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ORIGINAL ARTICLE

Isolation and Purification of Heterotetrameric Catalase from a Desiccation Tolerant Cyanobacterium *Lyngbya arboricola*

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The desiccation tolerant cyanobacterium *Lyngbya arboricola*, isolated from bark surfaces of *Mangifera indica*, possessed up to four stable isoforms of catalase in addition to other antioxidative enzymes, for several years under a dry state. Purification of the two most persistent isoforms of catalase (Cat) has been undertaken by employing acetone precipitation, ethanol: chloroform treatment, gel filtration and ion exchange chromatography. The two isoforms of catalase remained almost unchanged on varying matric and osmotic hydration levels of mats of the cyanobacterium. The purification procedures resulted in a 1.3 % yield of purified single isoform (0.22 mg mL^{-1} protein) with 709 Units mg^{-1} specific activity and a purity index of 0.83. Five millimolar of dithiothreitol (DTT) was observed to be pertinent in maintaining the optimum redox state of the enzyme. The purification procedures additionally facilitated the simultaneous elimination and procurement of phycoerythrins (PE) and mycosporine-like amino acids (MAA). Each purified isoform gave a single band (~45kDa) upon SDS-PAGE and denaturing urea isoelectric focusing (IEF) depicted the presence of 2 subunits each of CatA and CatB. The monoisotopic mass and pI value of CatA and CatB as revealed by LC-MS analysis and internal amino acid sequencing was 78.96, 5.89 and 80.77, 5.92, respectively, showing resemblance with CatA of *Erysiphe graminis* subs. *hordei* and CatB of *Ajellomyces capsulata*. The heterotetrameric monofunctional catalase (~320 kDa), due to its stability in the form of resistance to ethanol: chloroform, its thermoalkaliphilic nature and the presence of innumerable hydrophobic amino acid residues (~40%), thus exhibited its potential for biotechnological applications.

Key words: Chromatography; Denaturing isoelectric focusing; Desiccation; Electrophoresis; Lyngbya arboricola; Stable Catalase

Abbreviations: Cat, Catalase; DTT, dithiothreitol; IEF, isoelectric focusing; MAA, mycosporine like amino acids ; PE, phycoerythrins; KPB, potassium phosphate buffer; PMSF, phenylmethane-sulphonyl fluoride; ß-ME, beta mercaptoethanol

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Metabolic disorders in an oxygen-enriched environment often result in the generation of reactive oxygen species (ROS) such as superoxide, hydroxyl radical, and hydrogen peroxide leading to oxidation of cellular biomolecules causing breakdown of normal cellular, membrane, and

reproductive functions (Kranner and Birtic, 2005; Abele, 2002; Marx, 1985). In order to maintain normal growth and function, as well as to neutralize the lethal effects of ROS, organisms have evolved specific antioxidant defenses. Catalase (H_2O: H_2O oxidoreductase; EC 1.11.1.6), a ubiquitous enzyme that was one of the first enzymes isolated in a state of high purity (Schonbaum and Chance, 1976), efficiently catalyzes the decomposition of hydrogen peroxide into oxygen and water and thus is a significant component of antioxidative defense systems in aerobic organisms. Potential applications of catalases in medicine and industry have led to numerous attempts to engineering this protein (Shaked and Wolfe, 1988). Catalase has also gained attention in recent years because of its link to cancer, ageing in humans and animals (Melov *et al.*, 2000; Preston *et al.*, 2001; Turdi *et al*., 2007). Also the application of catalases for the elimination of H_2O_2 in textile bleaching effluents would not only allow the recycling of enormous amount of water used for dyeing but would also have advantage over the use of unfavorable high quantities of salt concentrations such as sodium bisulfite and act as a more environmentally friendly alternative to existing chemical treatments (Thompson *et al*., 2003; Tzanov *et al*., 2001; Gujelj *et al*., 2001). Most of the commercial catalases available in the market are optimally active at 20-50^{o}C and at neutral pH. This necessititates the requirement of new thermoalkalistable catalases which can act at temperatures above 50^{o}C and pH values above 9.0.

Catalases are not restricted to one protein type structurally, functionally, or by sequence. In general, there are three classes of catalase: the typical or monofunctional catalases, the catalase-peroxidases and the Mn-catalases or pseudocatalases (Zamocky and Koller 1999, Thompson *et al*., 2003). Additionally the monofunctional catalases are divided into three phylogenetic clades arising from a minimum of two genes duplication - two distint clades or subgroupings of small subunit enzymes and one clade of large subunit enzymes (Chelikani *et al.*, 2004; Klotz *et al.*, 1997). It has been demonstrated that catalases belonging to clade 2 mainly exhibit unusual resistances to physical and chemical denaturation. Catalase HPII was found to show thermal as well as pH stability by showing considerable activity till 60^{o}C which declined after 80^{o}C with a T_m value 83^{o}C (Goldberg and Hochman 1989; Switala *et al.*, 1999). The thermal stability as well as resistance property of catalase to treatment with chloroform and ethanol has been widely exploited for purification of the enzyme (Weiting *et al.*, 1990).

Cyanobacteria, one of the most primitive oxygenic phototrophs were probably the first organisms to develop elaborate mechanisms for the detoxification of partially reduced oxygen species. Desiccation tolerant terrestrial cyanobacteria, mainly exhibiting subaerial growth, due to oxygen rich atmosphere are supposed to generate considerable ROS and also to possess defense mechanisms against damages expected due to generation of ROS mainly during the phases of dry-down (dehydration) followed by desiccation (dry) and then subsequent recovery of metabolism (rehydration) at their natural habitats (Potts, 2001; Tripathi and Maurya, 2001). Cyanobacteria are mostly known to possess bifunctional catalase peroxidase (KatG), Mn-catalases (MnCat), and peroxiredoxins (Zamocky *et al.*, 2008). However, in spite of being the largest group of H_2O_2 degrading enzymes, typical catalases are very uncommon in cyanobacteria. Until now the only one complete

and nonfused gene in which all essential amino acids of typical catalase is conserved is found in *Nostoc punctiforme* PCC73102. Phylogenetic studies have futher revealed that it belongs to clade 3 of small subunit catalases that contain haem b as the active site and use NADPH as a second active cofactor (Bernroitner *et al.*, 2009). Also incomplete catalase genes have been reported in *Cyanothece* sp. ATCC51142 and *Synechococcus elongatus* PCC7942.

The genus *Lyngbya* has a prominent ecological role in marine ecosystems and is a group rich in bioactive secondary metabolites (Gerwick *et al.*, 2008; Hoffman, 1994). Recently the whole genome analysis of *Lyngba majuscula* 3L was done to gain insight into potential microbial interactions and gauge the natural product synthesis of this genus (Jones *et al.*, 2011). Also there are reports of purification and isolation of a catalase and its gene from other microbes like *Aspergillus* species (Chandrashekar, 2011) and *Bacillus* sp. (Wang *et al.*, 2011) but none have reported catalase from any cyanobacterial species yet. Tripathi and Srivastava in 2001 were the first to report the presence of active catalases, and dismutases in desiccation tolerant cyanobacterium *Lyngbya arboricola*.

Catalases are known to retain its stability for longer duration, being active in freeze-dried permafrost samples (Gilichinsky *et al.*, 1992) over a period of million years. But the presence of stable and active catalase in cells of this desiccation tolerant terrestrial cyanobacterium *Lyngbya arboricola* stored in desiccated state for two years is not only reflective of the ability of the cyanobacterium to maintain structural and functional integrity of their macromolecules including proteins under extremes of desiccation, but also signifies for better availability of stable catalase in dry mats of this cyanobacterium.

Due to the low structural stability of most of catalases much work in recent times has been devoted to increase their stability. This makes this cyanobacterium excellent source material for future exploitation of such enzymes for bioengineering and scientific research. This study will also be one of the first reports of presence and purification of catalases in a dessication tolerant cyanonbacteria which may serve as potential sources of thermophilic industrial catalases.

Unfortunately, structural, functional and molecular basis of stability of catalases from the cyanobacterium have not been explored at the desired level. In order to exploit this activity in the future, an understanding of the characteristics of the enzyme is required, necessitating the development of procedures for purification of catalase from the cyanobacterium.

Besides cyanobacteria being useful potential sources for antioxidants and antioxidative enzymes, they also contain significant amounts of unusual phycobiliproteins (PBPs), especially (PEs) (Tripathi *et al.*, 2007) and UV-absorbing pigments MAAs and scytonemins (Garcia-Pichel *et al.*, 1993) that are important in biotechnology but pose great problems for the purification of catalases. Thus, this work also describes a process of recovering these water soluble pigments as by-products of the catalase purification process. In addition we have also developed procedure(s) to maintain the pertinent level of redox state of the cell-free extract to minimize denaturation of the enzymes, i.e., catalases.

