J Ind Microbiol Biotechnol (2007) 34:27–34
DOI 10.1007/s10295-006-0158-4

ORIGINAL PAPER

Fermentative production of superoxide dismutase with *Kluyveromyces marxianus*

Clementina Dellomonaco · Alberto Amaretti · Simona Zanoni · Anna Pompei · Diego Matteuzzi · Maddalena Rossi

Received: 6 March 2006 / Accepted: 13 July 2006 / Published online: 15 August 2006

Abstract This work sought to develop a fermentative process for the microbial production of superoxide dismutase (SOD), to overcome extraction from animal tissues. Twenty-eight wild-type yeast strains were screened for SOD productivity. *Kluyveromyces marxianus* L3 showed the highest SOD activity (62 U mg^{-1}) and was used for process development. Oxidative stress conditions and parameters affecting oxygen transfer rate were exploited to improve production. The effects of dilution rate (0.067 vs 0.2 h^{-1}), aeration pressure (0.3 vs 1.2 bar) and H_2O_2 (0 vs 50 mM) were studied during chemostat experiments. Low dilution rate, high pressure and H_2O_2 resulted in an increase in CuZn–SOD up to 475 U mg^{-1}. When a regulation of oxygen saturation was applied during batch cultures, CuZn–SOD was progressively higher at 60, 80 and 90% dissolved oxygen tension (DOT) (250, 330 and 630 U mg^{-1}, respectively). Furthermore, the highest growth rate and biomass yield were achieved at 90% DOT, this being therefore the best DOT condition for high overall productivity. Growth and productivity on different carbon sources were compared. Specific activity was higher on glycerol than on lactose or glucose (496, 454 and 341 U mg^{-1}, respectively). The highest biomass yield was achieved on lactose. It may be therefore the best substrate for SOD production.

C. Dellomonaco · A. Amaretti · S. Zanoni · A. Pompei · D. Matteuzzi
Department of Pharmaceutical Sciences, University of Bologna, Bologna, Italy

M. Rossi (✉)
Department of Chemistry, University of Modena and Reggio Emilia, Via Campi 183, 41100 Modena, Italy
e-mail: rossi.maddalena@unimore.it

Introduction

Reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^·$) are toxic by-products of oxidative metabolism, since they are produced by the respiratory chain, by H_2O_2 generating oxidases or during stress conditions. Exposure to ionizing radiations or pro-oxidants, such as H_2O_2, paraquat or menadione, is also a source of ROS and leads to oxidative stress. Since ROS exert cytotoxic and mutagenic effects by peroxidation of the membrane fatty acids, protein oxidation and DNA damage, all aerobic or aerotolerant organisms have evolved defence mechanisms to prevent or repair ROS-mediated oxidative damages. Detoxification of ROS depends upon low molecular weight antioxidants and enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and several peroxidases.

SODs are metalloenzymes that detoxify superoxide radicals by conversion to hydrogen peroxide and oxygen. A wide range of therapeutic applications of SOD have been described. These include prevention of oncogenesis and tumor promotion [19], protection of tissues following infective, ischemic, traumatic or burn injuries [7, 23–25] and treatment of inflammatory diseases and arthritis [6, 26]. In addition to clinical purposes, SOD has been increasingly used in the last decade for the production of antioxidant cosmetics and dietary supplements such as capsules, tablets, milk, beer and beverages.

Since SOD is nowadays produced by extraction from animal tissues, mostly bovine liver or erythrocytes, a microbial production process could represent a significant improvement in terms of yields, costs, purity

and product safety. Some efforts had been already made toward production of SOD with filamentous fungi [1], but large-scale biotechnological processes have never been developed. The utilisation of GRAS (Generally Regarded as Safe) yeasts would prove very attractive, for industrial-scale fermentative production of food- or pharmaceutical-grade SOD.

Like most other eukaryotes, yeasts produce two SODs that protect mitochondrial and cytosolic constituents from oxidation. The copper- and zinc-containing SOD (CuZn–SOD) is encoded by *SOD1* and accounts for up to 90% of the total SOD. CuZn–SOD is primarily located in the cytosol, though a minor amount has been found in the mitochondrial intermembrane space [18, 22, 17]. The manganese-containing enzyme (Mn–SOD), encoded by the gene *SOD2*, is located in the mitochondrial matrix where it operates as the major superoxide scavenger, accounting for 5–15% of the total SOD [15, 17].

