Superoxide Dismutase during Glucose Repression of *Hansenula polymorpha* CBS 4732

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Hansenula polymorpha CBS 4732 was studied during cultivation on methanol and different glucose concentrations. Activities of Cu/Zn and Mn superoxide dismutase, catalase and methanol oxidase were investigated. During cultivation on methanol, increased superoxide dismutase and catalase activities and an induced methanol oxidase were achieved. Transfer of a methanol grown culture to medium with a high glucose concentration caused growth inhibition, low consumption of carbon, nitrogen and phosphate substrates, methanol oxidase inactivation as well as decrease of catalase activity ($21.8 \pm 0.61\ \Delta E_{240} \times min^{-1} \times mg\ protein^{-1}$). At the same time, a high value for superoxide dismutase enzyme was found ($42.9 \pm 0.98\ U \times mg\ protein^{-1}$, 25% of which was represented by Mn superoxide dismutase and 75% – by the Cu/Zn type). During derepression methanol oxidase was negligible ($0.005 \pm 0.0001\ U \times mg\ protein^{-1}$), catalase tended to be the same as in the repressed culture, while superoxide dismutase activity increased considerably ($63.67 \pm 1.72\ U \times mg\ protein^{-1}$, 69% belonging to the Cu/Zn containing enzyme).

Apparently, the cycle of growth inhibition and reactivation of *Hansenula polymorpha* CBS 4732 cells is strongly connected with the activity of the enzyme superoxide dismutase.

Introduction

The metabolism of methylotrophic yeasts has been intensively investigated as well as their physiology (Hopkins and Muller, 1987), biochemistry (Harder, 1990; van der Klei *et al.*, 1991) and genetics (Gellisen and Hollenberg, 1997; Gleeson and Sudbery, 1998; Sibirni *et al.*, 1998; Stasyk *et al.*, 1999). Some of them, especially *Hansenula polymorpha*, are reliable models for studying of glucose repression mechanism in yeasts, having a perfect glucose repressed metabolic system (enzymes and organelles involved in methanol oxidation) (Alamae *et al.*, 1996; van Dijk *et al.*, 2000; Gellisen *et al.*, 1999; Hansen and Hollenberg, 1996; Van der Bremt *et al.*, 1999). Regulation of methanol oxidase (MOX) through glucose inactivation was proved by Bruinenberg *et al.* (1982) and Roggenkamp (1988).

As the process of glucose repression of methanol grown cells is connected with disappearance of the peroxisomal structures abundantly present before, this damage of the organelles is a consequence of metabolic stress (Attfield, 1997). It is known that the stress-associated damages are connected with formation of reactive oxygen species (ROS). Detoxification of the cells depends upon some key factors among which is the very important enzyme defence system, responsible for scavenging of ROS – superoxide dismutase and catalase (Fridovich, 1972, 1973, 1986; Calabrese *et al.*, 1991). Having in mind the essential role of the enzymes SOD and CAT in the cellular defence system and in order to study the effect of glucose repression on these enzymes, the methylotrophic yeast *Hansenula polymorpha* CBS 4732 was investigated.

Abbreviations: CAT, catalase; MOX, methanol oxidase; SOD, superoxide dismutase.

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Materials and Methods

Microorganisms and culture media

All experiments were performed with *H. polymorpha* CBS 4732 strain from the collection of the Institute of Microbiology, Bulgarian Academy of Science. YNB (Yeast Nitrogen Base, Bacto®, Difco) supplemented with 1% methanol or different concentrations of glucose (1% to 8%), and YPD (1% yeast extract, 2% bacto-peptone, 2% glucose (Difco)) media were used.

Cultivation

A loopful of culture was used to inoculate YPD liquid medium and it was grown overnight. Cells were harvested by centrifugation, washed twice and suspended in distilled water. The suspension, containing 2–3 mg cells (dry weight) per ml was used to inoculate fresh YNB medium with 1% methanol. The culture was harvested in late exponential phase, transferred to YNB supplemented with 8% glucose, and cultivated for 28 h. Glucose inactivated cells were transferred after centrifugation to YNB, supplemented with 1% glucose. Samples were taken at 16, 20 and 24 h of cultivation and kept for further analyses.

Preparation of cell-free extracts

The harvested biomass was washed twice with distilled water and once with 0.5 M potassium phosphate buffer (PPB), pH 7.8, resuspended in a small amount of the same buffer followed by mechanical disruption in a vibration homogenizer VHG1 (B. Braun, Biotech International Melsungen, Germany) with glass beads. The homogenates were centrifuged at $3000 \times g$ for 10 min and after freezing and thawing – at $13000 \times g$ for 15 min at 4 °C, to obtain cell-free extracts for enzyme analyses.