This method of purification and isolation of antioxidant enzyme catalase with simultaneous

recovery of biotechnologically important PEs and UV screening MAAs, may also save the individual efforts and costs to extract these pigments from this cyanobacterium.

MATERIALS AND METHODS

(i) Cyanobacterial material: L. arboricola inhabiting bark surfaces of *Mangifera indica* from the campus of Banaras Hindu University faces 8-9 dry months during the hot summer and cold winter seasons due to a considerably low amount of rainfall (2-12%). However, 3-4 months of a warm and moist rainy season with torrential rainfall (90%) and intermittent breaks result in uncertain wetting and drying and provide relatively better conditions for growth of the cyanobacterium (Tripathi *et al.*, 1990/91). Collection of the cyanobacterial samples was performed by scraping cyanobacterial mats from bark at the end of the rainy season (during the last week of September); after removing any adherent soil particles, the intact mats were used for further analysis.

Under natural habitat, rehydration (wetting) of dry mats of *L. arboricola* is on direct availability of water; whereas, dehydration (drying) results due to loss of water from wet mats to the atmosphere. In other words, it can be said that under natural habitats, rehydration and dehydration of the cyanobacterium can be controlled by regulating the osmotic and matric availability of water, respectively. So, osmotic water potential of the cyanobacterial mat was controlled by incubating the mat to the solutes of the medium (water potential solutions) on filter papers; whereas, matric water potential of the mats was controlled by equilibrating the mats in the atmosphere of a solution of defined water potential (isopiestic control, Harris *et al.*, 1970; Potts and Friedman 1981).

In order to obtain the cyanobacterium under growing conditions, the dry cyanobacterial mats were placed over filter paper pre-soaked with double distilled water at 0 MPa, for 72 h at 25°C and 72 µmol photon $m^{-2}s^{-1}$ light intensity. To maintain hydration levels, the growing and dry natural mats of *L. arboricola* were incubated at different osmotic or matric water potentials by equilibrating the mats over sodium chloride solutions and saturated solutions of different salts equivalent to different water potentials (0, -2.8, -21, -39, -167, and -355 MPa) at 25°C as described by Brock (1975) and Tripathi and Maurya (2001). For rehydration of dry mat, the mat was incubated on the filter paper soaked with the required osmotic water potential solutions for the required incubation period; whereas, for dehydration the 72 h grown mat at 0 MPa osmotic water potential was in the atmosphere of the required matric water potential solution for the required time period.

(ii) Application of reducing agents: The impact of some reducing agents in optimization of redox state of the enzyme was studied by incubating catalase (0.05 mg protein per lane) with varying concentrations (0-20 mM) of cysteine, DTT and ß-ME, respectively, for 30 min at 4°C and monitoring changes in the enzyme activity by native-PAGE gel. The optimum redox state of the enzyme was evaluated by monitoring the concentration of reductant at which an optimum enzyme activity could be recorded. The levels of sharpness and intensity on native-PAGE gel were related to the state of the enzyme.

(iii) Purification of catalase: Purification of the enzyme was achieved by employing acetone precipitation, ethanol-chloroform treatment, gel filtration, ion-exchange chromatography and electroelution processes. These procedures were

carried out at 4°C unless otherwise stated, and the purity of catalase at each step of purification was determined using spectroscopy and PAGE.

(a) Preparation of cell-free extract: The cell-free extract used for purification of the enzyme was obtained by crushing cyanobacterial mats (25 g) to powder in liquid nitrogen and homogenizing the powder with 110 mL extraction buffer containing 100 mM potassium phosphate buffer (KPB, pH 7.5), 1 mM Na-EDTA and 1 mM phenylmethane sulfonyl fluoride (PMSF) at 4°C. The homogenate was sonicated at 130 W, 20 kHz for 5 min at 50% amplitude using an ultrasonicator (Sonic & Materials, USA) and centrifuged at 25,000 × g at 4°C for 1 h. The supernatant (cell-free extract) was filtered with 0.45 µm cellulose-acetate membrane filter (Millipore, USA) and was used to obtain catalase enriched fraction of the extract.

(b) Sequential acetone precipitation: The catalase enriched fraction was acquired by addition of acetone (20-60%) to the respective supernatants obtained after stepwise addition of acetone to cell free extract. The cell-free extract was mixed with chilled acetone (20%), kept overnight at 4°C for precipitation and the supernatant and precipitate were obtained by centrifuging the mixture at 17,000 × g for 30 min at 4°C. The supernatant was lyophilized, re-dissolved in extraction buffer and was further undertaken for second and third acetone fractionation (40& 60%) successively as before. Catalase enzyme activity was measured in the supernatants and pellets obtained at each step of acetone treatments.

(c) Ethanol: chloroform treatment: The enzyme-enriched fractions collected after acetone precipitations were mixed with cold ethanol (95%) and chloroform in a ratio of 10:5:3 (v/v). After vortexing vigorously for 2-5 min, the mixture was centrifuged at 17,000 × g at 4°C for 30 min. Out of the three layers formed, the top aqueous layer displaying high catalase activity was collected and further purified; the dense solid middle layer containing denatured proteins and some pigments was used for purification of PBPs.

(d) Column chromatography: Gel filtration - One milliliter of the enzyme extract (the top aqueous layer after lyophilization) containing 2 mg protein mL^{-1} was filtered with a 0.22 µm filter and loaded onto a Hi-Prep Sephacryl (16/60) S-300 HR column (Akta Prime Plus, GE Healthcare) that had been washed and pre-equilibrated with ~ 200 mL 100 mM KPB (pH 7.5) and extraction buffer, respectively. The enzyme was eluted at a flow rate of 30 mL h^{-1}. Two-milliliter fractions with maximum enzyme activity were pooled and concentrated using dialysis sacks (12 kDa cut off, Sigma, USA). After dialysis overnight against 10 mM Tris-HCl (pH 8.0) at 4°C, the pooled fraction was applied to a DEAE-Sephadex A-50 column.

Ion exchange chromatography - One milliliter of dialyzed sample (1.5 mg protein mL^{-1}) obtained after gel-filtration was applied to a DEAE-Sephadex A-50 column (20 × 1.5 cm) that was pre-equilibrated and was thoroughly washed with 10 mM Tris (pH 8.0) at a flow rate of 24 mL h^{-1} to remove any unbound proteins (mainly, MAA); bound proteins were eluted using a linear gradient of NaCl (0-1.0 M) in 10 mM Tris-HCl (pH 8.0). Ten milliliters (each fraction of 2.5 mL) was collected and desalted overnight at 4°C. Fractions showing high catalase activity at each gradient were pooled, concentrated using 10 kDa cut-off filters (Ultrafree-MC, Millipore, USA) and used for further analysis.

(iv) Determination of purity index: The purity index for catalase ($PI_{catalase}$) at each step of purification was expressed as A_{405}/A_{280}, where A_{405}

and A_{280} stand for absorbance at 405 & 280 nm, respectively. Also, the ratios of A_{565}/A_{280} and of A_{320}/A_{280} corresponding to PE and to MAA, respectively, were determined in order to monitor the level of pigment impurity persisting at each step of purification.

Protein concentration was also determined using the Lowry method, and lysozyme (1 mg protein mL^{-1}) was used as the standard (Lowry *et al.*, 1951).

(v) Native PAGE: The cell-free extract and samples obtained at each step of purification were evaluated by native- PAGE for catalase activity using a vertical slab gel apparatus (Miniprotein-II, Bio-Rad, USA) on 8% polyacrylamide gel supported with glycerol following the method of Davis (1964) with modifications as described by Tripathi and Srivastava (2001).

(vi) Electroelution of individual isoforms: Individual isoforms of the enzyme were electroeluted from the native-PAGE of the enzyme by adopting the procedure (with minor modifications) developed by Harrington (1990). The native-PAGE was carried out using the enzyme fraction obtained after 60% acetone precipitation. Packing of the gel slices in the electroeluter was performed by using loading buffer containing 20 mM Tris-HCl (pH 8.0), 100 µM DTT, 1 mM PMSF, 1 mM EDTA and 10 % (v/v) glycerol. The eluates were collected at 8-10 mA per tube for 3-4 h at 4˚C in elution buffer containing 20 mM Tris-HCl (pH 8.0) and 5 mM DTT and were further concentrated with a 10 kDa cut-off filter (Ultrafree-MC, Millipore USA) to the desired protein concentration.

