Characterization of a glutathione peroxidase-like protein from a diazotrophic cyanobacterium *Nostoc punctiforme* ATCC 29133 and its role in stress survival

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by

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DECLARATION

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February, 2019

I, Esther Lalnunmawii hereby declare that the subject matter is the record of the work done by me, that the contents of this thesis did not form the basis of the award of any previous degree to me or to the best of my knowledge to anyone else, and that the thesis has not been submitted by me for any research degree in any other University/ Institute.

This is being submitted to the Mizoram University for the degree of Doctor of Philosophy in Biotechnology.

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CERTIFICATE

I certify that the thesis entitled "**Characterization of a glutathione peroxidase-like protein from a diazotrophic cyanobacterium *Nostoc punctiforme* ATCC 29133 and its role in stress survival**" submitted to the Mizoram University for the award of a degree of Doctor of Philosophy in Biotechnology by **Esther Lalnunmawii** is a record of research work carried out by her during the period of 2010 to 2018 under my guidance and supervision, and that this work has not formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles in this University or any other University or institution of higher learning.

Signature of the Supervisor

(Dr. JYOTIRMOY BHATTACHARYA)

Dedicated to my father,

who had always wanted his daughter to excel.

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ABBREVIATIONS

μg ml^{-1}	microgram per millilitre
μm	micrometer
°C	degree Celcius
μmol	Micromole
bp	base pair
$CaCl_2$	*Calcium chloride*
$CaCl_2.2\ H_2O$	*Calcium chloride* dihydrate
$Co(NO_3)_2.6\ H_2O$	Cobalt (II) Nitrate Hexahydrate
$CuSO_4.5\ H_2O$	Copper (II) sulfate pentahydrate
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic acid
g	gram
H_3BO_3	Boric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
$K_2HPO_4.3H_2O$	Potassium phosphate dibasic trihydrate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
min(s)	Minute(s)

ml	milli litre
mM	milli Molar
$MnCl_2.4\ H_2O$	Manganese (II) chloride tetrahydrate
Na_2CO_3	Sodium carbonate
nm	nanometer
rpm	rotation per minute
sec(s)	Second(s)
sp	species
TEMED	Tetramethylethylenediamine
Tris (free base)	2-amino-2-hydroxymethyl propane-1,3-diol
w/v	weight to volume ratio
$ZnSO_4.7\ H_2O$	Zinc sulfate heptahydrate
µg	microgram
µl	micro litre
µM	micro Molar

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CHAPTER 1

INTRODUCTION

Cyanobacteria are oxygen-evolving photosynthetic prokaryotes and many of them fix atmospheric nitrogen. They are one of the most diverse groups of gram-negative prokaryotes. It is believed that they appeared on earth between 3.2 – 2.4 billion years ago and that the earth's atmosphere became oxygenated due to their ability to perform oxygenic photosynthesis (Dietrich *et al*., 2006). Cyanobacteria are believed to play a pivotal role in the conversion of early earths reducing atmosphere to an oxidizing one, thus playing an important role in the evolution of life forms. (Pommerville, 2010). One of the most impressive microbial innovations in Earth's history was said to be the emergence of oxygenic photosynthesis in ancient cyanobacteria as a result of which oxygenic photosynthesis is now the largest source of O_2 in the atmosphere today (Hamilton, 2016). The transition of the atmosphere from the anoxic to oxygenated state had two major effects. Firstly, the highly efficient aerobic respiration began to flourish over anaerobic respiration. Secondly, the risk of damage to metabolic machinery from the actions of partially reduced reactive oxygen species (ROS) was greatly increased. ROS such singlet oxygen ($^1O_2$), superoxide anion radicals($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) are inevitably generated during normal oxygen metabolism. ROS react indiscriminately with proteins, lipids and DNA causing oxidative damage and cell death (Imlay 2003, Latifi *et al*. 2009). Therefore, for cellular survival, a prompt and efficient ROS- scavenging mechanism is of critical importance.

Cyanobacteria are often subjected to harsh environmental conditions such as nutrient limitation, different intensities of light, herbicides, extreme temperatures and high salinity. These conditions often negatively affect their growth, photosynthesis and nitrogen-fixation ability in their natural habitats. One of the common reasons for this is

the production of reactive oxygen species (ROS) that can lead to cell death (Latifi *et al.*, 2009). For the detoxification of cell-toxic ROS, it is widely believed that the mechanisms evolved by cyanobacteria are probably amongst the first of its kind. The defense mechanism of cyanobacteria includes both enzymatic (superoxide dismutase, catalase, peroxidase) as well as non-enzymatic antioxidants (like carotenoids, tocopherols etc.). The following sections describe the intracellular production of ROS as well as the mechanisms cyanobacteria employ to combat it.

1.1 Cyanobacteria

Cyanobacteria are classified within the Kingdom Monera (Prokaryota), Division Eubacteria, class Cyanobacteria. However, there is still significant debate over classification at higher taxonomic levels regarding the composition of orders, families, genera and species (Percival *et al.*, 2013). There are an estimated 150 genera of cyanobacteria containing approximately 2000 species, of which around 46 have been reported as being toxicogenic (Hitzfeld *et al.*, 2000; Ernst, 2006).Cyanobacteria are found in a wide range of ecological habitats - water, soil as well as air. They have been found to inhabit places like hot springs, deserts and brackish water. Cyanobacterial habitats also include those that are considered extreme, such as frozen lakes, hot springs and salt works (Whitton, 1992). Cyanobacteria exist in different forms – they may be unicellular, filamentous, branched-filamentous or non-filamentous colonial (Carr and Whitton 1982; Bergman *et al*., 1997; Whitton and Potts, 2000). Many filamentous cyanobacteria are known to possess multiple cellular differentiation alternatives like heterocyst (for N_2- fixation), vegetative cells (for photosynthesis), motile trichomes

called hormogonia and spores / akinetes (perennating bodies) (Adams 2000, Rai *et al*., 2000). Some of them, particularly *Nostoc* possess the ability to fix atmospheric nitrogen which makes them a potential source of biofertilizers in paddy fields (Singh 1961; Roger and Ladha 1992). Even though nitrogen is one of the most abundant elements (predominantly in the form of N_2 gas) in the atmosphere, plants are able to utilize only the reduced form (Wagner, 2011). Nitrogen is a macronutrient which is required by plants including food crops (Broyer and Strout, 1959). Being part of all building blocks of life – nucleic acids, amino acids and proteins or metabolic products, nitrogen represents about 2% of plant dry tissue (Miller and Cramer, 2005).The ability to fix nitrogen thus becomes an important task. *Nostoc* also has the ability to form symbiotic associations with a variety of plants and animals, providing fixed nitrogen in the process to their hosts thereby fulfilling their nitrogen requirements (Rai *et al.*, 2000).

1.1.1 N_2- fixation and heterocystic cyanobacteria:

There are three different ways by which N_2 fixation occurs in nature, they are; (i) through geochemical processes like lightning, (ii) through the action of biological mechanisms like enzymes which is found only in some microorganisms (example - nitrogenase), and (iii) through industrial processes like the Haber-Bosch process (Hoffman *et al.*, 2014). Due to the fact that the availability of fixed nitrogen is the most common limiting factor in the field of agriculture in the world, it can be claimed that nitrogen fixation has a profound agronomic, ecological and economic impact (Smil, 2004). Filamentous cyanobacteria possess the ability to form nitrogenase containing cells called heterocysts. Between stretches of vegetative cells, terminally differentiated

nitrogen-fixing cells called heterocysts may be formed at semi-regular intervals. This produces a multicellular pattern of single heterocysts about every ten to twenty vegetative cells along the filaments (Kumar *et al.*, 2010). The enzyme nitrogenase converts molecular nitrogen into ammonia. Heterocysts are often reguarly spaced and they are formed when the organism is subjected to the absence of combined nitrogen sources such as ammonia, nitrate, nitrite and few amino acids. (Flores and Herrero 1994; Wolk *et al.*, 1994). Nitrogenase shows extreme sensitivity to oxygen, which affects its level of synthesis as well as activity (Gallon 1992). In filamentous heterocystous cyanobacteria, the heterocysts protect the enzyme nitrogenase from the activity of oxygen by safely harboring it. The round, large shape, diminished pigmentation, cell with thicker envelopes, and cyanophycin granules which are usually prominent at adjacent poles makes heterocysts typically distinguishable from the vegetative cells (Kumar *et al.*, 2010). Heterocysts are surrounded by additional envelope layers which help to protect the enzyme nitrogenase from oxygen (Fay 1992). The micro-oxic environment required for nitrogen fixation is provided by mature heterocysts by spatially providing separation between the oxygen-evolving photosynthesis in vegetative cells and nitrogen fixation. Many metabolic and morphological changes occur in differentiating cells (Golden and Yoon 1998). During differentiation, the heterocysts show an increased rate of respiration and oxygen-producing photosystem PSII is also dismantled (Wolk *et al.*, 1994). In non-heterocystous nitrogen-fixing cyanobacteria, nitrogenase is protected by resorting to temporal separation of N_2-fixation and photosynthesis (Gallon 1992).

1.1.2 Vegetative cells of Cyanobacteria:

There is a mutual interdependence between heterocysts and vegetative cells. Heterocysts lack PSII and the ability to fix carbon which makes them dependent on vegetative cells for a source of reductant and carbon. This is probably partially supplied as sucrose (Cumino *et al*., 2007; Marcozzi *et al.*, 2009). Vegetative cells in *Anabaena* PCC 7120 must also supply glutamate to heterocysts, where it is converted to glutamine and other amino acids (Martin-Figueroa *et al*., 2000). Vegetative cells in return obtain amino acids as a fixed nitrogen source from the heterocysts (Meeks and Elhai 2002).

Movement of molecules between cells along a filament (through a possibly continuous periplasm) is required for the intercellular signals that control the regulated spacing of the heterocysts and for the exchange of metabolites (Flores *et al*., 2006). The entire photosynthetic machinery of cyanobacteria is present in the vegetative cells. The phycobilisomes (a supramolecular assembly formed by the light harvesting phycobiliproteins) captures light energy and transmits it to the photosystems I and II (PSI and PSII) where it is converted to chemical energy. PSII, a thylakoid-embedded complex carries out the oxidation of water. For every two molecules of water, one oxygen molecule and four protons are evolved. Following this reaction, an electron is passed via a series of redox reactions to PSI. PSI is a large membrane-bound multi sub-unit pigment-protein complex. It carries out the light driven oxidation of plastocyanin and reduction of ferredoxin using the electrons obtained from PSII. $NADP^+$ is reduced to NADPH using a part of the reduced ferredoxin. The rest of the electrons carried by the reduced ferredoxin are channelled back to the cytochrome b_6/f complex in cyclic electron flow to form ATP around PSI. Through the Calvin cycle, ATP and NADPH then drives the fixation of CO_2 into carbohydrates (Golbeck 1992). Only PSI is present

in the case of heterocysts (Wolk *et al.* 1994).

1.2 ROS formation in cyanobacteria:

Although respiratory electron transport pathway contributes to the generation of toxic oxygen species, the major source of ROS in higher plants, oxygen-evolving photosynthetic cyanobacteria and green algae is the photosynthetic machinery (Mittler 2002; Apel and Hirt 2004; Simon *et al.,* 2005; Latifi *et al.,* 2009). In higher photoautotrophs, photosynthesis and respiration are carried out in separate compartments (chloroplast and mitochondria). Meanwhile in cyanobacteria, these processes occur simultaneously in its uncompartmentalized cells. Therefore, ROS poses a higher risk to cyanobacteria.

