

A role for antioxidants in acclimation of marine derived pathogenic fungus (NIOCC 1) to salt stress

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Abstract

Salinity tolerance a key factor helps in understanding the ionic homeostasis in general, which is a fundamental cellular phenomenon in all living cells. Here, a marine derived pathogenic fungus was examined for its adaptation under salt stress using antioxidant properties. The aqueous extracts of halophilic fungus exhibited different levels of antioxidant activity in all the *in vitro* tests such as α,α -diphenyl- β -picrylhydrazyl (DPPH), Hydroxyl Radical Scavenging Assay (HRSA), Metal chelating assay and β – carotene – linoleic acid model system. The antioxidant capacity of marine fungus exposed to high salt condition showed an increase in activity. In addition, the production of intra and extracellular antioxidant enzymes of the fungus at various salt stresses were analyzed and discussed for their possible role in the stress mechanism. The marine derived fungus was identified as *Phialosimplex* genus, which is associated with infections in dogs. Thus the present study elucidates that the scavenging activity is one of the protective mechanisms developed in the fungus to avoid the deleterious effect of salt stress. In addition, the study also helps in understanding how the pathogenic fungus tackles the oxidative burst i.e. hypersensitivity reaction performed by host to kill the pathogens.

Key words: acclimation; antioxidant activity; halophilic; marine derived fungus; salt stress.

1. Introduction

Cells living in natural saline systems, where high salt amounts cause high osmotic pressure, must maintain lower water potential than their surroundings in order to survive and proliferate [1]. In addition, sodium concentrations in the environment lead to water-deficient cells, this ionic stress resulting from the toxicity of high levels of cytosolic sodium causes metabolic inhibition of cellular activities. Thus, all living cells maintain an intracellular ionic composition compatible with their constituent molecules. This requires the regulation of multiple membrane transporters and signal transduction pathways that effect ionic homeostasis, which mostly remain unknown [2]. In this study, we focused on hyperosmotic and oxidative stress conditions because these have been shown to contribute to fungal virulence and their sensitivity to antifungal drugs [3].

Halophilic microorganisms are adapted to conditions of high salinity and require a certain concentration of NaCl for their optimum growth which forms a highly diverse group belonging to Archaea, Bacteria and Eukarya [4]. Fungi are now recognized as an integral part of indigenous halophilic microbial communities as they can grow and adjust across the whole salinity range, from freshwater to almost saturated NaCl solutions [5, 6]. Thus, some fungal species like *Debaryomyces hansenii*, *Hortaea werneckii*, and *Wallemia ichthyophaga* have been isolated globally from natural hypersaline environments [7]. Halophilic micro-organisms also have developed different strategies to counterbalance changes in osmotic pressure; one such change in membrane composition and its properties represent an important factor in adaption to high salinity [8]. In addition, the organic compounds accumulated in the cytoplasm act as osmoprotectants, also called as compatible solutes [9]. Compatible solutes at high concentrations, allow enzymes to function efficiently [10]. The most common compatible solutes are neutral or zwitterionic and include amino acids, sugars, polyols, betaines and ectoines, as well as derivatives of some of these compounds [9]. It has also been reported that when large amounts of various solutes (e.g. NaCl) are present extracellularly, cells are subjected to osmotic stress, but in order to ease this stress, salt-tolerant organisms intracellularly accumulate large amounts of substances known as compatible solutes. In yeast cells, these are mainly polyols (e.g. glycerol, arabitol and mannitol) [11]. Moreover, polyols can also serve as carbon storage compounds, which may help in balancing the cellular redox potential and they can act as scavengers of reactive oxygen species (ROS) [12]. The term “oxygen-free radicals” has been used to refer to all species of oxygen (reactive oxygen species, ROS) that are more reactive than O₂ in its ground state or triplet state (³O₂). These are dioxygen in its excited state singlet forms (¹O₂) and the partially reduced forms of

oxygen, that is superoxide radical ion and its protonated form ($O_2^{\cdot-}$ and HO_2^{\cdot} , respectively), hydroxyl radical (HO^{\cdot}) and hydrogen peroxide (H_2O_2) [13]. ROS influence molecular and biochemical processes and signal transduction pathways, which affect proliferation, differentiation, and death in fungi, and in a variety of other organisms [14]. Salinity stress results in increased production of ROS, and detoxifying enzymes such as superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase, are important components of the cellular response to salt stress [2]. Furthermore, earlier it was reported that HOG (High Osmolarity Glycerol) MAPK (Mitogen Activated Protein Kinase) pathway is required for responses to osmotic stress are essential in a pathogenic fungus for resistance to oxidative stress [3,15]. Thus, the present study was conducted using marine derived fungus, as the candidate species with the objective to assess the acclimation of fungus to salt stress using antioxidant properties, and to understand how the pathogenic fungus tackles the oxidative burst i.e. hypersensitivity reaction performed by host to kill the pathogens.

2. Materials and Methods

2.1. Chemicals

Sodium bicarbonate, Folin–Ciocalteu reagent, Gallic acid, Sodium nitrite ($NaNO_2$), Aluminium chloride ($AlCl_3$), Sodium hydroxide ($NaOH$), Quercetin, 1, Ethanol, xylenol orange, Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT) were obtained from Merck, Mumbai, India. α, α -diphenyl- β -picrylhydrazyl (DPPH) were purchased from Sigma Aldrich (Steinheim Germany). H_2O_2 , HPLC grade methanol, Ammonium ferrous sulphate, deoxy ribose, Ferric chloride, Ethylenediaminetetraacetic acid (EDTA), Ascorbic acid, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Potassium hexacyanoferrate, Ferric chloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ferrous sulphate, Ferrozine, Chloroform, Tween 80 were obtained from SD fine chemicals, India. β -carotene and Linoleic acid were purchased from HiMedia, Mumbai, India. Native PAGE protein molecular weight marker ranging from 240,000- 18,400 kD purchased from GeneiTM, Bangalore, India. All other reagents were of analytical grade.

2.2. Isolation and Growth characterization of the halophilic fungus

The sea water collected from Dona Paula beach (Goa, India) were poured (200 μ l) and spread on the Malt extract agar (MEA) media (Himedia, Mumbai, India) containing plates. The plates were screened under a stereomicroscope for the fungal structures at different intervals of incubation (2, 8, 16 and 32 weeks) and isolated using a sterile needle and transferred to Malt extract agar (MEA) medium

containing 10,000 units of sodium benzyl penicillin and 0.05 g of streptomycin sulfate per 100 ml of medium to inhibit bacterial growth as described earlier [16]. The optimization of media and salt concentration (for halotolerant) for marine derived fungus was done using plate assay and broth. Different commonly used media (Himedia, Mumbai, India) like MEA, potato dextrose agar (PDA), czapek dox agar (CDA) and corn meal agar (CMA) were tried. The salt concentration of the optimized medium was adjusted to distilled water, sea water, 10% NaCl, 15% NaCl, 25% NaCl. The fungal inoculum from the mother plate was placed at the center of the plate. The plates were made up in triplicate and incubated at room temperature. The growth of the mycelium was noted on each day and the rate of growth was plotted.

2.3. Determination of Wet and Dry weight

The growth of the cells was measured in terms of dry and wet weight from the cultures maintained in the broth. The mycelia were harvested after one month via filtration with pre-weighed whatman filter paper. Thus the wet weight was determined by the difference between the preweighed weight and weight of filter paper after filtration. This filter paper was then dried in hot air oven until a constant dry weight was obtained and weighed. The difference in dry weight was considered to be the dry weight of the mycelia. The cell-free culture filtrate obtained after filtration was collected and stored at -20°C for further processing.

2.4. Identification of fungus using microscopic and morphological characters, and sequencing analysis

The fungus was identified based on the colony morphology and microscopical features using standard taxonomic keys and monographs [17, 18]. For 18S gene analyses the DNA was extracted from the fungal mycelium following CTAB method [19]. PCR Amplification of the 18S region was achieved with the primers NS1, NS3 (forward) and NS4, NS8 (reverse) [19]. The conditions for reaction were as follows: The amplification was done with the following reaction conditions: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1.5 min. There was a final extension step of 10 min at 72°C with a 4°C soak at the end. The PCR products were purified by the MinElute PCR Purification Kit (Qiagen, USA) according to the manufacturer's instructions. The direct sequencing of 18S rRNA gene sequence data derived from the fungal strain using an automated multicapillary DNA sequencer (ABI Prism 3130xl Genetic analyzer, Applied Biosystems, Foster City, CA, USA), have been submitted and deposited at GenBank

under accession number HQ188293. To determine the most closely related halophilic fungus spp., the 18S was compared to other sequences in the GenBank database by NCBI BLAST analysis. The 18S sequences of morphologically and phylogenetically related halophilic fungus spp. obtained from GenBank were aligned in Clustal X 1.81 [20] with pair wise and multiple alignment parameters. The sequences were checked manually for large gaps and flushed at the ends to avoid missing information for any compared reference entries. The set of aligned sequences were then used to derive corrected Kimura two-parameter distance [21] estimates and infer phylogenetic relationships using both distance-based neighbor-joining (NJ) [22] and Maximum likelihood (ML) [23] methods, with analytical routines available in the software package MEGA4 (Molecular Evolutionary Genetics Analysis (MEGA)) software version 4.0. Node support is evaluated through 1000 bootstrap replicates [24]. For parsimony, a heuristic search was conducted with simple addition of sequences. Tree was rooted with *Chaetomium globosum*.

2.5. Total antioxidant and antioxidant activity assay

2.5.1. Sample Preparation

The culture filtrate was separated from the mycelium by filtration with Whatman No 1 filter paper (9 cm) under vacuum and centrifuged at 4000 rpm to separate the remaining mycelial mass from the filtrates. The obtained filtrate sample was stored at 4°C until use within 24 h. The filtrate was also concentrated and extracted with ethyl acetate to get the crude extract as described earlier [25].

