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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Characterization of the superoxide dismutase *SOD1* gene of *Kluyveromyces marxianus* L3 and improved production of SOD activity

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Abstract Superoxide dismutase (SOD) activity is one major defense line against oxidative stress for all of the aerobic organisms, and industrial production of this enzyme is highly demanded. The Cu/Zn superoxide dismutase gene (*KmSOD1*) of *Kluyveromyces marxianus* L3 was cloned and characterized. The deduced KmSod1p protein shares 86% and 71% of identity with *Kluyveromyces lactis* and *Saccharomyces cerevisiae* Sod1p, respectively. The characteristic motifs and the amino acid residues involved in coordinating copper and zinc and in enzymatic function were conserved. To the aim of developing a microbial production of Cu/Zn superoxide dismutase, we engineered the *K. marxianus* L3 strain with the multicopy plasmid YG-KmSOD1 harboring the *KmSOD1* gene. The production of KmSOD1p in *K. marxianus* L3 and *K. marxianus* L3 (pYG-KmSOD1) in response to different compositions of the culture medium was evaluated. The highest specific activity (472 U_{SOD} mg_{prot}^{-1}) and the highest volumetric yield (8.8×10^5 U_{SOD} l^{-1}) were obtained by the recombinant strain overexpressing *KmSOD1* in the presence of Cu^{2+} and Zn^{2+} supplements to the culture media. The best performing culture conditions were positively applied to a laboratory scale fed-batch process reaching a volumetric yield of 1.4×10^6 U_{SOD} l^{-1}.

Keywords *Kluyveromyces marxianus* · Cu/Zn superoxide dismutase · Expression · Productivity

Introduction

Superoxide dismutases (SOD, EC 1.15.1.1) catalyze the dismutation of superoxide anions($O_2^{\bullet-}$) into oxygen and hydrogen peroxide. They are metalloenzymes and represent an important antioxidant defense in nearly all aerobic and aerotolerant organisms, which evolved mechanisms to prevent or repair oxidative damages caused by oxygen exposure (Fridovich 1978). The superoxide anion is a by-product of enzymatic and non-enzymatic reactions involved in metabolic processes, such as oxidative phosphorylation and photosynthesis (Fridovich 1998), which can cause peroxidation of membrane fatty acid, protein oxidation, and DNA modification. The damages are generated by direct or indirect reactions: In fact, in the presence of Fe^{3+}, H_2O_2, and $^{\bullet}NO$, a superoxide anion can produce other reactive oxygen species (ROS) with high oxidizing potential and enhanced toxicity (Wink and Mitchell 1998).

Considerable experimental evidence has been accumulated on the therapeutical applications of SOD including prevention of oncogenesis (Nishikawa et al. 2001), treatment of inflammatory diseases, and arthritis (Corvo et al. 2002; Zhang et al. 2002), of infections (Emerit et al. 2006),

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and of ischemic or burn injuries for tissue protection (Vorauer-Uhl et al. 2001; Yabe et al. 2001; Yunoki et al. 2003).

At present, SOD is 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, and product safety. Several attempts to produce the enzyme utilizing *Escherichia coli* or yeasts have indeed been performed (Yoo et al. 1999; Goulielmos et al. 2003; Yu 2007).

In a recent study, the feasibility of a yeast fermentative production of SOD was explored (Dellomonaco et al. 2007). The screening of "generally recognized as safe" yeasts belonging to different genus and species pointed out *Kluyveromyces marxianus* L3 as a high SOD producer. The dairy yeast *K. marxianus*, besides the high SOD activity, presents a number of gainful large scale fermentation characteristics in comparison to other yeast species: fast and high biomass yield, high temperature of growth, and safety (not methylotrophic and food grade status) (Fonseca et al. 2007; Limtong et al. 2007). In addition, it is a Crabtree-negative yeast, with the capability to grow on a large variety of carbon sources, including whey, inuline, and pectine (Bender et al. 2006; Masoud and Jespersen 2006).

Yeast cells produce two forms of superoxide dismutase: the copper- and zinc-containing SOD (Cu/Zn SOD), encoded by *SOD1* gene, which is the predominant form and protects cytosolic constituents from oxidation (Moradas-Ferreira et al. 1996; Sturtz et al. 2001), and the manganese-containing enzyme (Mn SOD), encoded by the gene *SOD2*, which is located in the mitochondrial matrix (Luk et al. 2005; Moradas-Ferreira et al. 1996).

In this work, we report the characterization of the superoxide dismutase *SOD1* gene of *K. marxianus*. To improve SOD productivity, the *K. marxianus* L3 strain was engineered by increasing the gene dosage of *SOD1*. Specific activity and volumetric productivity were studied in batch cultures of wild-type and recombinant *K. marxianus* L3 cultures by varying media composition, carbon sources, and metal cofactor availability.