Energy in the form of light may also contribute as a source of major stress even though it essential for photosynthesis. Repair of photosystem II is believed to be inhibited by singlet oxygen ($^1O_2$) which is produced by an energy input to oxygen from photosensitized chlorophyll (Latifi *et al.,* 2009). When the redox potential of the QA quinone decreases, the production of $^1O_2$ and its impact on photoinhibition increases (Fufezan *et al*., 2007). If the intensity of light is higher than the level which can be normally managed by the photosynthetic electron flow, other ROS can be formed along with the increase of $^1O_2$ production This will lead to the inactivation of the photosystems the reason being that oxygen can be used as an electron acceptor instead of ferredoxin by which a superoxide anion is generated as a primary product (Latifi *et al.,* 2009). Termed as the Mehler reaction, the reaction on the donor side of the photosystem I (PSI) was first described in chloroplasts by Mehler (1951). It has been

assumed in all oxygenic phototrophs that there is occurrence of the reduction of oxygen by the PSI electron flow (Latifi *et al.,* 2009). However, there is controversy in the extent to which Mehler reaction takes place in cyanobacteria. There is a high degree of O_2 reduction in cyanobacteria as compared to algae and higher plants; 50% of the photosynthetic electrons are consumed while only 15% is consumed in plants (Badger *et al*., 2000).

The triplet ground state of molecular oxygen serves as an excellent oxidizing agent in aqueous solutions. It is reduced to several toxic intermediates like $O_2^{\bullet-}$,H_2O_2, $HO^{\bullet}$ and $^1O_2$. $O_2^{\bullet-}$, a moderately reactive membrane-impermeable ROS is produced by the transfer of electrons from reduced iron-sulphur centres of PSI to O_2(Mehler reaction). It is also produced as a result of excessive reduction of phaeophytin and plastoquinone (electron acceptors), particularly during limitation of electron transport between PSII and PSI. H_2O_2, a less reactive and membrane- permeable ROS is produced from either dismutation of $O_2^{\bullet-}$or from the incomplete oxidation of water in PSII. $HO^{\bullet}$ is the most reactive amongst the ROS and is generated through the Haber Weiss reaction:

$$O_2^{\bullet-} + H_2O_2 \longrightarrow OH^{\bullet} + HO^{\bullet} + O_2$$

Fe (II), released *in vivo* from the inactivation of Fe_4S_4 clusters by $O_2^{\bullet-}$ further catalyzes this reaction (Fenton reaction):

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^{\bullet} + HO^{\bullet}$$

$^1O_2$, an unusual ROS is not produced from the electron transfer to O_2 but from the excitation of the ground triplet state of oxygen ($^3O_2$) by the reaction center triplet excited state chlorophyll (Mittler 2002; Latifi *et al.*, 2009; Pospisil 2009; Gill and Tuteja 2010; Vass 2012).

1.3 Effects of ROS on cellular functions:

ROS have multiple effects on the cellular functions in aerobic organisms. They cause damage to nucleic acids, oxidize proteins and also cause lipid peroxidation. ROS such as singlet oxygen ($^1O_2$), hydroxyl radical (OH), superoxide anion ($O2^{\cdot-}$) and hydrogen peroxide (H_2O_2) has the ability to oxidize the thiol of the cysteine residues of proteins (–SH) into disulfides (–S–S–), sulfenic (–SOH), sulfonic acids ($–SO_3H$) or sulfinic acids ($–SO_2H$) (Imlay, 2013). Two cysteinyl residues from a protein and a molecule of the anti-oxidant tripeptide glutathione (γ-L-glutamyl-L-cysteinyl-L-glycine) or from the same or different proteins can be linked via disulfide bridges (Chauvat and Chauvat, 2015). At high concentrations, all ROS proves to be harmful to organisms even though there are some instances in which ROS function as important signaling molecules. In these cases, gene expression may be altered by the action of ROS and the activity of specific defense proteins may also be modulated (Apel and Hirt, 2004).

Photosynthesis is also a target of ROS in oxygenic photoautotrophic cyanobacteria. It has been shown that H_2O_2 cause disturbances in the cores of phycobilisomes and also in the transfer of light energy from allophycocyanin to the terminal emitter and thus finally to photosystems (Liu *et al.*, 2005). Phycobilisomes are

involved in the capture of light in cyanobacteria. They exist as large extrinsic complexes that are attached to the outer surface of thylakoid membrane. Phycobilisomes have a core from which six rods radiate. Allophycocyanin is the major core, while the rods have phycocyanin. Both phycoerythrin and phycocyanin may be present in some species (Latifi *et al*., 2009). The D1 protein which is a part of the reaction centre P_{680} of PSII is the principal target of ROS. Excess accumulation of ROS affects D1 by disturbing its *de novo* synthesis pathway. This disturbance is caused at the repair step thereby leading to PSII photoinhibition. Both H_2O_2 and $^1O_2$ has also been shown to possess ablilities for inhibiting the translation elongation step of *psbA* (which produces D1) (Nishiyama *et al*., 2006). EF-G which is the translation elongation factor G was identified to be the common target of ROS (Kojima *et al*., 2007). Many cyanobacterial strains possess the ability to replace D1 with a more resilient form (D1:2) when faced with PSII photoinhibition (Schaefer and Golden, 1989).

Hydroxyl radical ($HO^{\bullet}$), the most destructive of all ROS is also known to cause damage to the cyanobacterial membranes by lipid peroxidation. $HO^{\bullet}$ is the main initiator of lipid peroxidation and it triggers a reaction in the polyunsaturated fatty acids (PUFA) of the cyanobacterial membranes, producing lipid peroxide radicals ($LOO^{\bullet}$) in the presence of oxygen. $LOO^{\bullet}$ disturbs the structure and function of the membrane by causing a chain reaction which produces more $LOO^{\bullet}$ radicals (Maeda *et al.*, 2005). $HO^{\bullet}$ as well as H_2O_2, but not $^1O_2$ also cause damages to the purine, pyrimidine and deoxyribose backbones of nucleic acids.

Regulated production of free radicals in higher organisms and maintenance of ‘‘redox homeostasis’’ are essential for the physiological health of organisms (Ames *et al*., 1993). A small proportion 23 (2–3%) of free radicals may escape from the

protective shield of antioxidant mechanisms during these metabolic processes, which may subject the cellular components to oxidative damage (Valavanidis *et al*., 2006). Even though adequate enzymatic and non-enzymatic antioxidant mechanisms have been developed by biological systems during their evolution to protect their cellular components from oxidative damage, there is always an imbalance between the generation of ROS and its neutralization by antioxidant mechanisms within an organism; called oxidative stress (Davies, 1995).

1.4 Defense against ROS in cyanobacteria:

Mechanisms for coping with the oxidative stresses created by ROS are crucial for all organisms. Numerous strategies have been developed by cyanobacteria in order to avoid ROS production and also to scavenge them once they are produced. These mechanisms include a range of non-enzymatic and enzymatic antioxidants. A variety of metabolites (ascorbate, carotenoids, glutathione, vitamins, *etc.*), and enzymes (superoxide dismutase, catalase and peroxiredoxins) can detoxify the ROS oxidants by sequentially converting the superoxide anion to hydrogen peroxide and subsequently to water (Chauvat and Chauvat, 2015).

1.4.1 Non-enzymatic antioxidants:

Caroteniods and α-tocopherol (Vitamin E) are non-enzymatic molecules that act to minimize the damaging effects of ROS in cyanobacteria. Myxoxanthophyll, β-carotene and its derivatives (zeanthin, echinenone) are carotenoids that can dissipate energy from $^1O_2$ as well as from photosensitized chlorophyll. Zeanthin has been shown

to play a particularly important role in photoacclimation in *Synechocystis* sp. 7942 during ultraviolet-B stress (Gotz *et al.,* 1999). In higher plants, tocopherol (a lipid-soluble organic molecule) has been suggested to take part in scavenging of $^1O_2$ in PSII reaction centers (Havaux *et al.,* 2005).All green algae, plants and some cyanobacteria possess the ability to synthesize α-tocopherol (vitamin E) which is an organic lipid-soluble molecule. This molecule is synthesized exclusively by oxygenic phototrophs. α-tocopherol has also been found to prevent peroxidation in membrane lipids from a study in tocopherol-deficient mutant of *Synechocystis* sp. PCC 6803 when subjected to both high light and polyunsaturated fatty acids (Maeda *et al.,* 2005).

1.4.2 Enzymatic antioxidants:

All organisms that lead the aerobic lifestyle possess multiple antioxidant enzymes to scavenge different types of ROS (except $HO^{\cdot}$). Cyanobacteria also possess an effective stress combat system to cope with the pressure of ROS by the help of numerous antioxidants. The superoxide dismutases (SOD) acts initially as the first line of defence against oxidative stress by catalyzing dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2 (Imlay, 2003). The catalases, peroxiredoxins, and Dps proteins protect cells from H_2O_2 toxicity. Catalases dismutate H_2O_2 to water and oxygen, and free intracellular iron is scavenged by DPS proteins, which can otherwise interact with H_2O_2 to form $HO^{\bullet}$ (most toxic of all ROS) through Fenton reaction (Banerjee *et al*., 2013; Ekman *et al*., 2014). The peroxiredoxins (Prx) catalyze detoxification of a broad range of substrates, including H_2O_2, peroxynitrite and alkyl hydroperoxides to water or nitrate or corresponding alcohols, respectively. Unlike catalases, which mainly detoxify H_2O_2

(high levels) and exhibit photosynthetic electron transport independent catalysis, peroxiredoxins mainly scavenge endogenously generated H_2O_2 (low levels) by coupling catalysis to photosynthetic electron transport (Bernroitner *et al*., 2009; Latifi *et al*., 2009). However, ascorbate-dependent peroxidises which detoxifies H_2O_2 in plant chloroplasts seems to be absent in cyanobacteria as the gene encoding for this enzyme is not yet detected in any of the cyanobacterial genomes (Bernroitner *et al*., 2009; Latifi *et al*., 2009).

Although, catalase and peroxidase perform the same function they differ in their mechanism of action. Peroxidases are believed to play a crucial role in the detoxification of endogenous hydrogen peroxide, whereas catalases detoxify exogenous hydrogen peroxide. The enzymes of the former group rely on electrons donated by ascorbate, glutathione or thioredoxin. Peroxidases can also detoxify other peroxides, such as alkyl hydroperoxides and peroxynitrite. In general, catalases exhibit lower affinity for hydrogen peroxide and higher K_{cat} compared to peroxidases (Bernroitner *et al.*, 2009).

a) Superoxide dismutases:

Multiple types of SODs are present in cyanobacteria, and many cyanobacteria possess genes encoding for more than one SOD (Priya *et al*., 2007). SODs are classified into four groups according to the metal cofactor their active sites contain: iron- (FeSOD/SodB), manganese- (MnSOD/SodA), copper and zinc- (Cu-ZnSOD/SodC), and nickel- (NiSOD) (Latifi *et al*., 2009). In algae, plants and cyanobacteria, cambialistic SODs that can use either iron or manganese at the active site of the same

protein have not been detected (Latifi *et al.*, 2009; Pilon *et al.*, 2011; Regelsberger *et al.*, 2004, Simon *et al*., 2005). Regardless of the metal co-factor present, SODs catalyze the first dismutation of $O_2^{\cdot-}$ into hydrogen peroxide and oxygen. The reaction is as follows:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The state of the metal center i.e. whether oxidized or reduced does not affect the activity of SODs, they are active in both cases. For instance, SodB acts as a reductant when Fe (III) is present at the active site to produce H_2O_2. When Fe (II) is present at the active site, the enzyme acts as an oxidant and produces O_2. This perpetual switching of the redox state of the metal cofactor at the active site makes SOD catalytic reaction proceed at a near diffusion-limited rate of approximately 10^9 M^{-1} s^{-1}(Simon *et al.*, 2005).

