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Production, purification, and characterization of a novel cold-active superoxide dismutase from the Antarctic strain *Aspergillus glaucus* 363

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ABSTRACT

The Antarctic fungal strain *Aspergillus glaucus* 363 produces cold-active (CA) Cu/Zn-superoxide dismutase (SOD). The strain contains at least one gene encoding Cu/Zn-SOD that exhibited high homology with the corresponding gene of other *Aspergillus* species. To our knowledge, this is the first nucleotide sequence of a CA Cu/Zn-SOD gene in fungi. An effective laboratory technology for *A. glaucus* SOD production in 3 L bioreactors was developed on the basis of transient cold-shock treatment. The temperature downshift to 10 °C caused 1.4-fold increase of specific SOD activity compared to unstressed culture. Maximum enzyme productivity was 64×10^3 U kg^{-1} h^{-1}. Two SOD isoenzymes (Cu/Zn-SODI and Cu/Zn-SODII) were purified to electrophoretic homogeneity. The specific activity of the major isoenzyme, Cu/Zn-SODII, after Q-Sepharose chromatography was 4000 U mg^{-1}. The molecular mass of SODI (38 159 Da) and of SODII (15 835 Da) was determined by electrospray quadropole time-of-flight (ESI-Q-TOF) mass spectrometry and dynamic light scattering (DLS). The presence of Cu and Zn were confirmed by inductively coupled plasma mass spectrometry (ICP-MS). The N-terminal amino acid sequence of Cu/Zn-SODII revealed a high degree of structural homology with Cu/Zn-SOD from other fungi, including *Aspergillus* species.

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Introduction

Superoxide dismutases (SODs, EC 1.15.1.1) are antioxidant enzymes found in all aerobic cells as a first line of defense against superoxide anion radicals ($\cdot O_2^-$) (Fridovich 1998). At high levels, $\cdot O_2^-$ can affect physiological functions through changes in DNA, proteins, and, lipids, which lead to certain human pathologies including cancers, neurodegenerative

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disorders, and, cardiovascular disease, as well as ageing (see Rowe *et al.* 2008).

There are four types of SODs grouped according to their catalytic metal cofactor: Cu/Zn-SOD, Mn-SOD, Fe-SOD, and Ni-SOD (Youn *et al.* 1996; Fridovich 1998).

As is typical for eukaryotes, the filamentous fungi possess two different, evolutionary unrelated forms of SOD, Mn- and Cu/Zn-containing enzyme. Both isoenzymes have been determined in different fungal species, belonging to the genera *Aspergillus*, *Penicillium*, *Cladosporium*, *Mucor*, *Fusarium*, and *Alternaria* (Angelova *et al.* 2005), *Neurospora crassa* (Chary *et al.* 1994), *Humicola lutea* (Krumova *et al.* 2008), various pathogenic fungi (see Fernandes *et al.* 2008). Only Cu/Zn-SOD has been found in cells of *Aspergillus niger* 26 (Abrashev *et al.* 2008; Dolashki *et al.* 2008). Mn-SOD has been isolated as a single isoenzyme from the thermophilic fungus *Chaetomium thermophilum* (Guo *et al.* 2008), the entomopathogenic fungus *Beauveria bassiana* (Xie *et al.* 2012), *Candida albicans* (Lamarre *et al.* 2001), and several other fungi (Frealle *et al.* 2006).

The potential therapeutic applications of SOD are based on its scavenging action on the toxic $\cdot O_2^-$ that may occur in different pathological states. This enzyme might interrupt inflammatory cascades in the cells and thereby limit further disease progression. As has been reviewed by El Shafey *et al.* (2010), SOD is very useful for the treatment of systemic inflammatory diseases, skin ulcer lesions, degenerative diseases, radiation-induced side effects such as radiation-induced sclerosis and radiation-induced fibrosis (following irradiation for treatment of breast cancer), for stimulation of hair growth, and to reduce hair loss etc. Purified enzyme is used in many pharmaceutical compositions for treatment of diseases including myocardial ischaemia, Peyronie's Disease, multiple sclerosis, colitis, diabetic retinopathy, etc (El Shafey *et al.* 2010; Raimondi *et al.* 2010).

The use of exogenous antioxidant, for example SOD, in the assisted reproductive technology (ART) procedures prevents the deleterious effects of oxidative stress on sperm viability and protects functional parameters of spermatozoa (Alvarez 2012; Atig *et al.* 2012; Agarwal *et al.* 2014).

In contrast to the industrial use of SOD, the enzyme used therapeutically has to maintain its catalytic activity at lower temperatures. Cold-active (CA) enzymes are known to retain high conformational flexibility at low temperature (Feller & Gerday 2003; Feller 2013). In recent years these enzymes raised great interest in the area of science and biotechnology. Cold-adapted microorganisms are known as good producers of cold-tolerant enzymes (Brenchley 1996; Joseph *et al.* 2008; Margesin *et al.* 2008; Nam & Ahn 2011; Feller 2013). Filamentous fungi isolated from extreme cold environments synthesize CA amylase, xylanase, cellulase, phosphatase, pectinase, lipase, etc (Hou *et al.* 2006; Kim *et al.* 2010; Krishnan *et al.* 2011; Singh *et al.* 2012). High production of cold-tolerant chitinase by Antarctic fungus *Lecanicillium muscarium* CCFEE 5003 has been reported (Fenice *et al.* 2012; Barghini *et al.* 2013). These fungi *could* be suitable sources of antioxidant enzymes, especially SOD, because of enhanced level of free radicals caused by harsh conditions (very low temperatures, wide thermal fluctuations, frequent freeze/thaw cycles, extreme dryness, high salt concentrations, low nutrient availability, high radiation) (Russell 2003, 2006; Onofri *et al.* 2007; Chattopadhyay *et al.* 2011).

At the same time, CA SOD is very poorly investigated. Zheng *et al.* (2006) isolated and characterized CA Fe-SOD from the psychrophilic bacteria *Marinomonas* sp. NJ522. Later, Pedersen *et al.* (2009) and Merlino *et al.* (2010) reported the structure and flexibility of CA Fe-SOD produced by the cold-adapted bacteria *Aliivibrio salmonicida* and *Pseudoalteromonas haloplanktis*, respectively. But there are no published data about CA SOD from fungi. No information is available also on biotechnological production of such SOD and its application for human *in vitro* fertilization.

Our previous results showed that the filamentous fungi isolated from the permanent Bulgarian Antarctic base 'St. Kliment Ohridski' on Livingston Island synthesize CA SOD (Tosi *et al.* 2010). The best producer among thirty tested strains was *Aspergillus glaucus* 363. The present research was designed to study in more details the conditions for the production of a novel CA SOD by this Antarctic fungus. To develop an effective laboratory technology we used a new approach based on the relationship between oxidative stress and cold shock. Furthermore, the study's aim was to purify the novel enzyme and to characterize it structurally.

Materials and methods

Fungal strains, culture media, and cultivation

The fungal strain, *Aspergillus glaucus* 363 (having optimal growth temperature at 25 °C), isolated from Livingston Island (South Shetlands archipelago, Antarctica) (Tosi *et al.* 2010) was used for the experiments. The strain has been deposited in the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria (NBIMCC 8861). It is maintained at 4 °C on beer agar, pH 6.3. The composition of the seed and production media was as described previously (Angelova *et al.* 1996). Cultivation was performed in a 3 L bioreactor ABR-09 developed and constructed by the former Central Laboratory for Bioinstrumentation and Automatisation (CLBA) of the Bulgarian Academy of Sciences. The bioreactor was equipped with temperature, pH, and dissolved oxygen (DO) automatic monitoring and control equipment.