(vii) Enzyme characterization:

(a) SDS PAGE: 15% polyacrylamide resolving gel supported with 0.1 % SDS and 5% stacking gel) was used for further analysis of purity and estimation of molecular weight of the isoforms of catalase obtained in the eluate of DEAE-Sephadex column or after electroelution from native PAGE following the method of Sambrook (Sambrook *et al.*, 1989). The proteins on gels were visualized by staining with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 45% methanol and 10% acetic acid and destained with 45% (v/v) methanol and 7% (v/v) glacial acetic acid. Molecular weights were analyzed using low molecular weight markers as standards (MW-SDS-70, Sigma, USA).

(b) IEF: The isoform(s) of catalase purified by ion-exchange chromatography and by electroelution were further evaluated by denaturing urea-IEF on a 10% polyacrylamide gel with 2% (v/v) Ampholine (pH 3.5-10.0, Sigma, USA) (Robertson *et al.*, 1987) in 8 M urea using a vertical slab gel apparatus (Miniprotein-II, Bio-Rad, USA). Samples (10-30 µg protein/lane) were incubated with equal volumes of urea-IEF sample solution [8 M-urea, ampholytes (2%), β-ME- 50 µL, bromophenol blue- 50 µg in water] for 5 min at room temperature, applied to bottom of wells and overlaid with a 1% ampholyte, 15 % glycerol solution. Focusing was performed using 50 mM NaOH and 20 mM acetic acid as catholyte and anolyte solutions, respectively, and the gel was run at 250 V for 30 min followed by 450 V (constant current) for another 2 h at 4˚C. After electrophoretic separation, the gel was fixed for 20-30 min with constant shaking at room temperature in the IEF fixing solution [Trichloroacetic acid (12% w/v) and Sulfosalicyclic acid (4 % w/v)] in deionized water. Afterward, it was rinsed in destaining solution (the same as that used for SDS-PAGE gels) for 10 min. The protein bands in IEF gels were

visualized by Coomassie Blue staining as described for SDS-PAGE gels.

(c) Amino acid sequence analysis: The pI and molecular weights of individual subunits and internal amino acid sequence analyses were outsourced to The Centre for Genomic Application (TCGA), New Delhi, India. The internal amino acid sequences of catalases were analyzed using LC-MS by excising the gel bands after denaturing-IEF, performing tryptic digestion of the protein, and separating and fragmenting them on a reverse phase column. The data were analyzed by matching with Xcalibur/database/uniprot.fasta, and the deduced amino acid compositions of the catalases used as reference (with GenBank gene sequence primary accession numbers in parentheses) were CATA_ERYGR (Q8X1P0) EC.1.11.1.6) and CATB_AJECA (Q9Y7C2) EC 1.11.1.6).

(d) Enzyme assay: Catalase activity in enzyme samples (cell-free extract or purified) was determined by monitoring the decrease in absorbance of H_2O_2 as described by Tripathi and Srivastava (2001) with certain modifications. The reaction was initiated by addition of 4.4 mM H_2O_2 to a 3 mL reaction mixture containing 2.86 mL of KPB (50 mM, pH 7.5) and 100 µL of enzyme extract (1.4 mg protein mL^{-1}), and catalase activity was measured by recording the amount of H_2O_2 (extinction coefficient (ε) of H_2O_2=43.6 $M^{-1}cm^{-1}$) consumed. One unit (U) of catalase activity was defined as the amount of enzyme required to degrade 1 µmol of H_2O_2 min^{-1} at 25°C. The kinetic parameters were determined with the catalase solution using standard assay with varying substrate concentrations in the range of 3-460 mM. The apparent K_m value and V_{max} for the enzyme was estimated by analysis of data by Michaelis-Menten/ Lineweaver-Burk plots.

(e) Effect of pH and temperature: The effect of pH on catalase activity was evaluated in the range of pH 3.5-11.0 by employing different buffers, 50 mM sodium acetate buffer (pH 3.6-5.6), 50 mM K-phosphate buffer (pH 5.7-8.0) and 50 mM Glycine-NaOH buffer (pH 8.6-10.6). 100 µL enzyme solutions (1.4mg mL^{-1}) was mixed with 2.86 mL of buffer of desired pH and incubated at 25˚C for 1 h and enzyme activity was measured using the standard assay. Also, the effect of temperature (20-80˚C) on catalase activity was evaluated by incubating the enzyme solution in standard reaction mixtures at each temperature for 1 h. The effect of all the above was expressed as Enzyme Relative activity (%) by taking the pH and temperature at which maximum enzyme activity was recorded as optimum (100%).

The effect of different concentrations of reducing agent DTT (0 -20 mM) on catalase activity was determined by incubating the reaction mixture containing 100 µl of the enzyme solution along with DTT at 25˚C for varying time periods. However, relative enzyme activity (%) was calculated by taking the enzyme activity recorded in the native enzyme without the presence of any additive compound taken as control (100%).

RESULTS

(i) Selection of the catalase isoform(s) for purification: An apparent variability in the number of catalase isoforms from four in the present study, to three, reported earlier by Tripathi and Srivastava in 2001, in the mats of *L. arboricola* harvested from the same natural habitat poses a problem for selection of the isoform(s) to be selected for purification. Native PAGE reflecting CAT activity in the natural mats grown at 0 MPa osmotic water potential for 72 h when incubated matrically

showed only two major isoforms with very low intensity (Fig. 1 B inset, lane I) at 0 MPa and an enhancement in the intensity and number of the isoforms on further incubation at -2.8, -4.5, -11.2 and -21 MPa matric water potentials (Fig. 1 B inset, lanes II, III, IV &V) for 48 h. The gel was further supported by monitoring catalase activity spectrophotometrically. Catalase activity was higher at lower matric potentials (-2.8,-21 MPa than the activity recorded on osmotic treatment of the mats over a period of 24 hours.(Fig. 1 A, B). A general trend of initial increase was observed, followed by a decrease in the activity of catalase in the growing mats treated with lower matric water potentials with increasing incubation periods. Similarly, dry mats incubated at different osmotic water potentials also reflected similar trends, but with fewer variations in the enzyme activity as compared to the matrically treated mats. Nevertheless, on account of the variability observed with respect to isoforms as well as the activity of the enzyme on varying conditions of dehydration and rehydration, it can be inferred that isoforms 2 and 3 invariably were present under all conditions, thus providing impetus to purifying mainly these two isoforms.

(ii) Optimization of redox state of the enzyme: From the native PAGE of catalase isoforms on applying varying reducing agents, we observed that the level of sharpness and intensity of isoforms of catalase decreased from 10 mM onwards on treatment with both cysteine and DTT, with maximum being observed at 5 mM DTT/cysteine treated enzyme [Supplementary Fig. S2 (a) A, B, C)]. On the other hand, upon addition of ß-ME, the sharpness and intensity of the bands were comparatively greatly reduced. At a much lower concentration of 2 mM ß-ME, the bands of native enzyme were reduced to a thin diffuse band; increasing ß-ME concentration to 20 mM resulted in an almost complete loss of bands [Supplementary Fig. S2 (a) D]. Similar observations were recorded when measuring relative enzyme activity on incubation of the enzyme with varying concentrations of DTT. Maximum enzyme activity (~99 and 90%) was recorded at 5mM DTT on varying time period from 15 to 120 min [Supplementary Fig. S2 (b]. Significantly higher enzyme activity (~65 and 50%) than control was also observed on incubation of enzyme for 15 and 120 min. with 10mM DTT. Henceforth, DTT up to 5 mM was used to maintain the redox state to obtain structurally and functionally stable enzyme during this study.

(iii) *Purification steps*: Absorption spectra of the cell-free extract obtained from the cyanobacterium with different concentrations of proteins and the enzyme extract (upper aqueous layer) collected after ethanol: chloroform treatment is presented in Supplementary Fig. S3. The presence of peaks in UV region (260-360 nm) representing MAA and syctonemins, 440, 679 nm of chlorophylls,and 565, 620 nm of phycobiliproteins besides the heme peak (405 nm) in the crude extract (a) clearly indicated the presence of additional accessory pigments in this cyanobacterium. Removal/recovery of these was achieved through successive purification steps and the success of catalase purification was checked at each step in terms of purity index (A_{405}/A_{280}), decreasing ratio of A_{565}/A_{280} and of A_{320}/A_{280} in terms of protein content as illustrated in Supplementary Fig. S1. Also catalase activity at each step of purification and during fractionation procedures was checked through gel electrophoresis to ensure purity as given in Fig. 2a; , Fig.3 and Supplementary Fig. S4a, b.