SodB probably constitutes the most ancient SOD group and was thought to have originated when the earth's atmosphere was reducing and Fe(II) was in plenty (Alscher *et al.*, 2002). SodB is found in both prokaryotes and eukaryotes. While SodB is localized in the chloroplasts of higher plants, it is found to be present in the cytoplasm of *E.coli* and cyanobacteria (Imlay, 2003; Latifi *et al*., 2009; Pilon *et al.*, 2011). Both unicellular (e.g., *Synechocystis* PCC 6803, *Synechococcus* PCC 7942) and filamentous cyanobacteria (e.g., *Anabaena* PCC 7120, *Nostoc punctiforme*) were found to possess SodB using bioinformatics-based genome sequence analysis coupled with SOD-specific biochemical identification. But the opposite was found for marine unicellular forms (e.g., species of *Prochlorococcus*) (Bhattacharya *et al*., 2004; Moirangthem *et al*., 2014; Regelsberger *et al*., 2004). The physiological significance of SodB has been studied only in a few cyanobacteria. It has been demonstrated that mutation of SodB in

Synechococcus sp. PCC 7942 makes it sensitive to oxidative stress induced by methyl viologen and low temperature (Thomas *et al*., 1998). SodB overexpression was found to be detrimental for N_2-fixation dependent growth under oxidative stress conditions in *Anabaena* PCC 7120; a protective effect, however, was observed in non-N_2-fixing cultures against oxidative stress (Raghavan *et al*., 2011).

Synechocystis sp. PCC 6803 and *Synechococcus* sp. PCC 7942 do not possess SodA unlike SodB. However, most filamentous, N_2-fixing cyanobacteria do possess SodA (Priya *et al*., 2007). SodA are found in mitochondria but not in chloroplasts in higher plants (Pilon *et al.*, 2011). The role of SodA in preventing high light and methyl viologen-induced oxidative damage and cell death has been thoroughly studied in *Anabaena* PCC 7120 by insertional inactivation and overexpression (Raghavan *et al*., 2011; Zhao *et al*., 2007). The SODs other than SodA and SodB, i.e., Ni and Cu/Zn have been detected only in a few cyanobacteria, mostly marine forms. A cambialistic SOD (can use either Fe or Mn at its active site) has also not been found so far in these organisms (Priya *et al*., 2007).

b) Catalases:

Catalases dismutate the hydrogen peroxide produced from SOD activity to water and oxygen. Three different types of catalases, viz., typical monofunctional haem catalases, bifunctional haem containing catalase-peroxidases and monofunctional manganese-containing haem catalases have been described so far in literature (Bernroitner *et al.*,2009; Latifi *et al.*,2009; Perelman *et al.*, 2003; Tichy and Vermaas, 1999). The catalases commonly found in cyanobacteria are either haem- or manganese-dependent. A gene for haem-containing bifunctional catalase–peroxidise (*katG*) has

been found in over 30% of cyanobacterial genomes sequenced so far (Bernroitner *et al*., 2009). The KatG protein has been biochemically characterized, and its protective role against exogenous H_2O_2 has been demonstrated in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942 (Perelman *et al*., 2003; Regelsberger *et al*., 1999; Tichy and Vermaas, 1999). However, expression of *katG* was found to be down-regulated in response to iron starvation in both these cyanobacteria (Nodop *et al*., 2008; Singh *et al*., 2003). Monofunctional manganese-catalase (Mn-catalase) is only found in N_2-fixing cyanobacteria; for instance, two Mn-catalases are present in *Anabaena* PCC 7120 (Bernroitner *et al*., 2009). It has been shown that the overexpression of *alr0998*-encoded Mn-catalase protects *Anabaena* PCC 7120 from oxidative stress induced by methyl viologen and H_2O_2 (Banerjee *et al*., 2012). Similarly, a protective role of *alr3090*-encoded Mn-catalase has been demonstrated in desiccation and salinity tolerance in the same organism (Chakravarty *et al*., 2016; Katoh, 2012).

c) Peroxidases:

Peroxidases are another set of enzymatic antioxidants that can detoxify hydrogen peroxide. However, they are dependent upon electrons supplied by glutathione, ascorbate or thioredoxin for reducing H_2O_2. In plant chloroplasts, ascorbate-dependent peroxidases play a crucial role in H_2O_2 detoxification. The concentration of ascorbate is approximately 250-fold lower in cyanobacteria than in chloroplasts. No gene-encoding for ascorbate peroxidase has been identified from cyanobacterial genomes even though there have been reports of ascorbate dependent peroxidase activity (Bernroitner *et al.*, 2009; Latifi *et al.*, 2009).

Glutathione peroxidases are found in both plants and animals. They form an important part of the peroxide detoxifying mechanism. Glutathione peroxidases from plants use cysteine as a catalytic residue while those of animals uses selenocysteine as a conserved catalytic residue. Cyanobacterial genomes have been shown to contain millimolar concentrations of glutathione. Genes highly homologous to glutathione peroxidase have been found to exist in many cyanobacteria (Bernroitner *et al.*, 2009; Laltifi *et al.*, 2009; Pérez-Pérez *et al.*, 2009, Tichy and Vermaas, 1999). Biochemical characterization of two putative glutathione peroxidases, encoded by ORFs *slr1171* and *slr1992* from *Synechocystis* sp. PCC 6803 revealed that these proteins utilize NADPH as electron doner instead of glutathione (Gaber *et al*., 2001). These NADPH-dependent glutathione peroxidase-like proteins were found to play an active role against lipid peroxidation in *Synechocystis* sp. PCC 6803 suggesting that they play a vital role against oxidative stress (Gaber *et al*., 2004).

Peroxyredoxins(Prx), also called alkylhydroperoxides are present in multiple numbers in almost all cyanobacteria, suggesting that they may be crucial in defense against oxidative stress in this class of photosynthetic organisms (Latifi *et al*., 2009). They differ from catalases in two accounts: (i) they scavenge endogenously generated low levels of H_2O_2 and their activity depends on the photosynthetic electron transport, while catalases mainly detoxify exogenously added H_2O_2 (high levels) and their activity is not dependent on photosynthetic electron transport, (ii)they have a broad substrate specificity that includes alkyl hydroperoxides and peroxynitrites unlike catalases. There are five proteins representing peroxiredoxins in *Synechocystis* PCC 6803, six in *Synechococcus* PCC 7942 and seven in *Anabaena* PCC 7120 belonging to different sub-classes, namely, 1-Cys Prx, 2-Cys Prx, Type II Prx, and PrxQ (Bernroitner *et al*.,

2009). These sub-classes of Prx proteins mainly differ in the location and number of the conserved cysteine residues and, in subunit composition. Except 2-Cys Prx and a few representatives of Type II Prx that are dimeric, all other Prx proteins are monomeric (Banerjee *et al*., 2013). All Prx proteins share the same basic catalytic mechanism. The peroxidatic cysteine is oxidized to a sulphenic acid on reaction with the peroxide substrate. Subsequently, regeneration of the oxidized peroxidatic cysteine is carried out by a resolving cyeteine that forms a disulphide bond with peroxidatic cysteine, which then gets reduced by one of the several reductant molecules (glutaredoxin, thioredoxin etc.). This is followed by the beginning of the next catalytic cycle (Latifi *et al*., 2009). The transcript and protein levels of many of the Prx are up-regulated in response to stress imposed by various ROS generating agents in cyanobacteria (Banerjee *et al*., 2013).

d) DNA-binding protein from starved cells (Dps) proteins:

Dps, another class of proteins possess the ability to quench H_2O_2 and take part in ameliorating oxidative stress in cyanobacteria (Chiancone and Ceci, 2010; Narayan *et al.*, 2010; Wei *et al.*, 2007). The Dps proteins and bacterioferritins present in cyanobacteria are a part of the ferritin family proteins (Ekman *et al*., 2014). DPS proteins forms a subgroup of the ferritin family in which the fifth helix found in other ferritins is lacking (Andrews *et al*., 2003). Unlike other ferritins, DPS proteins utilize H_2O_2 to oxidize Fe (II) (Lewin *et al.*, 2005). The bacterioferritins store iron as Fe^{3+} by oxidizing Fe^{2+} through their ferroxidase centers (Andrews, 2010).

Even after the characterization of over 20 family members, Dps proteins are considered to be the major players in the bacterial response to oxidative stress

(Chiancone and Ceci, 2010). The ferroxidase activity of Dps proteins has remarkable differences when compared with other members of the ferritin superfamily. O_2 is generally used by ferritins as iron oxidant which results in the production of hydrogen peroxide (Zhao *et al.*, 2003). Bacterioferritins can employ both $O_2$and H_2O_2 (Bunker *et al*., 2005), while H_2O_2 is typically preferred by Dps proteins. H_2O_2 is usually about 100-fold more efficient in carrying out iron oxidation as compared to O_2 (Franceschini *et al*., 2006).

It has been demonstrated that FurA modulates the expression of DpsA in *Anabaena* PCC 7120 (Hernández *et al*., 2007). In a *Synechocystis* PCC 6803 mutant defective in MrgA (a Dps family protein), the intracellular iron-quota was not found to be affected as much as in the bacterioferritin mutant(s) (Keren *et al*., 2004; Shcolnick *et al*., 2009). The *mrgA* mutant of *Synechocystis* PCC 6803 was more sensitive to externally applied oxidative stress, similar to a mutant of *Nostoc punctiforme* lacking one of the DPS proteins (encoded by Npun_3730) (Ekman *et al*., 2014; Shcolnick *et al*., 2009, 2007). The intracellular level of DpsA has been shown to rise in *Synechococcus* PCC 7942 during stationary phase of growth, and during deficiency of nitrogen, sulphur or phosphorous. DpsA can bind and protect chromosomal DNA as well (Michel and Pistorius, 2004).

1.4.3 Energy dissipation:

Adverse conditions such as unsuitable environmental conditions are often encountered by cyanobacteria in their natural habitats. These conditions create an imbalance between the rate of electron consumption during fixation of CO_2 and the

photosynthetic electron transport leading to the increased generation of ROS in the system. Cyanobacteria possess different mechanisms to prevent or detoxify ROS efficiently and promptly as soon as they are produced. One of these strategies is by a preventive mechanism known as nonphotochemical quenching (NPQ), by which the excitation energy is safely dissipated as heat. The cyanobacterial orange carotenoid protein (OCP) has been shown to protect the system from photodamage by dissipating excess excitation energy collected by phycobollisomes (PBS) as heat in *Synechocystis* sp. PCC 6803(Wilson *et al.*, 2006). *In vivo* dissociation of the PBS - OCP complex is carried out by another protein which primarily exists as a dimeric complex - the fluorescence recovery protein (FRP) (Lu *et al.*, 2017). In *Synechocystis* sp. PCC 6803, it has also been show that the high-light inducible proteins (HLIPs) play a crucial role in photoprotection under stress created by high light (He *et al.*, 2001). An iron-starvation induced protein A (IsiA or CP43'), which is a homolog of the CP43 chlorophyll-binding protein, forms a part of the energy dissipation mechanism in some cyanobacteria and protects PSII (Sandstorm *et al.*, 2001). This protein has been shown to be induced under a variety of stress conditions (high salt, high temperature, oxidative, high light and iron-limitation) suggesting a protective role for IsiA against multiple stresses in cyanobacteria (Latifi *et al.*, 2005, 2009).