For the submerged cultivation, 74 mL of seed medium were inoculated with 6 mL spore suspension at a concentration of 2×10^8 spores mL^{-1} in 500 mL Erlenmeyer flasks. The cultivation was performed at 25 °C for 24 h on a rotary shaker (220 rpm). For bioreactor cultures, 200 mL of the seed culture was brought into the 3 L bioreactor, containing 1800 mL of the production medium. The cultures were grown at 25 °C with a stirrer speed of 400 rpm and air flow of 0.5 v.v.m. In the time of the middle exponential phase (18 h), the temperature was reduced to 4 or 10 °C. This downshift was reached in approx. 40 min. After an incubation of 6 h under cold stress conditions, the temperature was upshifted to the optimal value and the cultivation continued until the 72nd hour. The

control variants were grown at optimal temperature during the whole period.

Cell-free extract preparation and enzyme activity determination

The cell-free extracts were prepared as previously described in detail (Krumova *et al.* 2008). All steps were performed at 0–4 °C. SOD activity was measured by the nitro-blue tetrazolium (NBT) reduction method of Beauchamp & Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme required for inhibition of the reduction of NBT by 50 % (A_{560}) and was expressed as units per mg protein (U mg^{-1} protein).

SOD purification

Cell free extract from 36-h culture from *Aspergillus glaucus* was clarified by filtration through celite and Millipore's device Pellicon XL Durapore 0.1, concentrated and fractionated by ultrafiltration with Pellicon XL 10 (10 kDa). All purification steps were carried out at 4 °C using an FPLC system (ÄKTA purifier GE Healthcare Life Sciences, USA).

Step 1. Superdex 100 column chromatography (gel filtration). Active fractions were concentrated and applied to a column Superdex (10/300 GL), pre-equilibrated with 20 mM Tris–HCl buffer with pH 7.8 including 100 mM NaCl. The protein was eluted with the same buffer at a flow rate of 0.25 mL min^{-1} at 4 °C.

Step 2. Phenyl-Sepharose column chromatography (hydrophobic interaction chromatography). For additional removal of accompanying proteins, the active fraction from Superdex column was brought to 40 % ammonium sulphate saturation (0.02 M potassium phosphate buffer at pH 7.8). The dialysed enzyme solution was loaded onto a Phenyl-Sepharose column equilibrated with the above buffer at a flow rate of 120 mL h^{-1}. The residual protein was eluted with 15 % buffered solution of ammonium sulphate.

Step 3. Q-Sepharose column chromatography (ion-exchange chromatography). The active peak fractions from Phenyl-Sepharose column were pooled, concentrated, and applied to a column of Q-Sepharose pre-equilibrated with 20 mM Tris–HCl buffer with pH 7.8 including 100 mM NaCl. Unbound enzyme protein was eluted with the same buffer; bound enzyme protein (major isoenzyme) was eluted with buffer containing 0.6 M NaCl. The enzyme activity was recovered in both fractions (bound and unbound). At each stage of purification, active fractions were analysed for SOD activity.

DNA isolation procedure

Fifteen millilitre yeast extract peptone-dextrose (YEPD) medium (g L^{-1}: yeast extract 10.0, peptone 20.0, glucose 10) were inoculated with a single fungal colony and incubated in 100 mL Erlenmeyer flasks. After incubation for 48 h on a rotary shaker at 180–200 rpm and 25 °C the resulting pellet was collected and washed with 5 mL 0.98 % saline solution. The pellet was ground in a mortar with quartz sand. Two millilitre 1× Tris-EDTA (TE) buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) were added to the cell lysates and the homogenates dispensed in Eppendorf tubes (500 μL $tube^{-1}$). The DNA isolation was performed according to Maniatis *et al.* (1982). The DNA was purified *via* GFX columns (GE Healthcare, Little Chalfont, Buckinghamshire, England). Its quality and quantity was checked by measurement of the UV absorption, and used as a template for PCR amplification experiments.

PCR conditions and DNA sequencing

The amplification was performed on an Eppendorf Mastercycler personal thermocycler (Eppendorf AG, Hamburg, Germany) using PuReTaq™ Ready-To-Go™ PCR beads (Amersham Biosciences, Piscataway, NJ, USA). The final concentration of primers in the reaction mixture was 0.4 pmol μL^{-1}. The concentration of the DNA matrix was 50 ng/25 μL (final volume of the reaction mixture).

The universal primers used for fungal 18S rDNA amplification were PFf (AGGGATGTATTTATTAGATAAA AAATCAA) and PFr (CGCAGTAGTTAGTCTTC AGTAAATC) (Jaeger *et al.* 2000). PCR conditions: initial step, 95 °C, 5 min; amplification – 35 cycles, 95 °C, 30 s; 58 °C, 30 s; 72 °C, 45 s; extension step, 72 °C, 7 min.

The obtained PCR products were purified by the GFX™ PCR DNA and gel band purification kit (GE Healthcare). All DNA amplified fragments were sequenced on an ABI Prism 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The reaction mixture for DNA sequencing contained 2 μL of purified PCR product (2 ng μL^{-1}), 1.3 μL Big Dye® terminator kit v. 3.1, 2 μL 5× sequencing (Seq) buffer, 0.35 μL primers (10 pmol μL^{-1}). The volume was brought to 10 μL with distilled water.

Comparative sequence analysis

The raw data obtained from the sequencing were checked for errors by the program Sequence Scanner V1.0 (Applied Biosystems). DNA sequences were formatted in a form suitable for comparison by the BLAST analysis database of National Center for Biotechnology Information (NCBI). The corresponding protein sequences were established from the DNA sequence using DNASTAR Inc., Madison, WI software. The pair and multiple sequence alignment were performed using ClustalW2.

Analytical methods

Soluble reducing sugars were determined by the Somogyi–Nelson method (Somogyi 1952). Protein was estimated by the Lowry procedure (Lowry *et al.* 1951) using solution of bovine serum albumin as standard. The dry weight determination was performed on samples of mycelia harvested throughout the culture period. The culture fluid was filtered through a Whatman (Clifton, USA) No. 4 filter. The separated mycelia were washed twice with distilled water and dried to a constant weight at 105 °C.

The possible glycosylation of SODI and SODII was checked by a qualitative modification of the colourimetric method of

Dubois et al. (1956) in phenol–sulphuric acid. Dynamic light scattering (DLS) was performed on a DynaPro NanoStar instrument (Wyatt Technology Corporation) operated in batch mode at 20 °C and fitted with a laser beam emitting at 658 nm. Samples were filtered on Whatman Anotop 10 inorganic membrane (0.02 μm cut off) and loaded into a 10 μL quartz microcuvette. Scattering data were analysed using DYNAMICS v. 7.1.1.3 software (Wyatt Technology Corp.). A globular protein model was used for mass estimation. Mass determination was performed on an electrospray quadropole time-of-flight (ESI-Q-TOF) instrument (Waters, Micromass) in positive ion mode. Samples (10 μM) were analysed in 30 % acetonitrile, 0.5 % formic acid, 25 mM ammonium acetate. Spectra deconvolution technique of calculation was the maximum entropy (Max ent1). The metal content of the purified proteins was determined by inductively coupled plasma mass spectrometry (ICP-MS) at the Malvoz Institute, Laboratory of Environmental Chemistry (Liège, Belgium) for Cu, Zn, Mn, Fe, and Ni. Differential scanning microcalorimetry was performed using a MicroCal VP-DSC instrument at a scan rate of 60 K h^{-1} and under ∼25 psi positive cell pressure. Samples were dialysed overnight against 30 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 50 mM NaCl, pH 7.5. Both the sample and the reference buffer were brought to 0.5 M 3-(1-pyridinio)-1-propanesulfonate (i.e. a nondetergent sulfobetaine) in order to prevent aggregation. The N-terminal amino acid sequence was determined by automated Edman degradation using a pulsed-liquid-phase protein sequencer Procise 494 (Applied Biosystems) fitted with an on-line phenylthiohydantoin analyser.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, 12 % gel) was used to verify the protein purity of the enzyme under denaturing conditions, as was described by Laemmli (1970). Protein marker was from Pharmacia (Sweden) with a broad range of 14–97 kDa. Protein bands were visualized by Coomassie brilliant blue R-250 (Bio-Rad, USA) staining. Zones of SOD activity were stained with NBT using 10 % nondenaturing PAGE, as described by Beauchamp & Fridovich (1971).

