Process Biochemistry 46 (2011) 847–857

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Adaptation of marine derived fungus *Chaetomium globosum* (NIOCC 36) to alkaline stress using antioxidant properties

Chinnarajan Ravindran*, Thangaiah Naveenan

Biotechnology Laboratory, Biological Oceanography Division, National Institute of Oceanography, Council of Scientific and Industrial Research (CSIR), Donapaula, Panaji, Goa-403 004, India

ARTICLE INFO

Article history:
Received 27 July 2010
Received in revised form
17 November 2010
Accepted 12 December 2010

Keywords:
Antioxidants
Free radical scavenging
Marine fungi
pH stress

ABSTRACT

The fungi are an exceptionally useful model in elucidating the cell functions under extreme conditions (temperature, salinity, and pH values) and mechanisms underlying adaptation to those extreme environmental conditions. Here, *Chaetomium globosum* was examined for its adaptation mechanism under alkaline stress using antioxidant properties. The aqueous extracts of *C. globosum* exhibited different levels of antioxidant activity in all the *in vitro* tests such as α,α-diphenyl-β-picrylhydrazyl (DPPH•), hydrogen peroxide, hydroxyl radical scavenging assay (HRSA), ferric reducing/antioxidant power (FRAP) assay, metal chelating assay and β-carotene–linolic acid model system. The antioxidant capacity of marine fungi showed an increase in activity with increase in stress. In addition, the production of intra and extracellular antioxidant enzymes of the fungus at various pH stresses were analyzed and discussed for their possible role in the stress mechanism. The present study elucidates that the scavenging activity is one of the protective mechanisms developed to avoid the deleterious effect of stress. Furthermore, the *in vitro* assays also clearly indicate that fungal extracts are significant source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses.

1. Introduction

A variety of physiological signals and stresses may cause translation level controls to direct competent tissues of microorganisms to undertake specific differentiation processes [1]. The general theory of microbial eukaryotic cell differentiation postulates that this phenomenon is triggered by a hyperoxidant state, which induces the cell to isolate itself from molecular oxygen [1,2]. A hyperoxidant state is an unstable pro-oxidant (oxidative) state in which the amount of oxygen-free radicals inside the cell exceeds the cell's capacity to neutralize them. Although not all of them are radicals, the term "oxygen-free radicals" has been used to refer to all species of oxygen (reactive oxygen species, ROS) that are more reactive than O_2 in its ground state or triplet state ($^3O_2$). These are dioxygen in its excited state singlet forms ($^1O_2$) and the partially reduced forms of oxygen, that is superoxide radical ion and its protonated form ($O_2^{\bullet}$ and $HO_2^{\bullet}$, respectively), hydroxyl radical ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2) [1].

It has been known that 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 [3]. The fungal cell differentiation is related to different growth strategies, various forms of resistance to adverse environmental conditions, different ways of reproduction and differentiation. The physical factors that mostly influence the marine fungi are salinity and pH, low water potential, high concentrations of sodium ions, low temperature, oligotrophic nutrient conditions and high hydrostatic pressure (the last three parameters being unique to the deep-sea environment). The research on abiotic stress still emphasizes NaCl as main subject and it is deeply developing towards various aspects such as Na^+ metabolism, molecular biology of salt-resistance genes, and salt stress signal transduction. There are only a few reports about stress by alkali. There have been studies about alkaline soil [4] and alkaline salt stress [5], and also it has been clearly demonstrated that the existence of alkali stress is more severe than salt stress [6]. Ambient pH is thus one component that plays a major role in influencing growth, physiology and differentiation of many microorganisms. The diverse niches that fungi occupy vary greatly with respect to their pH, for example, in the marine water, mangrove wood litters alkaline soils, etc. This pH adaptation presumably addresses the impact of pH variation upon protein activities, nutrient availability and the proton gradient across the plasma membrane [7]. One of the central problems associated with growth in alkaline medium is to maintain the cytoplasmic pH at a level compatible with metabolic functions, i.e., more acidic than that of the external medium. To solve this problem, the cells have to evolve efficient mechanisms to sustain the pH gradient across the cytoplasmic plasma membrane [8].

* Corresponding author. Tel.: +91 08322450442; fax: +91 08322450602.
E-mail address: cravindran@nio.org (C. Ravindran).

1359-5113/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2010.12.005

Fungus *Aspergillus nidulans* is the first eukaryotic organism whose adaptation to ambient pH was characterized. This fungus can grow in a wide pH range from 2.5 to 9.0 and apparently disposes of a regulatory mechanism of controlling gene expression according to the circumstances [9,10], and the genes involved in the pH regulatory system of *A. nidulans* was found homologues with other major groups of fungi [11]. Further, in the last few years the alkaline response of the eukaryotic model organism such as *Saccharomyces cerevisiae* has been studied quite thoroughly, mainly through analysis of the transcriptional remodeling or the identification of mutants that grow poorly under such conditions [12]. In addition, it was also reported that the phenotypic effects including part of the transcriptional response derived from exposure to high pH must be result of an oxidative stress situation [13].