2.5.2. Determination of total phenolic content (TPC)

The total soluble phenolic content was estimated for each extract using a modified version of the Folin assay of Vatterm and shetty [26], and gallic acid was used as the phenolic standard. Briefly, 100 µl of sample and 2 ml sodium bicarbonate mix were incubated at room temperature for 2 min followed by addition of 100 µl of Folin-Ciocalteu reagent and incubated in dark for 30 min. The samples were vortexed and assayed for absorbance at $\lambda = 725$ nm using a spectrophotometer. Gallic acid 1mg/ml was used as standard and standard curve was obtained using various concentrations of gallic acid. We also used plain media (without fungus) as the controls where no phenols were observed.

2.5.3. DPPH Scavenging Assay

The fungal extracts containing antioxidants were aliquoted into different concentrations (50 – 200 µg/ml) to determine its ability to scavenge 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals using the

method of Yildirim et al. [27]. DPPH solution (1 mM DPPH radical solution in 95% ethanol) of 800 μ l was mixed with 200 μ l of sample extract, vortexed well, and then incubated for 30 min at room temperature in dark. After 30 min incubation, the samples were poured into microcentrifuge tubes and centrifuged for 5 min at 13,500 rpm (at room temperature). Then, the absorbance of each sample at $\lambda = 517$ nm was measured and 1 ml of 95% EtOH was used as a control. Butylated Hydroxy Anisole (BHA) and Butylated Hydroxy Toluene (BHT) were used as reference compounds. The antioxidant activity is given as percent (%) DPPH scavenging, calculated using the formula: [(control absorbance - extract absorbance) / (control absorbance) \times 100].

2.5.4. Hydroxyl Radical Scavenging Assay (HRSA)

In this assay the hydroxyl radical was produced by the Fe^{3+} ascorbate EDTA H_2O_2 system i.e. fenton reaction [28]. The reaction was performed in phosphate buffer (20 mM, pH 7.4) containing 2-deoxyribose (2.8 mM), FeCl_3 (100 μ M), H_2O_2 (1 mM), EDTA (100 μ M) and various concentrations (50–200 μ g/ml) of sample of the test sample and reference compound. The ascorbic acid (100 μ M) was added at last and incubated at 37 $^\circ\text{C}$ for 1 hr. The reaction volume of the above said mixture was 1 ml. After incubation 1 ml of 2.8 % TCA, then 1 ml of 1 % aqueous TBA was added and the mixture was incubated at 90 $^\circ\text{C}$ for 15 min to develop the color. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. The mannitol was used as reference compound. The percentage of scavenging was determined as, % scavenging = [1 - (sample absorbance / Blank absorbance) \times 100]

2.5.6. Metal chelating assay

The metal chelating activity was estimated by the method of Dinis et al. [29] with slight modification. The reaction was performed in HEPES buffer (20 mM, pH 7.2). Various concentrations (50–200 μ g/ml) of samples were mixed with solution of 12.5 μ M ferrous sulphate solution. The reaction was initiated by the addition of 75 μ M ferrozine and the mixture was shaken vigorously and incubated for 20 min at room temperature. After incubation the absorbance was measured at 562 nm. EDTA was used as the reference compound and the percentage chelating capacity was calculated as; % Chelating activity = [(A0 - A1)/A0] \times 100 where, A0 = Absorbance of the blank; A1= Absorbance of the sample.

2.5.7. Antioxidant assay using 1 β – carotene – linoleate model system

The antioxidant activity of the extract was evaluated by β -carotene–linoleate model system [30]. β -carotene–linoleate emulsion was prepared freshly. Aliquots of 2.8 ml of emulsion were transferred into assay tubes containing various concentration of extract (0.2 ml). As soon as the emulsion was added the initial absorbance was measured at 470 nm for each reaction and the tubes were incubated at 50 °C in a water bath for 2 hr. The absorbance after 2 hr incubation was also measured. A blank devoid of β -carotene was prepared for the background subtraction. The same procedure was used for synthetic antioxidant BHT (reference compound) at various concentrations (50–200 μ g/ml). The antioxidant activity was calculated using the following equation: Antioxidant activity = (β -carotene content after 2 hr / β -carotene content at initial stage) \times 100.

2.6. Antioxidant enzyme assay

Antioxidant enzyme was assayed in culture liquid filtrates (extracellular) and the mycelium (intracellular). The mycelium was separated from the medium on filter paper. Samples of mycelium were washed with distilled water and dissolved in 50 mM phosphate buffer (pH 7.0). The cells were destroyed by treatment with quartz sand in a porcelain mortar for 4–5 min and suspended in the buffer. The homogenate was centrifuged at 4000 rpm for 30 min. Protein was estimated by the procedure of Bradford's method [31].

2.6.1. Superoxide dismutase assay (SOD)

The method is based on the ability of superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium by superoxide [32]. An aliquot of 1ml of the reagent mixture (27 ml 50 mM Potassium phosphate buffer - pH 7.8, 1ml Nitroblue tetrazolium (NBT), 1.5 ml of Q-Methionine, 0.75ml of Triton x-100) was placed into small glass tubes. To which 20 μ l of enzyme extract to each tube was added along with 10 μ l of riboflavin and mixed properly. Then the cocktail was illuminated for 7 min's in aluminium foiled box (containing two 20 v fluorescent tubes). Control tubes were used without enzyme sample but with 20 μ l of buffer. The absorbance was taken at A560 nm. After reading, the test tube containing reagent mixture exposed to light for again 7 min's immersing in cylindrical glass container of $\frac{3}{4}$ th filled with clean water at 25°C and placed in between two 20 v fluorescent tubes. This increases the absorbance due to formazan formation, and the reading at 560 nm under each 30 seconds up to 5 min's was taken. Increasing in absorbance without enzyme extract (i.e. in control tube) was taken as 100% and the enzyme activity was calculated by determining the percentage incubation/min (A560 nm at 5 min's –

A560 nm at 0:30 sec/5 min's = A560 nm/Units). One unit is defined as that amount of enzyme causing half the maximum inhibition of NBT reduction and was expressed as units per mg protein. The reaction velocity will depend largely on somewhat variable assay conditions such as light intensity and reaction temperature.

2.6.2. Catalase assay

The catalase assay was performed based on the protocol described earlier [33]. The catalase assay principle was that the catalase enzyme degrades hydrogen peroxide to water and oxygen and hence the absorbance is reduced. The rate of change of absorbance was measured at 240 nm. The assay mixture was prepared by mixing 2.9 ml of the substrate solution i.e. 0.036% H₂O₂ and 100µl of the sample solution. The blank was prepared using phosphate buffer instead of the sample. The decomposition of the H₂O₂ (substrate) was measured as the reduction in absorbance at 240 nm. One unit is defined as the decomposition of 1 µM of H₂O₂ per minute per milligram of protein at pH 7.0 at 25°C. The rate of disappearance of H₂O₂ is followed by observing the rate of decrease in the absorbance at 240 nm.

2.6.3. Guaiacol Peroxidase assay

Based on the procedure described earlier [34], the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, was determined by measuring the rate of colour development spectrophotometrically at 436 nm. The assay mixture for peroxidase assay comprised of 2.8 ml of 0.1 M phosphate buffer (pH 7), 50 µl of 0.018 M guaiacol and 50 µl of substrate (H₂O₂, Dilute 0.1 ml 30% hydrogen peroxide with distilled water to 120 ml. Store the solution on ice and prepare fresh). The assay mixture was pipetted directly into the cuvette and 100 µl of sample was added. The rate of increase in absorbance per minute was observed at 436 nm. A blank was prepared without the sample and used for background subtraction. One unit is defined as the amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute.

2.6.4. Glutathione S Transferase assay:

The Glutathione S Transferase activity was measured by observing the conjugation of 1-Chloro, 2, 4-Dinitrobenzene (CDNB) with reduced glutathione (GSH). This is done by measuring an increase in absorbance at 340 nm. One unit of enzyme will conjugate 10 nmol of CDNB with reduced glutathione per minute at 25°C. This assay was performed based on the procedure described by Mannervik [35]. The

assay cocktail was prepared prior to the assay. For each reaction the assay cocktail composed of 980 μ l of Phosphate buffer saline (PBS) pH 6.5, 10 μ l of 100 mM CDNB (dissolved in ethanol), and 10 μ l of 100 mM glutathione. For each sample and a blank, 900 μ l of assay cocktail was added directly into the cuvette and 100 μ l of PBS buffer was added in case of blank to auto zero the instrument. For test reaction sample was added instead of PBS and the rate of increase in absorbance was measured at 340 nm.

2.7 Antioxidant enzyme activity staining assay

2.7.1. Superoxide dismutase (SOD)

Activity of SOD in the gels was visualized by the activity staining procedure of [36]. The extracellular cell free extracts and the cell grounded intracellular extracts subjected with salt stress were resolved on 12% and 10% non denaturing PAGE in Tris-glycine buffer. The resolved gel was incubated in staining buffer (50 mmol/L potassium phosphate buffer, pH 7.8, 0.1 mmol/L EDTA, 28 mmol/L TEMED, 0.003 mmol/L riboflavin and 0.25 mmol/L NBT) for 30 min in the dark at room temperature. Gels were then exposed under two fluorescent tubes until the white SOD activity bands became visible. After the appearance of the bands, exposure to light was avoided to prevent the darkening of the gel [37].