Results

Taxonomic identification of the fungal strain

Using classical taxonomy based on morphology, the producer of CA SOD was previously identified as *Aspergillus glaucus* (Tosi et al. 2010). Molecular methods have been widely applied to the identification of a large number of *Aspergillus* species. The study of the 18S rDNA sequences can provide important complementary information for the definition of species and their appropriate identification. In the present work, confirmation of the morphological identification by molecular methods was done.

The molecular taxonomic affiliation of the investigated Antarctic fungal strain was performed on the bases of the comparison with 18S rDNA sequences of reference organisms published in the gene sequence database of NCBI Data Bank. The validation of the genotypic *vs.* the phenotypic analyses indicated that the investigated strain is closely related to the species *A. glaucus*.

Sequence analyses of the SOD gene

For the nucleotide sequence of the Cu/Zn-SOD gene, a pair of oligonucleotide primers (AFCUF – 5′TTGCTGTCCTCCGCGGTGACTC CA3′ and AFCUR – 5′ATGACACCACAGGCGGGACGAG3′) was designed on the basis of the Cu/Zn-SOD gene of *Aspergillus fumigatus* Af293 (Nierman et al. 2005). The PCR product (about 650 bp) obtained from genomic DNA of the strain was electrophoretically analysed and found to have appropriate profile. It was purified and sequenced. The obtained partial DNA gene sequence consisted of 484 bp and is provided in Fig 1.

The BLAST analysis demonstrated similarity of the sequenced fragment with genes coding for enzymes with Cu/Zn-SOD activity. It comprised two putative introns consisting of 53 and 65 bases corresponding to the second and third introns identified in *A. fumigatus* (Lima et al. 2007). The coding region (Fig 1) was aligned with the sequences in the NCBI GenBank. There was 100 % identity of the nucleotide sequence of the Cu/Zn-SOD gene obtained with the corresponding *A. fumigatus* Af293 sequence NCBI Acc. No. XM_748622.1 and *A. fumigatus* AF128886.1. Furthermore, the sequence identity of the investigated gene with the Cu/Zn-SOD gene of *Neosartoria fishery* (NCBI Acc. No. XM_001259765.1) was 98 % and the identity with the closest similar gene in *Penicillium* (*Penicillium chrysogenum* Wisconsin 54-1255 NCBI Acc. No. XM_002568648.1) was 84 %.

The deduced protein sequence consisted of 121 amino acids (Fig 2). The protein sequence comparison by the BLASTP 2.2.26+ program revealed 100 % identity with Cu/Zn SOD (SOD1) of the referent strain *A. fumigatus* Af293. The similarities established with other members of the Cu/Zn-SOD enzyme's superfamily varied between 100 % and 71 %.

```
TTGCTGTCCTCCGTGGTGACTCCAAGATCACCGGCACTGTCACCTTCGAGCAGGC
CGACGAGAACTCTCCCACCACCGTCTCTTGGAACATCAAGGGCAACGACCCCAAC
GCCAAGCGTGGCTTCCATGTCCACCAGTTCGGTGACAACACCAACGGCTGCACCT
CCGCTGGTCCTCACTgtatgtcctcttcccagtggtcactctgagaaccagaactgaccgacctccagTCAACCCC
TATGGCAAGACCCATGGAGCTCCTGAGGACTCCGAGCGCCATGTCGGTGACCTTG
GTAACTTCGAGACCGATGCTGAGGGTAACGCCGTCGGCTCCAAGCAGGACAAGC
TTATTAAGCTGATTGGTGCCGAGAGCGTTCTGGGCgtaagtttttttttttctgcagatggtaatgcataga
gttaacagtgagctgacaacaatgttagCGGACCTTGGTCGTTCACGCCGGTACCGACGACC
```

Fig 1 – Partial nucleotide sequence of Cu/Zn-SOD gene from the *Aspergillus glaucus* 363 (GenBank: JN206685.1). The two introns are indicated by lowercase letters.

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A.glaucus363         : WNIKGNDPNAKRGFHVHQFGDNTNGCTSAGPHFNPYGKTHGAPEDSERHV
A.fumigatusAf293     : WNIKGNDPNAKRGFHVHQFGDNTNGCTSAGPHFNPYGKTHGAPEDSERHV
A.fumigatus          : WNIKGNDPNAKRGFHVHQFGDNTNGCTSAGPHFNPYGKTHGAPEDSERHV
A.japonicus          : ----NSSSVPLHGFHVHALGDTTNGCMSTGPHFNPTGKEHGAPQDENRHA
A.nidulans           : WNITGNDPNAERGFHIHQFGDNTNGCTXAGPHFNPFGKTHGAPEDEVRHV
N.fischeriNRRL181    : WNIKGNDPNAKRGFHVHQFGDNTNGCTSAGPHFNPYGKTHGAPEDSERHV
P.chrysogenumW.54-12 : WNITGHDANAERAFHVHQFGDNTNGCTSAGPHFNPFGKTHGAPTDSERHV

A.glaucus363         : GDLGNFETDAEGNAVGSKQDKLIKLIGAESVLGRTLVVHAGTDD---
A.fumigatusAf293     : GDLGNFETDAEGNAVGSKQDKLIKLIGAESVLGRTLVVHAGTDDLGR
A.fumigatus          : GDLGNFETDAEGNAVGSKQDKLIKLIGAESVLGRTLVVHAGTDDLGR
A.japonicus          : GDLGNITAGADGVANVNVSDSQIPLTGAHSIIGRAVVVHADPDDLGK
A.nidulans           : GDLGNFKTDAEGNSKGSKTDKLIKLIGAESVLGRTLVVHAGTDDLGR
N.fischeriNRRL181    : GDLGNFETDAEGNAVGSKQDKLIKLIGAESVLGRTLVVHAGTDDLGK
P.chrysogenumW.54-12 : GDLGNFKTDAEGNSNGSKQDELIKLIGAESVLGRTLVVHAGTDDLGK
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Fig 2 – Multiple alignment of the amino acid sequences of the metal binding region of Cu/Zn-SOD enzymes from different fungi.

The multiple sequence alignment analysis showed the existence of key amino acids' residues responsible for Cu^{2+} (His43, His45, His59, His116) and Zn^{2+} (His59, His67, His76, Asp79) binding in positions cited by other researchers (Lin *et al.* 2008). The comparison of the deduced amino acid sequence of *Aspergillus glaucus* strain 363 Cu/Zn-SOD protein fragment with the similar proteins in different fungi demonstrated very conservative structure of the active site regions (Fig 2).

Production of CA SOD

During the course of previous investigation on Antarctic fungi, a high level of CA SOD in *Aspergillus glaucus* 363 was detected (Tosi *et al.* 2010). Furthermore, short-term treatment of exponentially growing culture of *A. glaucus* 363 with low temperatures enhanced the level of oxidative stress that resulted in the activation of antioxidant enzyme defence (Kostadinova *et al.* 2012). Based on the above mentioned findings, a transient cold-stress was applied in order to increase the yield of the first antioxidant enzyme SOD. Changes in biomass content, glucose consumptions, intracellular protein synthesis, and SOD production after 6 h temperature downshift and subsequent recovery at the optimum temperature were evaluated (Fig 3). In the control experiment (Fig 3A), the growth (maximum d.w. 2.16 g/100 mL) and protein synthesis rose simultaneously until the 48 h of cultivation coinciding with a rapid glucose uptake. Maximum SOD activity (about 29 U mg^{-1} protein) was recorded 36 h after inoculation. The transient exposure to 10 or 4 °C (Fig 3B and C, respectively) caused a delay in biomass formation and prolongation of the exponential growth phase, a decrease in the protein level and activation of antioxidant defence. Maximum mycelia growth of 1.9 and 1.8 g/100 mL, respectively, was achieved after 72 h of incubation. Thus, the biomass content was between 11 % and 17 % lower than in the control variant. The same trend of decreasing was observed for the intracellular protein content. In contrast, short-term treatment with cold temperatures resulted in significant increase in SOD activity compared to the control and this increase remained even after recovery of the optimal temperature. The cultures exposed to 10 °C demonstrated 34 % higher specific SOD activity, while the antioxidant response of the cold-treated cells at 4 °C resulted in 71 % increase in SOD level. It should be noted that the maximum enzyme activity in the cultures exposed to 4 °C was realized after the 48th hour.

