growth of *Penicillium variable* (P16) was reduced (Petruccioli *et al.*, 1995).

It is important to note that the production of intracellular protein was in significant response to the DO level. Controlling DO at 60% caused a 2-fold lower in protein content compared with that of DO at 20%. Several studies suggested that the oxygen radicals generated under hyperoxic conditions caused a rapid protein damage and degradation (Davies, 1987).

To evaluate the effect of DO level on the antioxidant enzyme synthesis, activities of SOD and catalase in *H. lutea* mycelium were determined (Fig. 3). It is evident from the data that both en-

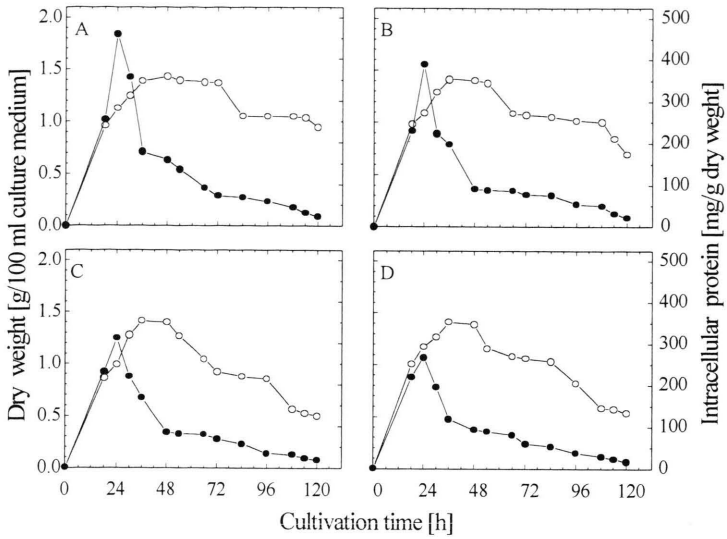


Fig. 2. Effect of increasing dissolved oxigen concentration (DO) levels on the biomass production (○) and intracellular protein content (●). A – 20% DO; B – 35% DO; C – 50% DO; D – 60% DO.

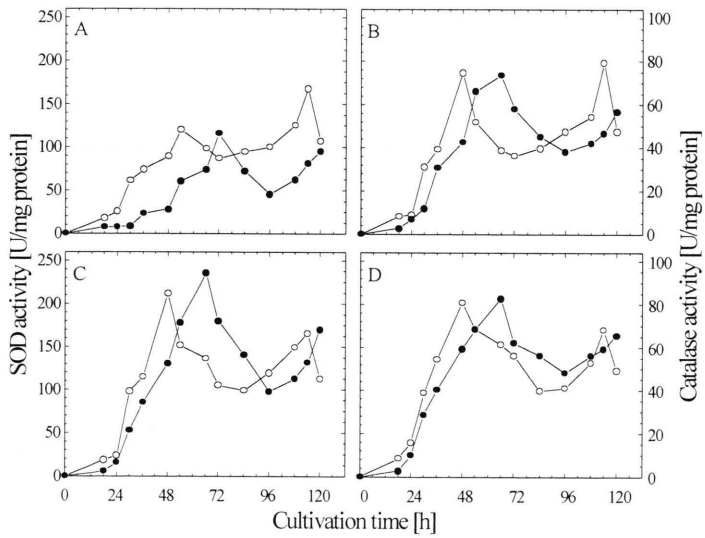


Fig. 3. SOD (○) and catalase (●) production depending on the controlled dissolved oxigen concentration (DO) levels. A – 20% DO; B – 35% DO; C – 50% DO; D – 60% DO.

zyme activities increased with increasing DO level from 20 to 50%. The maximum value was attained when DO level was controlled at 50%. At this DO, maximum SOD and catalase activities were 211 U/mg protein and 94 U/mg protein respectively, and above 50% DO level, they remained almost unchanged. In all cases, the time course of SOD production shows two maxima. The increasing DO level resulted in an earlier achievement of the 1-st maximum value (from 54-th h at 48-th h), as well as almost 2-fold increase in the activity in this point. The secondary increase in SOD activity during the late stationary stage is at first sight surprising. A similar phenomenon has been observed for SOD and catalase production by *Candida maltosa* (Shilova *et al.*, 1989). It can be explained by an intensification of the processes of $O_2^-$ generation when the cells utilize endogenous sources of carbon and nitrogen (organic or amino acids).