2.7.2. Catalase assay (CAT)

Cell free extracts for extracellular, and the fungal cells grounded with PBS buffer for intracellular catalase subjected to different salt stress was resolved in 8% non-denaturing PAGE in Tris-glycine buffer using the method of Shukla et al. [38] with minor modification. Briefly, the resolved gel was washed twice with distilled water immediately and rinsed with 50 mM phosphate buffer (pH 7.8) containing 0.01M H₂O₂. Gentle rocking was given for 20 min at room temperature in dark. Phosphate buffer was removed and the gel was rinsed with distilled water to remove any residual H₂O₂. Finally the gel was immersed in 2% of 30 ml potassium ferric cyanide and 2% of 30 ml of ferric chloride and gentle rocking was made until the appearance of bands over light box. The yellow catalase bands on the blue background of the gel appear. The ferricyanide mixture was decanted and the gel was rinsed with distilled water immediately on the appearance of the catalase bands.

2.7.3. SOD gene analyses

The DNA was extracted from the fungus using the CTAB method (19). The SOD gene fragments were amplified through PCR by using the primers, sodF (5'-AGG TCA CTG GCT CCA TCA TC-3') and sodR (5'-CTA CTG CGA GAT GCG AAT GA-3') with the required cycle numbers at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The SOD genes of fungal strain NIOCC 1 were determined by using a BigDye Terminator v3.1 Cycle sequencing kit and an ABI Prism 3130xl automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The gene sequences were compared with relative gene sequences available at the GenBank database (<http://www.ncbi.nlm.nih.gov/blast/>) and the Gen Bank accession number (JX157150) was obtained.

2.7.4. RNA isolation and quantitative RT-PCR analysis

Total RNA contents were extracted from fungal mycelia using Tri Reagent according to the manufacturer's protocols (SIGMA-ALDRICH, USA). The RNA preparations were treated with ribonuclease-free deoxyribonuclease (Dnase) I (Invitrogen) to remove residual genomic DNA. Total RNAs were then subjected to quantitative RT-PCR for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen) and oligo (dT) primer for 1 h at 50°C. Thereafter, equal amounts of cDNA products were amplified using the above SOD primers and 18S rRNA (forward, 5'-GTA GTC ATA TGC TTG TCT C -3'; reverse, 5'-CTT CCG TCA ATT CCT TTA AG-3') (19), with the required cycle numbers at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The amplified PCR products were fractionated on a 1% agarose gel in 1 x tris-acetate-EDTA (TAE) buffer for 30 min at 100 V and visualized by staining with 0.6 µg/mL ethidium bromide. The intensities of the bands were measured with IMAGE GUAGE V.12 analysis software, and the ratios to 18S rRNA signal were determined.

2.8. Statistical analysis:

The data were subjected to a one way analysis of variance and the significance of the difference between means was determined by Kruskal wallis test ($p < 0.05$) using Statistica for, Version 6 (Statsoft Inc., Tulsa, USA) followed by a post-test Bonferroni. All data are given as the mean \pm SD of triplicates ($n = 3$). The EC₅₀ values were calculated from the linear regression equation determined by XY scatter plot in Excel.

3. Results and discussion

3.1. Identification and growth optimization of marine derived halophilic fungus

The marine derived fungus was grown in different media containing sea water and found Malt extract agar (MEA) was best suited for its growth. The colony morphology of the fungus was white to cream colored, cottony at the center, and membranous towards the edge (Fig. 1a). The reverse was colorless, but became pale brown at the center with age. The vegetative hyphae were hyaline, 1 to 2 μm wide, smooth walled, and usually formed strands from which chlamydo spores rose perpendicularly (Fig. 1b). An abundant production of chlamydo spores, was a typical feature of this fungus. The chlamydo spores were solitary, usually arising on short stalks, hyaline, smooth and thick walled, unicellular, globose, subglobose, or obpyriform, occasionally broadly ellipsoidal, and 5 to 8 μm long by 4 to 7 μm wide (Fig. 1b). The morphological characters of the marine derived halophilic fungus agree well with the genus *Phialosimplex* of Sigler et al. [38], but differ from not having the conidia. Thus, the 18S rRNA gene sequence from marine derived halophilic fungus was subjected to NCBI BLAST search. Phylogenetic analyses based on the 18S rRNA gene sequence variation, irrespective of the clustering algorithm (NJ and ML), robustly establish the halophilic fungus species (HQ188293) of this study, to be a genetically closest to 18S small subunit ribosomal RNA gene sequences of *Phialosimplex sclerotalis* (AB024592) and *Phialosimplex chlamydo sporus* (GQ169326) (Fig. 2a -b). The genus *Phialosimplex* is a medically important genus for its potential to cause opportunistic disseminated mycoses in dogs [39], and this is the first report for the species isolated from the marine habitat.

The fungus of the present study exhibited very slow growth in sea water media, and no growth was observed under distilled water containing media (Fig 1a). Thus, the fungus was grown on solid malt extract medium amended with increasing concentration of NaCl (10%, 15% and 25%) for determining its growth rate and salt tolerant. The growth was observed in high concentration of salts amended media, but there was no any improvement in growth rate was observed. To improve the growth rate of the fungus, different amino acids were added to the above salt concentrations and tested. Fungus showed fast growth in malt extract broth of 10%, 15% and 25% salt concentration amended with lysine ($p=0.006$) (Fig. 3a). But there was no growth had been observed in distilled water media amended with lysine (Fig. 3a). Further, the fungus grown under broth condition using lysine was determined for its dry weight and wet weight, where the growth rate was found higher in 15% followed by 10% and 25% ($p=0.002$) (Fig. 3b). The fungus grown in 25% showed decrease in growth than 15% may be due to the

lack of tolerance level at higher concentration. It had been reported that all salt tolerant fungi described to date can grow well in media without salt, but *Wallemia ichthyophaga* is the most halophilic eukaryote cannot grow in media without NaCl concentrations < 1.5 M [40]. Here, the isolated marine derived fungus, grows only in media containing salt. Thus, the fungus isolated in the present study may be a new species of *Phialosimplex* sp., and also a novel halophilic fungus.

3.2. Antioxidants and antioxidant activity of extracts of halophilic fungus

3.2.1. Total antioxidant content from extracts of halophilic fungus

In this study we determined the total phenolic contents of halophilic fungal extracts (Fig. 4a). The total phenols of the halophilic fungus found maximal with 15% NaCl of extracellular and 25 % NaCl of intracellular (p=0.02) (Fig. 4a). However, the total phenolic contents of extracellular were found high when compared to that of intracellular extracts of the fungus (p=0.02) (Fig. 4a). Earlier, many phenolic compounds have been found to be effective antioxidants in many strains of moulds belonging to species of *Aspergillus*, *Penicillium*, *Rhizopus* and *Mortierella* [41, 42], *Chaetomium*, *Cladosporium*, *Torula*, *Phoma* [43], Mushrooms [44], and marine derived fungus, *Chaetomium globosum* [45], *Acremonium* sp. [46], *Wardomyces anomalus* [47]. Furthermore, it had been reported that the lifespan of a fungal organism is tightly connected with its stability to the environmental factors, which is determined by the state of the cell antioxidant defense components e.g. phenolic compounds [14]. And also the phenolic compounds are very important plant and fungal constituents because of their hydroxyl groups confer scavenging ability [48, 49]. Therefore, in this study we determined the total phenolic contents of halophilic fungal extracts (Fig. 4a) for their antioxidant activity against different concentrations of salt stresses using various antioxidant assays.

3.2.2. DPPH scavenging activity

The free radical-scavenging activities of halophilic fungus along with reference standard BHA were determined by the DPPH assay. DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants [50]. The fungal extracts of the present study showed a concentration dependant antiradical activity by inhibiting DPPH radical (p=0.02) (Fig. 4b). The scavenging activity of all fungal extracts obtained from different salt concentrations was found increased with higher salt concentration and was maximal with 15% (p=0.02) (Fig. 4b). This is because of decreased production of total antioxidant than 15%, as the fungus showed less growth in 25% NaCl. This may be due to the inability of the fungus to tolerate to grow in higher level concentrations. The percentage scavenging values were

almost equal at different concentration (50, 100, 150 and 200 µg/ml) tested with standard, BHT (25.14%, 40.32%, 45.95%, 54.61%) and 15% salt (32.7%, 37.8%, 41.48%, 43%) (Fig. 4b; Table 1). However, scavenging activity of BHT, a known antioxidant used as positive control, was relatively more pronounced than that of sea water and 25% NaCl ($p=0.02$) (Fig. 4b). The decrease in absorbance of the DPPH radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. It is visually noticeable as a colour change from purple to yellow. Also a lower value of EC_{50} indicates a higher antioxidant activity at lower concentration (Table 1). The EC_{50} is nothing but the concentration at which the 50% scavenging activity is obtained. The fungal extract of the present study was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., p-phenylene diamine, p-aminophenol), reduce and decolorise α,α -diphenyl- β -picrylhydrazyl by their hydrogen donating ability [51]. Further, antioxidant compounds such as 3,3-di-OH terphenyllin, 3-OH terphenyllin, and candidusin B obtain from extracts of *Aspergillus candidus* filtrate, [42] and four compounds including chaetopyranin isolated from marine derived *C. globosum*, are identified for their DPPH scavenging activity [45]. Here, the results of the halophilic fungal extracts for their hydrogen donating capabilities and acts as an antioxidant is in agreement with previous study of marine derived algicolous fungus, *C. globosum* [45], *Xylaria* sp. [52].