Comparison of the efficiency of SOD biosynthesis by Aspergillus glaucus *363 grown under different conditions*

Comparison of kinetic relations (substrate consumption and product formation parameters) for CA SOD production between three variants of bioreactor cultures (control, cold stress at 10 °C, and cold stress at 4 °C) is given in Table 1. The results indicated that the highest value of the kinetic parameters for biomass and yield of protein were observed in the control cultures. But, the application of 6 h cold stress resulted in enhanced both specific and total SOD production. Moreover, enzyme yield coefficient (U g^{-1}) and SOD productivity (U kg^{-1} h^{-1}) were significantly higher than in the unstressed cells. Despite the reduced yield of biomass (g L^{-1}) and protein (mg L^{-1}) after temperature downshift, the increased specific SOD activity led to the enhanced value of total SOD activity and enzyme yield coefficient (Yp/x). The Yp/x demonstrated 25 % and 28 % increase at 10 and 4 °C, respectively, compared to the control. It should be noted however that this increase at 4 °C was detected later (after 48 h *vs.* 36 h at 10 °C). Thus, the most effective SOD production was observed after cold stress at 10 °C. As seen in Table 1, the SOD productivity is highest after exposure at 10 °C, compared to the control and to the cold stress at 4 °C.

Purification of Aspergillus glaucus *SODs*

In a previous paper we reported the presence of three SOD isoenzymes in the cell-free extract from *A. glaucus* 363 (Kostadinova *et al.* 2012). They were designated as Mn-SOD, Cu/Zn-SODI, and Cu/Zn-SODII in the order of their migration towards the anode. The most abundant isoforms are Cu/Zn-SODI and Cu/Zn-SODII. In the present study, the cell-free extract (1500 mL) obtained from 150 g fresh biomass (29 g d.w.) was used in the experiments. The purification protocol included concentration by ultrafiltration, gel filtration on Superdex 100, hydrophobic interaction chromatography on Phenyl-Sepharose, followed by a final ion-exchange chromatography on Q-Sepharose to produce a homogenous enzyme. Summary

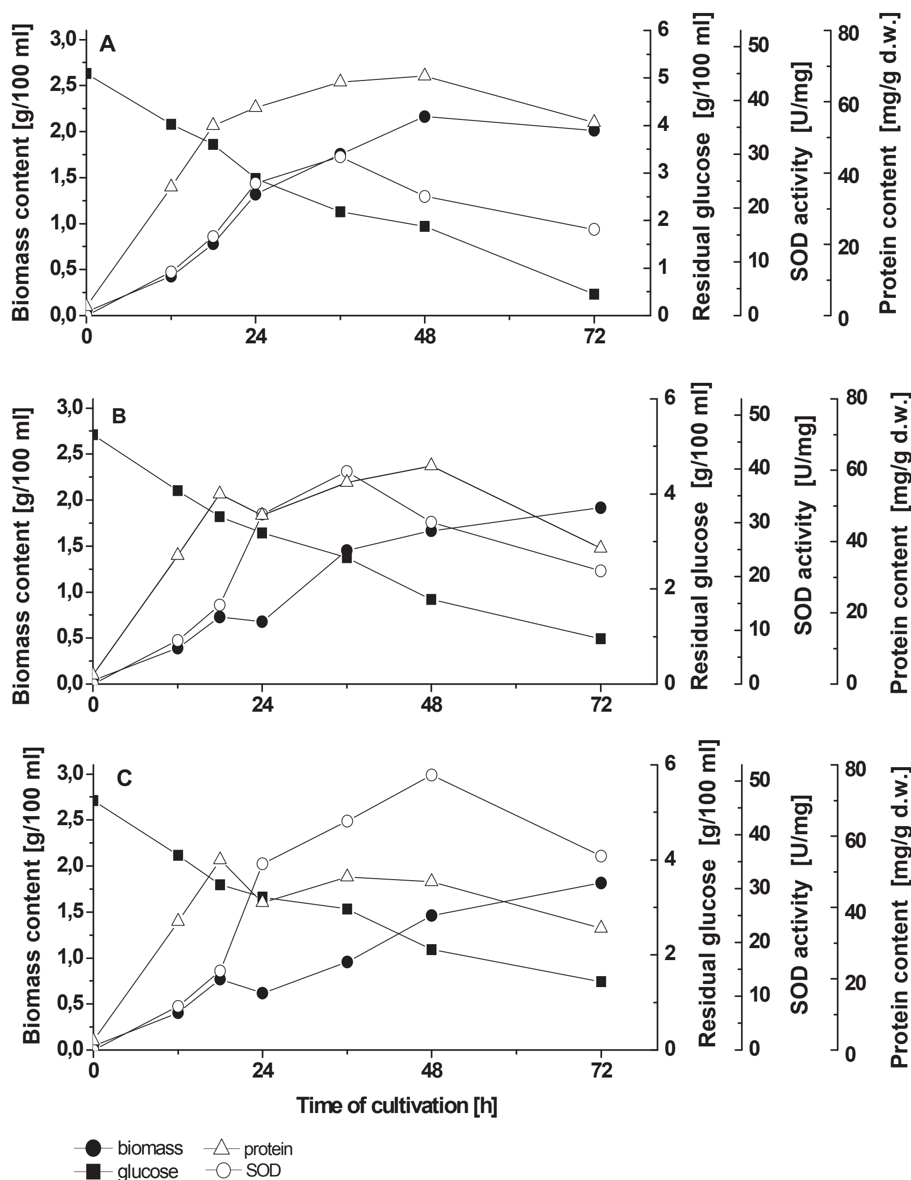

Fig 3 – Time courses of mycelia growth (△), SOD production (○), residual glucose (■), and intracellular protein content (●) in the fungal cultures during fermentation in 3 L bioreactor. A – growth at optimal temperature; B – growth after 6 h cold stress at 10 °C and the subsequent restoration of the normal conditions; C – growth after 6 h cold stress at 4 °C and the subsequent restoration of the normal conditions.

of purification procedures is presented in Table 2 and Fig 4A, B, C. In the first step, Superdex fast flow gel filtration successfully separated ultrafiltered solution into fractions by their molecular mass (Fig 4A). This chromatography enables the achievement of an 11-fold increase in the specific activity and enzyme yield of 35 %. Further purification was achieved by hydrophobic interaction chromatography on Phenyl-Sepharose column, giving rise to one active peak (Fig 4B) with specific activity of 749 U mg^{-1} protein, which was 28.8-fold higher than that of the crude enzyme with 8.1 % yield. PAGE analysis after Superdex and Phenyl-Sepharose column showed that the active peak contained also the above mentioned SOD bands (Cu/Zn-SODI and Cu/Zn-SODII), except for Mn-SOD (Fig 5A, lane 1; B, lane 1). These two enzymes were separated by chromatography on Q-Sepharose (Fig 4C). The major isoenzyme, Cu/Zn-SODII, was purified to homogeneity with a specific activity of 4000 U mg^{-1}, 153-fold purification, and an overall activity yield of 9.7 % (Fig 5A, lane 2; B, lane 2). The minor isoenzyme, Cu/Zn-SODI, gave a specific activity of 610 U mg^{-1} with 23-fold purification and 0.65 % yield (Fig 5A, lane 3; B, lane 3).

Characterization of Aspergillus glaucus *SODs*

Table 3 summarizes the results of SODI and SODII characterization. The possible protein glycosylation was checked by a qualitative colourimetric method in phenol–sulphuric acid. SODI responded positively to this test whereas SODII

Table 1 – Comparison of SOD productivities in unstressed and cold-stressed cultures[a].