Fungi were reported for using both the enzymatic and non-enzymatic defense systems against oxidative injury caused by ROS due to external factors [14]. But there was no study on the eukaryotic organism for its adaptation to ambient pH using antioxidant properties. Thus, in the present study, specific adaptations of the filamentous marine derived fungus, *Chaetomium globosum*, which grows in a wide range of pH from 4 to 12 [15] was investigated for its adaptive mechanisms under different pH conditions using antioxidant properties. *C. globosum*, the type species of the genus, *Chaetomium* (Phylum *Ascomycota*, Class *Sordariomycetes*) can be isolated easily from decaying plant material, seeds and other cellulosic substrates. It is the most frequently isolated and cosmopolitan genus of over 150 species [16]. It has also been reported that some isolates of *C. globosum* produce antibiotics that can suppress plant diseases like damping-off of sugar beet [17], leaf spot disease of corn, rice blast, sheath blight of rice and tomato wilt [18]. *C. globosum* has also been proven to be an important source of novel bioactive compounds (like ergosterol, ergosteryl palmitate, chrysophanol, chaetoglobosin C, alternariol monomethyl ether, echinuline, isochaetoglobosin D), further chaetomanone and echinulin has been shown to have activity towards *Mycobacterium tuberculosis* [19] and chaetopyranin, shown to exhibit moderate to weak cytotoxic activity towards several tumor cell lines [20]. Some of the above compounds are also noted for their antioxidant properties for example chaetopyranin for its DPPH radical scavenging property [20]. In addition, the whole genome of *C. globosum* sequence project is in progress with part of the Broad Institute of Harvard and MIT Fungal Genome Initiative (http://www.broadinstitute.org/). Therefore, the present study was conducted using marine derived fungus, *C. globosum*, as the candidate species with the objective to assess the pH responses of the fungus under alkaline stress using antioxidant defense components.

2. Materials and methods

2.1. Chemicals

Sodium bicarbonate, Folins-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. All other reagents were of analytical grade.

2.2. Isolation of alkaline stress tolerant fungi

The isolation of stress tolerant fungi was done as described earlier [15]. Briefly, the wood pieces were collected from mangrove swamps (Sal, Chorao and Zuari in Goa, India), washed with sterile sea water and were incubated (25 ± 2 °C) in sterile polythene bags. The wood pieces 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 (Himedia, Mumbai, India) containing 10,000 units of sodium benzyl penicillin and 0.05 g of streptomycin sulphate per 100 ml of medium to inhibit bacterial growth. The isolated fungus was inoculated at the centers of MEA medium plates of different pH viz., 4, 6, 8, 10, and 12. The plates were made up in triplicates and incubated at room temperature. The growth of the mycelium was noted on each day and the time course of growth was plotted.

2.3. Identification of fungi

The fungus was identified based on the colony morphology and microscopical features using standard taxonomic keys [16] as described earlier [15]. In addition, the sequence data derived from the fungal strain using an automated multicapillary DNA sequencer (ABI Prism 3130xl Genetic analyzer, Applied Biosystems, Foster City, CA, USA), has been submitted and deposited at GenBank under accession number GQ412081.

2.4. Total antioxidant and antioxidant activity assay

2.4.1. Sample preparation

The fungus grown in the MEA broth for 6–7 days at different pH conditions was separated into mycelial mat and culture filtrate by filtration with Whatman No 1 filter paper (9 cm) under vacuum. Culture filtrate thus obtained was further centrifuged at 4000 rpm for 10–15 min to separate the remaining mycelial mass from the filtrates. The clear final culture filtrate sample was stored at 4 °C until use within 24 h. The mycelial mat was extracted with ethyl acetate to get the crude extract.

2.4.2. 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 Vattem and Shetty [21], 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 λ = 725 nm using a spectrophotometer. Gallic acid 1 mg/ml was used as standard and standard curve was obtained using various concentrations of gallic acid.

2.4.3. Determination of total flavonoid content

The total flavonoid content of the extract was estimated by colorimetric method described by Zhishen et al. [22] with some modifications. The extract (100 μl) was mixed with 300 μl distilled water and 30 μl of 5% $NaNO_2$. After 5 min 30 μl of 10% $AlCl_3$ was added and mixed well. The mixture was incubated for 5 min and 0.2 ml of 1 mM NaOH was added. Finally, the volume was made to 1 ml using distilled water and mixed well. The absorbance was measured at 510 nm. Quercetin was used to calculate the standard curve. The concentration versus absorbance was plotted and the slope value was determined.

2.4.4. DPPH scavenging assay

The fungal extracts containing antioxidants was aliquoted into different concentrations (50–200 μg/ml) to determine its ability to scavenge 1, l-diphenyl-2-picrylhydrazyl (DPPH) radicals using the method of Yildirim et a1. [23]. DPPH solution (1 mM DPPH radical solution in 95% ethanol) of 800 μl was mixed with 200 μl 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 micro-centrifuge tubes and centrifuged for 5 min at 13,500 rpm (at room temperature). Then, the absorbance of each sample at λ = 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) × 100].

2.4.5. Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging activity was determined using Long et al. [24] method with minor changes. An aliquot of different concentration of sample (50–200 μg/ml) and 50 mM H_2O_2 was mixed in the ratio of 1:1 (v/v) and incubated for 30 min at room temperature. After 30 min, 90 μl of H_2O_2 – sample solution was mixed with 10 μl HPLC grade methanol and 0.9 ml of previously prepared FOX reagent (Ferrous Oxidation Xylenol (FOX) reagent was prepared by mixing 9 volumes of 4.4 mM BHT dissolved in HPLC grade methanol mixed with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulphate in 0.25 M H_2SO_4. It was prepared before the assay) was added. The reaction mixture was mixed well and incubated at room temperature for 30 min. The reaction mixture without the sample was used as blank. The absorbance of ferric xylenol orange complex was measured at 560 nm. The scavenging activity leads to the inhibition of ferric xylenol orange complex formation and the scavenging activity was calculated based on the formula: % hydrogen peroxide scavenging = [(control absorbance − extract absorbance)/(control absorbance) × 100].