The regulation of oxidative stress defences in yeasts has been deeply studied and many factors affecting the expression of *SOD1* and *SOD2* genes have been characterized: respiratory metabolism, glucose depletion and oxidative or osmotic stress were found to be involved in SOD increase [5, 9–13, 17, 20–22]. Nevertheless, yeasts have not yet been proposed for the industrial production of SOD. The aim of this work is the development of a fermentative process for the production of SOD by yeasts. A preliminary screening of 28 wild-type strains sought to identify the yeast with the highest SOD activity to be used for the process optimization. Oxidative stress conditions and parameters affecting oxygen transfer rate were exploited to improve SOD production: specific activity was studied through chemostat and batch cultures as a function of carbon source, growth rate, dissolved oxygen tension (DOT), aeration pressure and presence of a ROS generator in the medium. Data concerning yield improvements in pilot-scale bioreactor are reported.

Materials and methods

Yeast strains and maintanance

Yeast strains were obtained from ATCC (American Type Culture Collection, Rockville, USA), CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands), NCYC (National Collection of Yeast Cultures, Norwich, UK) and DBVPG (Industrial Yeasts Collection, Department of Vegetal Biology, Perugia, Italy) or from our own collection. Yeasts were maintained on YPD-agar slants.

Chemicals and media

All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. Microbiological products were purchased from Difco Laboratories (Sparks, MD, USA).

Complex YPD medium contained: glucose, 20 g l^{-1}; Yeast extract, 10 g l^{-1}; Bacto-peptone, 20 g l^{-1}; autoclaved for 30 min at 110°C. Mineral SM medium contained: $(NH_4)_2SO_4$, 5 g l^{-1}; KH_2PO_4, 9.52 g l^{-1}; K_2HPO_4, 3.48 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1}; NaCl, 0.1 g l^{-1}; $CaCl_2 \cdot 2H_2O$, 1 mg l^{-1}; $MnSO_4 \cdot 7H_2O$, 0.4 mg l^{-1}; boric acid, 0.5 mg l^{-1}; KI 0.1 mg l^{-1}; $ZnSO4 \cdot 7H_2O$, 0.4 mg l^{-1}; $Na_2MoO_4 \cdot 2H_2O$, 0.2 mg l^{-1}; $FeCl_3 \cdot 6H_2O$, 0.2 mg l^{-1}; $CuSO_4 \cdot 5H_2O$ 40 μg l^{-1}; biotin 2 μg l^{-1}; Ca panthotenate, 0.4E-3; folic acid, 2 μg l^{-1}; niacin, 0.4 mg l^{-1}; *p*-aminobenzoic acid, 0.2 mg l^{-1}; pyridoxine HCl, 0.4 mg l^{-1}; riboflavin, 0.2 mg l^{-1}; thiamine HCl, 0.4 mg l^{-1}; autoclaved for 30 min at 110°C. Glucose, lactose or glycerol were used as carbon sources at the concentration of 24.0, 22.8 or 24.5 g l^{-1}, respectively, in order to achieve the same 0.8 M carbon atoms. The carbon source was autoclaved separately and added into the cultural medium.

Shake-flask cultivation

Batch cultures were carried out in 1 l Erlenmeyer flasks with 100 ml sterile medium. Flasks were inoculated (10% v/v) with exponential phase cultures grown in the same medium. Cultures were incubated at 30°C for 24 h in an orbital shaking incubator at 180 rpm, then broths were harvested for biomass measurement and SOD analysis.

Bioreactor cultivation

Batch and chemostat experiments were performed in a BM-PPS3 bioreactor (Solaris Biotech, Porto Mantovano, Italy). The fermenter was stirred by two Rushton turbines and was sparged using compressed air via a ring-shaped sparger at the base of the vessel. The bioreactor was equipped with InPro6800 and InPro3030 probes (Mettler Toledo, Switzerland) for the continuous measurement of dissolved oxygen concentration (DOT, expressed as percentage saturation) and pH, respectively. Foaming was controlled by the automatic addition of PPG2000 (Cyanamid, Catania, Italy). Temperature was kept to 30°C.

A 2.0 L working volume was used for batch cultures. Exponential phase seed-cultures grown in the same medium inoculated (10% v/v) the bioreactor. pH was controlled at 6.3 by automatic titration with 4 M NaOH. Stirring was kept to 500 rpm, air flow to

1.0 vvm (volume/volume/minute) and pressure to 0.3 bar. When a DOT cascade regulation was applied, stirring and air-flow were automatically adjusted in the range of 200–900 rpm and 0.5–2.0 vvm.

A 1.0 L working volume was used in chemostat experiments. Stirring was kept to 500 rpm and air-flow to 1.0 vvm. Dilution rates of 0.067 h^{-1} versus 0.2 h^{-1}, pressures of 0.3 versus 1.2 bar and 0 versus 50 mM H_2O_2 in the feeding tank were applied to study their effects on SOD specific activity during steady-state cultures. The culture grew batch-wise for 12–14 h before the feed and the waste pumps were started. Five residence times were allowed to elapse and steady-state was considered attained when pH and biomass concentration were constant for at least two residence times. Samples for SOD analysis were collected at steady-state and after a further residence time.