Cultivation system[a]	Cultivation time [h]	DW[b] [g L^{-1}]	Yield of protein[c] [mg L^{-1}]	Specific SOD activity[d] [U (mg protein)$^{-1}$]	Total SOD activity[e] [U L^{-1}] $\times 10^3$	Yield coefficient[f] (Yp/x) [U g^{-1}] $\times 10^3$	SOD productivity[g] [U kg^{-1}] h^{-1} $\times 10^3$
Unstressed culture	36	17.52	1095.00	29.5	32.3	1843.7	51.3
Stressed culture							
10 °C	36	14.52	845.38	39.5	33.4	2299.7	63.89
4 °C	48	14.74	680.72	51.1	34.7	2363.1	49.2

a *A. glaucus* was cultivated as unstressed and stressed (4 h at 10 or 4 °C; see Materials and methods) cultures.
b Biomass content as dry weight at the time of harvest.
c Yield of protein is calculated as mg intracellular protein in the dry biomass, produced per litre of culture medium.
d Activities at the time of harvest.
e Total SOD activity is calculated as amount of enzyme per dry weight biomass, produced per litre of culture medium.
f SOD yield coefficient is calculated as amount of enzyme per kg dry biomass.
g SOD productivity is calculated as amount of enzyme per kg dry biomass produced per hour.

was found to be free of glycosylation. The size distribution of both SODs in solution was analysed by DLS. The hydrodynamic radius of SODI (2.9 nm) can accommodate a modelled globular protein of about 40 kDa and the radius of SODII (1.8 nm) corresponds to a globular protein of about 14 kDa. Furthermore, the low polydispersity parameter (distribution of sizes) below 10 % for both SODs indicates monodisperse and very homogenous monomer populations, without evidence for dimers or higher oligomers. The mass of both SODs was determined more precisely by ESI-Q-TOF mass spectrometry. The mass of SODI (38 159 Da) and of SODII (15 835 Da) confirmed the estimates provided by DLS.

The metal content of both SODs was further determined by ICP-MS. In the case of SODII, ICP-MS indicates a 1/1 molar ratio (metal/protein) for Cu and Zn, without significant amounts of Fe, Mn or Ni. Accordingly, this low molecular weight (MW) SOD clearly belongs to the Cu/Zn-SOD type. For SODI, the following molar ratios (metal/protein) were found: Cu = 0.7; Zn = 0.4; Mn = 0.16. These ratios suggest that the high MW SOD is also a Cu/Zn-SOD. These low ratios also suggest that its metal binding affinity is weak, resulting in the loss of metal ions during the purification process as both metals were lacking in the buffers. Finally, the melting temperature T_m was recorded by differential scanning calorimetry. SODII displays a T_m value of 80 °C and therefore its structure is rather stable to temperature. By contrast, SODI is more heat-labile (T_m = 54 °C). This can explain the low metal content of SODII as heat-labile psychrophilic proteins generally bind their cofactors with low affinity (Feller 2013).

The N-terminal amino acid sequence of SODII determined by automated Edman degradation corresponded to VKAVAVLRGDSKITG. A BLAST search (http://web.expasy.org/blast/) of this sequence against the whole databases retrieved only Cu/Zn SODs from fungi, including *Aspergillus* species. All these SODs are small proteins (153–155 amino acids, including the initial formyl-methionine), with masses in the range 15 800–15 900 Da and binding one Cu ion and one Zn ion. More specifically, this sequence is 100 % identical to N-terminal sequences of the fungi *Humicola lutea*, *Aspergillus fumigatus*, *Thermoascus aurantiacus*, and *Aspergillus fischerianus*. Attempts to determine the N-terminal sequence of SODI were unsuccessful.

Table 2 – Purification of Cu/Zn-SOD from *A. glaucus* cells.

Step	Total SOD activity (units)	Specific SOD activity (U mg^{-1})	Yield (%)	Purification (fold)
Cell-free extract	66 838	26	100	1.0
Superdex	7887	142	35	11.8
Phenyl-Sepharose	5416	749	8.1	28.8
Q-Sepharose I peak	6500	4000	9.7	153.4
Q-Sepharose II peak	430	610	0.65	23.5

Discussion

Antarctica constitutes a large unrevealed reservoir of cold-adapted organisms, including fungi that can be exploited as producers of valuable thermo-sensitive enzymes. Our previous studies evidenced that Antarctic fungi possess powerful enzymatic systems and synthesized CA antioxidant enzymes, SOD, and catalase (Tosi *et al.* 2010). The present paper reports that *Aspergillus glaucus* 363 is an effective producer of Cu/Zn-SOD. The strain contains at least one gene encoding Cu/Zn-SOD that exhibited high homology with the corresponding gene of *Aspergillus fumigatus* Af293 and *A. fumigatus* AF128886. To our knowledge, this is the first nucleotide sequence of a CA Cu/Zn-SOD gene in fungi. Identification of genes encoding CA SOD synthesis is very seldom published. Until now, such identification has been carried out in bacterial strains *Aliivibrio salmonicida* (Fe-SOD, *sodB*) and *Psychromonas arctica* (Pedersen *et al.* 2009; Na *et al.* 2011).

Although *A. glaucus* 363 was isolated from samples taken from Antarctica, this strain belongs to the mesophilic temperature group (Tosi *et al.* 2010). Mesophilic fungi have been found in different cold ecosystems (Margesin *et al.* 2008). According

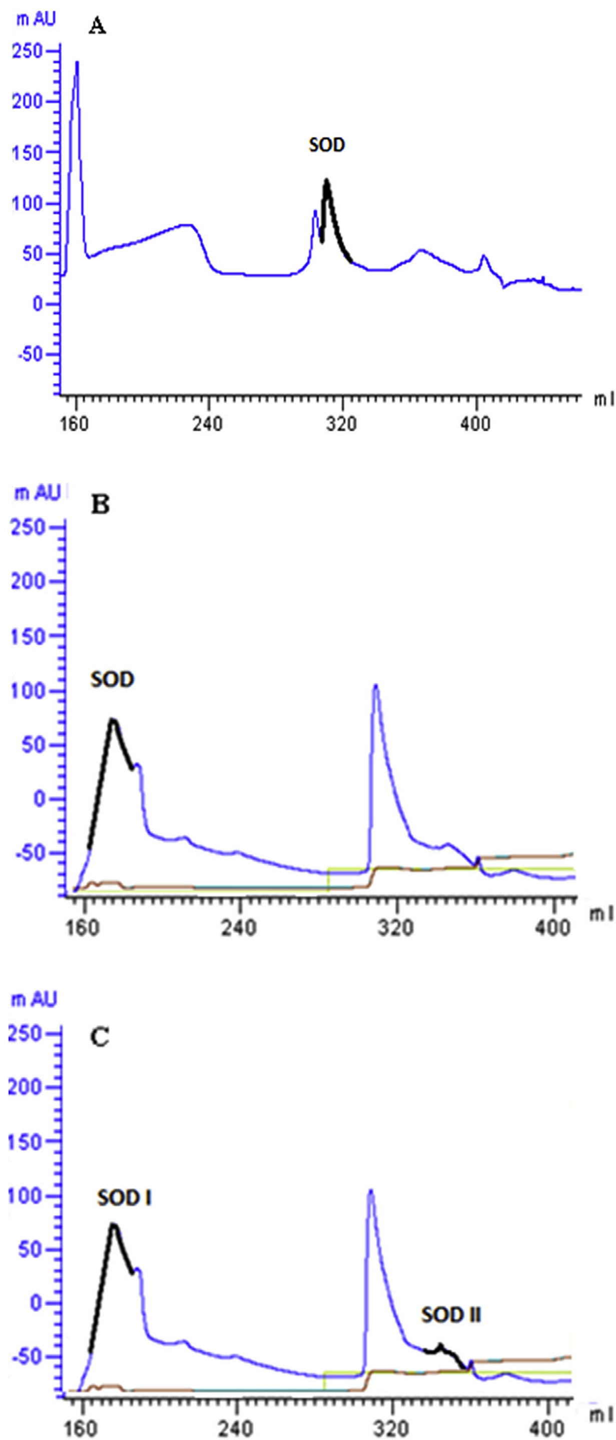

Fig 4 – Chromatographic patterns observed during purification of *A. glaucus* SOD. SOD samples were applied to a column of Sephadex (A), Phenyl-Sepharose (B), and Q-Sepharose (C).