The data of purification of catalase from *Lyngbya arboricola* are summarized in Table 1. The purified catalase was stored as a concentrated solution at -20°C without any significant loss in enzyme activity. Purification of the two most persistent isoforms [2 and 3; See Fig. 2 (a), E] of catalase with purity index of 0.83 and 0.6, respectively, contributing to 45 % and 40% of the total proteins and yield of 1.3 % was achieved by this procedure. Fig. 3 shows the SDS-PAGE profile of the subsequent purification steps of through which successful procurement of two major isoforms (2 and 3) of catalase was achieved. However electroelution of catalase isoforms from the native PAGE of the partially purified enzyme (after 60% acetone precipitation) supported the isolation of all the four individual isoforms separately (Fig. 2b) if required.

(iv) *Characterization of catalase*:

(a) *Electrophoresis:* The purity of the each of the isoforms isolated by precipitation steps and by electroelution was verified by presence of a single band at ~45kDa in SDS-PAGE (Fig. 4a). Urea-IEF and internal amino acid sequencing of subunits of individual isoforms obtained after ion-exchange chromatography and electroelution revealed the presence of 4 subunits in a single isoform, with the fourth subunit overlapping the third (Fig. 4c). The subunits 1 & 4 and 2 & 3, when analyzed for their internal amino acid sequences, molecular weight and pI revealed greatest homology and sequence matching with CatB (80.77 kD, pI:5.92) of *Ajellomyces capsulata* and CatA (78.96 kD,pI:5.89) of *Erysiphe graminis* subs. *hordei*, respectively (Fig. 5). Thus, an individual isoform of the catalase enzyme is heterotetrameric (MW equivalent to approximately 320 kDa), and each of its dimers is composed of two heteromeric subunits, where each one resembles either CatA or CatB, as depicted by the line diagram in Supplementary Fig. S5.

(b) *Kinetic Properties*: Catalase activity recorded with varying H_2O_2 concentration indicated a gradual but steady increase in the enzyme activity till 150 mM attaining almost a stationary state at 200 mM. Nevertheless, on further increasing the concentration from 200 mM to 450 mM a gradual lowering was observed with ~ 50 % of enzyme activity being recorded at 450 mM. Linear curve fitting to the Lineweaver-Burk equation yielded a K_m value of 10.27 mM and V_{max} of 11.70 mM min^{-1} (Fig.6).

(c) *Effect of pH and temperature*: The activity of catalase as a function of pH and temperature showed that the catalase from the cyanobacterium being a typical catalase was active over a broad pH range (pH 6.0-10.5); optimal activity (100%) being recorded at pH 8.0. Noticeably, nearly 40% and 80 % of the optimum catalase activity was also recorded at lower (below 5.5) and higher (above 8.0) pH (Fig. 7).

The impact of temperature on enzymatic activity showed that the cyanobacterium catalase exhibited considerable activity in the range 20-70°C with optimum temperature for activity recorded at 50°C. Nearly 80% of the optimum activity was recorded in the range 20°C-55°C which gradually declined to 60% and 40% at 70°C and 80°C respectively (Fig. 7)

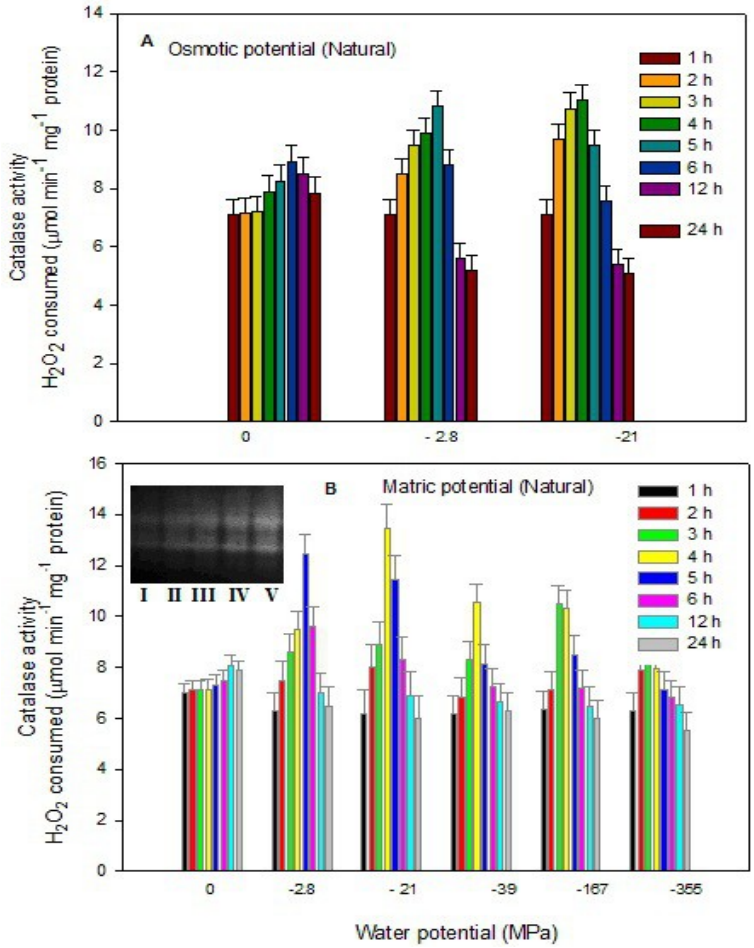

Figure 1: Catalase activity in the dry mats of *L. arboricola* incubated at different time periods, (A) osmotically and also in its osmotically grown mats on further (B) matric treatment. (Inset B: Lanes I, II, III, IV & V represent native PAGE of catalase isoforms in the osmotically (0 MPa for 72 h) grown mats on matric incubation for 48 h at 0, -2.8, -4.5, -11.2 and -21 MPa, respectively.

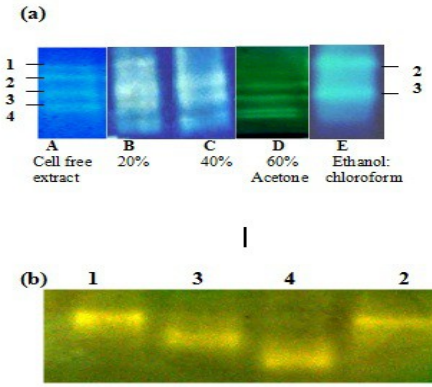

Figure 2: Native-PAGE (8%) of catalase activity in (a) respective enzyme enriched fractions at each step of purification; catalase activity in (A) cell free extract, in supernatant fraction after (B) 20% , (C) 40% acetone fractionation, in pellet fraction after (D) 60% acetone precipitation, (E) uppermost aqueous ethanol fraction (E2, see-Supplementary Fig. S1);.(b) four electroeluted catalase isoforms (corresponding to isoforms 1, 3, 4, 2 respectively procured from the native PAGE of the enzyme fraction obtained after 60% acetone precipitation as described in Materials and Methods

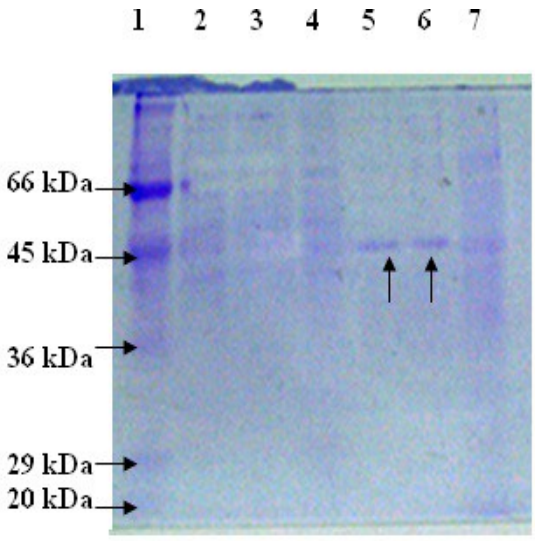

Figure 3: SDS-PAGE of catalase containing fractions after each purification step: (Lane 1) molecular mass standards; (Lane 2) cellular extract; (Lane 3) pellet fraction after 60% acetone precipitation; (Lane4) ethanol fraction after ethanol: chloroform treatment; (Lane 5, 6) purified isoform 2 and 3 of catalase separated by DEAE-ion exchange and (Lane 6) peak fraction after gel filtration.