Preparation of cell-free extracts

Biomass was harvested by centrifugation (5,000×*g* for 10 min at 4°C), washed twice with PP buffer (potassium phosphate buffer pH 7.8, 50 mM; EDTA, 0.1 mM) and resuspended in the same buffer 1:1 w/v. Cells were disrupted for 30 min at 4°C by 0.3 mm glass beads in a vibration homogenizer at 1,800 rpm. Whole cells and debris were removed by centrifugation at 13,000×*g* for 15 min at 4°C and the supernatant was dialyzed for 16 h against PP buffer. Protein concentration was assayed according to Lowry [14] using bovine serum albumin as standard.

Enzyme activity

SOD activity was assayed by measuring the inhibition of epinephrine autoxidation, as described by Misra and Fridovich [16]. Bovine SOD (Sigma-Aldrich) was used as standard for the calibration curve in the range between 0 and 10 U ml^{-1}. One unit is defined as the amount of SOD that inhibits by 50% the reduction of nitro-blue tetrazolium [3]. Specific activity of cell-free extracts was expressed as enzymatic units per milligram of protein. CuZn–SOD was inactivated by adding 30 mM KCN into the reaction mixture in order to discriminate Mn-SOD activity. CuZn–SOD activity was calculated as difference between total and Mn–SOD activities.

Results

Screening of yeasts

SOD production was analyzed for 28 strains of 19 different species, after 24 h shake-flask cultivation on YPD medium. SOD specific activities are reported in Table 1 Fifteen strains produced less then 4 U mg^{-1} whereas 10 ranged among 4 and 16 U mg^{-1}. The highest activities ($P \leq 0.01$) were measured in the cell-free extracts of *Zygosaccharomyces mellis* DBVPG6928 (27.0 U mg^{-1}), *Saccharomyces exiguus* L10 (55.0 U mg^{-1}) and *Kluyveromyces marxianus* L3 (62.0 U mg^{-1}).

Batch cultures of *K.* marxianus L3

Biomass concentration, DOT and SOD specific activity were monitored during pH regulated batch fermentations of *K. marxianus* L3 in order to study growth and production kinetics. YPD and SM media containing 24 g L^{-1} glucose were compared. The culture grew on YPD medium with a specific growth rate of 0.28 h^{-1} and entered the stationary phase at about 12 h (Fig. 1). A peak in SOD specific activity was observed during the early exponential phase, then SOD decreased and, in the late exponential phase, increased again. The maximum specific activity (50 U mg^{-1}) was observed

Table 1 SOD specific activity of 28 yeast strains measured on cellular extracts after a 24 h growth in YPD medium containing 10 g L^{-1} glucose

Strain	SOD U mg^{-1}
Hansenula anomala HN5	0.5
Hansenula bejerinkii HN3	3.5
Hansenula bimundalis HN8	1.5
Hansenula ciferri HN6	0.1
Hansenula jadinii HN9	3.5
Kluyveromyces lactis L1	6.0
Kluyveromyces lactis L2	5.5
Kluyveromyces marxianus L3	62.0
Kluyveromyces lactis L4	10.0
Pichia angusta L8	13.0
Pichia anomala L6	0.6
Pichia anomala L7	1.7
Pichia burtomi P8	0.2
Pichia farinosa L9	1.4
Pichia fermentans P1	5.0
Pichia fermentans P1/2	5.7
Pichia guillermondi P4	0.1
Pichia stipitis L11	1.8
Saccharomyces cerevisiae L12	0.8
Saccharomyces cerevisiae L17	0.2
Saccharomyces cerevisiae L18	8.0
Saccharomyces cerevisiae L19	0.8
Saccharomyces cerevisiae L28	1.2
Saccharomyces exiguus L10	55.0
Zygosaccharomyces mellis DBVPG6928	27.0
Zygosaccharomyces rouxii DBVPG6187	11.0
Zygosaccharomyces rouxii DBVPG6399	14.0
Zygosaccharomyces rouxii DBVPG6459	7.0

The results are mean values from three separate experiments (standard deviations always less than 10%)

after 12 h, at the transition between exponential and stationary phases, achieving 140×10^3 U L^{-1}, corresponding to 11×10^3 U $(L\ h)^{-1}$ as the maximum volumetric productivity. *K. marxianus* L3 grew on SM medium with an early specific growth rate of 0.18 h^{-1}, gradually decelerated and entered the stationary phase at about 20 h (Fig. 1). The trend of specific activity was similar to the one observed on YPD medium. The maximum specific activity (245 U mg^{-1}) was measured after 20 h, at the transition between exponential and stationary phases, achieving 49×10^3 U $(L\ h)^{-1}$ as the maximum volumetric productivity. In both the media SOD specific activity was strongly related to DOT, as elicited by the comparison between SOD and DOT curves. The initial peak in SOD activity decreased during the exponential phase, until dissolved oxygen reached its minimum concentration. Then, the highest specific activity was observed in correspondence with DOT increase at the shift to stationary phase. Characterization of SOD activity of *K. marxianus* L3 demonstrated that CuZn–SOD accounted for more than 95% of total activity, independent of the growth phase and the culture medium.