3.2.3. Hydroxyl radical scavenging activity

This assay shows the abilities of the fungal extracts and standard mannitol to inhibit hydroxyl radical mediated deoxyribose degradation in a Fe^{3+} EDTA ascorbic acid and H_2O_2 reaction mixture. The scavenging activity of all fungal extracts of different salt concentrations of the present study was found increased with higher salt concentration, and 15% NaCl showed maximal ($p<0.001$) (Fig. 4c). This is because of decreased production of total antioxidant than 15%, as the fungus showed less growth in 25% NaCl. This may be due to the inability of the fungus to tolerate to grow in higher level concentrations. In addition, the percentage scavenging values of 15% NaCl (13.68%, 34.9%, 35%, 36%) was found high at different concentration (50, 100, 150 and 200 µg/ml) than the known antioxidant, standard mannitol (12.8%, 20.5%, 21.84%, 29.1%) ($p<0.001$). This is supported by the EC_{50} value of the fungal extracts of 15% NaCl (244.65 ± 8.68) was found less when compared to that of the standard mannitol (346.48 ± 8.25) tested (Table 1).

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [53]. Hydroxyl radical were produced in this study by incubating ferric-EDTA with ascorbic acid and H₂O₂ at pH 7.4 and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA)-like product. This compound forms a pink chromogen upon heating with TBA at low pH [54]. When fungal extracts of the samples was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. The EC₅₀ value indicates that the halophilic fungal extract of 15% NaCl is a better hydroxyl radical scavenger than the standard mannitol. Earlier fungi had been reported for scavenging hydroxyl radical [55]. Graphis lactone A, an antioxidant compound of fungus *Cephalosporium* sp. had been identified for its pronounced activity in eliminating hydroxyl radicals in a dose-dependent manner [56]. Thus, the *in vitro* study clearly elucidates that the antioxidants present in the fungus may attenuate the process of metal ion-dependent hydroxyl radical formation and may reduce hydroxyl radical-induced cell injury.

3.2.4. β -carotene–linoleate model system antioxidant activity

The antioxidant activities of the extracts from halophilic fungus and of reference (BHT), as measured by the bleaching of β -carotene, were shown in Figure 4d. It had been observed that inhibition values of all the salt concentration of fungal extracts and the standards increased with higher concentration, and almost all the fungal extracts of different salt concentration showed better antioxidant activity than the standard BHT ($p < 0.001$) (Fig. 4d). Furthermore, the EC₅₀ value indicates that the fungal extracts obtained from all salt concentrations are better hydroxyl scavenger than the standard BHT (Table 1). In this model system β -Carotene undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β -carotene and linoleic acid, which generates free radicals. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β -carotene molecules. As a result, β -carotene will be oxidized and broken down in part; subsequently, the system loses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically [57]. The antioxidant compounds, isopestacin isolated from culture fluid of the fungus *Pestalotiopsis microspora* [58] and Graphis lactone A from extracts of *Cephalosporium* sp. was demonstrated clearly for the antioxidant activity against linoleic acid free radical [57]. The ability of these fungal antioxidants to scavenge free radicals may be comparable to that of the present halophilic fungal extracts. Thus, the presence of different antioxidants in the fungus hinder the extent of β -carotene bleaching by neutralizing the

linoleate-free radical and other free radicals formed in the system aiding the fungus to acclimatize to the stress conditions.

3.2.5. Metal chelating activity

Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation. In the present study, the absorbance of Fe^{2+} -ferrozine complex was decreased dose-dependently; otherwise the activity increased with the increasing concentration from 50 to 200 $\mu\text{g/ml}$ in all the NaCl concentrations tested ($p=0.05$) (Fig. 4e), and the EC_{50} values of the fungal extracts decreased with increased NaCl concentration compared to the standard, EDTA (Table 1). The fungal extracts of 15% NaCl showed the higher chelating activity than the other extracts of NaCl concentrations compared to that of the standard, EDTA (Fig. 4e). However, the chelating activity of the standard mannitol was found more pronounced than that of the fungal extracts ($p=0.05$) (Fig. 4e). In this assay, both fungal extract and EDTA, interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. Ferrozine can quantitatively form complexes with Fe^{2+} . In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complexes decreases. Measurement of the rate of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator [49]. It was reported that chelating agents which form r-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [59]. Chelating activity of earlier reports in fungus, *Trametes hirsuta*, which includes secondary antioxidants like transferrin and ceruloplasmin [60, 61] and *Aspergillus* sp. [62], for ferrous ion was assayed by the inhibition of formation of red coloured ferrozine and ferrous complex as in present study.

3.3. Antioxidant enzyme assay

3.3.1. Superoxide Dismutase (SOD) assay and analysis

We examined the extra and intracellular antioxidant enzyme synthesis of the fungus at different salt concentrations as it was earlier reported that the production of toxic oxygen derivatives increased in biotic and abiotic stresses. Superoxide dismutase (EC 1.11.1.6) enzymes scavenge radicals by catalyzing the conversion of radicals into hydrogen peroxide and molecular oxygen, thus being involved in the cellular defense strategies against oxidative damage by ROS. In the present study, the SOD activity was maximal with 15% NaCl in intracellular ($p=0.03$) and extracellular ($p=0.02$) conditions respectively

(Fig. 5). The SOD production levels are low in intracellular condition compare to that of the extracellular (Fig. 5). This may be due to the fungal release of the intracellular SOD to act against the extracellular ROS created by the salt stress conditions. In eukaryotic cells, the predominant forms are the copper-containing enzyme and the zinc-containing enzyme, located in the cytosol. Thus, we confirmed this by SOD staining assay using native page and found Cu/Zn SOD bands (Fig. 6a). In addition, the Cu/Zn SOD was identified using sequencing analysis. The Cu/Zn SOD gene sequence (JX157150) similarities of strain NIOCC 1 to *Paecilomyces tenuipes* (AY176060.1), *Cordyceps militaris* (AY195841.1), *Beauveria bassiana* (GQ304735.1), *Myceliophthora thermophila* (XM_003659991.1), *Chaetomium globosum* (XM_001222494.1) were 97, 88, 84, 79 and 78 % respectively. Further, quantitative RT PCR analysis revealed that the Cu/Zn SOD expression levels of 15% and 25% NaCl grown fungus was approximately fifteen fold higher than the sea water or 3.5% salt concentrations (Fig.7). Our results congruent with earlier observations on SOD induction by Zn, Cd, and menadione has been described in other fungi such as *A. niger* [63], *Candida intermedia* [64], *A. nidulans* [65], *Candida albicans* [66], and *Cryptococcus neoformans* [67].

3.3.2. Catalase and peroxidase assay

Catalase is a homotetrameric ferriheme-containing enzyme, which decomposes H₂O₂, but not other peroxides, to water and oxygen. It is localised mainly in peroxisomes, but may also be detected in the cytoplasm and mitochondria [68, 69]. The enzyme catalase (EC 1.11.1.6) activity was maximal with 15% NaCl and 25% NaCl in intracellular (p=0.02) and extracellular (p=0.02) conditions respectively (Fig. 5). This result is in agreement with the previous study of microscopic fungi from various taxonomic groups [70]. In our catalase staining assay using native page the fungus showed one band in lower salt concentration and two band patterns in higher salt concentrations of 15% and 25% (Fig. 6b).

It was also reported that catalase activity in fungi would be high if the selected strains exhibited no pronounced activity of peroxidase (1.11.17), which catalyzes the oxidation of organic compounds by using hydrogen peroxide [70, 25]. Thus, we also examined the peroxidase activity in marine derived halophilic fungus and its production was very less compared to that of the catalase production. But, we could observe the intracellular (p=0.02) and extracellular (p=0.02) peroxidase levels high in 15% and 25% NaCl stress (Fig. 5). The antioxidant enzymes catalase and peroxidase serve as the primary line of defense in destroying free radicals. Further, it was reported that several genes of the antioxidant enzyme catalase are expressed at different metamorphic states of *Aspergillus nidulans* and *Neurospora crassa*

[12]. Thus, we assayed the above enzymes production in marine derived halophilic fungus under different salt conditions. Catalase (EC 1.11.1.6) catalyzes the degradation of hydrogen peroxide with release of molecular oxygen. It has been found that over expression of SOD and CAT provides protection from lethal heat shock in *S. cerevisiae* and *Candida albicans* [71, 72]. In addition, a protective role of antioxidant enzymes (SOD, CAT, and peroxidase) against temperature treatment of fungal cells (*Neurospora crassa* and *A. nidulans*) has also been demonstrated [73, 74] suggesting that the production of toxic oxygen derivatives increased in biotic and abiotic stress conditions. This agrees with our experiments by the production of higher level of antioxidant enzymes in high salt stress conditions (Fig 5).

3.3.3. Glutathione S Transferase assay

Catalase (CAT) reduces H_2O_2 to water and molecular oxygen, and Glutathione peroxidase (GPX) catalyzes the reduction of H_2O_2 to water and organic peroxide to alcohols at the expense of reduced glutathione (GSH). Enzyme Glutathione-S-transferase (GST) (EC 2.5.1.18) conjugates xenobiotics with GSH for excretion. Thus, we assayed the GST activity levels in marine derived halophilic fungus under different salt stress conditions. The enzyme activity was maximal with 25 % in both the intra ($p=0.02$) and extracellular ($p=0.02$) conditions (Fig. 5). The results suggest that GST activity of the fungus differs according to their various salt stress tolerance. The GST activity has been identified in several fungal species and they have only been purified with very few fungal species [75].

4. Conclusion

In conclusion, we identified and reported a novel halophilic fungus, *Phialosimplex*, from marine habitat. The stress tolerance mechanism of the marine derived fungus for its amount of antioxidant components and scavenging activity at different level of stress confirms that at an increased level of stress of NaCl, higher amount of ROS is generated. The fungus that can tolerate the stress produced increased amount of antioxidants to scavenge ROS. Thus, the present study elucidates that the scavenging capability is one of the phenomenon that help the fungus to survive in extreme stress conditions. In addition, the study also helps in understanding how the pathogenic fungus tackles the oxidative burst i.e. hypersensitivity reaction performed by host to kill the pathogens.