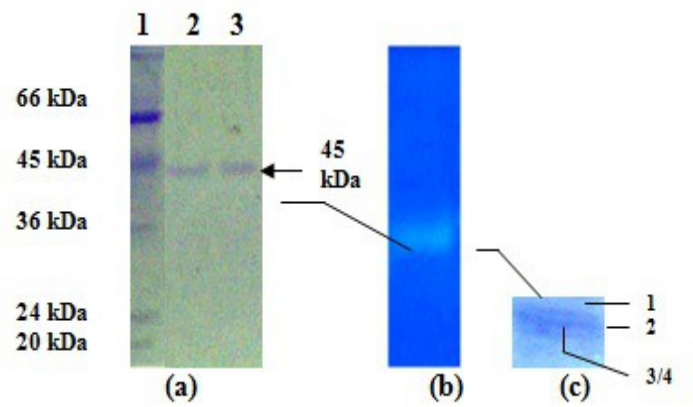

Figure 4: SDS PAGE, native PAGE and denaturing urea isoelectric focusing of single isoform of purified catalase. **(a)** Denaturing SDS (15%) polyacrylamide gel stained with Coomassie Brilliant Blue. Lane 1 -Molecular mass standard proteins; Lane 2 & 3, purified catalase isoforms (second and third) showing single band at 45 kDa, **(b)** Native (8%) PAGE stained for catalase activity showing electroeluted single isoform of catalase **(c)** Urea (8 M) IEF gel with Coomassie Brilliant Blue R-250 staining showing presence of four subunits (3 & 4 are superimposed) in the individual isoform of purified catalase.

A. Sequence of CatA

	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100
1	MRLSLWNLLG	LSGLVVASCP	YMSGEDQEYH	ISHIQARGTD	SEFLDQFKVE	DSNSYLTTDA	GGPIQDDASL	KAGERGPTLL	EDFIFRQKIQ	HFDHERVPER
101	AVHARGAGAY	GIFTSYADWI	NITAASFLNS	KGKETPVFVR	FSIVAGSRGS	ADIVRDVHGF	AIRFYTDEGN	FDIVGNNIPV	FFIQDAILFP	DLVHAVKPSP
201	DSEIPQAAIG	HDSAWDFFSQ	QPSTLHTLFW	AMSGHGIPRS	YRHMDGFGVH	IMRLVTDDGK	SKLVKWHWKT	KQGKASLVWE	EAQILAGKNP	DFHRQDLWDD
301	INAGNGPEWE	LGVQIVDEED	VQAFGFDLLD	PTKFLPEELV	PVTILGKMKL	TDNPTNYFAE	TEQVMFQPGH	IVRGVDFSDD	PLLQGRIYSY	LDTQLNRNGG
401	PNFEQLPVNR	PRTKVHNNNR	DGAGQMFIHT	NKAPYSPNSL	SGGNPKQANQ	TKGRGFFTAP	SRKVVGSLHR	GTASSFADVW	SQPRMFYNSL	IPSEQQFLVN
501	AIRFEISQLK	SDLIKKNTLM	QLNRVSNDLA	TRVAAVIGYK	PLDPSPEFYT	NAITDYVTIF	GKPLPSVVGF	TVGILASTSS	STSISQAAQL	ATSFSSRGIR
601	AVIVGESLLS	GTDQTYSSAD	ATAFDAVVVT	MGAETLFGPV	AKPNTLFPSG	RPSQILHDAY	RWGKPVGAVS	KASVVLEPLP	GTKNQGGVYR	VESVNELATS
701	IAKGLETFRF	VDRFPLDS								

B. Sequence of CatB

	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100
1	MRSLKLILAS	ASVVSAICPY	MSGEMPNSQN	GPLDRRHDTL	SDPTDQFLSK	FYIDDEQSVL	TTDVGGPIED	QHSLKAGNRG	PTLLEDFIFR	QKIQHFDHER
101	VPERAVHARG	AGAHGVFTSY	NNWSNITAAS	FLNAAGKQTP	VFVRFSTVAG	SRGSVDSARD	IHGFATRLYT	DEGNFDIVGN	NVFVFFIQDA	IQFPDLIHAV
201	KPQPDSEIPQ	AATAHDTAWD	FFSQQPSSLH	ALFWAMSGHG	IPRSMRHVDG	WGVHTFRLVT	DEGNSTLVKF	RWKTLQGRAG	LVWEEAQALG	GKNPDFHRQD
301	LWDAIESGRY	PEWELGFQLV	NEADQSKFDF	DLLDPTKIIP	EELVPFTPIG	KMVLNRNPKN	YFAETEQIMF	QPGHVVRGID	FTDDPLLQGR	LYSYLDTQLN
401	RHGGPNFEQL	PINRPRIPFH	NNNRDGAGQM	FIPLNTAAYT	PNSMSNGFPQ	QANRTHNRGF	FTAPGRMVNG	PLVRELSPSF	NDVWSQPRLF	YNSLTVFEKQ
501	FLVNAMRFEN	SHVRSEIVRK	NVIIQLNRVD	NDLARRVALA	IGVEPPSPDP	TFYHNKTIVP	IGIFGTNLLR	LDGLKIALLT	RDDGSFTIAE	QLRAAFNSAN
601	NKVDIVLVGS	SLDPQRGVNM	TYSGADGSIF	DAVIVVGGLL	TSASTQYPRG	RPLRIITDAY	AYGKPVGAVG	DGSNEALRDV	LMAAGGDASN	GLDQPGVYIS
701	NDVSEAYVRS	VLDGLTAYRF	LNRFPLDR							

Figure 5: Protein sequencing of subunit 1&4 (B) and subunit 2&3 (A) [Ref. Fig. 4 (c)], showing sequence similarity with CatB and CatA of *Ajellomyces capsulata* and *Erysiphe graminis* subs. hordei, respectively.

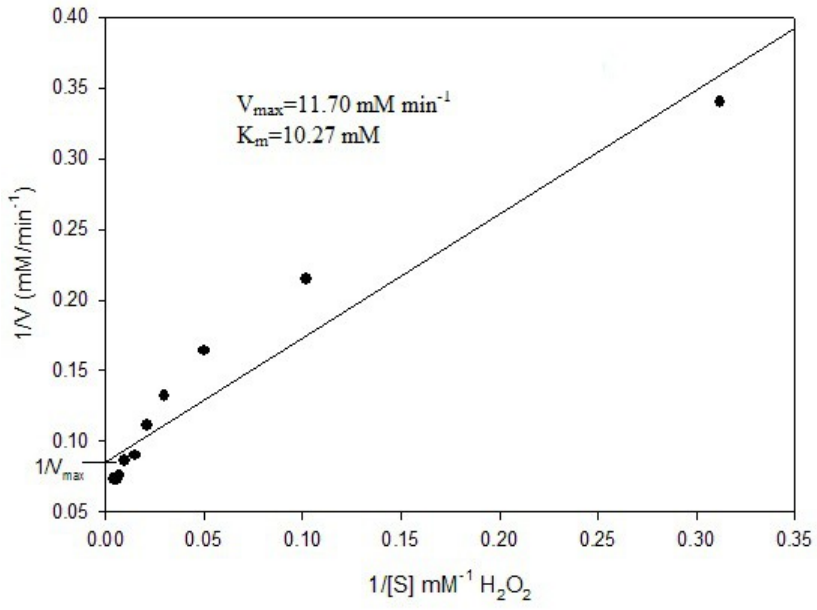

Figure 6: Lineweaver-Burk plot of reaction velocity of catalase purified from *L. arboricola* for estimation of kinetic parameters of enzyme. The enzyme assays were conducted at various hydrogen peroxide concentrations under standard assay conditions, as described in the Materials and Methods section.

Table 1: Purification characteristics of catalase from *L. arboricola*.

Purification Steps	Volume (ml)	Total Protein (mg)	Enzyme activity (U/ml)	Specific activity (U/mg)	Purification fold	Yield %
Crude Extract	65	159.75	4620	29.05	1	100
1st acetone precipitation (20:80)	51	99.20	4200	42.30	1.45	90
2nd acetone precipitation (40:60)	40	88.7	3900	43.90	1.51	84.4
3rd acetone precipitation (60:40)	29	63.6	2850	44.81	1.54	61.6
Ethanol: chloroform (5:3)	21	39.6	2070	52.2	1.79	44.8
Sephacryl S-300HR	12	19.1	1900	99.4	3.42	41.12
DEAE-Sephadex A-50	5	1.1	780	709.09	24.43	1.3