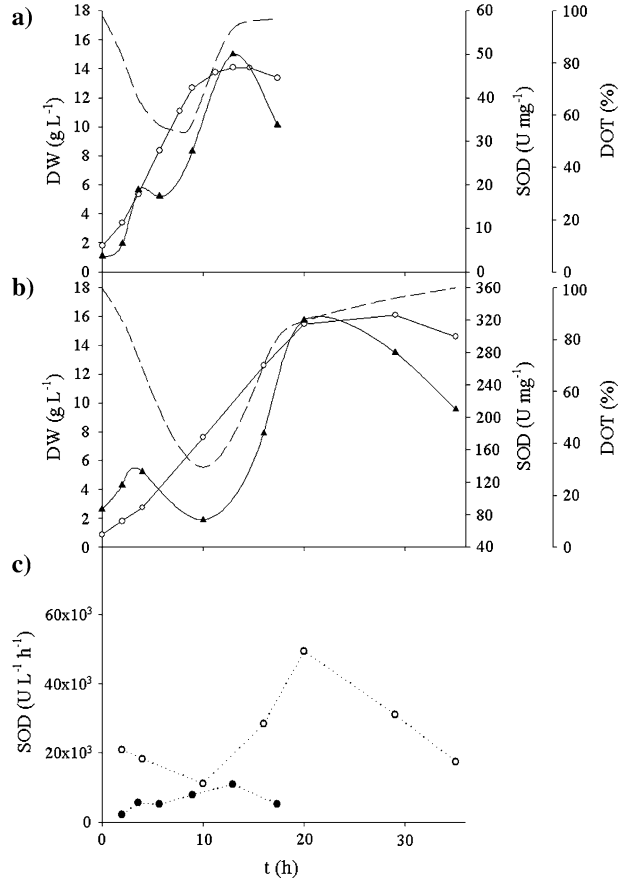

Fig. 1 **a** pH controlled batch fermentation of *K. marxianus* L3 in YPD medium with 24 g L^{-1} glucose. **b** pH controlled batch fermentation of *K. marxianus* L3 in SM medium with 24 g L^{-1} glucose. Symbols: *Open circle* DW, *Filled triangle* SOD specific activity in cell-free extracts, *Solid lie* DOT. **c** SOD volumetric productivity of *K. marxianus* L3 in YPD (*Filled circle*) and SM (*Open circle*) medium

Chemostat cultures of *K.* marxianus L3

Chemostat cultures of *K. marxianus* L3 were carried out in SM medium with glucose as the sole carbon source. The effects of dilution rate (0.067 vs 0.2 h^{-1}), aeration pressure (0.3 vs 1.2 bar) and H_2O_2 (0 vs 50 mM) on steady-state SOD activity were investigated (Table 2). SOD specific activity rose with the decrease in dilution rate or the increase in aeration pressure. Fifty mM H_2O_2 improved the specific activity. Dilution rate, pressure and H_2O_2 displayed additive effects: the highest SOD activity (475 U mg^{-1}) was obtained with 1.2 bar air pressure, 0.067 h^{-1} dilution rate and 50 mM H_2O_2. For all the steady-state samples, CuZn–SOD accounted for more than 95% of total activity.

Effects of DOT on SOD activity in *K.* marxianus L3

Cascade regulation of DOT was applied to batch cultures of *K. marxianus* L3 in order to study the relationship between oxygen concentration and SOD specific activity. Fermentations were carried out on SM medium with glucose. Throughout the fermentations DOT was kept constant to 60, 80 or 90%. As reported in Fig. 2 biomass growth rate and yield were positively affected by DOT. Specific growth rate was 0.07, 0.13 and 0.18 h^{-1} at 60, 80 and 90%, respectively. Biomass yield coefficients were 0.28, 0.40 and 0.54 at 60, 80 and 90 %, respectively. A relationship between DOT and SOD activity was observed. When DOT was changed from 60 to 80 and 90%, SOD activity increased from 250 to 330 and 620 U mg^{-1}, respectively. More than 95% activity was due to CuZn–SOD and was almost constant during the whole exponential phase and the beginning of stationary phase, and decreased with the