1.5 Present study:

As discussed earlier, peroxidase detoxifies cell-toxic molecules into simpler and less toxic molecules, thus it represents an important part of the antioxidant machinery of cyanobacteria. In chloroplasts of higher plants, ascorbate-dependent peroxidase is

the principle scavenger of hydrogen peroxide, and ascorbate concentrations are high (millimolar range) (Pérez-Pérez *et al.,* 2009). In contrast, ascorbate concentration in cyanobacteria is low (micromolar range), and genes which code for ascorbate peroxidase is missing from the already sequenced cyanobacterial genomes. The concentration of glutathione in cyanobacteria range from 2-4 mM, and two glutathione peroxidase-like proteins have been identified in *Synechocystis* sp. PCC 6803 and characterized (Gaber *et al.*, 2001). However, glutathione peroxidase like genes/ proteins has not been characterized in other cyanobacteria.

Nostoc punctiforme ATCC 29133 (hereafter *Nostoc punctiforme*) is a filamentous, heterocystous, nitrogen-fixing cyanobacterium the genome of which has been completely sequenced. The objectives of the present study are as follows:

1. To detect glutathione peroxidase activity in *Nostoc punctiforme* ATCC 29133 and to evaluate its response to oxidative stress.

2. Bioinformatics driven identification of gene encoding for glutathione peroxidase and *in silico* characterization of the corresponding protein.

3. To clone and overexpress the glutathione peroxidase-like gene in *Escherichia coli.*

CHAPTER 2

MATERIALS AND METHODS

2.1 Organisms:

Nostoc punctiforme ATCC 29133, the filamentous N_2-fixing cyanobaterium (hereafter referred to as *Nostoc punctiforme*) was used for the present study. The cyanobacterium - *Nostoc punctiforme* used was obtained from Prof. Peter Linbald, Uppsala University, Sweden. *Escherichia coli* (hereafter referred to as *E.coli*) strain DH5α (genotype: F^-, *endA, hsdR17, supE44, recA1, gyrA96, relA1,* argF) was used for cloning purposes and *E.coli* strain BL21 (DE3) (genotype: F^-, *ompT,* r_B^-, m_B^-(DE3) was used for overexpression of the glutathione peroxidase-like gene (Npun_R4660).

2.2 Culture methods:

2.2.1 Culture medium: *Nostoc punctiforme* was grown from anexic stock cultures in BG-11o medium (Rippka *et al.*,1979). The concentrations of macronutrients in BG-11o medium were (mM): $K_2HPO_4.3H_2O$, 0.18; Na_2CO_3, 0.19; $MgSO_4.7H_2O$, 0.30; $CaCl_2.2$ H_2O, 0.25;EDTA (disodium salt), 0.003; Citric acid, 0.029; Ferric ammonium citrate, 0.030. The concentrations of micronutrients in BG-11o medium were (μM): H_3BO_3, 46; $MnCl_2.4$ H_2O, 9.2; $ZnSO_4.7$ H_2O, 1.6; $CuSO_4.5$ H_2O, 0.32; $Co(NO_3)_2.6$ H_2O, 0.17. The medium was always buffered with equimolar concentration of HEPES. The pH of the medium was adjusted to 7.5 before autoclaving. *Nostoc* sp. was also maintained on agar slants as well as in liquid BG-11o. The temperature of the culture room was maintained at 25°C and light was provided at a photon fluence rate of 20μmol.$m^{-2}s^{-1}$ on the vessel surface.

Both strains of *E.coli* - DH5α and BL21 (DE3) were grown in Luria Bertani (LB) medium. The composition of the LB medium for 1 liter is as follows:

10g tryptone, 10g sodium chloride (NaCl) and 5g yeast extract. Then the pH was adjusted to 7.0 using 5N NaOH. For solid medium, 2% agar was added to the nutrient broth. Whenever needed, ampicillin was added to a final concentration of 100μg/ml from a stock solution of 25mg/ml made in sterile double distilled water. High quality water (Millipore) was used for preparation of all media.

2.2.2 Sterilization: All media, solutions glasswares and plasticwares were sterilized by autoclaving for 15mins at 121 °C (15 pounds per square inch) before use. The heat labile chemicals were sterilized by ultrafiltration using membrane filters with pore size of 0.45 μm.

2.2.3 Culture conditions: Anexic cultures of *Nostoc punctiforme* was routinely grown in liquid BG11o –medium and maintained on slants containing 1.5% (w/v) agar. Culture room temperature was maintained at 25 °C and continuous illumination was provided to the cultures at a photon fluence rate of 20μmol.$m^{-2}s^{-1}$. The bacterial cultures of *E.coli* DH5α and BL21 (DE3) were cultured in LB-medium at 37 °C, shaking the culture vessels at 180 rpm. Cells harboring recombinant plasmid were cultured and maintained with the addition of appropriate antibiotics (kanamycin or ampicillin).

2.3 Growth measurement of *Nostoc punctiforme*:

The growth of *Nostoc punctiforme* was measured by estimating the concentration of chlorophyll *a*. Chlorophyll *a* was measured using the method

described by Mackinney (1941). In brief, 5ml samples were extracted in equal volume of 100% methanol and incubated for 10 min. The chlorophyll *a* concentration was measured spectrophotometrically using Cary 60 spectrophotometer (Agilient Technologies, USA) in the supernantant of centrifuged extracts at 663 nm by using the formula: Chlorophyll *a* ($\mu g\ ml^{-1}$) = Absorbance at 663 nm x 12.63.

2.4 Preparation of cell free extracts by cell disruption for protein determination:

The *Nostoc punctiforme* culture was harvested by centrifugation at 2300×*g* for 5min at room temperature. The cell pellets were then washed twice with 36mM potassium phosphate buffer (pH 7.4). To prepare the cell-free extracts, the cells were resuspended in the same buffer with protease inhibitors (phenylmethylsulphonyl fluoride, 1 mM leupeptin and 5 μM pepstatin). The cells were disrupted at 4°C by ultrasonication using a Soniprep 150 (MSE) fitted with a microprobe. The resulting crude extracts were then centrifuged at 10000×*g* for 30 min at 4°C. The supernatant was used for protein determination.

2.5 Protein estimation by Bradford assay:

Using a kit provided by Biorad, the protein concentration was measured in cell-free extracts. 20 μl of the protein sample was mixed with 1 ml of Bradford reagent (supplied by the manufacturer) and the mixture was incubated for 5 min. Absorbance was measured at 595 nm (Bradford, 1976). A calibration curve was prepared by using Bovine serum albumin (BSA) solution as standard.

2.6 Native (non-denaturing) Polyacrylamide gel electrophoresis:

Non-denaturing polyacrylamide gel electrophoresis (Native PAGE) of protein samples obtained from *Nostoc punctiforme* culture was performed using BioRad mini gel apparatus (BioRad Laboratories, California, USA). The 10% resolving and 5% stacking Native-PAGE gels were prepared by following the protocol described in Sambrook and Russel (2001), except that the gels contained 10% glycerol but no sodium dodecyl sulphate (SDS). The 10% resolving gel contained 2 ml of Millipore water, 1.6 ml of 30% acrylamide and N,N'-methylenebisacrylamide mix (1:29), 1.3 ml of 1.5 M Tris buffer (pH 8.8), 0.05 ml of 10% ammonium persulphate, and 0.002 ml of TEMED. The composition of a stacking gel was as follows: 1.7 ml of Millipore water, 0.42 ml of 30% acrylamide and N, N'-methylenebisacrylamide mix (1:29), 0.34 ml of 0.5 M Tris buffer (pH 6.8), 0.025 ml of 10% ammonium persulphate, and 0.002 ml of TEMED. SDS was also omitted from sample loading dye (50mM Tris, pH 6.8; 10% Glycerol; 0.1% Bromophenol blue) and electrophoresis buffer (25 mM Tris; 250 mM Glycine, pH 8.3). The cell-free extracts obtained as described above were loaded at equal concentration of protein and electrophoresis was performed at 4 °C for 2-3h under constant amperage.

2.7 In-gel glutathione peroxidase assay for identification and evaluation of its response to oxidative stress:

Nostoc punctiforme cultures were grown in $BG11_0$ medium under conditions discussed in the previous sections. 1M stock solutions of H_2O_2 and *tert*-butyl hydroperoxide were made for glutathione peroxidase activity assay.7 day old *Nostoc*

punctiforme cultures were collected and inoculated at a concentration of 1µg chla/ml into fresh BG 11_0 medium. Different culture vessels were subjected to a range of concentrations of hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide taken from the stock culture made. The different concentrations of H_2O_2 used were of 0 µM (control), 250 µM, 500 µM, 1mM and 5mM and those of *tert*-butyl hydroperoxide were of 0 µM (control), 25 µM, 50 µM, 100 µM. The cultures were then incubated at a photon fluence rate of 20µmol.$m^{-2}s^{-1}$. The cells were harvested after a period of 2hours, broken down, 8µg of the protein was made to run on a 12% NATIVE-PAGE gel and glutathione peroxidase assay was performed using the protocol used by Das and Bagchi (2012). The gel was washed with 2.5% Triton X-100 and distilled water for 15mins each. It was then immersed in 10mM in potassium phosphate buffer (pH 7.2) containing 2mM o-dianisidine dihydrochloride for 1 hour. It was then washed with potassium phosphate buffer for 15mins and 0.1mM H_2O_2. Brown bands indicating the presence and activity of glutathione peroxidase (EC 1.11.1.9) appeared against a pale yellow background.

2.8 Bioinformatics analysis:

A bioinformatics-based approach was used for identification and characterization of the open reading frames encoding for glutathione peroxidase protein from the complete genome sequence of *Nostoc punctiforme* as described in Chapter 3. Two previously characterized glutathione peroxidase-like proteins from *Synechocystis* sp. PCC 6803 (slr1171 and slr1992) were used as query sequence.

2.9 Isolation of genomic DNA from *Nostoc punctiforme*:

Genomic DNA was isolated from approximately 10 days old culture. Cells were harvested and resuspended in 1ml freshly prepared solution 1(0.05 M NaCl, 0.05 M Na_2EDTA, 0.05 M Tris-HCl, pH 8.5), depending on the size of the pellet. 10mg/ml lysozyme was added and kept at 37°C for 2 hrs. After incubation, equal volume of solution1 with 1% SDS was added. Lysate thawed first at 37°C for 15 mins in water bath; then it was frozen at -20°C for 15 more minutes. Freezing and thawing was performed 3 times. Equal volume of Tris-saturated phenol was added to the tubes and the lysate then allowed to shake gently at 4°C for 15mins. The bacterial lysate was centrifuged at 10,000 rpm for 15mins. 500µl of the supernatant was transferred to fresh micro-centrifuge tubes. 1ml (twice the volume) ice cold 100% ethanol was added to the supernatant in each tube and kept overnight at -80°C. The solution was centrifuged at 12,000rpm for 15 mins and the supernatant was discarded. 1ml of 70% ethanol was added to the pellet for washing and the tubes were centrifuged again at 12,000 rpm for 10 mins. Supernatant was again removed and kept in an incubator at 37°C until the ethanol has evaporated and there is no visible fluid inside the tube. The pellet was then resuspended in 100µl of solution 2(0.1 M NaCl, 0.001M Na_2EDTA, 0.01 M Tris-HCl, pH 8.5) and 400µg/ml RNase and was incubated for 1 hr at 45°C. 50µl phenol and 50µl chloroform (in 1:1 ratio) were added to each tube (both phases were mixed well) and centrifuged at 12,000 rpm for 10 mins. 500µl of aqueous phase from each tube was taken and transferred to fresh tubes. Double the volume of ice cold ethanol (1ml) was added alongwith 1/10 volume (50µl) 3M Sodium acetate (pH 5.2) and kept overnight at -80°C. The tubes were centrifuged again at 12,000 rpm for 15 minutes and the supernatant was discarded. The pellet was washed with 70% ethanol and was

centrifuged at 12,000 rpm for 15 minutes. Supernatant was removed gently and the tube was stored at 37°C in an incubator till all the ethanol has evaporated and there was no fluid visible inside the tube. The pellet was dissolved in 20µl HPLC grade water.