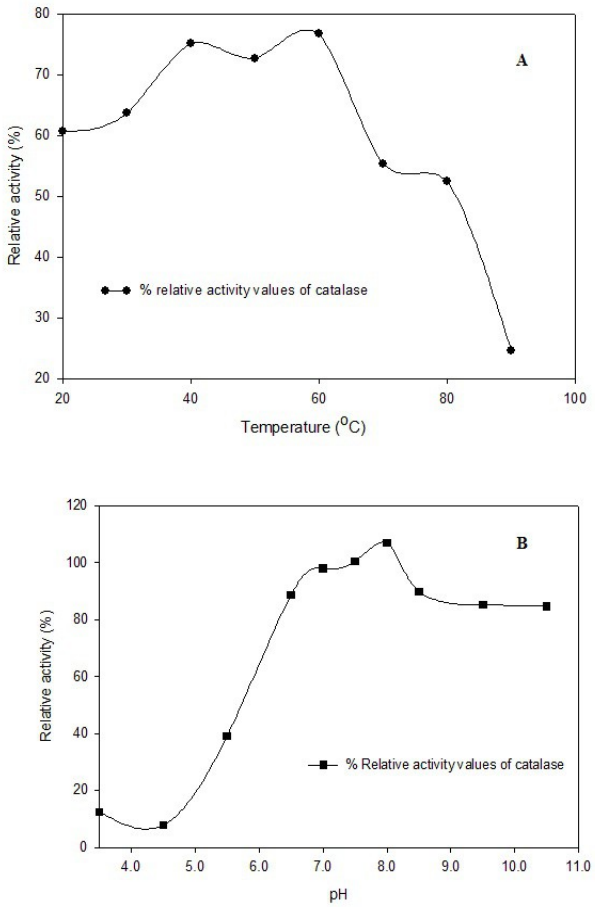

Figure 7: Effect of pH, temperature on purified catalase from *L. arboricola*. Catalase activity was assayed at different temperature (A) and pH (B) as described in Materials and methods.

DISCUSSION

Catalases from different organisms have a broad range of subunit sizes, a variety of quaternary structures, at least two different prosthetic groups, and even substantially different sequences. A number of catalase isoforms are reported to be present in organisms depending on the growth phases, types of cells and tissues, and also on genetic variability. Loewen and Switala (1987) have shown multiple numbers of catalases upon varying the growth conditions of *Bacillus subtilis*. Also catalases have been reported to exist in multiple

forms in many higher plants, such as loblolly pine, spinach, cotton, wheat, tobacco (Mullen and Gifford, 1993; Garcia *et al.*, 2000). Tissues from different parts of castor seedling exhibited variability in the catalase subunits (Ota *et al.*, 1992). Chandlee and colleagues and Skadsen and Scandalios have shown that maize has three genetically different catalase molecules observed in a tissue specific and age-dependent manner (Chandlee *et al.*, 1983; Skadsen and Scandalios, 1987). Such qualitative and quantitative variations in catalases, mainly monofunctional ones, are commonly attributed to cellular stress responses that are performed by regulating the enzyme, most commonly at the transcriptional level, and occasionally at the translational level, as well as through post-translational modifications and proteolysis. Zamocky *et al.*, 2004 have also investigated the response of soil bacterium *Commamonas terrigena* N3H to various forms of oxidative stress and have isolated and studied the most abundant isoform. In the present study as evident from the observations made regarding activity (Figs. 1 A, B) and also regarding the number of isoforms (Fig. 1 B inset) of catalase upon varying the level of hydration (osmotic as well as matric), the cyanobacterium also displayed variability in its activity and in the number of isoforms. Such variability in the isoforms appears to be due to variations in the abiotic conditions of the habitats along with the oxygen-rich habitat that the cyanobacterium may encounter at its habitat (Talpasayi and Tripathi 1982). Such observations can also be correlated to the possession of higher levels of ROS enzymes in the cavity of *Azolla* (Canini *et al.*, 1991) and bubbling cultures of *Anabaena cylindrica* with higher levels of O_2-rich air (Tel-Or *et al.*, 1986) Nevertheless, there are few reports regarding the occurrence of a varying number of isoforms in other cyanobacteria. Most of the studies until date have been related to activity of the enzyme catalase- peroxidase. Mutsuda and colleagues have shown the presence of a single isoform of 150 kDa catalase-peroxidase in *Synechococcus* PCC 7942 (Mutsuda *et al.*, 1996) and Obinger and coworkers demonstrated the presence of catalase and o-dianisidine peroxidase activity in the cytosolic extracts of the cyanobacterium *Anacystis nidulans* with similarity to prokaryotic catalase-peroxidase in having single isoform (Obinger *et al*., 1997). Thus our study will be the first of its kind to report multiple isoforms of catalase in a desiccation tolerant terrestrial cyanobacterium *Lyngbya arboricola* which also shows variation in its isoforms on exposure to desiccation stress.

Cyanobacteria have evolved different mechanisms to withstand extremes of desiccation. It has already been seen that the cyanobacteria change the level of – SH content, mainly protein – SH, on varying the hydration level, and thus maintain the redox state of their cells during different degrees of dehydration and rehydration. In general, DTT, ß-ME, Na-Asc, cysteine and $NaBH_4$ are used for regulation of thiol reactions (Habeeb, 1972). Compared to other reducing agents, a minimum concentration of DTT (10 mM) was recorded be more effective in maintaining high levels of –SH in dry cells of a desiccation-tolerant cyanobacterium *Scytonema geitleri* obtained from the rooftop of a building (Paul, 1998). This finding was consistent with earlier findings by Cleland (1964) and Wolf (1993). Nevertheless, in the present study activity of catalase detected on native PAGE as well as spectrophotometrically reflected DTT (5mM) was more effective in bringing

the redox state of the enzyme closer to the functionally stable state, and higher concentrations of DTT resulted in functionally unstable enzyme (Supplementary Fig. S2a,b). Also similar reports of effect of thiol stress on *Streptomyces coelicolor* (Vekaria *et al.*, 2007) showed an induction of catalaseA on exposing the organism to 10 mM DTT. In this study it was shown that specific activity of catalase increased by a factor of >8 upon exposure to 15mM DTT whereas there was no significant change in activity of superoxide dismutase.

Interestingly it was observed that in eukaryotes catalaseA, an enzyme normally observed in oxidative stress, was induced under thiol stress too. Thus, to maintain the redox state of cell-free extract of a cyanobacterium growing in subaerial habitats and to obtain significantly high yield of purified catalase , addition of 5mM DTT is recommended prior following the process of purification of proteins in particular.

Due to a lack of awareness about the type of catalase, and also the abundance of a number of interfering biomolecules in desiccation-tolerant cyanobacteria, selection of the procedure for purification of the enzyme was a critical aspect. As seen from the absorption spectrum (Supplementary Fig. S3), the Soret peak at 405 nm representing the heme group of the enzyme is not so prominent. The phenomenon of shielding of absorption peaks attributed to acetone soluble photosynthetic pigments has been observed in these terrestrial cyanobacteria (Tripathi, 1983). The shielding of the absorption peaks of the photosynthetic pigments was further observed due to the large number of substances with absorption maxima in the UV region that were later recognized as MAAs and scytonemins. It is likely that these UV pigments are responsible for the shielding of absorption peaks of heme in the present case also. In addition, PBPs (mainly PE, as shown in the inset of Supplementary Fig. S3), which have more or less same molecular weight as some isoforms of catalase, are also needed to be removed during enzyme purification.

Monitoring the catalase enzyme activity by observing loss of absorbance at 240 nm and also on native PAGE in the presence of pyrogallol (20 mM), guaiacol (5 & 10 mM), and NADH and NAD(P)H_2 (results not shown) did not suggest the presence of catalase-peroxidase, but rather favors the possibility of the presence of monofunctional catalases in this cyanobacterium. Compared with other proteins, the typical catalases have been reported since 1923 by Tsuchihashi to be much stable against ethanol: chloroform as observed for some bacterial catalases (Nadler *et al.*, 1986) with the exception of a report on the denaturation of beef erythrocyte catalase under the same treatments (Bonnichsen, 1955). Thus in the present study treatment with ethanol: chloroform resulted in further enrichment of almost all (four) catalase isoforms, with a minor loss of isoform 1 and & 4 and the transfer of PEs to the middle papery fraction for subsequent removal and purification. This treatment not only consolidated a view about the existence of the typical and stable catalases in this cyanobacterium but was also found to be significantly applicable in the purification of PEs. Further , ion exchange with DEAE-Sephadex with NaCl gradient (0-1.0 M) could successfully eliminate MAAs from the enzyme fraction that were in turn further purified (not shown here) after collecting them from the flow through of the ion-exchange chromatography in accordance with the method adopted by Bohm and colleagues in 1995. Overall, the procedure of catalase purification by employing acetone precipitation (20-60%) and fractionating

the pellet (after 60%) further with ethanol: chloroform, gel filtration and ion-exchange chromatography facilitated the procurement of two isoforms of catalase with maximum purity of a single isoform of catalase (0.22 mg protein mL^{-1}) as evidenced by the one band obtained by SDS PAGE with a PI (A_{405}/A_{280}) equivalent to 0.83, a specific activity of 709 Units mg^{-1} protein, and a yield of 1.3%. Compared to the procedure adopted for the purification of catalase-peroxidase from *Synechococcus* PCC 7942 (Mutsuda *et al.*, 1996) and from *Anacystis nidulans* (Obinger *et al.*, 1997) which gave 12.8% and 11.8 % yields, 0.54 and 0.41 - 0.48 PI values and 6670 and 785 U mg^{-1} protein specific activity, respectively, the purification procedure adopted for catalase purification from *L. arboricola* may be considered a comparatively suitable procedure for purifying the enzyme from terrestrial cyanobacteria. Besides catalase, the purification procedure described herein also aided in procuring the biotechnologically important PEs and MAAs from this cyanobacterium, so that they may be further characterized and exploited industrially.