Table 2 Steady-state values of SOD specific activity (U mg^{-1}) in chemostat cultures of *K. marxianus* L3 under the following conditions: SM medium with 24 g L^{-1} glucose; dilution rate 0.067 or 0.2 h^{-1}; air pressure 0.3 or 1.2 bar; 0 or 50 mM H_2O_2

D (h^{-1})	H_2O_2 (mM)			
	0		50	
	p (bar)		*p* (bar)	
	0.3	1.2	0.3	1.2
0.2	3	12.6	37	65
0.067	109	342	195	475

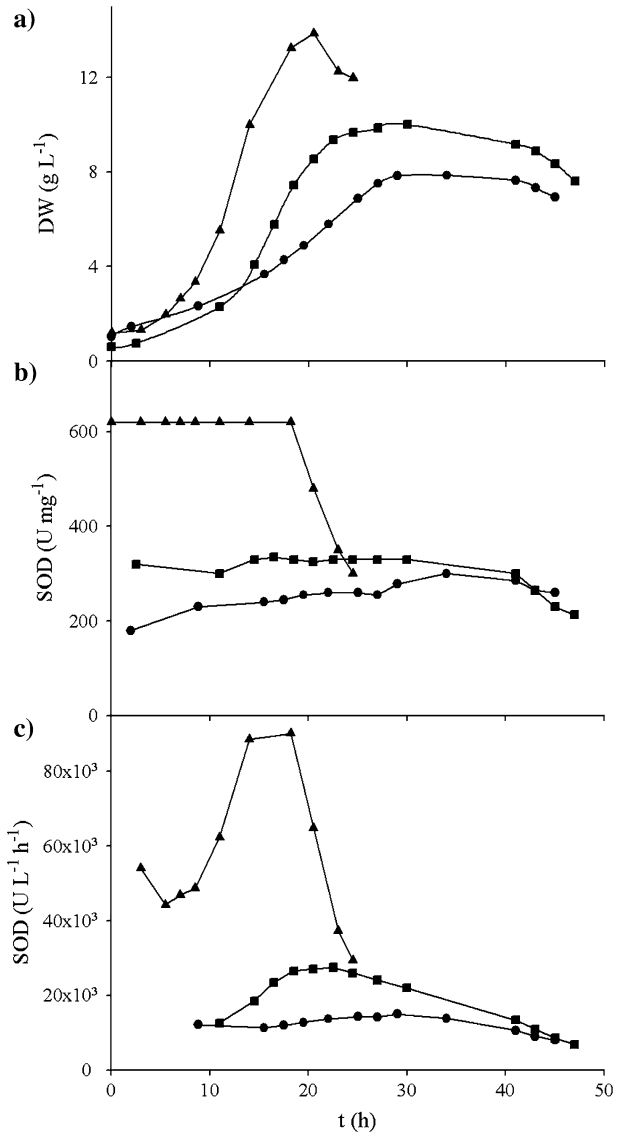

Fig. 2 Growth curves (**a**), SOD activity in cell-free extracts (**b**) and SOD volumetric productivity (**c**) in pH controlled batch fermentations of *K. marxianus* L3 in SM medium with glucose. DOT was regulated to 60% (*Filled circle*), 80% (*Filled square*), and 90% (*Filled triangle*)

biomass concentration during the death phase. Maximum volumetric productivity was 15×10^3 U $(L\ h)^{-1}$ at 27 h, 28×10^3 U $(L\ h)^{-1}$ at 22 h and 90×10^3 U $(L\ h)^{-1}$ at 18 h for 60, 80 and 90% DOT, respectively. Although 90% DOT positively affected biomass growth and SOD yields, oxygen exerted a high toxicity as demonstrated by the absence of any stationary phase and the rapid entry into the death phase.

Effects of the carbon source

Shake-flask cultures of *K. marxianus* L3 on SM medium were carried out using glucose, lactose or glycerol to monitor biomass concentration and SOD specific activity throughout exponential and stationary phases (Fig. 3). The same growth rate was obtained on all the carbon sources ($0.13\ h^{-1}$), but significant differences were observed in biomass yield coefficients: 0.58, 0.50 and 0.42 on lactose, glucose and glycerol, respectively. As observed in batch fermentations without DOT regulation, on all carbon sources SOD specific activity exhibited a peak during early exponential phase, then decreased and, at the late exponential phase, increased again, reaching the highest value at the stationary phase onset. Maximum specific activity on glycerol was higher ($496\ U\ mg^{-1}$) than on lactose and glucose (454 and $341\ U\ mg^{-1}$, respectively). CuZn–SOD accounted for more than 95% of total activity independently by the growth phase on all the carbon sources. Maximum volumetric productivity was 42×10^3 U $(L\ h)^{-1}$, 49×10^3 U $(L\ h)^{-1}$ and 70×10^3 U $(L\ h)^{-1}$ at 18 h for glucose, glycerol and lactose, respectively.