2.10 PCR-based amplification of Npun_R4660:

Gene amplification was carried out by PCR using cyanobacterial genome as template and gene specific primers. Amplification of Npun_R4660 was carried out by using genomic DNA as a template, in a total volume of 40µl reaction mixture. The sequence of the primers used was:

Forward primer- 5'-CAT ATG AGT AAC ACA ATT TCG GAT ATC-3'

Reverse primer- 5'- CTC GAG CTA TTT TGC TAA TTC TTT CTC A-3'

The primers alongwith buffer (1X), dNTP mix (0.2mM), $MgCl_2$ (0.2mM) and 1U Pfu polymerase was used for DNA amplification. This was carried out in a thermal cycler with the following temperature profile: Initial denaturation at 95°C for 3 mins, 35 cycles of denaturation for 1min at 95°C, annealing at 50°C and 55°C for 1min and extension at 72°C for 2mins, and final extension at 72°C for 10mins. The amplified product was run on a 1% agarose gel alongwith 100bp molecular weight marker at a constant voltage and visualized following staining with ethidium bromide.

2.11 Purification of DNA fragment:

The DNA was recovered from the agarose gel using Fermentas DNA extraction kit as follows -

The DNA was excised from the stained 1% agarose gel with a razor blade. Agarose was cut as much as out as to get the product band. An approximate volume of gel slice was determined by weight and the slice was placed into a plastic tube. 3 volumes of binding solution (125ml of 6M sodium iodide solution) was added to 1 volume of gel and incubated for 5mins at 55°C in a water bath to dissolve agarose. 5µl of silica powder suspension (1.5 m of specially prepared glass bead suspension in water) was added to the DNA and incubated at 55°C for 5mins. The incubated solution was mixed by vortexing every 2mins to keep silica powder in suspension. Silica powder/DNA complex was spun at 10,000 rpm for 5mins to form a pellet and supernatant was removed. 500µl of ice-cold wash buffer (15ml of concentrated solution of Tris, NaCl and EDTA) was mixed by spinning for 5mins at 5500 rpm and the supernatant was removed. This was repeated 3 times. During each wash, the pellet was completely resuspended and after the supernatant was removed from the last wash, the tube was spun again and the remaining liquid was removed with micropipette. DNA was then eluted into water. The pellet was resuspended in an aliquot of sterile deionised water and the tube was incubated at 55°C for 5mins followed by centrifugation. Avoiding the pellet, the supernatant was transferred into a new tube. The elution was repeated with another aliquot of water. The tube was spun for 30secs in a table-top centrifuge in order to remove any silica powder remaining in small amounts. The supernatant was transferred into a new tube. An aliquot was made to run on an agarose gel of 0.8% and DNA amount was determined.

2.12 Ligation of DNA fragments:

Ligation of the vector with the insert was done by using Fermentas Ligation kit as follows:

50ng of pJET1.2 vector was combined with PCR product. Volume was adjusted to 10µl with distilled water. 10µl of 2X reaction buffer was added and mixed. 1µl of T4 DNA ligase was added and mixed thoroughly. Ligation mix was vortexed briefly and centrifuged for 3-5secs. The ligation mix was incubated at room temperature (22°C) for 5mins. The ligation mix was used directly for transformation.

2.13 Restriction gel:

Sequential digestion was performed in order to retrieve the insert/gene in vectors, pJET1.2/pET20B. After checking the orientation, the plasmid was digested by *Nde*I and *Xho*I. The component of digestion reaction is as follows:

DNA : 10µl

10x buffer : 10µl

RNase : 3µl

Water (DDW) : 75µl

Enzyme : 2µl

Total : 100µl

After confirming the complete digestion (by checking in 0.8% agarose gel as described before), the solution was precipitated by adding 1/10 volume 3M sodium acetate (pH 5.2) + 3 times ethanol and stored overnight at -20°C.

The plasmid was then digested again with *Xho*I. The component of digestion reaction is as follows:

DNA : 10µl

10x buffer : 10µl

RNase : 3µl

Water (DDW) : 75µl

Enzyme : 2µl

Total : 100µl

2.14 Preparation of competent cells:

Competant cells of DH5α and BL21 (DE) were prepared as described by Sambrook and Russel (2001) using $CaCl_2$ method with some modifications. A single DH5α colony was inoculated into 5ml of LB and then it was incubated overnight at 37°C while subjecting it to vigorous shaking at 120 rpm until OD590 = 0.3-0.5(approx. 2 hrs). The culture was transferred to 2 pre-chilled sterile 250ml centrifuge tubes. The bacterial cells were centrifuged at 5000 rpm for 10mins at 4° to form pellets. The supernatant was removed and the pellets were kept in ice. The pellets were washed in cold 0.1M $CaCl_2$ and spun at 5000rpm for 10 mins at 4°C.The cells were resuspended

in ½ volumes ice-cold 0.1M $CaCl_2$ solution and the cells were kept in ice for half an hour. The cells were again spun at 5000 rpm for 10mins at 4°C. The supernatant was removed and the cells were resuspended in 1ml ice-cold 0.1M $CaCl_2$ solution.

2.15 Transformation of DH5α and BL21 (DE3):

Transformation was carried out according to the protocol of Sambrook and Russell, 2001. Plasmid DNA/ligation mix was added to 100µl competent cells of DH5α and BL21 (DE3) and kept in ice for half an hour. The cells were heat shocked at 42°C for 2mins. Then the cells were placed back on ice. 1ml LB was added to cells/DNA and resuspended into it. The cells/DNA was incubated at 37°C for an hour. The transformed cells were plated onto LB+ Amp plate and the plates were kept inverted at 37°C overnight.

2.16 Isolation of plasmid:

Plasmid DNA was isolated by alkaline lysis method. 25ml of LB medium containing ampicillin was inoculated with a single colony of transformed bacteria. The culture was incubated overnight at 37°C with vigorous shaking. The culture was transferred into a 15ml tube and the bacteria were recovered by centrifugation at 10,000 rpm for 10mins at 4°C. The medium was removed and the bacterial pellet was resuspended in 500µl of ice-cold alkaline lysis solution1 [50mM glucose, 25mM Tris-Cl (pH 8)(from 1M stock) and 10mM ethylene diamine tetraacetic acis (EDTA) (pH 8) (from 0.5M stock)] by vortexing vigorously, and the suspension was transferred to a

microcentrifuge tube. 1ml of alkaline lysis solution 2[0.2N sodium hydroxide (NaOH) (from 5N stock), which was freshly prepared 1% (w/v) sodium dodecyl sulphate (SDS) (from 10% SDS stock)] was added to the bacterial suspensions and the contents in the tubes were mixed by inverting them. The tubes were kept at room temperature for 5mins. 750µl of ice-cold alkaline lysis solution 3 [60ml of 5M potassium acetate, 11.5M glacial acetic acid, 28.5ml H_2O stored at 4°C] was added to each tube and mixed by inverting many times. The tubes were kept in ice for 10 mins. The bacterial lysates were centrifuged at 10,000 rpm for 10mins at 4°C. The supernatant was transferred into fresh microcentrifuge tubes. An equal volume of phenol:chloroform (300µl phenol and 300µl chloroform) was added to the supernatant. The organic and aqueous phases were mixed by vortexing and then centrifuged the emulsion at 10,000 rpm for 10mins at 4°C. The aqueous upper layer was then transferred into fresh tubes. 2 volumes of ice-cold ethanol were added to the supernatant. The solution was mixed by vortexing and then allowed to precipitate by keeping at 80°C for 1 hour (or -20°C for overnight). The precipitated nucleic acid was centrifuged at 10,000 rpm for 15mins and the supernatant was removed. 500µl of 70% ethanol was added to the pellet for washing and the DNA was retrieved by centrifuging it at 10,000 rpm for 10mins. The supernatant was again removed and any beads of ethanol that form on the sides of the tube were removed by keeping in an incubator at 37°C until all the ethanol has evaporated and no visible fluid is seen inside the tube. The DNA was stored at -20°C.

2.17 Chemicals:

The chemicals used during the present study were obtained from Sigma ,Himedia, and Merck India.

CHAPTER 3

RESULTS

3.1 Detection of glutathione peroxidase activity in *Nostoc punctiforme* and its response to H_2O_2 and *tert*-butyl hydroperoxide.

Glutathione peroxidase is an enzyme that catalyzes the reduction of hydroxyperoxides. Its main function is to protect the cells from endogenously produced hydroxyperoxides. It forms an important part of the cells machinery which enables it to cope with oxidative stress. In organisms like cyanobacteria, in which abiotic stress in the form of ROS is in abundance even in its natural environment, it plays a pivotal role in the survival of the organism. Glutathione peroxidase proteins have not yet been characterized from the diazotrophic cyanobacterium *Nostoc punctiforme*. Its role and presence have not yet been established experimentally in *Nostoc* sp. or any cyanobacteria except *Synechocystis* sp. PCC 6803 (Gaber *et al.*, 2001). Thus, the task of finding out so will shed more light on the stress combat system of cyanobacteria and is of prime significance.

Anexic clonal cultures of *Nostoc punctiforme* were grown photoautrophically in $BG11_0$ medium, pH 7.5 and exposed to different conditions as described in Chapter 2. The growth of the cyanobacterial cultures was measured at regular intervals at an optical density of 750nm. An assay to detect the presence of glutathione peroxidase activity was carried out using the protocol by Das and Bagchi (2012) as described in Chapter 2.

The N_2- fixing cultures of *Nostoc punctiforme* produced a single glutathione peroxidase activity on Native gels. The activity of glutathione peroxidase enzyme was

identified as a brown band against a pale yellow background. The intensity of the band could be assumed to be directly proportional to the activity of the enzyme. Upon treatment with different concentrations of hydrogen peroxide, I found that the glutathione peroxidase enzyme activity was present in cultures treated with different concentrations of H_2O_2 up to 1mM. AT 5mM, however, no enzyme activity could be detected. These results indicate that glutathione peroxidase activity was constitutively produced in *Nostoc punctiforme* and the cultures treated with low levels of H_2O_2 (1mM) were able to retain glutathione peroxidase activity similar to control cultures. Further, the loss of activity at 5mM H_2O_2 suggests that the enzyme is unable to tolerate high concentrations of H_2O_2 (Fig 3.1.1).

When cultures of *Nostoc punctiforme* were treated with a different range of concentrations of *tert*-butyl hydroperoxide (25 - 100µM), I found an activity band of glutathione peroxidase enzyme in all cultures without any change in increase in intensity compared to control. This suggested that the enzyme is not sensitive to *tert*-butyl hydroperoxide and therefore may be involved in the detoxification of the same (Fig 3.1.2).