Though most of the catalases purified from animals, plants, and microorganisms have four subunits of equal size and molecular weights in the range of 200-300 kDa, some large monofunctional catalases such as those isolated from E. coli HPII and *Neurospora crassa* Cat-1 having molecular mass of subunits and total molecular weight of enzyme being 84.3 kDa, 80 kDa and 337 kDa 320 kDa , respectively have also been reported (Loewen *et al.*, 2000; Michan *et al.*, 2002). The heterotetrameric monofunctional catalase from *L. arboricola* with large subunits (~79 & 81 kDa) of the individual isoforms (~320 kDa) based on resemblance with CatA and CatB of fungal species *Erysiphe graminis* subs. *hordei* and *Ajellomyces capsulata* (as observed from internal amino acid sequencing), can be placed into clade 2 of catalases that are phylogenetically of bacterial and fungal origin (Chelikani *et al.*, 2004). The monofunctional catalase purified from *L. arboricola* bears similarity to some bacterial and fungal catalases, but the heterotetrameric nature of the subunits has not yet been reported. It seems that CatA and CatB differentially possess the capacity to scavenge H_2O_2 and also to maintain their structural and functional integrity in adverse habitats where the organisms grow, as transcripts of CatA (*catA* gene) are most commonly induced during sporulation and in response to different stresses, whereas transcripts of CatB (*catB* gene) are most prominent in vegetative mycelia and are almost undetectable in spores (Calera *et al.*, 2000). This differential potentiality of CatA and CatB under different circumstances might be one of the reasons for their presence in *L. arboricola*, as this cyanobacterium encounters the phenomena of frequent wetting and drying in its natural habitat and undergoes variation in the catalase isoforms (shown earlier in Fig. 1).

The cyanobacterial catalase behaves like a typical catalase showing a true Michaelis Menten behaviour with saturation kinetics at 100 mM and above. Though there was inhibition in catalase activity above 250 mM till a H_2O_2 concentration of 450 mM but (50%) of activity was still recorded showing that there was not complete inhibition/inactivation of catalase at high substrate concentrations. This is in accordance with the behaviour of some typical catalases which are not saturable even up to 200 mM H_2O_2 (Jang *et al.*, 2004; Yumoto *et al.*, 2000). This may also be due to the thermostability of the cyanobacterium catalase as its reactions to substrate concentrations are

similar to the novel thermo-stable catalase from *Thermus brockianus* which shows no substrate inhibition and inactivation of the enzyme at H_2O_2 concentrations up to 450 mM (Thompson *et al.*, 2003).

The ability of catalases to remain stable in a desiccated state may be attributed to the presence of hydrophobic amino acids (Supplementary Fig. S6), which contribute nearly 40% of the total amino acid residues in catalase, as determined by internal protein sequences of catalase (results not shown). The stability may also be due to formation of disulfide bonds between two molecules of cysteine in the different subunits. Further catalase activity over a broad pH and temperature range (Fig.7) are indicative of the typical and monofunctional nature as well as the thermostability and pH stability of the enzyme. Similar observations were reported from catalase-peroxidases of therrmoalkaliphilic bacteria *Bacillus* sp. and catalases from *Thermus brockianus* (Gudelj *et al.*, 2001; Thompson *et al.*, 2003) which shows promising stabilities at high pH and temperature. Nearly 80% of the optimum catalase activity recorded at pH 9.0-10.5 of the cyanobacterium catalase is higher than the 4-6% of activity observed in *Bacillus* sp. at pH 9-10 and similar to the substantial catalase activity recorded over a broad pH range of 6-10 in *Thermus brockianus* catalase. The observations of temperature are also comparable to those recorded in *Bacillus* sp. (Gudelj *et al.*, 2001) which showed temperature optima at 55°C and nearly 50% of enzyme activity at 70°C as compared to temperature optima at 50°C and 40% of enzyme activity at 70°C for *L. arboricola* catalase. The optimum activity is lower than that reported for *Thermus brockianus* which recorded a temperature optima at 90°C (Thompson *et al.*, 2003) but higher than the Mn-catalase from *Thermoleophilum album* which had activity over a temperature range of 25-60°C with an optimum temperature for activity at 35°C (Allgood and Perry 1986).

On the basis of the above observations, it can be said that the heterotetrameric, monofunctional catalase with dimers of CatA and CatB, purified from *L. arboricola* with its high number of isoforms is novel among catalases detected so far in cyanobacteria. The detection of enzyme activity and the persistence of isoforms under extreme dehydration, resistance to ethanol: chloroform, presence of many hydrophobic amino acid residues along with the observations of pH and temperature demonstrated the stability of catalase and makes it a valuable source material for industrial applications, particularly by implementing the purification procedures of the enzyme with mass cultures of the cyanobacterium on a large scale. Besides the purification procedure described herein helped in simultaneously purifying 3 biotechnologically important biomolecules which could be a significant and cost effective protocol as compared to other methods. Further study of the structural and functional characteristics of this enzyme and its amino acid sequence analysis would give us a greater insight into the possible mechanisms of its stability and allow exploitation of its survival strategies for its biotechnological potential.