Enzyme assays

SOD assay was performed according to Beauchamp and Fridovich (1971). One unit of activity was expressed as the amount of cell-free protein that caused 50% inhibition to the reduction rate of nitroblue tetrazolium (NBT) to blue formazan under the test conditions (reaction mixture, containing riboflavin, L-methionine and NBT, 25 °C, pH 7.8). Cu/Zn and Mn type of SOD enzyme were distinguished by inhibition with 2 mM KCN.

Catalase activity was determined spectrophotometrically according to Aebi (1981). Enzyme activity was expressed as $\Delta E_{240} \times \text{min}^{-1} \times \text{mg protein}^{-1}$.

Methanol oxidase activity was measured, using Nash reagent (Chrastil and Wilson, 1975). One unit was expressed as the amount of cell-free protein that caused formation of 1 μmol HCHO within 1 min in the test system (Nash reagent, 0.25–10 μmol methanol, cell-free extract) at 25 °C, pH 7.5.

Analysis of glucose, nitrogen, phosphate and methanol

Glucose was determined by the method of Somogy (1952) and Nelson (1944) on samples with cell mass removed by centrifugation. Nitrogen and phosphate concentrations were measured according to the procedures described by Herbert *et al.* (1971). Residual methanol concentration was determined according to Dawes *et al.* (1971).

Assay methods

Growth was monitored by the optical density at 610 nm (OD_{610}). Cell dry weight was determined gravimetrically, after drying washed cells to constant weight at 105 °C. Growth yield (Y_s) and maintenance coefficient (m_s) values were calculated, as described by Pirt (1975). Protein concentration was determined according to Lowry *et al.* (1951) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis

PAGE was performed on 10% vertical gel according to the method of Davis (1964), using Mighty Small II vertical electrophoresis (Hoefer). SOD activity was visualized by soaking the gels in 10 mM PPB, pH 7.2, containing 0.1 mM riboflavin and 2 mM dianisidine-HCl for 1 h at room temperature in dark, followed by brief rinsing with distilled water and illumination for 15 min, as described by Misra and Fridovich (1977). As a result, brown bands of SOD isoenzymes appeared on a pale yellow background. The type of SOD enzyme was detected by inhibition with 2 mM KCN for one hour before staining of the gels. Mn SOD from

Bacillus stearothermophilus (Sigma St. Louis, MO, USA) was used as a standard.

Results and Discussion

Growth of Hansenula polymorpha CBS 4732 on methanol

Hansenula polymorpha CBS 4732 was cultivated on YNB medium with 1% methanol, and growth yield Y_s was found out to be 0.418 ± 0.01 g × g^{-1}. The activities of methanol oxidase (MOX), catalase (CAT) and superoxide dismutase (SOD) were also determined (Fig. 1).

There is a correlation between growth and the above mentioned activities. The value of CAT activity at the end of the exponential growth phase (16 h) was $63.1 \pm 1.26\ \Delta E_{240} \times min^{-1} \times mg\ protein^{-1}$ and that of MOX – 0.6 ± 0.015 U × mg $protein^{-1}$. Such correlation between MOX and CAT activities and growth has been observed by other authors (Eggeling and Sahm, 1978). A similar relationship between growth and SOD activity with the maximal value at the end of the exponential growth phase was shown for *Candida boidinii* (Kujumdzieva-Savova *et al.*, 1985).

A control experiment has been performed through cultivation on YNB medium with 1% glucose. Total SOD activity was determined at the end of the exponential growth. As shown in Fig. 2, a 2-fold higher SOD activity was measured in methanol grown cultures compared to that grown with glucose. Inhibition experiments with KCN showed the presence of Cu/Zn and Mn SOD during cultivation on both substrates. The percentage of Mn SOD increased nearly two fold (43.5% on methanol against 27.8% on glucose). Obviously,

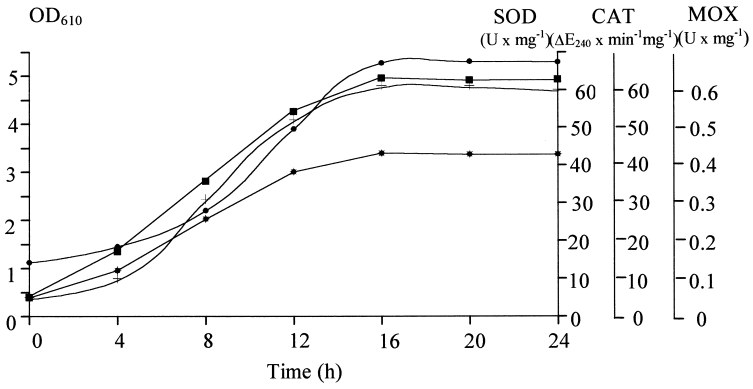

Fig. 1. Growth of *H. polymorpha* CBS 4732 on YNB + 1% methanol (-•-) and specific enzyme activities: MOX (-+-), SOD (-*-) and CAT (-■-).