Discussion

Superoxide dismutase detoxifys superoxide radicals originating from respiratory metabolism and oxidative stress conditions. Hence, SOD from bovine liver and red blood cells is increasingly used for pharmaceutical applications and as a component of antioxidant dietary supplements. This work is aimed to develop a fermentative process for the production of SOD, in order to overcome extraction from animal tissues. Data pointed out the parameters that affect SOD production in *K. marxianus* L3 and represent a contribution toward application of yeasts for the biotechnological production of SOD. Twenty-eight wild-type yeast strains, representing 19 species, were preliminary screened for SOD production. *K. marxianus* L3 yielded the highest SOD activity ($62\ U\ mg^{-1}$) and was used for the process development.

Most information on SOD production in yeasts came from studies on *Saccharomyces cerevisiae*, stating that it was generally associated to the respiratory metabolism, as a consequence of ROS release in the mitochondrial electron transfer chain. In fact, the synthesis of CuZn–SOD and Mn–SOD was repressed during exponential growth of aerobic batch cultures of *S. cerevisiae*, growing mainly by fermentation [5, 17]. Enzyme activities increased during the diauxic shift, when respiratory adaptation occurred between ethanol production and consumption, and at the stationary phase, when all energy sources were depleted. Glucose depletion was involved in transcriptional activation of many genes encoding proteins involved in antioxidant

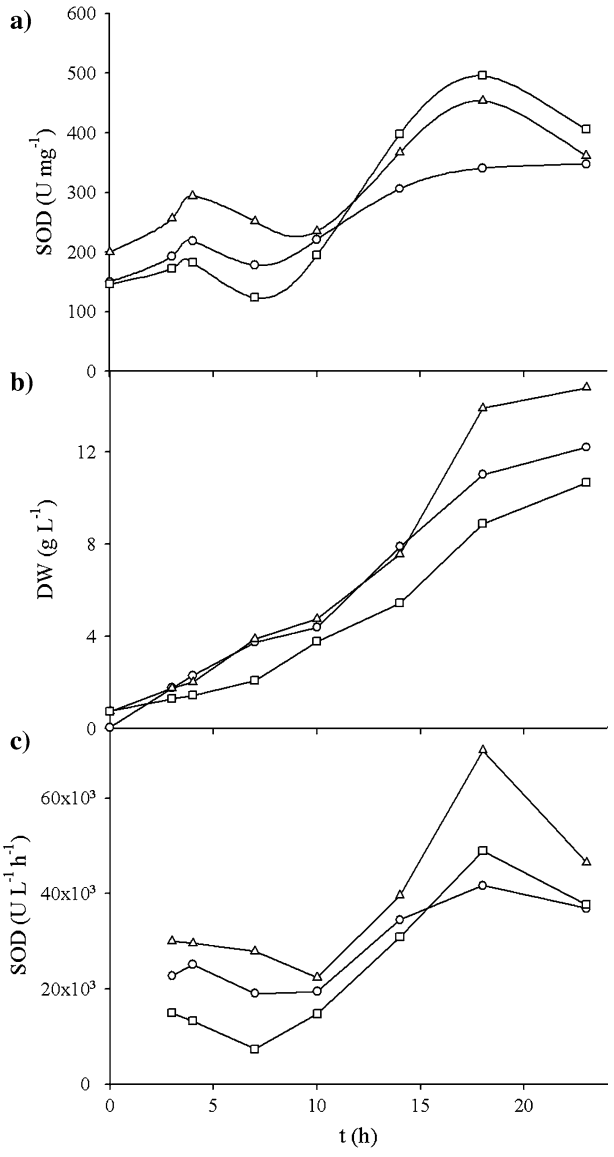

Fig. 3 SOD specific activity in cell-free extracts (**a**), growth curves (**b**) and SOD volumetric productivity (**c**) in shake-flask batch cultures of *K. marxianus* L3 in SM medium with glucose (*Open circle*), lactose (*Open triangle*) and glycerol (*Open square*) as sole carbon source

defences and it was responsible for the increase in both SOD activities at the diauxic shift [5, 17]. Oxygen limitation also decreased the translation of both *SOD1* and *SOD2* genes and resulted in low activities during the exponential phase in *S. cerevisiae* [9]. In stationary phase, *S. cerevisiae* mitochondria exhibited a burst of ROS production, and SODs were involved in protecting against aging process and stationary phase death [13, 22]. Moreover, post-translational changes were involved in increasing SOD activity at the shift between fermentative and respiratory phases, since O_2 or superoxide were required for activation of CuZn–SOD apoprotein by CCS, the copper chaperone for SOD1 in *S. cerevisiae* [4, 8].