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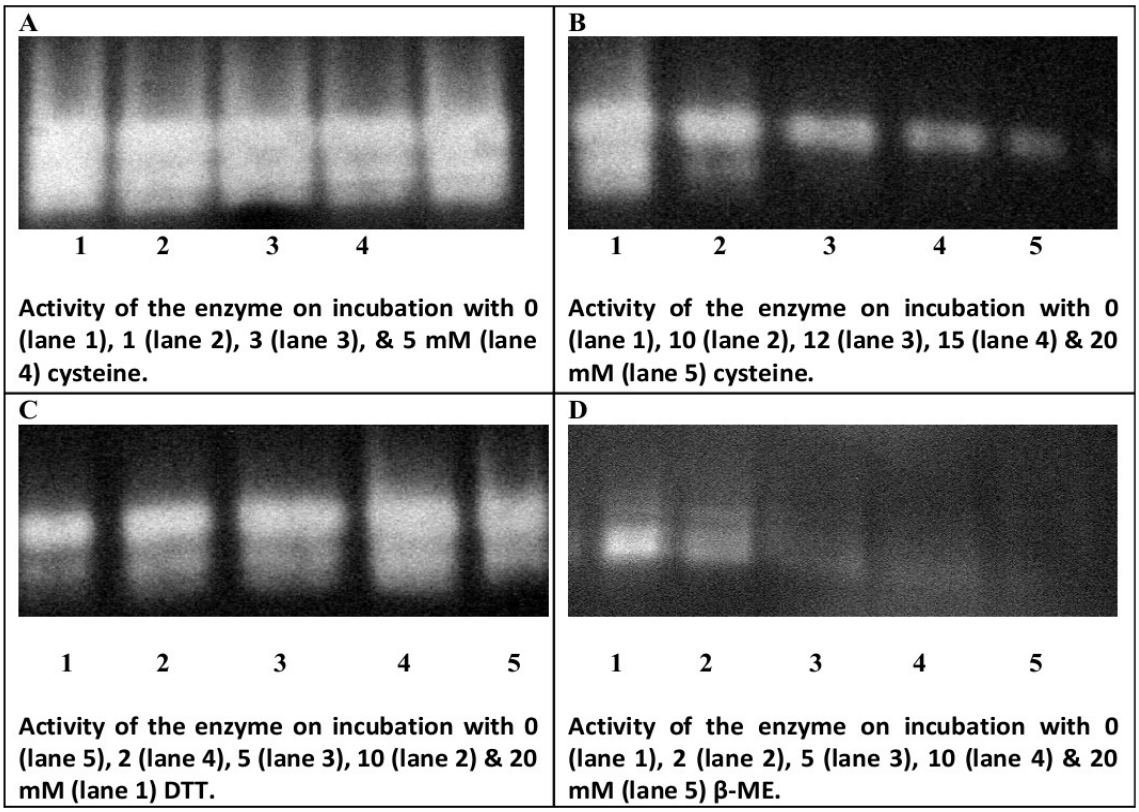
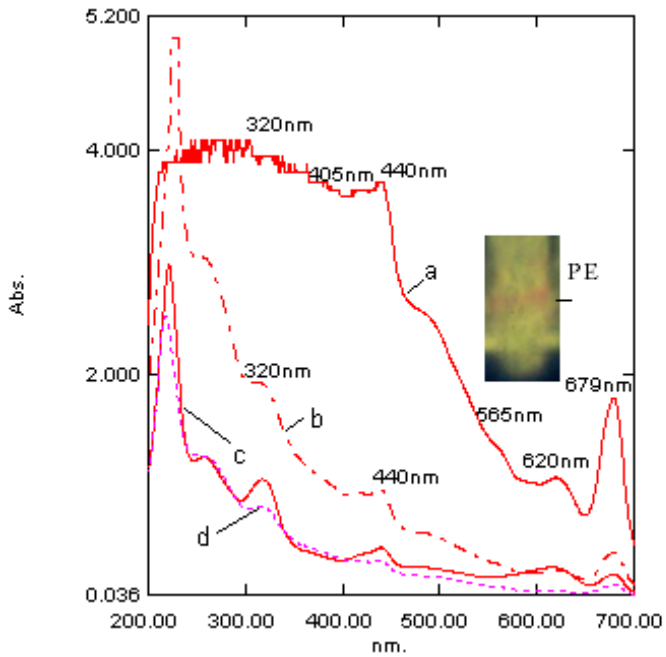
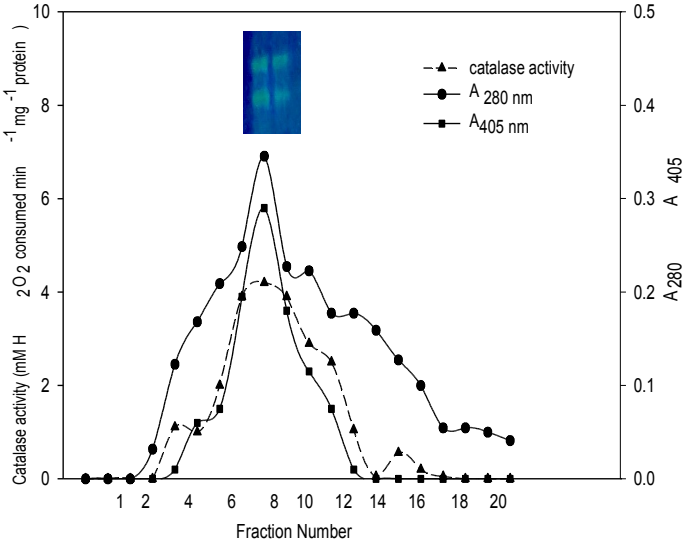


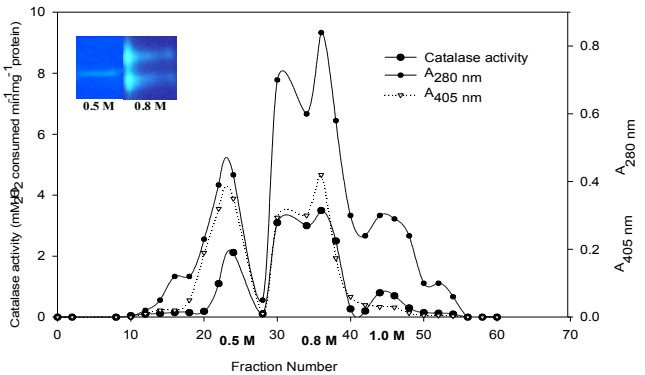
Supplementary Fig.1. Native-PAGE of catalase (0.05 mg enzyme per lane) activity on incubation for 30 min at 4°C with (A) 0-5 mM cysteine & (B) 10-20 mM cysteine, (C) 0- 20 mM dithiothreitol (DTT) and (D) 0- 20 mM Mercaptoethanol (β-ME).



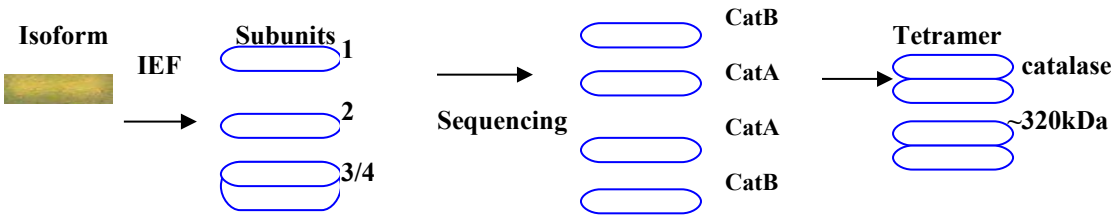
Supplementary Fig. 2. UV-visible overlay absorption spectra of cell free enzyme extract (0.5 mg protein ml^{-1}) of *L. arboricola* having at each step of purification. (a) crude extract, (b) 60% acetone precipitation (c) after ethanol: chloroform treatment, (d) after ion exchange chromatography . (Inset: native-PAGE of catalase isoforms along with PE in the cell-free extract of the cyanobacterium).



Supplementary Fig.3a. Gel-filtration chromatography of ethanol fraction obtained after ethanol: chloroform treatment during purification of catalase. The enzyme was eluted with 100 mM KPB (pH 7.0) at 30 ml h^{-1} flow rate and 2 ml fraction volume. (Inset: Native-PAGE of catalase isoforms from pooled fractions 7-12 with maximum enzyme activity).



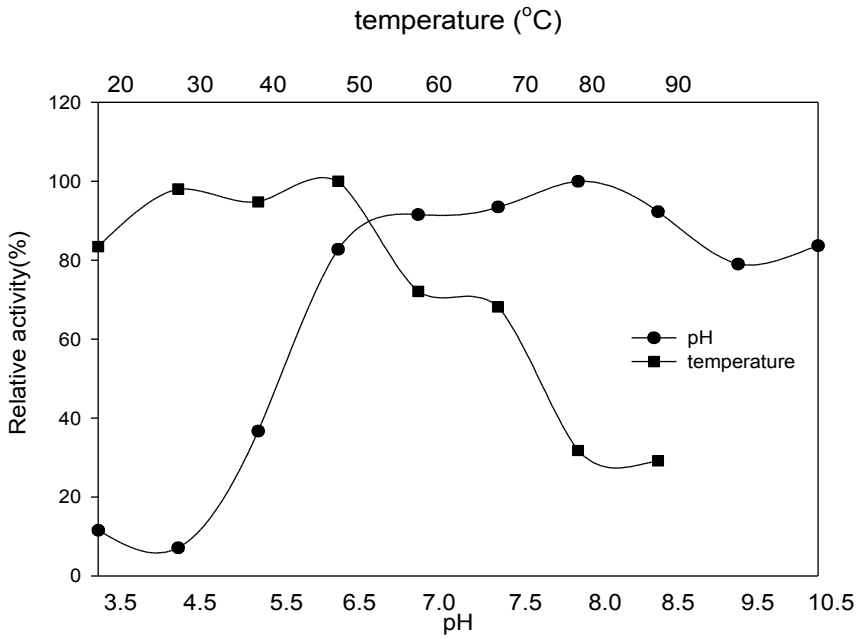
Supplementary Fig.3b. Ion-exchange chromatography of enzyme enriched fractions procured after gel- filtration. The enzyme was eluted by a linear gradient of NaCl (0-1.0 M) in 10 mM Tris-HCl (pH 8.0) at a flow rate of 24 ml h^{-1} collecting 10 ml at each gradient. (Inset: Native-PAGE of catalase isoforms in the fractions pooled using 0.5 M and 0.8 M NaCl).



Supplementary Fig. 4. Schematic representation of heterotetrameric structure of catalase on the basis of internal amino acid sequence analysis.

Amino acids	CATA	CATB	Total	% of total amino acids
Tryptophan	10	9	19	1.31
Tyrosine	17	16	33	2.28
Alanine	54	55	109	7.53
Valine	51	50	101	6.98
Isoleucine	27	34	61	4.21
Leucine	56	60	116	8.022
Methionine	11	12	23	1.59
Phenylalanine	42	42	84	5.80
Cysteine	1	1	2	0.138

Supplementary Fig. 5. Hydrophobic amino acids in catalase structure of *L.arboricola* as revealed from amino acid sequencing



Supplementary Fig.6. Effect of pH, temperature on purified catalase from *L. arboricola*. Catalase activity was assayed at different pH and temperature as described in Materials and methods.