Materials and methods

Biochemicals and reagents

All chemicals, unless otherwise specified, were provided by Sigma-Aldrich (Steinheim, Germany). The Ex-Taq kit used for polymerase chain reaction (PCR) reactions was supplied by TaKaRa (Otsu, Higa, Japan). The other enzymes and the reagents used for molecular biological procedures were from Roche. Primers were supplied by MWG-Biotech (Ebersberg, Germany). Microbiological products were purchased from Difco Laboratories (Sparks, MD, USA).

Strains and growth conditions

Kluyveromyces marxianus L3 (Dellomonaco et al. 2007) was the source of genomic DNA for the isolation of the Cu/Zn superoxide dismutase gene (*SOD1*) and was used as homologous expression host. Yeast cells were grown in YPD (consisting of yeast, peptone, and dextrose) medium (10 g l^{-1}yeast extract, 10 g l^{-1} peptone, and 20 g l^{-1} glucose). Supplement of antibiotic G418 (0.2 g l^{-1}) was added for the maintenance of recombinant plasmids.

The yeast strains *Saccharomyces cerevisiae* BY4741 (*MAT a his3 leu2 met15 ura3*) and *Kluyveromyces lactis* MW98-8C (*MATα ura A arg-lys*) were used to prepare cell extracts to assess the antibody specificity for yeasts Cu/Zn superoxide dismutases.

Escherichia coli DH5α (φ80*lacZ*Δ*M15*, *recA1*, *end A1*, *gyr A96*, *thi-1*, *hsd R17*, *rel A1*) was used for general cloning purposes and was grown at 37°C in Luria broth. Ampicillin was utilized to select *E. coli* transformants at 0.1 g l^{-1}. 5-Bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) was utilized at concentrations of 0.04 g l^{-1}.

DNA isolation and manipulation

A 10-ml culture was grown overnight in YPD broth to isolate *K. marxianus* L3 chromosomal DNA according to the method described by Sherman et al. (1986).

DNA manipulations were carried out under the conditions specified by the manufacturer or according to standard procedures as described by Sambrook et al. (2001).

DNA fragments from agarose gel were purified using the QIAquick Kit (Qiagen, Valencia, CA, USA).

PCR amplification

The entire *SOD1* coding region was amplified from *K. marxianus* L3 genome using the couple of degenerated primers Deg*SOD1*-for (AAGCTTATGGTTMATGCAG TYGCAGTK) and Deg*SOD1*-rev (AAGCTTTTAGTTRG AKAKACCAATRACA), designed on the basis of *K. lactis* NRRL Y-1140 (XM_454197) and *S. cerevisiae* S288C *SOD1* (NC_001142) sequences alignment. Both 5′ and 3′ oligonucleotide primers contained the recognition site for the restriction endonuclease *Hin*dIII (underlined).

DNA amplification was performed using a thermocycler (Robocycler Gradient 96, Biorad, La Jolla, CA, USA) programmed with the following temperature profile: 94°C for 5 min (1 cycle); 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min (30 cycles) and 72°C for 7 min (1 cycle).

Plasmid construction and transformation

The PCR fragment encoding the *SOD1* gene was ligated within pCR2.1-TOPO vector (Invitrogen, USA) according to the manufacturer instructions, giving pCR-KmSOD1.

The gene was excised from pCR-KmSOD1 by *Hin*dIII digestion and ligated into the vector pYG131 linearized with *Hin*dIII and dephosphorylated. pYG131 derives from the plasmid pKD1 (Chen et al. 1986; Saliola et al. 1999) and carries the gene for the resistance to antibiotic G418. In the resulting plasmid pYG-KmSOD1, the gene *SOD1* was under the control of the *K. lactis KlADH4* inducible promoter and of the *K. lactis PGK* terminator.

Plasmids were transformed into *E. coli* DH5α competent cells (Sambrook et al. 2001). *K. marxianus* L3 was transformed by electroporation with a Biorad Gene-Pulser apparatus, as described by Wesolowsky-Louvel et al. (1996).

Shake-flask cultivation

To determine SOD activity, *K. marxianus* L3 and *K. marxianus* L3 harboring pYG-KmSOD1 were aerobically grown in 25 ml of complex media YP (10 g l^{-1}yeast extract, 10 g l^{-1} peptone) or minimal media YKK (6.7 g l^{-1} yeast nitrogen base without amino acids, 8.5 g l^{-1} KH_2PO_4 and 3.4 g l^{-1} K_2HPO_4) containing glucose (YPD and YKKD), glycerol (YPG and YKKG), or lactose (YPL and YKKL). Carbon source solutions were filtered through a 0.22-μm pore-size filter and added to the sterile basal medium at the final concentration of 20 g l^{-1}.