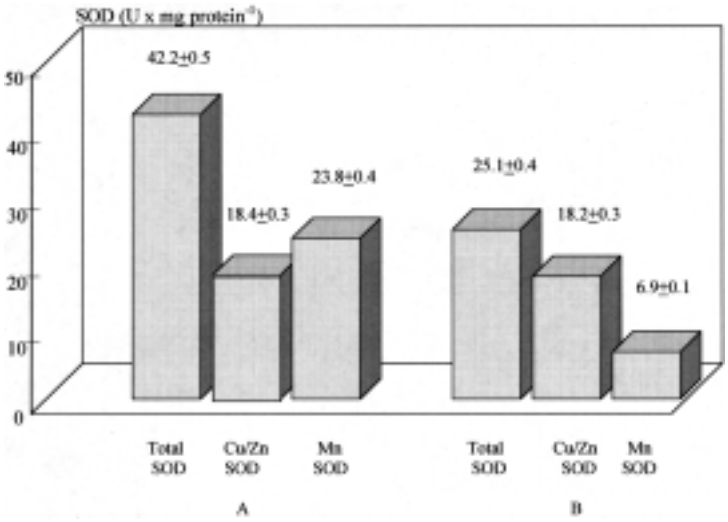

Fig. 2. Cu/Zn and Mn SOD activity during cultivation of *H. polymorpha* CBS 4732 on YNB + 1% methanol (A) and YNB + 1% glucose (B).

the Mn SOD is connected with oxidation of methanol by yeasts (van Dijken *et al.*, 1975).

Effect of high glucose concentration on methanol grown culture

A methanol grown culture was used as a model system to investigate the fate of the antioxidant enzyme system (SOD / CAT activity), when cells were subjected to high glucose concentration. Roggenkamp (1988) proved a catabolic inactivation of MOX during the transfer of methanol grown *Hansenula polymorpha* wild type to YNB medium with 5% glucose. To achieve the same status of the culture, methanol grown cells of *Hansenula polymorpha* CBS 4732 were transferred to YNB medium with different glucose concentrations (from 1 to 8%). After a 20 h cultivation under aeration a glucose concentration of 8% caused complete disappearance of MOX activity (Fig. 3). The kinetics of cell growth at these conditions indicated growth inhibition. Utilization of glucose was very low (only 33.7% at the 28 h). The same tendency was observed for the consumption of phosphate and nitrogen sources (15.1% and 18% respectively). Approximately 97.5% of the cells were viable and budding of the culture was negligible. Apparently, disappearance of MOX activity caused by 8% glucose concentration in the nutrient medium was connected with the growth inhibition.

The total activity of superoxide dismutase was determined. With 42.01 ± 0.38 U × mg protein^{-1}, it was found similar to that measured in the preculture on methanol. After inhibition with 2 mM KCN the residual activity was about 25% from the total one, i.e. 9.89 ± 0.23 U × mg protein^{-1} for the Mn SOD enzyme. Compared with the percentage of Mn SOD on methanol grown culture, it decreased about two-fold after cultivation on 8% glucose. The major part of the SOD activity after growth inhibition belongs to Cu/Zn type (33.1 ± 0.76 U × mg protein^{-1}). For investigation of both types of enzyme, a PAGE was performed (Fig. 4). Two bands of Mn SOD with relative mobility (Rm) values of 0.24 and 0.4 and a major band of Cu/Zn SOD (Rm = 0.13) were observed. Apparently, a high glucose concentration, causing growth inhibition and disappearance of the MOX enzyme did not influence the total SOD biosynthesis. Presumably, the activity decrease of the Mn SOD enzyme is due to reduction of the number of mitochondria, where this enzyme is located in the eukaryotic cell (Fridovich, 1978). A considerable reduction of mitochondria by high glucose concentration was observed by electron-microscope studies of Veenhuis and Harder (1988). As the total SOD activity remained on a high level, it could be speculated that the high glucose concentration caused formation of some reactive oxygen radicals in the cells. Thus, the maintained high SOD enzyme activity may protect cellular structures against reactive oxygen species.