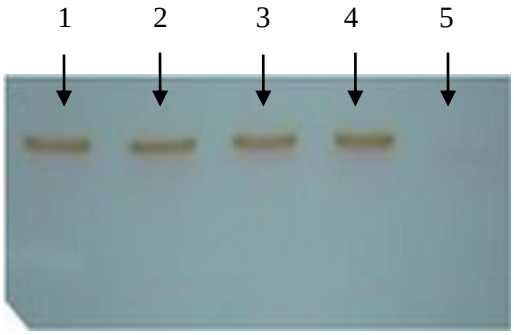


Fig 3.1.1 : In-gel assay of glutathione peroxidase assay upon treatment with H_2O_2. 8µg of protein was loaded in 12% NATIVE PAGE gels. The enzyme was found to be active only at lower levels of exposure i.e. up to 1mM hydrogen peroxide treatment. At higher concentrations of hydrogen peroxide, the activity of glutathione peroxidase was not seen. Lane 1- Control (*Nostoc punctiforme* cell free extract); Lane 2 - *Nostoc punctiforme* cell free extract + 250 µM H_2O_2; Lane 3 - *Nostoc punctiforme* cell free extract + 500 µM H_2O_2; Lane 4 - *Nostoc punctiforme* cell free extract + 1 mM H_2O_2; Lane 5 - *Nostoc punctiforme* cell free extract +5 mM H_2O_2

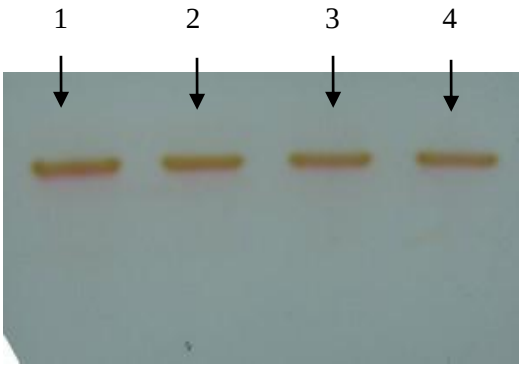


Fig 3.1.2: In-gel assay of glutathione peroxidase assay upon treatment with *tert*-butyl hydroperoxide showing its activity. 8µg of protein was loaded in 12% NATIVE PAGE gels The enzyme was found to be active in all concentrations of *tert*-butyl hydroperoxide used, showing its activity.Lane 1-Control (*Nostoc punctiforme* cell free extract); Lane 2 - *Nostoc punctiforme* cell free extract + 25µM *tert*-butyl hydroperoxide ; Lane 3 - *Nostoc punctiforme* cell free extract + 50 µM *tert*-butyl hydroperoxide; Lane 4 - *Nostoc punctiforme* cell free extract + 100 µM *tert*-butyl hydroperoxide.

3.2 Analysis of glutathione peroxidase-like protein from *Nostoc punctiforme* ATCC 29133 using bioinformatics-based approach:

The presence of a glutathione peroxidase activity has been detected as described in the previous section using in-gel assays. Therefore, the next step was to identify the gene or protein that may be responsible for producing this activity. Since the genome sequence is completely known for this organism, bioinformatics approach was taken to identify the gene/ protein.

The amino acid sequences of two previously characterized glutathione peroxidase-like proteins (slr1171 and slr1992) from *Synechocystis* sp. PCC 6803 (Gaber *et. al.*, 2001) were retrieved from cyanobase (www.genome.microbedb.jp/cyanobase). Using both these sequences as query, the glutathione peroxidase-like protein from *Nostoc punctiforme* ATCC 29133 was identified as Npun_R4660. The percentage identity of the proteins was calculated using ALIGN (www.uniprot.org/align/) (Fig. 3.2.1). Npun_R4660 showed the highest percentage of identity to slr1992 (43.478%) followed by *Chlamydomonas reinhardtii* (43.293%). Npun_R4660 showed 42.775% identity with slr1171. Multiple sequence alignment with several other glutathione peroxidase proteins from known organisms including those from Eukaryotes as well as Prokaryotes was performed using T-Coffee (Tommaso, 2011). The alignment revealed a conserved cysteine residue at position 65 of Npun_R4660 marked in red. The catalytic triad of the enzyme was also marked with red hashtags and the amino acid in similar positions as those in Npun_R4660 were highlighted as white against a black background (Fig 3.2.2).

The physico-chemical properties of Npun_R4660 protein was analyzed using the ProtParam tool at Expasy (www.expasy.ch/tool/ProtParam) and compared with other known glutathione peroxidase-like proteins, belonging to both Eukaryotes and Prokaryotes, the amino acid sequences of which were retrieved from NCBI. Analysis of the Npun_R4660, slr1171 and slr1992 using ProtParam revealed several characters of the proteins including pI, expected molecular weight and the stability to be similar (Fig. 3.2.3). The prediction of signal peptide in these proteins were carried out using Phobius software (Käll *et al*., 2004). The analysis of the three proteins (Npun_R4660, slr1171 and slr1992) revealed that they do not possess any signal peptide, which would be required for their localization in the membrane (Fig. 3.2.4 A, B &C).

The 3D structure of Npun_R4660 was predicted using SWISS-MODEL (Schwede *et al*. 2003) or Phyre2 (**P**rotein **H**omology/analog**Y** **R**ecognition **E**ngine V 2.0) (Kelley and Sternberg, 2009). The predicted three dimensional structures were evaluated using Rampage (Lovell *et al*. 2003). PyMOL (Delano, 2005) and Swiss PDB Viewer (Guex and Peitsch, 1997) were used for visualization of the modelled structures. Homology modelling was performed on the basis of significant sequence similarity using the templates identified. PBD ID 2v1m chain A (1.0 Å) (Dimastrogiovanni *et al*. 2010) with 56.4 % sequence similarity was used as the template by Phyre2 and PDB ID 2p5r chain B (2.45 Å) (Koh *et al.* 2007) with a sequence similarity of 52.8% was used as the template by SWISS-MODEL server. The structure which was predicted consisted of 5 α-helices and 6 β-strands (Fig. 3.2.5). The monomeric subunit is represented in Fig 3.2.5. The modelled glutathione peroxidase structure showed remarkable resemblance to the previously described eubacterial glutathione peroxidase structures in overall fold (Dimastrogiovanni *et al*. 2010). The

predicted models form both SWISSMODEL and Phyre2 was assessed using the Ramachandran plot. The modelled structures had ~97.5% residues in the most favourable regions and 2.5% residues occurring in the outlier regions (Fig. 3.2.6). To define a reliable model, the G factor score (a log odds score based on the observed distribution of stereochemical parameters like bond length, phi-psi torsion angles and main chain bond angles) should be above -0.50. The degree of unusualness in the predicted model can be determined using this. The overall average values of G-factors below -0.5 and -1.0 corresponds to unusual and highly unusual properties of the model, respectively. The overall average G-factor score was -0.21 and -0.06 for models predicated by SWISSMODEL and Phyre2, respectively. When the modelled structures were validated by VERIFY3D, it showed that at least 80% of the amino acids have scored >= 0.2 in the 3D/1D profile, which is also an indication of good quality of the modelled structure. The secondary structures of the Npun_R4660 protein were analysed using PDBsum (www.ebi.ac.uk/thornton-srv/database/egi-bin/pdbsum). Two beta sheets, one beta alpha beta unit, two beta hairpins, one psi loop, four beta bulges, six beta strands, eight helices, five helix-helix interacts, eleven beta turns and one gamma turn were found. The secondary structure is depicted in Fig. 3.2.7. The overall topology of the modelled protein was deduced (Fig. 3.2.8).

Phylogenetic trees were constructed to bring about light into the relationship between the glutathione peroxidase-like protein of *Nostoc* and of those in *Synechocystis* using Mega7 (Kumar *et al.*, 2016). Three trees were made according model recommendation by the model test of Mega 7. The evolutionary history was inferred using Neighbor joining (NJ), Maximum Likelihood(ML) and Maximum Parsimony methods . The following describes the criteria for the trees constructed:

Neighbor-joining method: The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 7.08065418 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.. The evolutionary distances were computed using the JTT matrix-based method (Jones *et al*., 1992*)* and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 12 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 149 positions in the final dataset. [Fig. 3.2.9 (A)] Evolutionary analyses were conducted in MEGA7 (Kumar *et al*.,2016).

Maximum Likelihood: The evolutionary history was inferred by using the Maximum Likelihood method based on the Whelan and Goldman model, 2001. The tree with the highest log likelihood (-2897.28) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.7748)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 12.28% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12

amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 149 positions in the final dataset. [Fig. 3.2.9 (B)]. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

Maximum Parsimony method: The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 539 is shown. The consistency index is (0.768293), the retention index is (0.518987), and the composite index is 0.409220 (0.398734) for all sites and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 12 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 149 positions in the final dataset [Fig. 3.2.9 (C)]. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016).

In all of the phylogenetic trees obtained, there were two clades which were divided into sub-clades and sub sub-clades. The clustering suggested that Npun_R4660 is more closely related to slr1992 than slr1171. It may be deduced from the evolutionary trees that the slr1992 and NpunR_4660 might have originated from a common ancestor. The phylogenetic trees deduced from MP showed difference from the trees given by NJ and ML methods. This may be attributed to the fact that both NJ and ML methods used the parameters given by the Mega 7 model test while none was given for MP method.

GPX source	Percentage identity with Npun_R4660 (%)	Identical positions	Similar positions
slr1171(*Synechocystis*)	42.775	74	50
slr1992 (*Synechocystis*)	43.478	70	45
Citrus	41.618	72	54
Tobacco	38.857	68	53
Chlamydomonas reinhardtii	43.293	71	47
Yeast	43.03	71	49
Human PHGPX	31.863	65	51
Rat	29.851	60	50
Escherichia coli	42.077	77	48
Bacillus subtilis	39.157	65	60
Saccharomyces cerevisiae	42.169	70	55

Fig 3.2.1: Table showing the percentage of identity of the proteins of different organisms including Eukaryotes and Prokaryotes with the glutathione peroxidase-like protein from *Nostoc punctiforme* ATCC 29133. Npun_R4660 showed the highest similarity (expressed in terms of percentage) to slr1992, a glutathione peroxidase-like protein from *Synechocystis* sp. PCC 6803.