For the maintenance of the expression plasmid pYG-KmSOD1, YP and YKK media contained 0.2 g l^{-1} and 0.3 g l^{-1} of G418, respectively.

To evaluate the effect of the metal cofactors, SOD specific activity in cultures non-supplemented or supplemented with 0.1 mM $CuCl_2$ and 0.1 mM $ZnCl_2$ was compared.

The flask were inoculated (10% *v*/*v*) with exponential phase pre-cultures grown in the same medium and placed in an orbital shaking incubator (180 rpm) at 30°C for 48 h. Induction with 2% *v*/*v* of ethanol was performed after 24 h of growth.

Fed-batch cultivation

Functional SOD expression was studied in fed-batch experiments performed in a Sixfors bioreactor (Infors AG, Bottmingen, Switzerland). Vessels of 0.5-l working volume were equipped with InPro6820 and 405-DPAS probes (Mettler Toledo, Switzerland) for the continuous measurement of dissolved oxygen concentration (expressed as percentage saturation) and pH, respectively. Temperature was kept at 30°C, stirring at 1,000 rpm and air flow at 0.5 *v*/*v*/*m* (volume per volume per minute). To prevent nutrient limitation, YPG medium was supplemented with 0.5 g l^{-1} $MgSO_4$, 8.0 g l^{-1} KH_2PO_4, 0.1 mM $CuCl_2$, and 0.1 mM $ZnCl_2$. The medium after addition of 0.2 g l^{-1} G418, was inoculated (10% *v*/*v*) with exponential phase seed-cultures of *K. marxianus* L3(pYG-KmSOD1), grown in the same medium. Foaming was controlled by the initial addition of PPG2000 (Cyanamid, Catania, Italy) at the concentration of 0.5 g l^{-1}. Feeding rate of 0.5 ml h^{-1} of 200 g l^{-1} glycerol solution was applied when the batch culture reached the late-exponential phase and the concentration of glycerol decreased below 3.0 g l^{-1}. Induction of KmSOD1 expression was obtained with the addition of ethanol to a final concentration of 2% *v*/*v*. Culture samples were collected during batch and feeding phase to estimate biomass yield, substrate consumption and SOD activity.

Preparation of cell-free extracts

Biomass was harvested by centrifugation (5,000×*g* for 10 min at 4°C), washed twice in potassium phosphate buffer pH 7.8, 50 mM; ethylenediaminetetraacetic acid 0.1 mM and resuspended 1:1 *w*/*v* in the same buffer. Cells were disrupted for 30 min at 4°C by using 0.5 mm glass beads (Sigma Aldrich, cat. no. G8772) 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. Protein concentration of the cell-free extract was assayed according to Lowry (Lowry et al. 1951) using bovine serum albumin as standard.

Superoxide dismutase activity

SOD activity was assayed by measuring the enzymatic inhibition of cytochrome *c* reduction, as described by McCord and Fridovich (McCord and Fridovich 1969). One unit of superoxide dismutase was defined as the amount of enzyme that inhibited the reduction of cytochrome *c* by 50% in a coupled system, using xanthine and xanthine oxydase to generate superoxide anion at pH 7.8 and 25°C.

To determine Mn-SOD activity, Cu/Zn-SOD was inactivated by adding 30 mM KCN to the samples. Cu/Zn SOD activity was calculated as the difference between total and Mn SOD activities.

Specific activity of cell-free extracts was expressed as enzymatic units per milligram of total proteins.

Western-blot analysis of Cu/Zn SOD

Proteins (20 μg/lane) of each cell-free extracts were separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis using the buffer system of Laemmli

(Laemmli 1970) and 12% acrylamide gels. The gels were then electroblotted onto a polyvinylidene difluoride membrane (Biorad). Proteins were detected with polyclonal rabbit antibodies raised against rat Cu/Zn SOD (Stressgene, Victoria, Canada) in dilution 1:1,000. The secondary antibodies were monoclonal anti-rabbit IgG conjugated with peroxidase (Promega). Immunologically active proteins were visualized with the electrochemiluminescence detection system (Amersham) according to the manufacturer's instructions.

DNA sequencing and sequence analysis

Sequencing service was supplied by MWG-Biotech (Ebersberg, Germany). The DNA sequence data were submitted to the GenBank nucleotide databases under the accession number AM748536.

The nucleotide sequence of *K. marxianus* L3 *SOD1* was analyzed with the discontinuous Mega BLAST program [National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST]).