Catalase activity was also investigated under the same cultivation conditions. The CAT activity in repressed cells was 21.8 ± 0.61 $\Delta E_{240} \times \text{min}^{-1} \times$ mg protein^{-1}. It strongly decreased in comparison to that measured in the methanol grown culture (about 3 fold). This considerable decrease may be connected with degradation of peroxisomes during

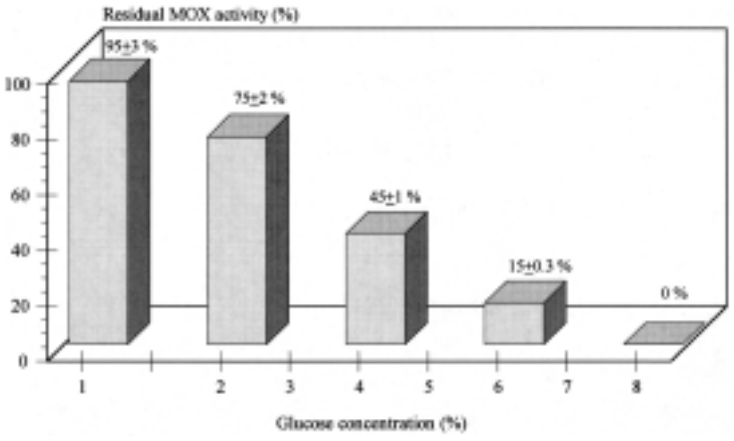

Fig. 3. Determination of glucose concentration causing catabolite repression of *H. polymorpha* CBS 4732 after cultivation for 20 h, using MOX as a marker enzyme.

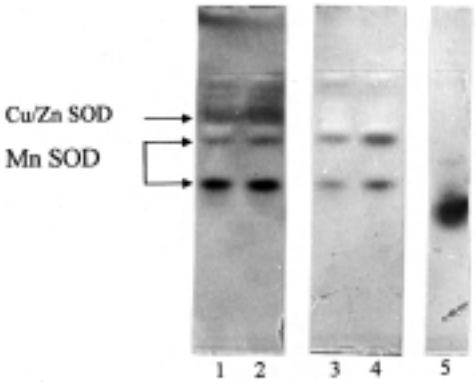

Fig. 4. Electrophoretic migration patterns of SOD of *H. polymorpha* CBS 4732 cultivated on 1: 8% glucose for 20 h; 2: 1% glucose for 16 h; 3 and 4: the same like 1 and 2 inhibited with 2 mM KCN as described in Materials and Methods; 5: control, Mn SOD from *B. stearothermophilus* inhibited in the same way.

the cultivation on 8% glucose (Roggenkamp, 1988).

Superoxide dismutase and catalase in derepressed cells of H. polymorpha CBS 4732

As shown by Eggeling and Sahm (1978), after repression of yeast cells with a high glucose concentration, a derepression takes place in batch culture with 1% glucose as a carbon source. *H. polymorpha* CBS 4732 already repressed with 8% glucose, was transferred to YNB medium with 1% glucose and growth was determined for 24 h. Glucose was consumed slowly, and the Y_s was about $0.24 \pm 0.01\ g \times g^{-1}$. As *H. polymorpha* is considered an oxidative type of yeast (Kappeli, 1986), this low growth yield was not due to fermentative utilization of glucose, as it is generally observed with the well-known facultative anaerobe *Saccharomyces cerevisiae*. The high value of the maintenance coefficient (m_s) $0.44\ g \times g^{-1} \times h^{-1}$ indicated that a considerable part of the substrate was utilized for maintenance purposes (Pirt, 1975). After 16 h MOX activity was negligible (0.005 ± 0.0001 U × mg protein^{-1}), and the total SOD activity increased considerably (63.67 ± 1.72 U × mg protein^{-1}). During the same period CAT activity tended to a plateau (about 20 $\Delta E_{240} \times min^1 \times$ mg protein^{-1}). Having in mind the very slow growth, high m_s value and a very high SOD activity, probably a repair process takes place along the formation of substrate for the SOD enzyme.

Thirty one percent of the total SOD activity belonged to Mn SOD enzyme (24.06 ± 0.17 U × mg protein^{-1}), indicating some repair of mitochondria in comparison to the repressed cells. There was no change in the type, number and Rm values of the isoenzymes compared with the repressed culture (Fig. 4). Again, the major activity was due to Cu/Zn SOD enzyme.

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