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Figure legend

Figure 1a-b. Microscopic and morphological characteristics of halophilic fungus. **1a** shows the growth ability of the fungus at different salt concentrations, where no growth was observed in distil water media and pronounced growth in salt contained media. **1b** shows the microscopic structures of the fungus with sterile mycelia ($\times 100$ x). Values are means \pm SD (n=3).

Figure 2a-b. Phylogenetic tree based on sequence data from the nuclear ribosomal 18S region derived with maximum parsimony analysis (a) and neighbor joining (b). Numbers above the nodes are bootstrap values in percent (based on 1000 bootstrap samples). Values below 50 are not shown. The bar represents number of changes. The tree is rooted with *Chaetomium globosum*.

Figure 3a-b. Growth optimization. **3a.** Fungus was grown on different salt concentrations and distil water as control amended with lysine, where distil water doesn't showed any growth. The 15% NaCl showed the maximal growth. *Days indicated in the graph on x-axis are at interval of 5 days (i.e. 1=5 days and 6=30 days). **3b.** In both the fresh and dry weight 15% NaCl showed the maximal growth. Values are means \pm SD (n=3).

Figure 4a. Total phenolic content. Extracellular total phenolic content was found more than that of the intracellular. 15% and 25% NaCl concentration conditions showed higher phenolic content in extra and intra cellular respectively. The results are \pm S.D. of three parallel experiments

Figure 4b-e. **4b.** DPPH scavenging activities of the halophilic fungus extracts of different salt concentrations and the reference compound BHT. The fungal extracts of 15% NaCl showed maximal activity in all the salt concentrations tested. **4c.** Hydroxyl scavenging activities of the halophilic fungus extract and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation, where the fungal extracts of 15% NaCl showed the scavenging activity better than the standard, Mannitol. **4d.** Antioxidant using β -carotene linoleate model system. The fungal extracts of all NaCl concentrations showed better antioxidant activity than the standard, BHT. **4e.** Chelating effect of different salt concentration of fungal extracts and EDTA (50- 200 μ g/ml) on Fe^{2+} ion. Values are means \pm SD (n=3).

Figure 5. Production of antioxidant enzymes by the halophilic fungus at different salt concentrations. Extracellular and intracellular SOD and catalase levels are maximal at 15% and 25% NaCl respectively,

but extracellular SOD and catalase production levels are significantly higher than that of intracellular catalase. Extracellular and intracellular peroxidase production was less comparative to that of the extra and intracellular SOD, catalase and GST, but a significant increased production was observed at higher salt concentrations. Extra and intracellular GST production was more when compared with that of the other antioxidant enzymes. Values are means \pm SD (n=3).

Figure 6. Antioxidant enzyme activity staining assay. Native PAGE was performed for the catalase and SOD activity staining assays. The crude protein of the intracellular fungal samples were analysed with native PAGE protein molecular weight marker (6a-A; 6b-A) for the assays of SOD bands (**6a-B**) and catalase bands (**6b-B**).

Figure 7. Expression profile of copper/ zinc superoxide dismutase (Cu/Zn SOD) at the transcriptional level by quantitative RT-PCR using total RNAs from cells of *Phialosimplex* sp. (NIOCC 1) exposed to different salt stress. Amplification of 18S rRNA was used as a control to evaluate the expression level.

Figure 1a

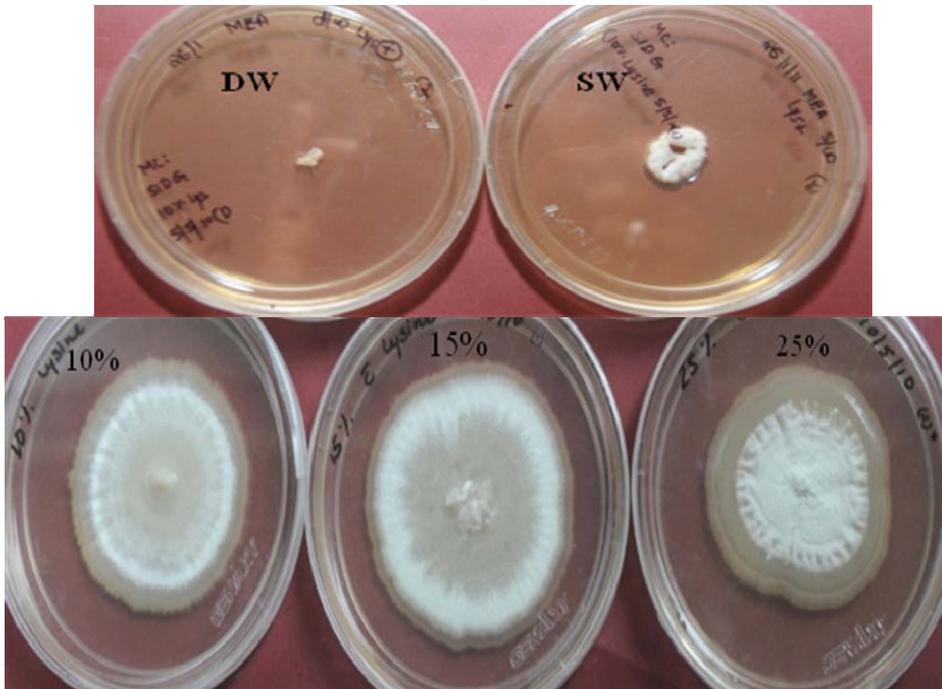


Figure 1b

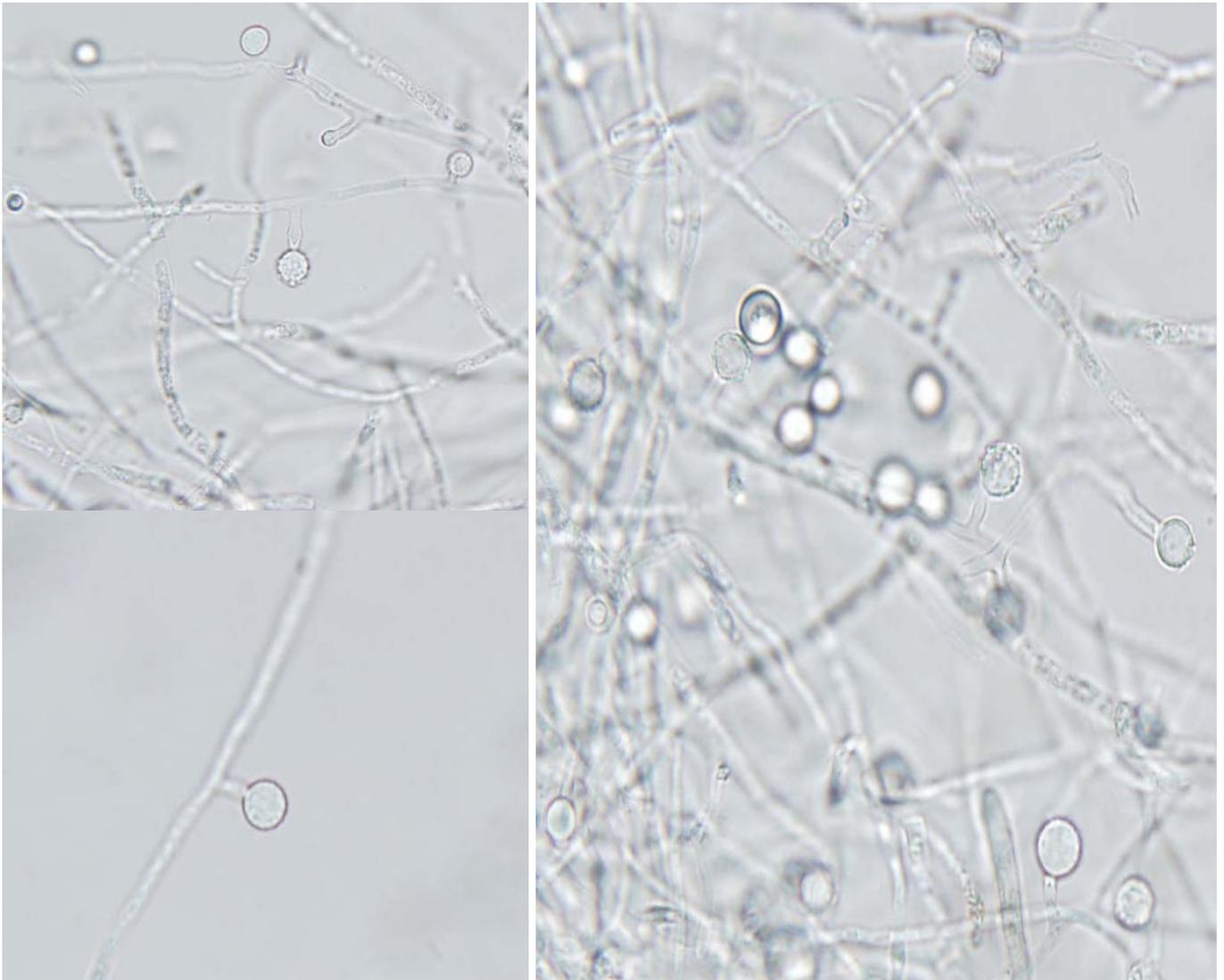
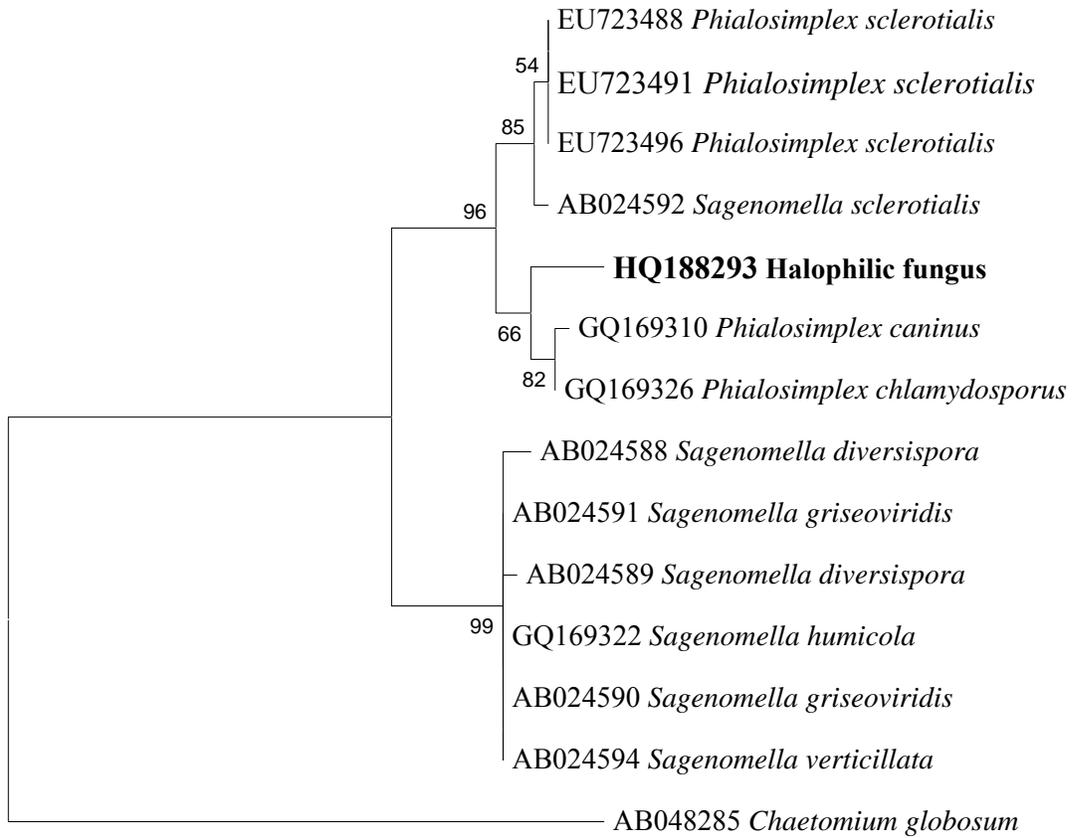