The protein sequence alignment was performed by using the ClustalW program at the Biology Workbench [version 3.2; the San Diego Supercomputer Centre, the University of California, San Diego (http://www.workbench.sdsc.edu)]. The similarity/identity scores were calculated by the open-source freeware MatGAT [version 2.02; Indiana University Biology Archive (http://iubio.bio.indiana.edu/soft/molbio/evolve/)] (Campanella et al. 2003).

Results

Characterization of the *K. marxianus SOD1* gene

The SOD1-related ORF of *K. marxianus* L3 was amplified by using primers designed on the basis of nucleotide sequences of known *SOD1* genes from *K. lactis* and *S. cerevisiae*. The sequences coding for the N- and C-terminal region of these proteins were highly conserved, and after alignment, degenerated oligomers were designed. Amplification of *K. marxianus* L3 genomic DNA produced a single DNA band of 0.5 kb that was cloned into the plasmid pCR2.1-TOPO resulting in pCR-KmSOD1 construct.

The DNA sequences determined from three independent clones were identical and contained one open reading frame (ORF) of 465 bp encoding a putative protein of 154 amino acids with a calculated molecular mass of 15,882 Da and an isoelectric point of 6.39. The deduced amino acid sequence was compared with the Swiss-Prot protein database. The analysis showed a strong closeness with Sod1p from other yeasts, among them Sod1p of *S. cerevisiae* (72% identity and 82% of similarity) and the corresponding protein of *K. lactis* (86% of identity and 92% of similarity). The relationship with human SOD1 was also fairly high (58% of identity and 68% of similarity) (Fig. 1). On the basis of sequence similarities and of activity assays (see later), the ORF was hereinafter named *KmSOD1*.

The *K. marxianus* Sod1p contained the highly conserved histidine residues (H-47, H-49, H-64, H-72, H-81, and H-121) involved in the interaction with the metallic cofactors, which are essential for activity and folding in all the Sod1 enzymes (Hart et al. 1999). In addition, the residues involved in critical interactions were present in the proper positions: these include the aspartic residue D-84 involved in Cu^{2+} binding, the arginine residue R-144 that participate in leading the substrate to the active site, and the two cysteine residues C-58 and C-147, involved in the formation of a disulfide bond. In the C-terminal region, relevant for the Sod1 activation mechanism (Carroll et al. 2004), KmSod1p presented a glycine in position 143 and a valine in position 145, instead of the conserved residues of serine S-143 and leucine L-145 of mammalian Sod1.

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K. marxianus    -MVNAVAVLK GDSNVSGIVR FEQESEDQST KISWEITGND ANALRGFHIH EFGDNSNGCT 59
K. lactis       -MVNAVAVLK GDSSVSGIVR FEQESEDQQT KISWEITGND ANALRGFHIH TFGDNTNGCT 59
S. cerevisiae   -MVQAVAVLK GDAGVSGVVK FEQASESEPT TVSYEIAGNS PNAERGFHIH EFGDATNGCV 59
H. sapiens      MATKAVCVLK GDGPVQGIIN FEQKESNGPV KVWGSIKG-L TEGLHGFHVH EFGDNTAGCT 59
                  .:**.*** **. *.*::. *** ...  . .:  .* *   .:. :***:*  *** : **.

K. marxianus    SAGPHFNPYK KTHGAPGDET RHVGDLGNIS TDAQGVAKGS VTDKHVKLLG PLSVIGRTVV 119
K. lactis       SAGPHFNPFN KNHGAPEDEE RHVGDLGNIP TDAQGISKGS LTDKHIKLLG PLSIVGRTVV 119
S. cerevisiae   SAGPHFNPFK KTHGAPTDEV RHVGDMGNVK TDENGVAKGS FKDSLIKLIG PTSVVGRSVV 119
H. sapiens      SAGPHFNPLS RKHGGPKDEE RHVGDLGNVT ADKDGVADVS IEDSVISLSG DHCIIGRTLV 119
                ******** . :.**.* **  *****:**:  :* :*::. * . *. :.* *   .::**::*