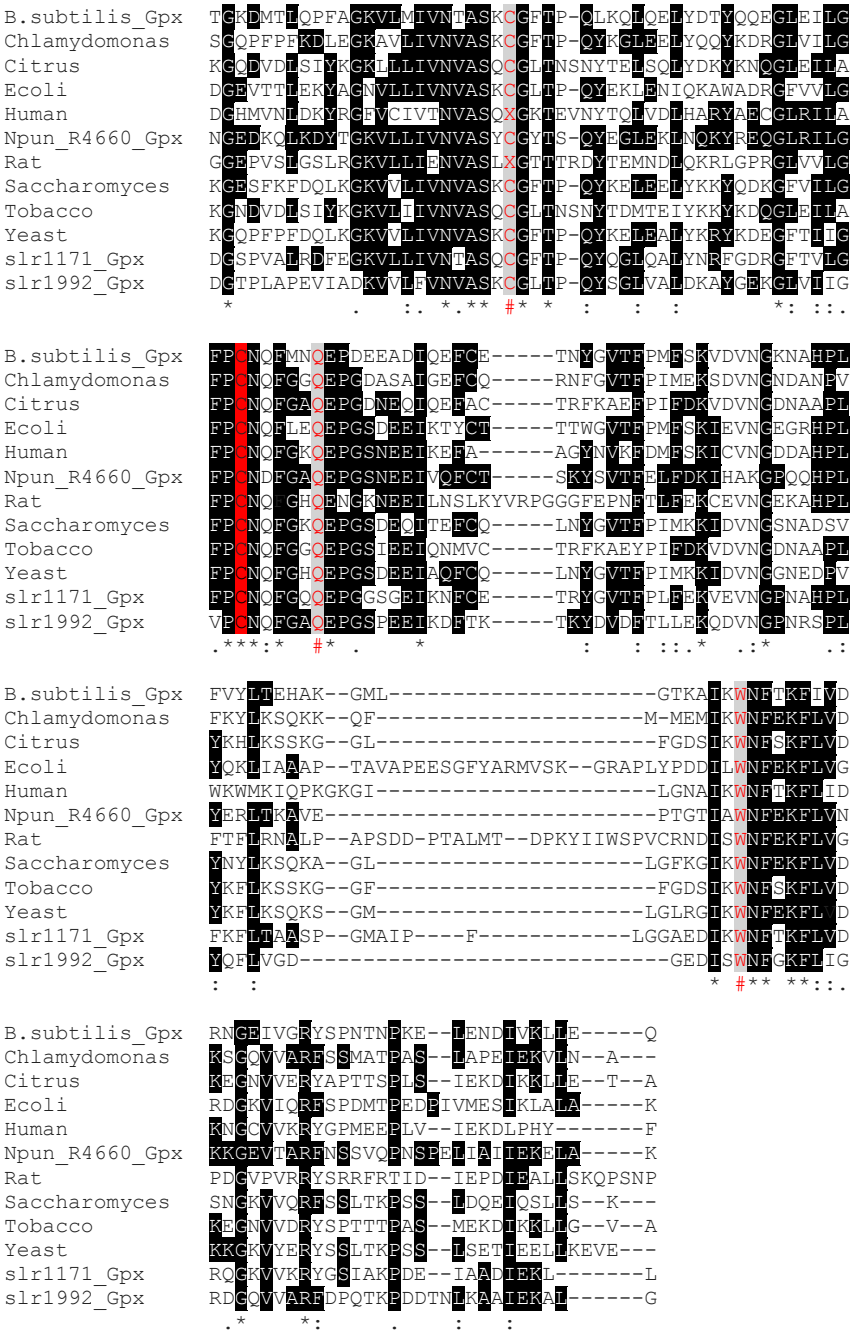


Fig 3.2.2: Multiple sequence alignment of the amino acid sequence of Npun_R4660 with the GPX-like proteins from eukaryotic algae, yeast, higher plants and animal GPXs. Partial alignment of the deduced Npun_R4660 sequence with the GPX of *Bacillus subtilis*, *Chlamydomonas reinhardtii*, Citrus, *E.coli*, rat, tobacco, yeast (*Saccharomyces cerevisiae*), slr1171 and slr1992 from *Synechocystis* PCC 6803 and human PHGPX using T-Coffee. The red hashtags in the rows marked with red letters against a grey background indicate the catalytic triad (X indicates Sec, as Cys is replaced by Sec in Eukaryotes). Residues found in the same position as Npun_R4660 are shown as white letters over a black background.

Protein id	Npun_R4660	slr1171	slr1992
Protein name	Glutathione peroxidase-like protein	Glutathione peroxidase-like protein	Glutathione peroxidase-like protein
Source (organism)	*Nostoc punctiforme* (Cyanobacteria)	*Synechocystic* sp.PCC 6803 (Cyanobacteria)	*Synechocystic* sp.PCC 6803 (Cyanobacteria)
Number of amino acids	161	169	154
Molecular Weight	18.1 KDa	18.45KDa	16.64 KDa
Theoretical pI	6.16	6.58	4.63
Instability index (IT)	27.45 (Protein is stable)	20.73 (Protein is stable)	28.39 (Protein is stable)
Aliphatic index	81.74	76.80	89.22
Localization	Non-cytoplasmic	Non-cytoplasmic	Non-cytoplasmic
Signal peptide	Not present	Not present	Not present

Fig. 3.2.3 - Physico-chemical properties of Npun_R4660, slr1171 and slr1992 as derived from ProtParam & Phobius software showing the Protein id, protein name, organism from which it is derived, number of amino acid residues, molecular weight, theoretical pI, instability index, aliphatic index, localization and whether or not signal peptide is present.

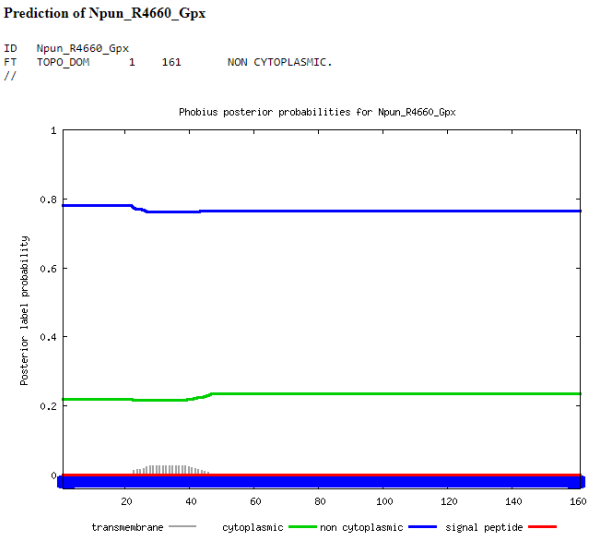


Fig 3.2.4 (A) : Prediction of Npun_R4660 using Phobius software

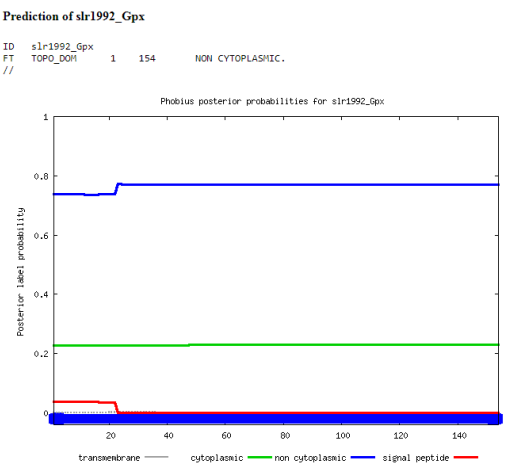


Fig 3.2.4 (B): Prediction of slr1992 using Phobius software

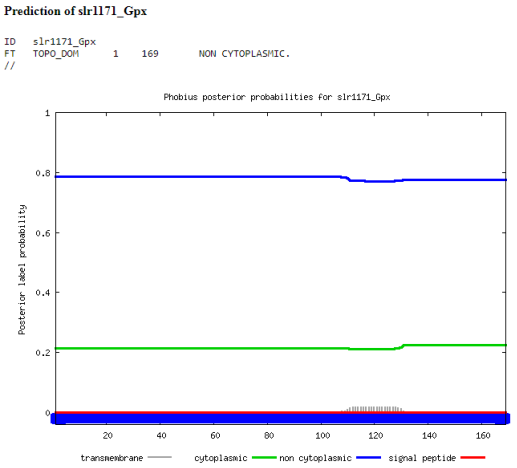


Fig 3.2.4 (C): Prediction of slr1171 using Phobius software

Fig 3.2.4 : Analysis of the sequence of glutathione peroxidase-like proteins (Npun_R4660) from *Nostoc punctiforme* ATCC 29133, slr1171 and slr1992 from *Synechocystis* PCC 6803 using Phobius software showed that all three proteins are non-cytoplasmic and lack signal peptides.

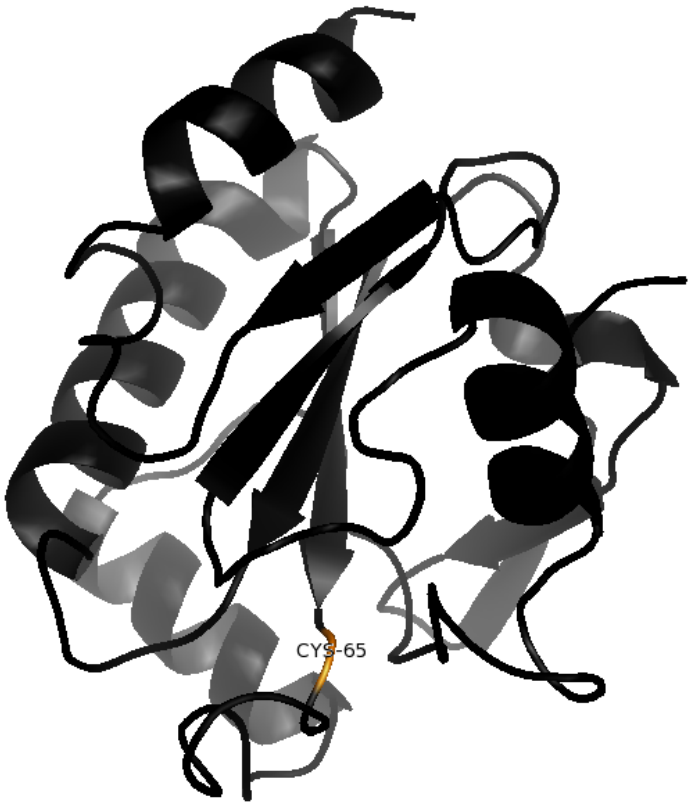


Fig 3.2.5: Modelled Npun_R4660 protein showing the conserved cysteine residue at position 65.

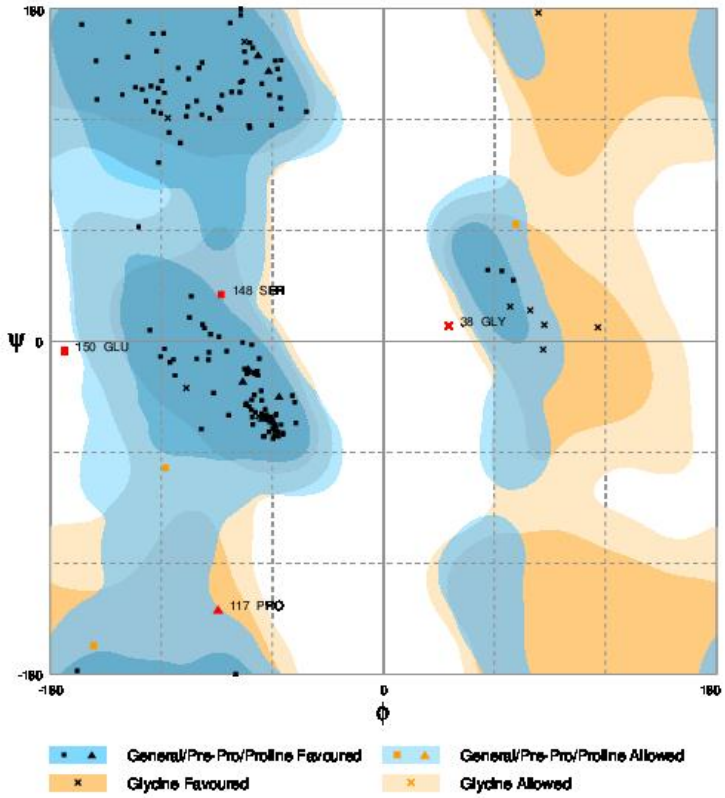


Fig 3.2.6: Ramachandran plot of the modelled Npun_R4660 protein from *Nostoc punctiforme* ATCC 29133. The modelled structures had ~97.5% residues in the most favorable regions and 2.5% residues occurring in the outlier regions.