During batch fermentations of *K. marxianus* L3 high specific activities were observed at the early exponential phase and at the onset of the stationary phase, suggesting that regulation of SOD in *K. marxianus* L3 may be triggered mostly by oxygen concentration. The highest specific activity was observed at the onset into stationary phase, as soon as DOT rose toward saturation. The sudden increase of SOD, as a response to the enhanced oxygen availability, could be consistent with a mechanism of post-translational activation of an existing apo-pool of CuZn–SOD [4].

Growth and productivity curves on mineral and complex medium were compared in this study. Both specific activity and volumetric productivity were 5-fold higher on mineral medium than on complex medium, hence the former was used for further process optimization.

Oxidative stress conditions and parameters affecting the oxygen transfer rate were analyzed in chemostat cultures and related to SOD production. Dilution rate (0.067 vs 0.2 h^{-1}), aeration pressure (0.3 vs 1.2 bar) and H_2O_2 (0 vs 50 mM) were combined in 8 different chemostat cultures for a comparison of steady-state SOD specific activity. Cultures growing at low dilution rate, at high pressure or in the presence of H_2O_2 showed the highest SOD levels. Such conditions increased specific activity up to 475 U mg^{-1}, when simultaneously applied. These results are in agreement with previous studies demonstrating that ROS-generating agents and/or conditions, in addition to the respiratory metabolism, caused oxidative stress and positively affected the production of SOD in yeasts. The exposure to H_2O_2, paraquat, pure oxygen or air pressure induced the oxidative stress response and increased both SOD activities in *S. cerevisiae* [9, 10], *K. marxianus* [20, 21] and filamentous fungi [2]. Since CuZn–SOD was also involved in resistance to ethanol toxicity [5] and hyperosmotic shocks in *S. cerevisiae* [10, 11], the investigation of the effects of both ethanol and hyperosmotic treatments on SOD production in *K. marxianus* L3 is of great interest too.

Although evidences of O_2 effectiveness in positive regulation of SOD were reported, the effects of DOT on SOD synthesis in yeasts have never been investigated. The present study seeks to fill this gap by comparing SOD specific activity during DOT controlled processes. The results demonstrated that DOT strongly affect the production of CuZn–SOD. In cultures with DOT controlled to 90%, SOD specific activity was higher than in the ones kept to 80 or 60% (630, 330 and 250 U mg^{-1}, respectively). Moreover SOD was

substantially constant throughout the whole exponential phase and the shift into stationary phase, confirming that glucose did not repress the enzyme production, while oxygen availability exerted a major role on SOD regulation. In addition to the highest specific activity, 90% of oxygen saturation led to the highest growth rate and biomass yield, and was therefore the best DOT condition to obtain a high overall volumetric productivity of CuZn–SOD [90×10^3 U $(L\ h)^{-1}$]. Nevertheless high oxygen concentration exerted a toxic effects and caused the culture to rapidly enter into the death phase.

A comparison on different carbon sources was established. The substitution of glucose with lactose or glycerol as the sole carbon source caused the maximum SOD specific activity to increase from 341 to 454 and 496 U mg^{-1}, respectively. Glycerol is a non-fermentable carbon source and it is expected that a greater amount of ROS is produced in mitochondria during growth on this substrate than on fermentable sugars. Even though it was a good substrate for growth of *K. marxianus* L3, glycerol displayed lower biomass yield if compared to glucose or lactose. Lactose was the best carbon source for growth of *K. marxianus* L3 and also yielded high SOD specific activity, corresponding to a productivity of 70×10^3 U $(L\ h)^{-1}$. Thus lactose is the best substrate to be used in a SOD-producing process.

The results of this study provide a better understanding of the parameters which affect SOD production in *K. marxianus* L3 and represent a first significant contribution toward the application of yeasts for an optimal biotechnological production of SOD.