K. marxianus    VHGGQDDLGK GGNEESLKT GNAGGRVACG VIGISN- 154
K. lactis       VHAGQDDLGK GGDAESLKT GNAGARHACG VIGISNA 155
S. cerevisiae   IHAGQDDLGK GDTEESLKT GNAGPRPACG VIGLTN- 154
H. sapiens      VHEKADDLGK GGNEESTKT GNAGSRLACG VIGIAQ- 154
                :*   ***** *.  ** ** **** * *** ***:::
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Fig. 1 Alignment of the deduced amino acid sequence of *K. marxianus* L3 Cu/Zn SOD with other SOD1 sequences: *S. cerevisiae* S288C (CAA89634), *K. lactis* NRRL Y-1140 (CAG99284), and *H. sapiens* (CAG46542). *Asterisk* Identical residues in that position of all sequences in the alignment; *Colon* Conserved substitutions according to Venn diagram (Taylor 1986); *Period* Semi-conserved substitutions. *Bold letters* indicate conserved istidine and aspartic residues for Cu and Zn binding, cysteine residues involved in intrachain disulfite bond, arginine residue R-144 leading the substrate to the active site. Amino acid motifs responsible for the Cu chaperone CCS independent activation are *boxed*

Improvement of *Km*SOD1 production

The *KmSOD1* gene was introduced in the pYG131 plasmid under the control of the *K. lactis ADH4* inducible promoter, resulting in the pYG-KmSOD1 construct. The construct was transformed into *K. marxianus* L3 cells, and the recombinant strain obtained was named *K. marxianus* L3 (pYG-KmSOD1). *K. marxianus* L3 and *K. marxianus* L3 (pYG-KmSOD1) were inoculated in YPD; after 24 h, 2% *v*/*v* of ethanol was added to culture media to induce protein expression under the control of the *KlADH4* promoter. Cultures of *S. cerevisiae* and *K. lactis* were employed as control strains.

After 48 h of growth, cell-free extracts were analyzed by Western blot; a Coomassie-stained electrophoresis gel of the same samples was used to normalize the proteins (Fig. S1). The experiment established the capability of the antibody to recognize not only ScSod1p (Harris et al. 2005) but also KlSod1p and KmSod1p. The *K. marxianus* L3 cell extracts presented a single band with the same electrophoretic mobility of ScSod1p and KlSod1p (Fig. 2).

The content of the protein Sod1p in the cell-free extracts of *K. marxianus* L3 was higher when cells were grown in YPG than in YPD. The presence in *K. marxianus* L3 of the expression vector pYG-KmSOD1 lead to an efficiently increased production of immunologically active Sod1p, as observed in the recombinant strain (Fig. 2).

SOD activity and productivity as a function of growth parameters

Batch cultures of *K. marxianus* L3 and *K. marxianus* L3 (pYG-KmSOD1) were carried out under different growth conditions. SOD specific activity (U_{SOD} mg_{prot}^{-1}) and SOD productivity (U_{SOD} l^{-1}) were compared to evaluate the effect of different media and carbon sources. Complete (YP) and minimal (YKK) media, containing glucose (YPD and YKKD), lactose (YPL and YKKL), or glycerol (YPG and YKKG) as carbon source, were tested. Furthermore, SOD activity and productivity were measured in the cultures grown in all the media either non-supplemented or supplemented with 0.1 mM $CuCl_2$ and 0.1 mM $ZnCl_2$.

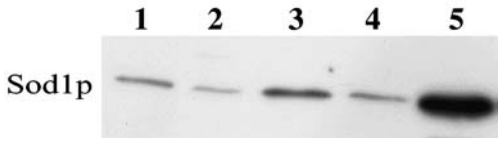

Fig. 2 Western-blot analysis of SOD1p content in the indicated cell cultures. Each well was loaded with 20 μg of cell-free extract proteins. *Lane 1*, *S. cerevisiae* BY4741 (antibody control); *lane 2*, *K. lactis* MW98-8C grown in YPD; *lane 3*, *K. marxianus* L3 grown in YPG; *lane 4*, *K. marxianus* L3 grown in YPD; *lane 5*, *K. marxianus* L3 (pYG-KmSOD1) grown in YPD

The expression of recombinant *KmSOD1* was induced with 2% *v*/*v* ethanol after 24 h of growth. Biomass yield and SOD activity were determined in 48-h cell cultures.

For *K. marxianus* L3 and *K. marxianus* L3(pYG-KmSOD1), only marginal differences in the biomass yields were observed between the various combinations of media and carbon sources. Biomass yields ranged between 5.4±0.3 and 6.1±0.3 g DW l^{-1}. Neither the Cu^{2+}/Zn^{2+} supplement nor the presence of the plasmid pYG-KmSOD1 affected the biomass yields (Supplementary Table 1).

For *K. marxianus* L3, SOD specific activity was influenced by medium composition. Maximum specific activity was observed for cells grown on glycerol as carbon source both on complex and minimal media (Fig. 3a,b). The addition of the Cu^{2+}/Zn^{2+} supplement to the complex and minimal media produced an overall positive effect on *K. marxianus* L3 specific activity. All of the cultures supplemented with 0.1 mM $CuCl_2$ and 0.1 mM $ZnCl_2$ (indicated with the name of the medium followed by +) presented higher specific activities than the corresponding ones without the supplement. This was suggestive of a limited availability of Cu^{2+}/Zn^{2+} for the optimal functionality of Sod1p in standard media.