Figure 2a



10

Figure 2b

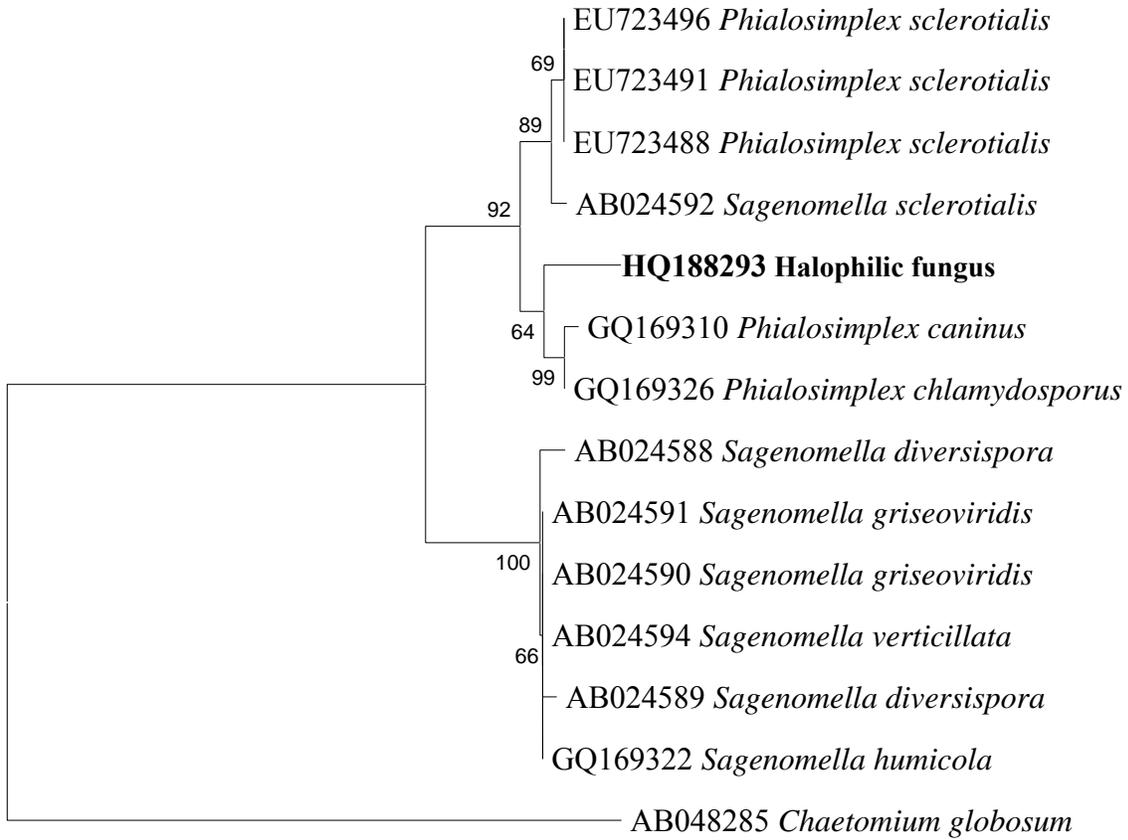


Figure 3a

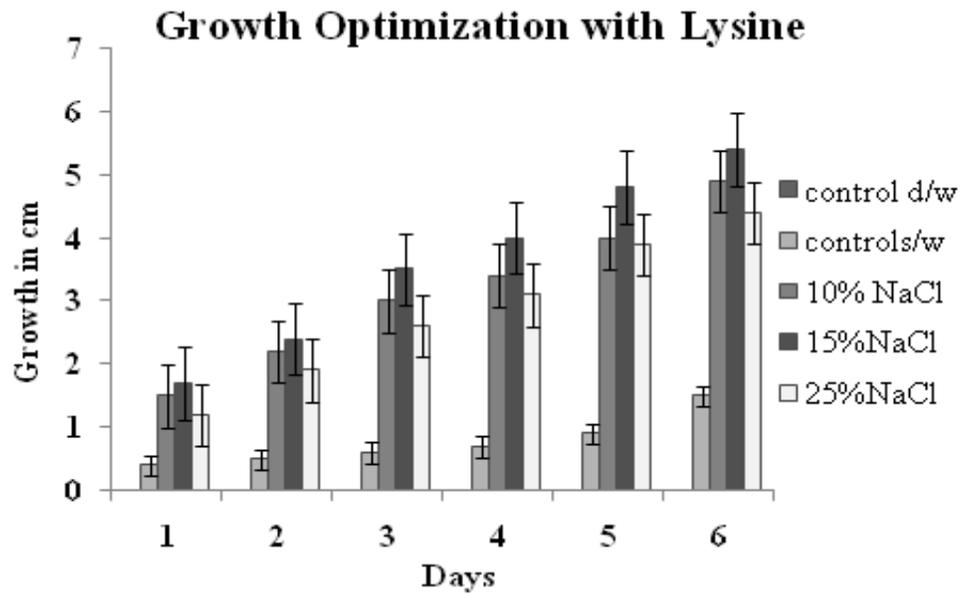


Figure 3b

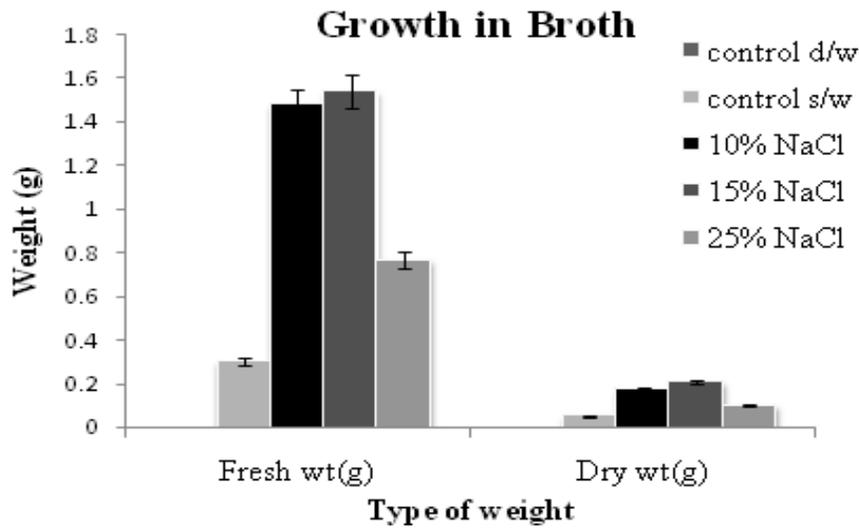


Figure 4a

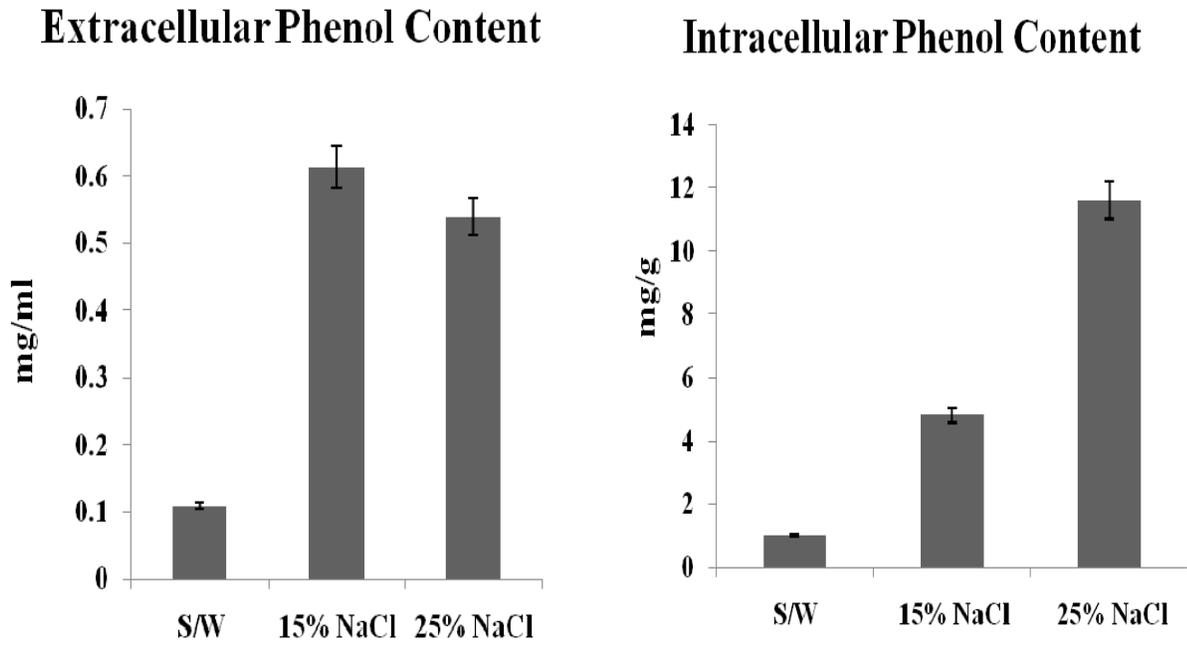


Figure 4b

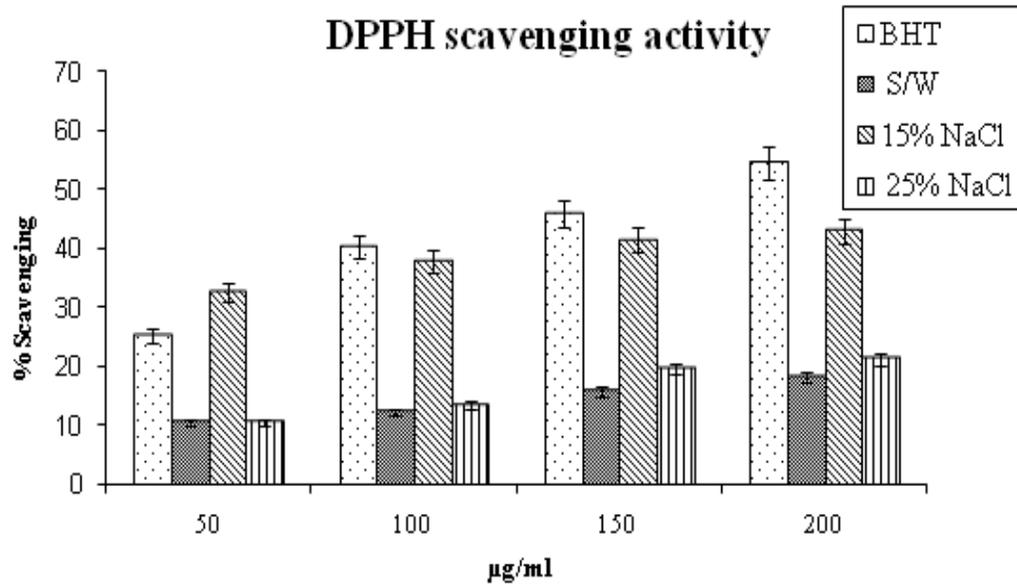


Figure 4c

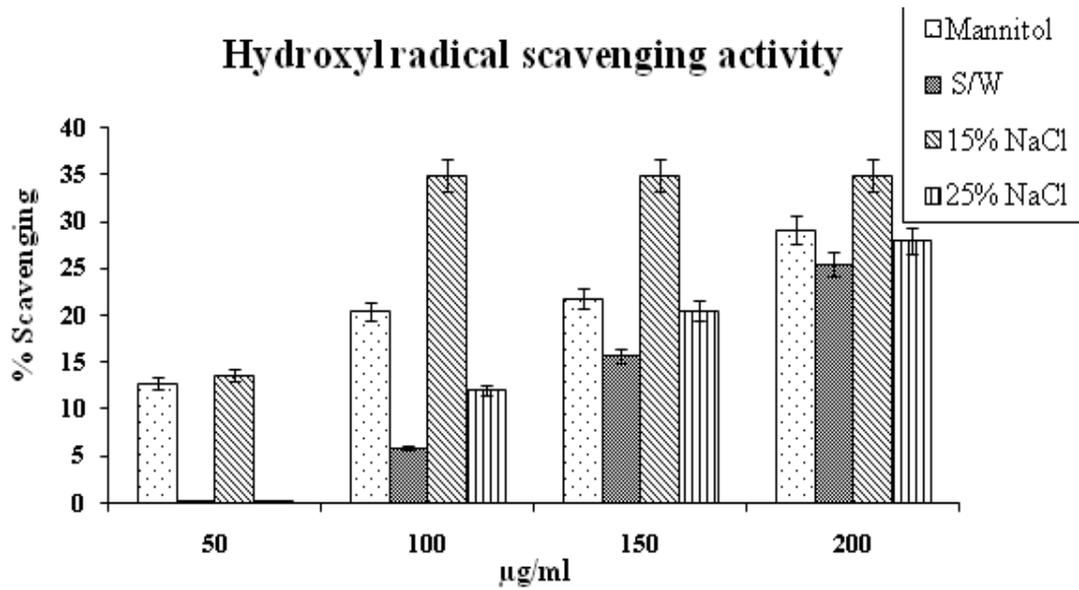


Figure 4d

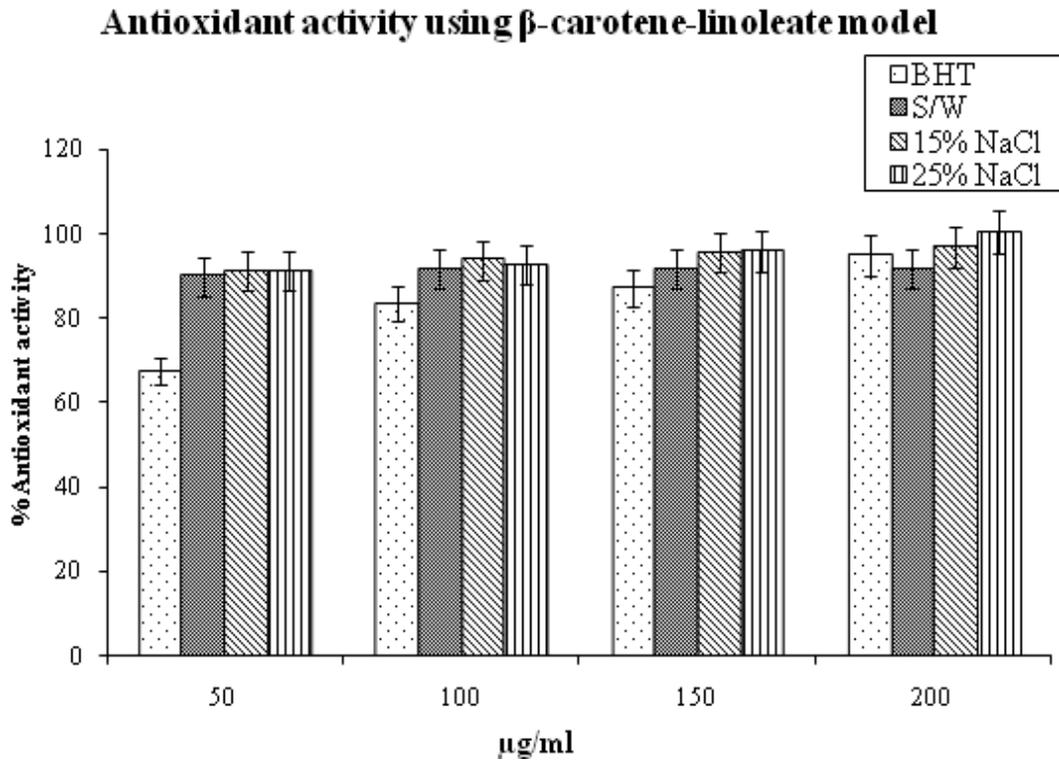


Figure 4e

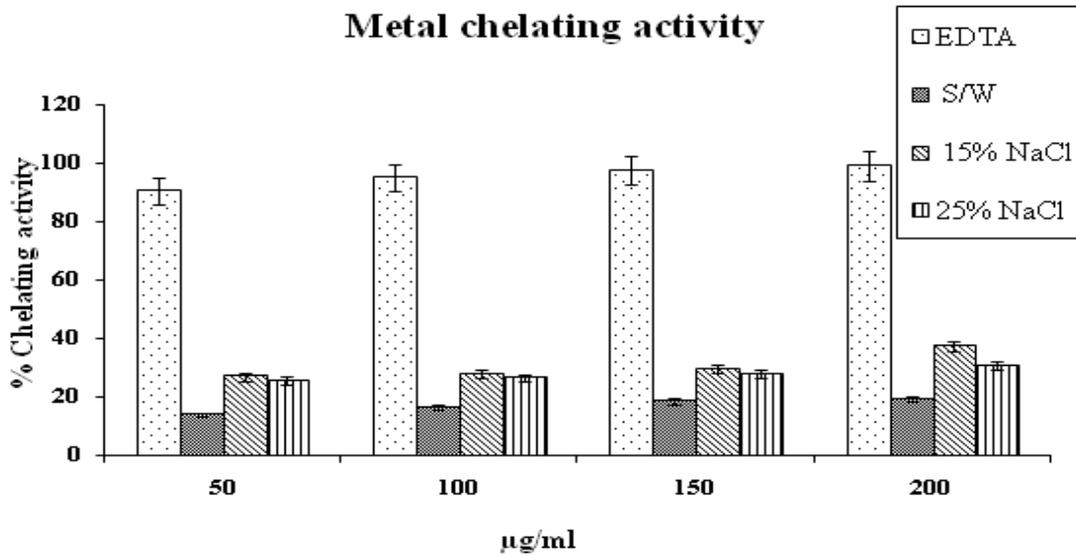