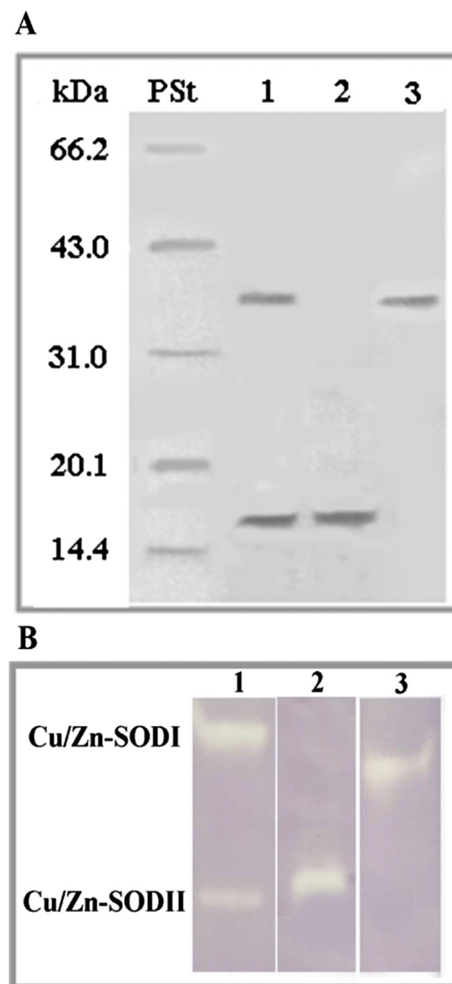

Fig 5 – PAGE analyses of *A. glaucus* SOD isoenzymes after different step of purification. (A) SDS-PAGE and protein staining; (B) native PAGE staining for activity. PSt – protein standards; 1 – after Phenyl-Sepharose column; 2 and 3 – after Q-Sepharose chromatography.

to Zucconi *et al.* (1996), such fungi can be assigned to dominant indigenous or mesophilic psychrotolerant species. Moreover, the produced SOD by *A. glaucus* 363 retains psychrophilic characteristics. The majority of CA enzymes of microbial origin are produced by psychrophilic or psychrotolerant strains. However, some mesophilic microorganisms can also synthesize thermo-sensitive enzymes. A similar phenomenon has been reported for CA lipase found in mesophilic bacteria, yeasts, and fungi (see Yuan *et al.* 2010; Bae *et al.* 2014). The mesophilic fungal strain *Trichoderma reesei* produces several thermo-sensitive hydrolases (Nevaleinen *et al.* 2012).

Generally, more reactive oxygen species (ROS) are produced in stressed cells, which cause cellular damages. The overexpression of SODs can lead to protection against specific stresses, implying that SOD may be the first line of defense against ROS (Fridovich 1998). The present results suggest that manipulation of the cell redox state allows the development of fungal cultures with improved antioxidant defence (Fig 3). Besides the transient character of the cold stress, the enhancement of SOD activity was observed until the end of cultivation. Our previous studies have demonstrated that the increased level of ROS generated during the temperature

Table 3 – Characterization of *A. glaucus* SODs.

	SODI	SODII
Glycosylation	+	−
Hydrodynamic radius	2.9 nm	1.8 nm
Polydispersity	10 %	6 %
Mass by ESI-Q-TOF	38 159 ± 4 Da	15 835 ± 2 Da
Metal content (molar ratio)		
Cu	0.7	1
Zn	0.4	1
Melting point	54 °C	80 °C

downshift induced SOD biosynthesis in *A. glaucus* 363 (Kostadinova *et al.* 2012). Similar approach has been used for improving SOD activity in different pro- and eukaryotic organisms (Smirnova *et al.* 2001; Baek & Skinner 2012; Kayihan *et al.* 2012; Xu *et al.* 2013). High ROS concentrations generated by exposure to H_2O_2, high DO, and superoxide-generating chemicals were reported to cause activation of SOD synthesis (Angelova *et al.* 2005; Li *et al.* 2009). According to Baez & Shiloach (2013), superoxide stress regulator (SoxRS) and SOD are the main defence mechanism that protects bacteria from the toxic effects of high oxygen saturation.

Due to the wide range of SOD applications, numerous direct and indirect methods have been developed for production of mesophilic enzyme. Both native and recombinant SODs are now available (Xie *et al.* 2010; Zhu *et al.* 2013; Tuteja *et al.* 2015). Despite great efforts, efficient production of recombinant SOD in heterologous host has failed because of deficit in its metal cofactors (Bafana *et al.* 2011). Thus, more efficient microbial technologies need to be elaborated. Indeed, there is no information also about technology for CA SOD production. We used the relationship between low temperature treatment and enhanced antioxidant defense to develop a laboratory technology for effective production of CA SOD by the fungus *A. glaucus* 363. Our results showed that the cold-stress bioprocessing strategy led to significant increase in enzyme yield. This strategy could be useful for large-scale efficient production of CA SOD. There is no information about the transient temperature downshift application for improvement of SOD yield in biotechnological process. Zhu *et al.* (2013) reported the positive effect of NH_4^+ and Mn^{2+} ions on the production of hyperthermostable Mn-SOD in 5 L bioreactors. This is another bioprocessing strategy that is based on the metal ions-induced oxidative stress. Our previous studies demonstrated a significant increase in the yield of mesophilic SOD in bioreactor cultures by using both an exposure to 20 % DO level or glucose-feeding technology (Angelova *et al.* 2001; Krumova *et al.* 2007). Our study offers a simple and effective procedure for CA SOD purification. The specific activity of the major isoenzyme Cu/Zn-SODII was improved by 150 % while total protein content was decreased by more than 90 %. Such specific activity (4000 U mg^{-1}) is comparable or even higher than the activity of commercial mesophilic Cu/Zn-SOD.

In addition to the partial gene sequence of Cu/Zn-SODII, all structural parameters (mass, metal content, N-terminal sequence) relate this major SOD enzyme from *A. glaucus* to homologous SODs specifically found in fungi. For instance, the closest homologue from *A. fumigatus* Af293 (based on nucleotide and N-terminal sequences) is a small Cu/Zn-SOD (153 amino acid residues, formyl-Met removed) of 15 835 Da, in perfect agreement with the experimentally determined mass of Cu/Zn-SODII. By contrast, identification of the high MW Cu/Zn-SODI from *A. glaucus* requires further investigations.

The experiments with purified enzyme demonstrated its protective effect on normozoospermic and oligo-asthenoteratozoospermic male partner's semen samples undergoing manipulation procedures for *in vitro* fertilization (unpublished data).

Conclusion

Taken together, our results presented an effective laboratory technology for production of CA SOD by the Antarctic fungus *Aspergillus glaucus* 363. The major isoenzyme Cu/Zn-SODII is a 15.8 kDa protein, closely related to some homologous SODs from fungi. The purification protocol allows obtaining enzyme with 4000 U mg^{-1} specific activity that is comparable or even higher than the activity of commercial mesophilic SOD. These findings make *A. glaucus* 363 worthy of further investigation in larger scale operations. The purified CA enzyme SOD reported in the present work would be boon for application in ART.

Conflict of interest

The authors declare that they have no conflict of interest.

Informed consent

All named authors declare that they are agreed to publish this manuscript.