In the absence of the Cu^{2+}/Zn^{2+} supplement, the overexpression of recombinant *KmSOD1* did not significantly increase the SOD specific activity, and in some cases, a net reduction of the enzymatic activity could be instead observed. However, also for the recombinant cells, the addition of the Cu^{2+}/Zn^{2+} supplement resulted in a significant increase of the SOD activity. In some combinations of media and supplement, a consistent improvement of the productive capabilities of the strain was observed. The highest specific activity was obtained in complex medium with glycerol as sole carbon source, with the Cu^{2+}/Zn^{2+} supplement (YPG^{rec}+448.4 U_{SOD} mg_{prot}^{-1}). For all tested conditions, Cu/Zn SOD accounted for more than 95% of total activity.

SOD productivity, expressed as U_{SOD} l^{-1} of culture, were calculated and compared to summarize the influence of medium composition on both biomass yields and SOD specific activity. Bearing in mind the applicative outcomes of this study, we focused our attention on complex media. In YPD and YPL, SOD productivity was rather low and not significantly influenced by the presence of the Cu^{2+}/Zn^{2+} supplement for both *K. marxianus* L3 and *K. marxianus* L3 (pYG-KmSOD1).

Finally, the results obtained on YPG were more promising, and the highest productivity was obtained with the recombinant strain *K. marxianus* L3(pYG-KmSOD1) grown in the complex medium with glycerol as carbon source, supplemented with 0.1 mM $CuCl_2$ and 0.1 mM $ZnCl_2$ (YPG^{rec}+8.8×10^5 U_{SOD} l^{-1}; Fig. 4).

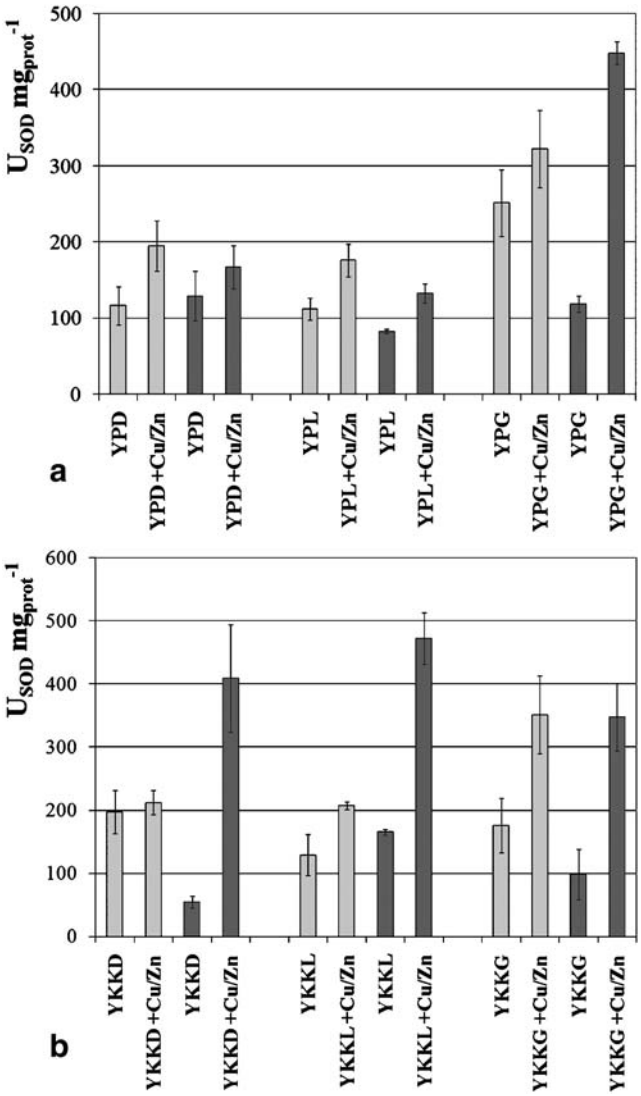

Fig. 3 Comparison of SOD specific activities (U_{SOD} mg_{prot}^{-1}) from *K. marxianus* L3 and *K. marxianus* L3(pYG-KmSOD1) grown on various media and with supplement, measured by spectrophotometric assay. *+Cu/Zn* Addition of 0.1 mM CuCl2 and 0.1 mM ZnCl2 in the culture media; *gray bar* wild-type strain; *black bar* recombinant strain harboring pYG-KmSOD1 plasmid. **a** YP-based (complex) media, **b** YKK-based (minimal) media