References

1. Angelova M, Dolashka-Angelova P, Ivanova E, Serkedjieva J, Slokoska L, Pashova S, Toshkova R, Vassilev S, Simeonov I, Hartmann HJ, Stoeva S, Weser U, Voelter W (2001) A novel glycosylated Cu/Zn-containing superoxide dismutase: production and potential therapeutic effect. Microbiology 147:1641–150
2. Angelova MB, Pashova SB, Spasova BK, Vassilev SV, Slokoska LS (2005) Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. Mycol Res 109:150–158
3. Beuchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to polyacrylamide gels. Anal Biochem 44:276–287
4. Brown NM, Torres AS, Doan PE, O'Halloran TV (2004) Oxygen and the copper chaperone CCS regulate posttranslational activation of Cu,Zn superoxide dismutase. Proc Natl Acad Sci USA 101:5518–5523
5. Costa V, Amorim MA, Reis E, Quintanilha A, Moradas-Ferreira P (1997) Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. Microbiology 143:1649–1656
6. Corvo ML, Jorge JC, van't Hof R, Cruz ME, Crommelin DJ, Storm G (2002) Superoxide dismutase entrapped in long-circulating liposomes: formulation design and therapeutic activity in rat adjuvant arthritis. Biochim Biophys Acta 1564:227–236
7. Emerit J, Samuel D, Pavio N (2006) Cu-Zn super oxide dismutase as a potential antifibrotic drug for hepatitis C related fibrosis. Biomed Pharmacother 60:1–4
8. Galiazzo F, Ciriolo MR, Carri MT, Civitareale P, Marcocci L, Marmocchi F, Rotilio G (1991) Activation and induction by copper of Cu/Zn superoxide dismutase in *Saccharomyces cerevisiae.* Presence of an inactive proenzyme in anaerobic yeast. Eur J Biochem 196:545–549
9. Galiazzo F, Labbe-Bois R (1993) Regulation of Cu,Zn- and Mn-superoxide dismutase transcription in *Saccharomyces cerevisiae*. FEBS Lett 315:197–200
10. Garay-Arroyo A, Covarrubias AA, Clark I, Nino I, Gosset G, Martinez A. (2004) Response to different environmental stress conditions of industrial and laboratory *Saccharomyces cerevisiae* strains. Appl Microbiol Biotechnol 63:734–741
11. Garay-Arroyo A, Lledias F, Hansberg W, Covarrubias AA (2003) Cu,Zn-superoxide dismutase of *Saccharomyces cerevisiae* is required for resistance to hyperosmosis. FEBS Lett 539:68–72
12. Hristozova T, Rasheva T, Nedeva T, Kujumdzieva A (2002) Superoxide dismutase during glucose repression of *Hansenula polymorpha* CBS 4732. Z Naturforsch C 57:313–318
13. Jakubowski W, Bilinski T, Bartosz G (2000) Oxidative stress during aging of stationary cultures of the yeast *Saccharomyces cerevisiae*. Free Radic Biol Med 28:659–664
14. Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
15. Luk E, Yang M, Jensen LT, Bourbonnais Y, Cizewski Culotta V (2005) Manganese activation of superoxide dismutase 2 in the mitochondria of *Saccharomyces cerevisiae.* J Biol Chem 280:22715–22720
16. Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 247:3170–3175
17. Moradas-Ferreira P, Costa V, Piper P, Mager W (1996) The molecular defences against reactive oxygen species in yeast. Mol Microbiol 19:651–658
18. Nedeva TS, Petrova VY, Zamfirova DR, Stephanova EV, Kujumdzieva AV (2004) Cu/Zn superoxide dismutase in yeast mitochondria - a general phenomenon. FEMS Microbiol Lett 230:19–25
19. Nishikawa M, Nagatomi H, Nishijima M, Ohira G, Chang BJ, Sato E, Inoue M (2001) Targeting superoxide dismutase to renal proximal tubule cells inhibits nephrotoxicity of cisplatin and increases the survival of cancer-bearing mice. Cancer Lett 171:133–138
20. Pinheiro R, Belo I, Mota M (2000) Air pressure effects on biomass yield of two different *Kluyveromyces* strains. Enzyme Microb Technol 26:756–762
21. Pinheiro R, Belo I, Mota M (2002) Oxidative stress response of *Kluyveromyces marxianus* to hydrogen peroxide, paraquat and pressure. Appl Microbiol Biotechnol 58:842–847
22. Sturz LA, Diekert K, Jensen LT, Lill R, Cizewski Culotta V (2001) A fraction of yeast Cu,Zn-superoxide dismutase and its metallochaperone, CCS, localize to the intermembrane space of mitochondria. A physiological role for sod1 in guarding against mitochondrial oxidative damage. J Biol Chem 41:38084–38089
23. Vorauer-Uhl K, Furnschlief E, Wagner A, Ferko B, Katinger H (2001) Topically applied liposome encapsulated superox-

ide dismutase reduces postburn wound size and edema formation. Eur J Pharm Sci 14:63–67
24. Yabe Y, Kobayashi N, Nishihashi T, Takahashi R, Nishikawa M, Takakura Y, Hashida M (2001) Prevention of neutrophil-mediated hepatic ischemia/reperfusion injury by superoxide dismutase and catalase derivatives. J Pharmacol Exp Ther 298:894–899
25. Yunoki M, Kawauchi M, Ukita N, Sugiura T, Ohmoto T (2003) Effects of lecithinized superoxide dismutase on neuronal cell loss in CA3 hippocampus after traumatic brain injury in rats. Surg Neurol 59:156–601
26. Zhang Y, Wang JZ, Wu YJ, Li WG (2002) Anti-inflammatory effect of recombinant human superoxide dismutase in rats and mice and its mechanism. Acta Pharmacol Sin 23:439–444