According to some reports (Vercellone *et al.*, 1990; Valdiva *et al.*, 1983) DO can influence production of antioxidant enzymes in the microbial cells. SOD and catalase activity increased 1.5- and 2.2-fold respectively in the cells of *Campylobacter*

*jejuni* MC711–01, cultured at 21% DO compared to the effects of 6% DO (Vercellone *et al.*, 1990). These results are in contrast to studies of *Candida maltosa*, in which the cells exhibited decreased level of SOD at high DO concentration compared to the low DO concentration (Shilova *et al.*, 1989). Our results showed that under hyperoxic conditions, when *H. lutea* cells were exposed to an atmosphere of 20–60% $O_2$, the level of antioxidant enzymes significantly increased. The alterations observed could be result of altered respiratory chain activity with a concomitant change in the rate of $O_2^-$generation (Hassan, 1983). Several investigators (Hassan, 1983, Gort and Imlay, 1998) have proposed that $O_2^-$ directly or indirectly may regulate SOD biosynthesis under conditions of oxidative stress (high steady-state concentrations of partially reduced oxygen intermediates).

*Comparison of the efficiency of SOD biosynthesis under conditions of uncontrolled and controlled DO level*

A comparison of DO controlled cultures with a DO uncontrolled culture is given in Table I. The yields of protein and total SOD activity were highest at the 1st maximum of activity in the culture grown under DO uncontrolled conditions. Though specific SOD activity was higher at the 2nd maximum, the total enzyme activity was only 77% of that in the 1st maximum. Furthermore, the protein content and cell mass productivity were markedly lower (2.5- and 1.9-fold respectively) as compared with the 1st maximum. A similar efficiency has been reached at the 1st maxima in every variant with DO controlled level. Interestingly, while the specific SOD activity at 1st and 2nd maxima in DO controlled cultures were comparable, the total SOD activity at the 2nd maxima decreased sharply, because of reduction of intracellular protein content.

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Table I. Comparison of the efficiency of SOD biosynthesis[a].

| Cultivation system | Cultivation time [h] | D. W. [g/l] | Cell mass productivity[b] [g d.w./lxh] | Yield of protein [mg/g d.w.] | Specific SOD activity[c] [U/mg protein] | SOD productivity[b] [U/lxh]x$10^2$ | Total SOD activity [U/l] x$10^4$ |
|---|---|---|---|---|---|---|---|
| Uncontrolled DO | | | | | | | |
| 1st maximum[d] | 54 | 19.4 | 0.36 | 369.5 | 96.2 | 127.9 | 69.1 |
| 2nd maximum[e] | 108 | 22.8 | 0.21 | 149.3 | 156.2 | 49.4 | 53.4 |
| Controlled DO 20% | | | | | | | |
| 1st maximum[d] | 54 | 13.9 | 0.26 | 134.6 | 120.2 | 41.8 | 22.6 |
| 2nd maximum[e] | 114 | 10.4 | 0.09 | 30.8 | 168.2 | 4.7 | 5.4 |
| Controlled DO 35% | | | | | | | |
| 1st maximum[d] | 48 | 14.1 | 0.29 | 89.7 | 186.7 | 49.1 | 23.6 |
| 2nd maximum[e] | 114 | 8.44 | 0.07 | 29.9 | 198.3 | 4.4 | 5.0 |
| Controlled DO 50% | | | | | | | |
| 1st maximum[d] | 48 | 14.0 | 0.29 | 85.2 | 211.9 | 52.7 | 25.3 |
| 2nd maximum[e] | 114 | 5.2 | 0.05 | 22.1 | 165.4 | 1.7 | 1.9 |
| Controlled DO 60% | | | | | | | |
| 1st maximum[d] | 48 | 13.9 | 0.29 | 92.6 | 201.5 | 54.0 | 25.9 |
| 2nd maximum[e] | 114 | 5.7 | 0.05 | 22.5 | 170.2 | 1.9 | 2.2 |

[a] The data are calculated from Fig. 1, 2 and 3; [b] The mean of values over the period "cultivation time"; [c] Activities at the time of harvest; [d] 1st maximum of SOD activity; [e] 2nd maximum of SOD activity. d. w. – dry weight of biomass.

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