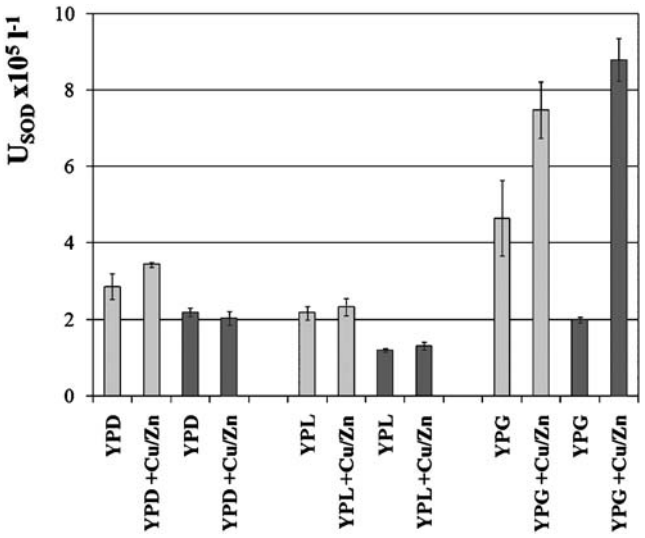

Fig. 4 SOD productivity ($U_{SOD}\times10^5$ l^{-1}): performance comparison of control and recombinant strains on complex culture media. *+Cu/Zn* Addition of 0.1 mM CuCl2 and 0.1 mM ZnCl2 in the culture media; *gray bar* wild-type strain; *black bar* recombinant strain harboring pYG-KmSOD1 plasmid

Fed-batch cultivations were carried out with the recombinant strain *K. marxianus* L3(pYG-KmSOD1) in the best performing culture medium (YPG supplemented with Cu^{2+} and Zn^{2+} ions). Experiments were carried out in triplicate, and the results presented are representative of the three fermentations (Fig. 5). The culture was initiated batchwise in the mineral-supplemented YPG medium. The ethanol pulse was applied after 47 h, when biomass concentration was 9.3 DW g l^{-1} and SOD specific activity was 221 U mg^{-1}, corresponding to a 5.4×10^5 U l^{-1} productivity. The highest SOD specific activity (412 U mg^{-1}) was observed 6 h after the induction of KmSOD1 expression. Once glycerol concentration decreased below 3 g l^{-1}, the culture was fed with glycerol, allowing biomass to increase up to 16.6 DW g l^{-1}. Both the improved specific activity and biomass concentration enhanced SOD productivity up to 1.4×10^6 U l^{-1}.

Discussion

Superoxide dismutase is one of defense mechanisms that organisms developed for the detoxification from ROS and for preventing oxidative damages. This activity is at the base of increasing pharmaceutical and nutraceutical appli-

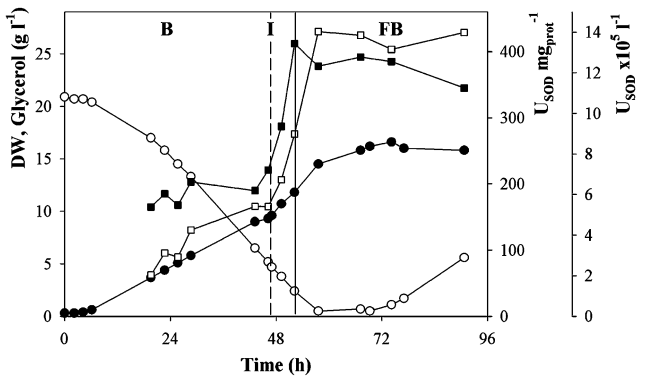

Fig. 5 *K. marxianus* L3(pYG-KmSOD1) fed-batch cultivation: *filled circle* biomass yield (g DW l^{-1}); *open circle* glycerol (g l^{-1}); *filled square* SOD specific activity (U_{SOD} mg_{prot}^{-1}); *open square* SOD productivity ($U_{SOD}\times10^5$ l^{-1}). *B* Batch phase; *FB* fed-batch phase; *I* ethanol pulse for induction of KmSOD expression

cations. The aim of this work is to improve the knowledge on the Cu/Zn SOD from *K. marxianus* L3, a promising dairy yeast that we proposed in an earlier paper for the fermentative production of SOD (Dellomonaco et al. 2007). Furthermore, the characterization of the gene coding for KmSod1p allowed molecular approaches for genetic engineering of the wild-type strain, aimed at improving SOD1 production.