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REFERENCES

Abrashev RI, Pashova SB, Stefanova LN, Vassilev SV, Dolashka-Angelova PA, Angelova MB, 2008. Heat-shock-induced oxidative stress and antioxidant response in *Aspergillus niger* 26. *Canadian Journal of Microbiology* **54** (12): 977–983.

Agarwal A, Durairajanayagam D, du Plessis SS, 2014. Utility of antioxidants during assisted reproductive techniques: an evidence based review. *Reproductive Biology and Endocrinology* **12**: 112.

Alvarez JG, 2012. Loss of intracellular antioxidant enzyme activity during sperm cryopreservation: effects on sperm function after thawing. In: Parekattil SJ, Agarwal A (eds), *Male Infertility*

Contemporary Clinical Approaches, Andrology, ART & Antioxidants. Springer, New York, Heidelberg, Dordrecht, London, pp. 237–244.

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**: 1641–1650.

Angelova M, Genova L, Pashova S, Slokoska L, Dolashka P, 1996. Effect of cultural conditions on the synthesis of superoxide dismutase by *Humicola lutea* 110. *Journal of Fermentation and Bioengineering* **82** (5): 464–468.

Angelova MB, Pashova SB, Spasova B, Vassilev SB, Slokoska LS, 2005. Oxidative stress response of filamentous fungi induced by hydrogen peroxide and paraquat. *Mycological Research* **109**: 150–158.

Atig F, Raffa M, Ben Ali H, Abdelhamid K, Saad A, Ajina M, 2012. Altered antioxidant status and increased lipid per-oxidation in seminal plasma of Tunisian infertile men. *International Journal of Biological Sciences* **8** (1): 139–149.

Bae J-H, Kwon Mi-Hyun, Kim In-Hwan, Hou Ching T, Kim Hak-Ryul, 2014. Purification and characterization of a cold-active lipase from *Pichia lynferdii* Y-7723: pH-dependant activity deviation. *Biotechnology and Bioprocesses Engineering* **19**: 851–857.

Baek K-H, Skinner DZ, 2012. Production of reactive oxygen species by freezing stress and the protective roles of antioxidant enzymes in plants. *Journal of Agricultural Chemistry and Environment* **1**: 34–40.

Baez A, Shiloach J, 2013. *Escherichia coli* avoids high dissolved oxygen stress by activation of SoxRS and manganese-superoxide dismutase. *Microbial Cell Factories* **12**: 12–23.

Bafana A, Dutt S, Kumar S, Ahuja PS, 2011. Superoxide dismutase: an industrial perspective. *Critical Reviews in Biotechnology* **31** (1): 65–76.

Barghini P, Moscatelli D, Garzillo AMV, Crognale S, Fenice M, 2013. High production of cold-tolerant chitinases on shrimp wastes in bench-top bioreactor by the Antarctic fungus *Lecanicillium muscarium* CCFEE 5003: bioprocess optimization and characterization of two main enzymes. *Enzyme and Microbial Technology* **53**: 331–338.

Beauchamp C, Fridovich I, 1971. Superoxide dismutase: improved assay and an assay applicable to polyacrylamide gels. *Analytical Biochemistry* **44** (1): 276–287.

Brenchley JE, 1996. Psychrophilic microorganisms and their cold-active enzymes. *Journal of Industrial Microbiology* **17**: 432–437.

Chary P, Dillon D, Schroederf AL, Natvig DO, 1994. Superoxide dismutase (*sod-I*) null mutants of *Neurospora crussu*: oxidative stress sensitivity, spontaneous mutation rate and response to mutagens genetics. *Genetics* **137**: 723–730.

Chattopadhyay MK, Raghu G, Sharma YVRK, Biju AR, Rajasekharan MV, Shivaji S, 2011. Increase in oxidative stress at low temperature in an Antarctic bacterium. *Current Microbiology* **62**: 544–546.

Dolashki A, Abrashev R, Stevanovic S, Stefanova L, Abasid A, Velkova L, Hristova R, Angelova M, Voelter W, Devreese B, Van Beeumen J, Dolashka-Angelova P, 2008. Biochemical properties of Cu/Zn-superoxide dismutase from fungal strain *Aspergillus niger* 26. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy* **71**: 975–983.

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F, 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**: 350–356.

El Shafey HM, Bahashwan SA, Alghaithy AA, Ghanem S, 2010. Microbial superoxide dismutase enzyme as therapeutic agent and future gene therapy. In: Mendez Vilas (ed.), *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. *Microbiology Book Series A*, vol. 1. Formatex, Spain, pp. 435–443.

Feller G, 2013. Psychrophilic enzymes: from folding to function and biotechnology. *Scientifica* **2013**: 28 Article ID 512840. http://dx.doi.org/10.1155/2013/512840 Hindawi Publishing Corporation.

Feller G, Gerday C, 2003. Psychrophilic enzymes: hot topics in cold adaptation. *Nature Reviews Microbiology* **1**: 200–208.

Fenice M, Barghini P, Selbmann L, Federici F, 2012. Combined effects of agitation and aeration on the chitinolytic enzymes production by the Antarctic fungus *Lecanicillium muscarium* CCFEE 5003. *Microbial Cell Factories* **11**: 12. http://dx.doi.org/10.1186/1475-2859-11-12.

Fernandes L, Bocca AL, Ribero AM, Silva SS, Paes HC, Amaral AC, Polez VLP, Martins NF, Soares CMA, Felipe MSS, 2008. Regulatory networks in the host fungal pathogen interactions. In: San-Blas G, Calderone RA (eds), *Pathogenic Fungi: insights in molecular biology*. Caister Academic Press, Norfolk, UK, pp. 77–111.

Frealle E, Noel C, Nolard N, Symoens F, Felipe MS, Dei-Cas E, Camus D, Viscogliosi E, Delhaes L, 2006. Manganese superoxide dismutase based phylogeny of pathogenic fungi. *Molecular Phylogenetics and Evolution* **41**: 28–39.

Fridovich I, 1998. Oxygen toxicity: a radical explanation. *Journal of Experimental Biology* **201**: 1203–1209.

Guo FX, Shi-jin E, Liu SA, Chen J, Li DC, 2008. Purification and characterization of a thermostable MnSOD from the thermophilic fungus *Chaetomium thermophilum*. *Mycologia* **100**: 375–380.

Hou YH, Wang TH, Long H, Zhu HY, 2006. Novel cold-adaptive *Penicillium* strain FS010 secreting thermo-labile xylanase isolated from Yellow Sea. *Acta Biochimica et Biophysica Sinica* **38**: 142–149.

Jaeger E, Carroll N, Choudhury S, Dunlop A, Towler H, Matheson M, Adamson P, Okhravi N, Lightman S, 2000. Rapid detection and identification of *Candida*, *Aspergillus*, and *Fusarium* species in ocular samples using nested PCR. *Journal of Clinical Microbiology* **38**: 2902–2908.

Joseph B, Ramteke PW, Thomas G, 2008. Cold active microbial lipases: some hot issues and recent developments. *Biotechnology Advances* **26**: 457–470.

Kayihan C, Eyidogan F, Afsar N, Oktem HA, Yucel M, 2012. Cu/Zn superoxide dismutase activity and respective gene expression during cold acclimation and freezing stress in barley cultivars. *Biologia Plantarum* **56**: 693–698.

Kim HR, Kim IH, Hou CT, Kwon KI, Shin BS, 2010. Production of a novel cold-active lipase from *Pichia lynferdii* Y-7723. *Journal of Agricultural and Food Chemistry* **58**: 1322–1326.

Kostadinova N, Krumova E, Stefanova T, Dishliyska V, Angelova M, 2012. Transient cold shock induces oxidative stress events in Antarctic fungi. In: Lushchak VI, Stoliar O (eds), *Oxidative Stress/Book 3*. InTech, pp. 75–99.

Krishnan A, Alias SA, Wong CMVL, Pang KL, Convey P, 2011. Extracellular hydrolase enzyme production by soil fungi from King George Island, Antarctica. *Polar Biology* **34**: 1535–1542.