Figure 5

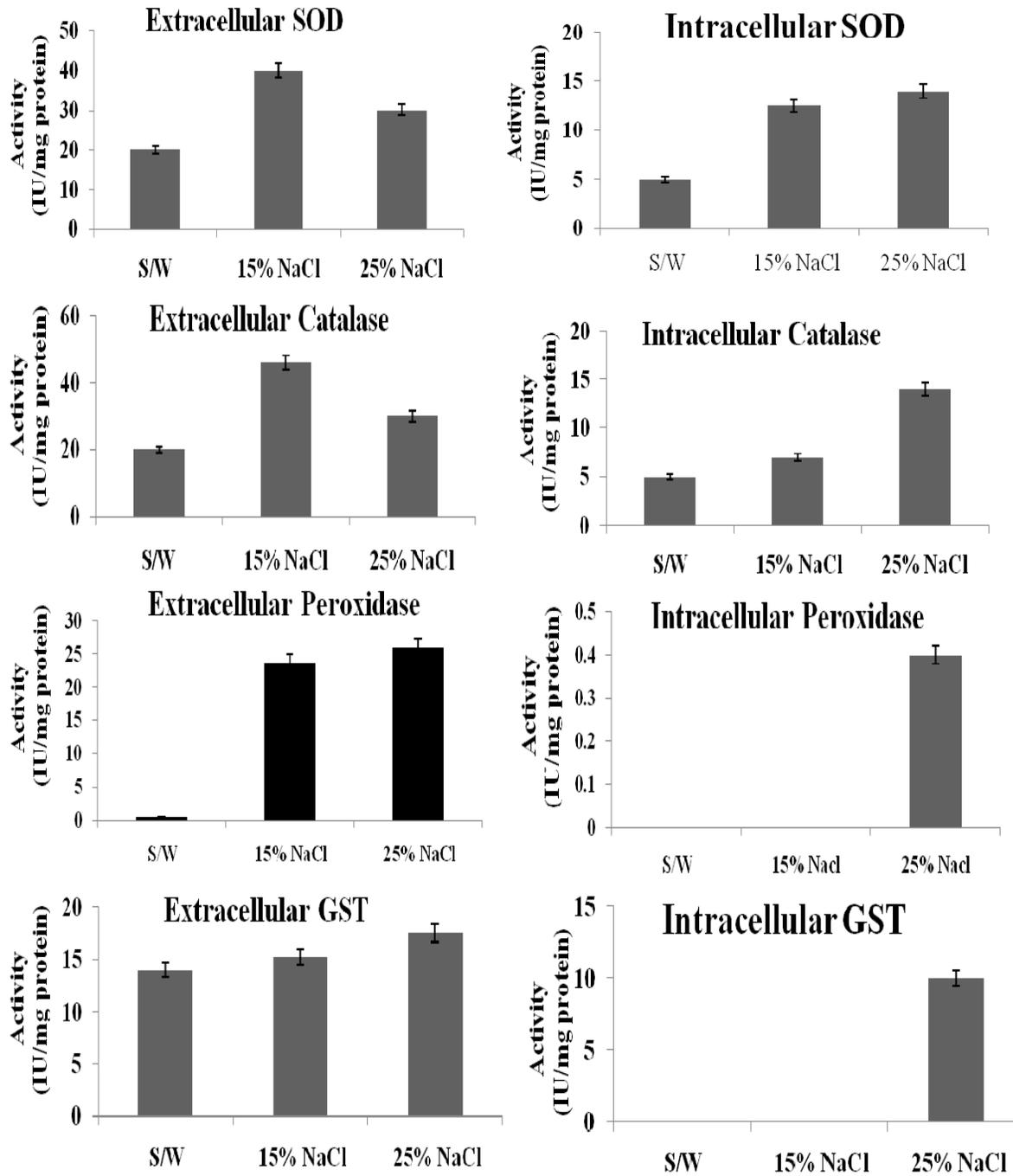


Figure 6a

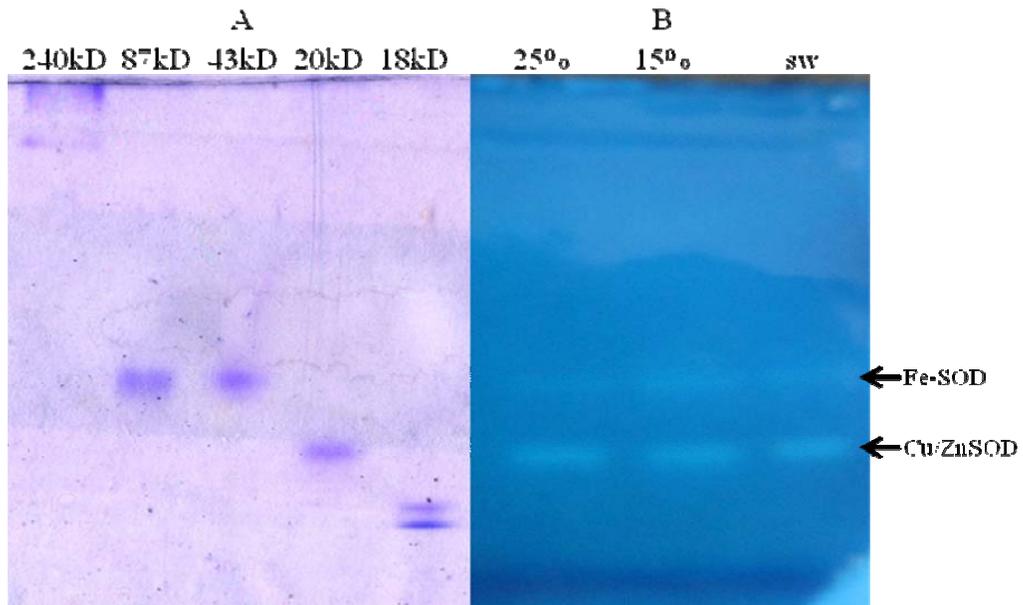


Figure 6b

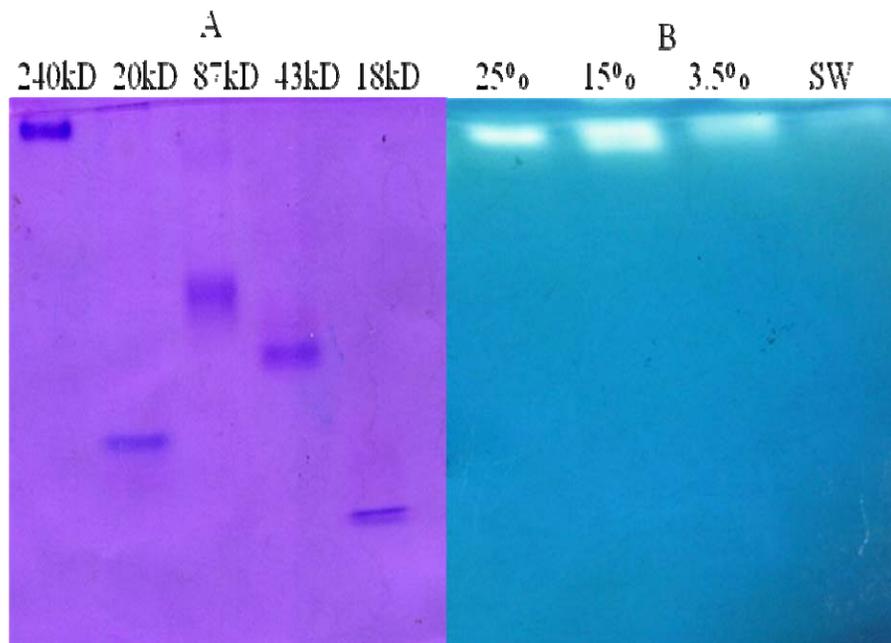


Figure 7

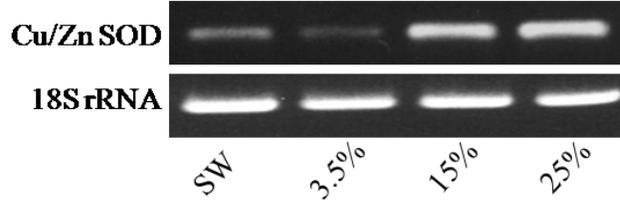


Table 1: Scavenging of reactive oxygen species and iron chelating activity (EC_{50} ($\mu\text{g/ml}$) values) of halophilic fungus and reference compounds

Samples	DPPH Scavenging ($\mu\text{g/ml}$)	OH^\cdot Scavenging ($\mu\text{g/ml}$)	β Carotene antioxidant ($\mu\text{g/ml}$)	Iron chelation ($\mu\text{g/ml}$)
Standard (BHT)	123.50 \pm 1.15	(Mannitol) 346.48 \pm 8.25	(BHT) 60.28 \pm 1.5	(EDTA) 35.59 \pm 0.63
S/W	564.20 \pm 0.59	403.51 \pm 7.35	37.82 \pm 3.42	538.37 \pm 17.21
15% NaCl	205.12 \pm 7.31	244.65 \pm 18.68	35.61 \pm 1.13	319.89 \pm 6.03
25% NaCl	450.92 \pm 6.73	390.97 \pm 3.97	36.59 \pm 2.94	362.64 \pm 3.59