In eukaryotes, the Cu/Zn SOD mainly acquires copper through the chaperone CCS (copper chaperone for superoxide dismutase; Rae et al. 1999). Mammalian SOD1 present a CCS-independent secondary pathway of activation involving reduced glutathione, and reliant on the residues of serine S-143 and leucine L-145. *S. cerevisiae* enzyme can receive Cu only through CCS, and this total dependence on CCS requires the presence of two residues of proline near the C terminus (P-143 and P-145; Carroll et al. 2004). Both *K. marxianus* and *K. lactis* Sod1 do not present any conservation of the indicated residues, and those positions are embedded in an otherwise exceptionally conserved region of the compared enzymes. This may suggest that, in *Kluyveromyces* yeasts, different mechanism(s) for cation interactions are present.

In this study, we compared recombinant and wild-type *K. marxianus* L3 strains performances, measuring biomass yields, SOD specific activity of cell-free extract, and SOD productivity. Effects exerted by culture media composition were registered by changing from complete media to minimal synthetic media, by adding three different carbon sources (glucose, lactose, and glycerol) and by varying the availability of the metal cofactors Cu^{2+} and Zn^{2+} with a supplement of 0.1 mM of each ion in the cultures.

Surprisingly, *K. marxianus* L3 grew in batch cultures with similar biomass yields both on minimal synthetic and complete medium; in addition, no significant differences were observed on the three carbon source tested: glucose, lactose, and glycerol. Despite the fact that glycerol is a respiratory carbon source for yeast cells and it was expected that a sustained oxidative stress could occur in cells growing on this substrate, it turned out to be a good carbon source for *K. marxianus* L3, similar to fermentable sugars. Furthermore, the presence of recombinant plasmid pYG-KmSOD1 and of the supplement of 0.1 mM Cu^{2+} and 0.1 mM Zn^{2+} ions, which are 50 times lower with respect to the minimal inhibitory concentrations of this strain (data not shown), did not significantly affect biomass yields.

On the other hand, the addition of the Cu^{2+}/Zn^{2+} supplement to the culture media noticeably increased SOD specific activity and SOD productivity, both in the wild-type and in the recombinant strains. The overexpression of recombinant KmSOD1 leads to a real increase of the specific activity only in presence of the supplement of Cu^{2+} and Zn^{2+} ions.

For recombinant strain cultures, Western-blot analysis indicated a clear increase of SOD1 protein in the presence of plasmid pYG-KmSOD1, as compared to the control. Perhaps, the overexpression conditions produced an excess of recombinant protein that was not activated to the corresponding holoenzyme; this could be due to a limiting supply of Cu^{2+} and Zn^{2+} ions under the culture conditions studied. This is supported by the observation that increasing metal factor availability in the culture media resulted in enhanced SOD activity.

Recently, *SOD1* gene of *S. cerevisiae* was expressed in *Pichia pastoris* to obtain a recombinant SOD production (Yu 2007). However, the specific activity, determined on total cell extracts, did not favorably compare with the one obtained by the *K. marxianus* recombinant strain (83 U mg^{-1} in *P. pastoris* vs 500 U mg−1 in *K. marxianus*). The difference could be due at least in part to the *P. pastoris* expression system and in part to a limited availability of metal cofactors for the SOD enzyme produced by using standard media. Moreover, even the *K. marxianus* L3 untransformed strain showed threefold more SOD specific activity as compared to *P. pastoris* counterpart, suggesting that *K. marxianus* L3 overexpressing *KmSOD1* could indeed represent a good candidate for large-scale SOD production.

Metal ions are highly toxic due to their redox capabilities, and their concentration and availability in the cell compartments is tightly controlled. In the yeast *S. cerevisiae*, Cu^{2+} ions enter the cell via the high-affinity uptake protein Ctr1p (Dancis et al. 1994). The specific chaperones CCS, Cox17p, and Atx1p are involved in the distribution of Cu^{2+} to intracellular proteins and to the secretory pathway compartments, respectively (Culotta et al. 1997; Field et al. 2002; Glerum et al. 1996; Lin and Culotta 1995). In the meantime, the sequestration of excess Cu^{2+} by metallothioneins prevents cellular damage (Butt et al. 1984; Rae et al. 1999). Future investigations aimed at studying the expression levels of those proteins in different culture conditions may help to explain the values of SOD activity observed in *K. marxianus* L3 cultures.

The results of this study provide a better knowledge of *K. marxianus* L3 Cu/Zn superoxide dismutase. The culture parameters that positively affect active enzyme production were identified, the best performing culture conditions were positively applied to a laboratory scale fed-batch process, and a first promising indication for a biotechnological production of recombinant *K. marxianus* SOD was obtained.

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