Krumova E, Dolashka-Angelova P, Pashova S, Stefanova L, Beeumen JV, Vassilev S, Angelova M, 2007. Improved production by fed-batch cultivation and some properties of Cu/Zn-superoxide dismutase from the fungal strain *Humicola lutea* 103. *Enzyme and Microbial Technology* **40**: 524–532.

Krumova E, Dolashki A, Pashova S, Dolashka-Angelova P, Stevanovic S, Hristova R, Stefanova L, Voelter W, Angelova M, 2008. Unusual location and characterization of Cu/Zn-containing superoxide dismutase from filamentous fungus *Humicola lutea*. *Archives of Microbiology* **189**: 121–130.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lamarre C, LeMay JD, Deslauriers N, Bourbonnais Y, 2001. *Candida albicans* expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. *Journal of Biological Chemistry* **276**: 43784–43791.

Li Q, Harvey LM, McNeil B, 2009. Oxidative stress in industrial fungi. *Critical Reviews in Biotechnology* **29**: 199–213.

Lima OC, Larcher G, Vandeputte P, Lebouil A, Chabasse D, Simoneau P, Bouchara JP, 2007. Molecular cloning and biochemical characterization of a Cu,Zn-superoxide dismutase from *Scedosporium apiospermum*. *Microbes and Infection* **9**: 558–565.

Lin YC, Vaseeharan B, Chen JC, 2008. Identification of the extracellular copper–zinc superoxide dismutase (ecCuZnSOD) gene of the mud crab *Scylla serrate* and its expression following β-glucan and peptidoglycan injections. *Molecular Immunology* **45**: 1346–1355.

Lowry OH, Rosenbrough HJ, Faar AL, Randall RJ, 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**: 265–275.

Maniatis T, Fritsch E, Sambrook J, 1982. *Molecular Cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Margesin R, Schinner F, Marx JC, Gerday C, 2008. *Psychrophiles: from biodiversity to biotechnology*. Springer, Berlin.

Merlino A, Krauss IR, Castellano I, De Vendittis E, Rossi B, Conte M, Vergara A, Sica F, 2010. Structure and flexibility in cold-adapted iron superoxide dismutases: the case of the enzyme isolated from *Pseudoalteromonas haloplanktis*. *Journal of Structural Biology* **172**: 343–352.

Na J, Im H, Lee K, 2011. Expression and purification of recombinant superoxide dismutase (PaSOD) from *Psychromonas arctica* in *Escherichia coli*. *Bulletin of the Korean Chemical Society* **32**: 2405–2409.

Nam ES, Ahn JK, 2011. Isolation and characterization of cold-adapted bacteria producing lactose hydrolyzing enzyme isolated from soils of Nome area in Alaska. *International Research Journal of Microbiology* **2** (9): 348–355.

Nevaleinen H, Bradner R, Wadud S, Mohammed S, McRae C, Te'o J, 2012. Enzyme activities and biotechnological applications of cold-active microfungi. In: Anitori RP (ed.), *Extremophiles: microbiology and biotechnology*. Caister Academic Press, Norfolk, UK, pp. 89–108.

Nierman WC, Pain A, Anderson MJ, Wortman JR, Kim HS, Arroyo J, *et al*., 2005. Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature* **438**: 1151–1156.

Onofri S, Selbmann L, de Hoog GS, Grube M, Barreca D, Ruisi S, Zucconi L, 2007. Evolution and adaptation of fungi at boundaries of life. *Advances in Space Research* **40**: 1657–1664.

Pedersen HL, Willassen NP, Leiros I, 2009. The first structure of a cold-adapted superoxide dismutase (SOD): biochemical and structural characterization of iron SOD from *Aliivibrio salmonicida*. *Acta Crystallographica Section F: Structural Biology and Crystallization Communications* **65**: 84–92.

Raimondi S, Uccelletti D, Amaretti A, Leonardi A, Palleschi C, Rossi M, 2010. Secretion of *Kluyveromyces lactis* Cu/Zn SOD: strategies for enhanced production. *Applied Microbiology and Biotechnology* **86**: 871–878.

Rowe LA, Degtyareva N, Doetsch PW, 2008. DNA damage-induced reactive oxygen species (ROS) stress response in *Saccharomyces cerevisiae*. *Free Radical Biology and Medicine* **45**: 1167–1177.

Russell N, 2006. Antarctic microorganisms: coming in from the cold. *Culture (Oxoid)* **27**: 1–4.

Russell NJ, 2003. Psychrophily and resistance to low temperature. In: Gerday C, Glansdorff N (eds), *Extremophiles (Life Under Extreme Environmental Condition), Encyclopedia of Life Support Systems (EOLSS)*. EOLSS Publishers, Oxford Contribution number 6-73-03-00. www.eolss.com.

Singh SM, Singh SK, Yadav LS, Singh PN, Ravindra R, 2012. Filamentous soil fungi from Ny-Ålesund, Spitsbergen, and screening for extracellular enzymes. *Arctic* **65**: 45–55.

Smirnova GV, Zakirova ON, Oktiabr'skiĭ ON, 2001. Role of the antioxidant system in response of *Escherichia coli* bacteria to cold stress. *Mikrobiologiia* **70**: 55–60 (in Russian).

Somogyi M, 1952. Notes on sugar determination. *Journal of Biological Chemistry* **195**: 19–23.

Tosi S, Kostadinova N, Krumova E, Pashova S, Dishliiska V, Spassova B, Vassilev S, Angelova M, 2010. Antioxidant enzyme activity of filamentous fungi isolated from Livingston Island, Maritime Antarctica. *Polar Biology* **33**: 1227–1237.

Tuteja N, Mishra P, Yadav S, Tajrishi M, Baral S, Sabat SC, 2015. Heterologous expression and biochemical characterization of a highly active and stable chloroplastic CuZn-superoxide dismutase from *Pisum sativum*. *BMC Biotechnology* **15**: 3.

Xie XQ, Li F, Ying SH, Feng MG, 2012. Additive contributions of two manganese-cored superoxide dismutases (MnSODs) to antioxidation, UV tolerance and virulence of *Beauveria bassiana*. *PLoS ONE* **7**: e30298.

Xie XQ, Ying SH, Feng MG, 2010. Characterization of a new Cu/Zn-superoxide dismutase from *Beauveria bassiana* and two site-directed mutations crucial to its antioxidation activity without chaperon. *Enzyme and Microbial Technology* **46**: 217–222.

Xu J, Duan X, Yang J, Beeching JR, Zhang P, 2013. Coupled expression of Cu/Zn-superoxide dismutase and catalase in cassava improves tolerance against cold and drought stresses. *Plant Signaling and Behavior* **8**: e24525.

Youn HD, Kim EJ, Roe JH, Hah YC, Kang SO, 1996. A novel nickel-containing superoxide dismutase from *Streptomyces* spp. *Journal of Biochemistry* **318**: 889–896.

Yuan BH, Cai YJ, Liao XR, Yun LH, Zhang F, Zhang D, 2010. Isolation and identification of a cold-adapted lipase producing strain from decayed seeds of *Ginkgo biloba* L. and characterization of the lipase. *African Journal of Biotechnology* **9**: 2661–2667.

Zheng Z, Jiang YH, Miao JL, Wang QF, Zhang BT, Li GY, 2006. Purification and characterization of a cold-active iron superoxide dismutase from a psychrophilic bacterium, *Marinomonas* sp. NJ522. *Biotechnology Letters* **28**: 85–88.

Zhu H, Liu J, Qu J, Gao X, Pan T, Cui Z, Zhao X, Lu JR, 2013. Stress fermentation strategies for the production of hyperthermostable superoxide dismutase from *Thermus thermophilus* HB27: effects of ions. *Extremophiles* **17**: 995–1002.

Zucconi L, Pagano S, Fenice M, Selbmann L, Tosi S, Onofri S, 1996. Growth temperature preferences of fungal strains from Victoria Land, Antarctica. *Polar Biology* **16**: 53–61.