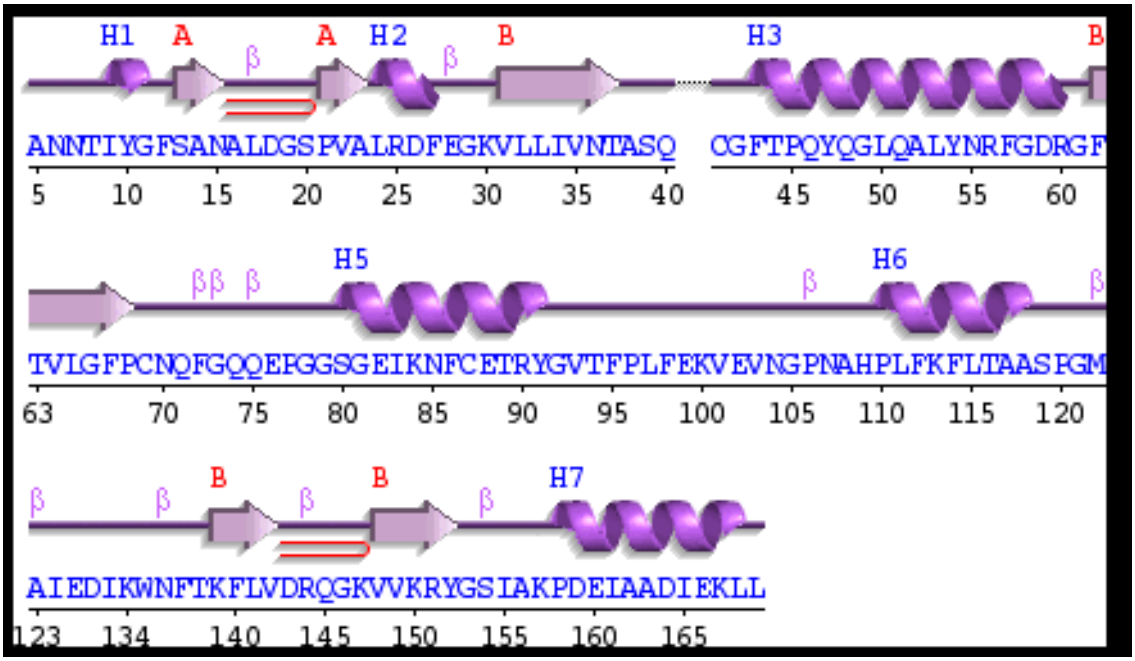


Fig 3.2.7: Secondary structure of the modelled protein Npun_R4660, a glutathione peroxidase-like protein from *Nostoc punctiforme* ATCC 29133 revealed the presence of two beta sheets, one beta alpha beta unit, two beta hairpins, one psi loop, four beta bulges, six beta strands, eight helices, five helix-helix interacts, eleven beta turns and one gamma turn.

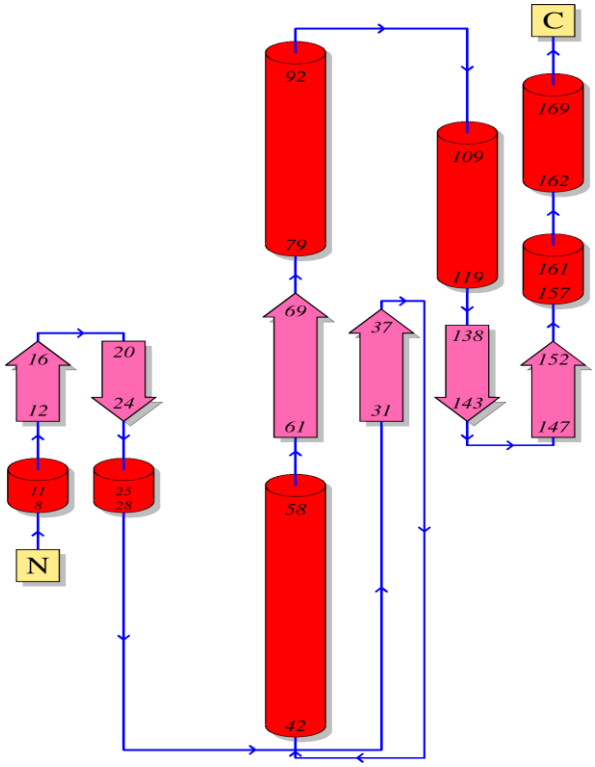


Fig 3.2.8 - Topology of the modelled glutathione peroxidase-like protein (Npun_R4660) from *Nostoc punctiforme* ATCC 29133.

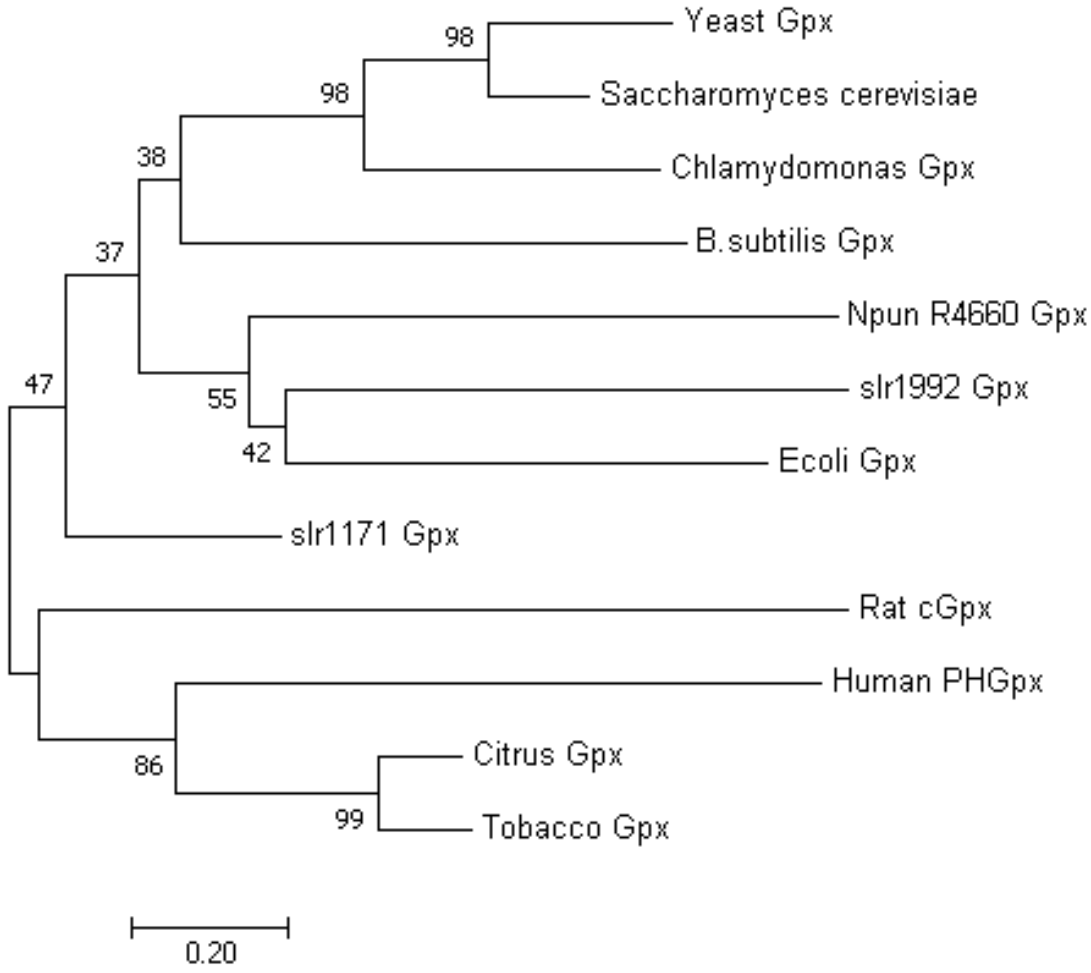


Fig 3.2.9 (A) - Phylogenetic tree constructed using Neighbor-joining method with a bootstrap value of 500 showing the distance i.e. identity of the protein coded by Npun_R4660 to other queries.

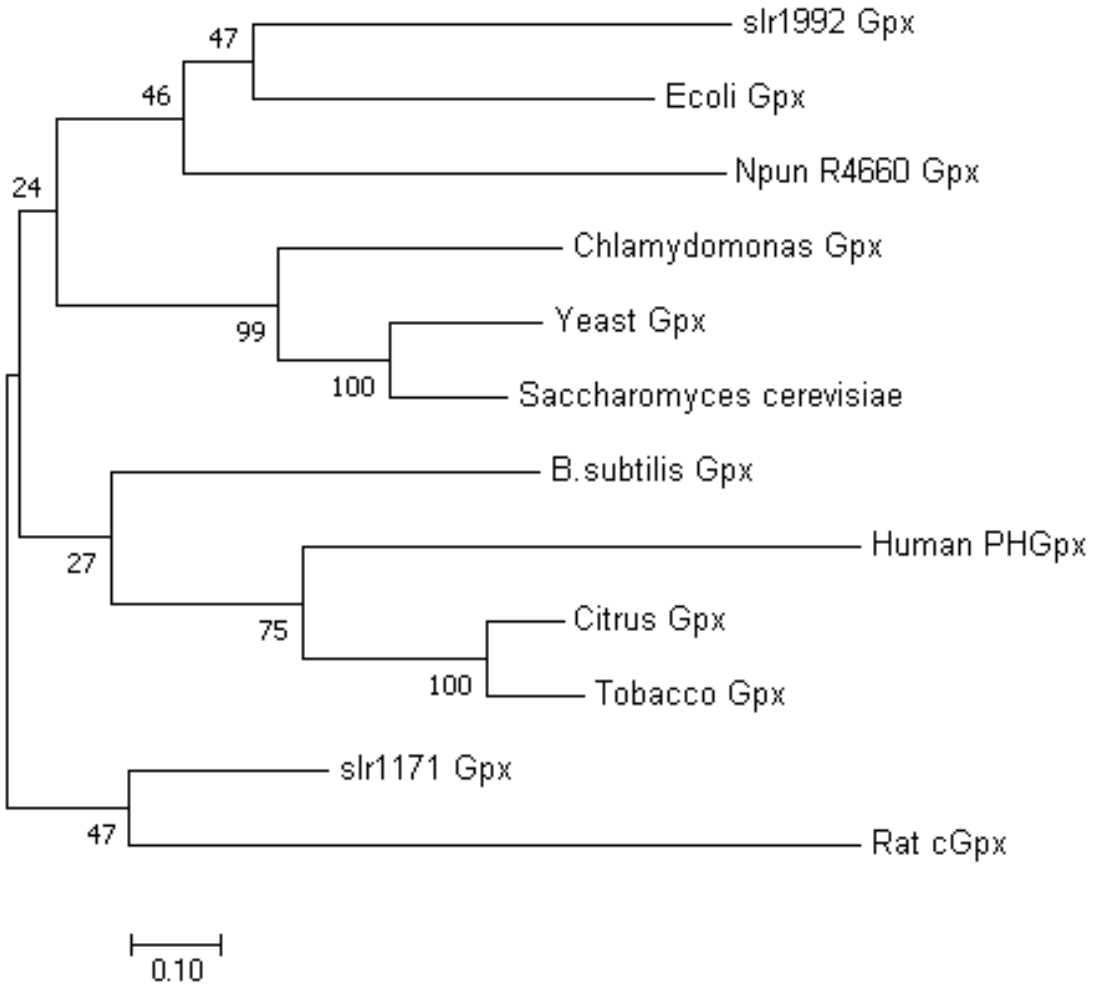


Fig 3.2.9 (B) - Phylogenetic tree constructed using Maximum Likelihood method with a bootstrap value of 500 showing the distance i.e. identity of the protein coded by Npun_R4660 to other queries.

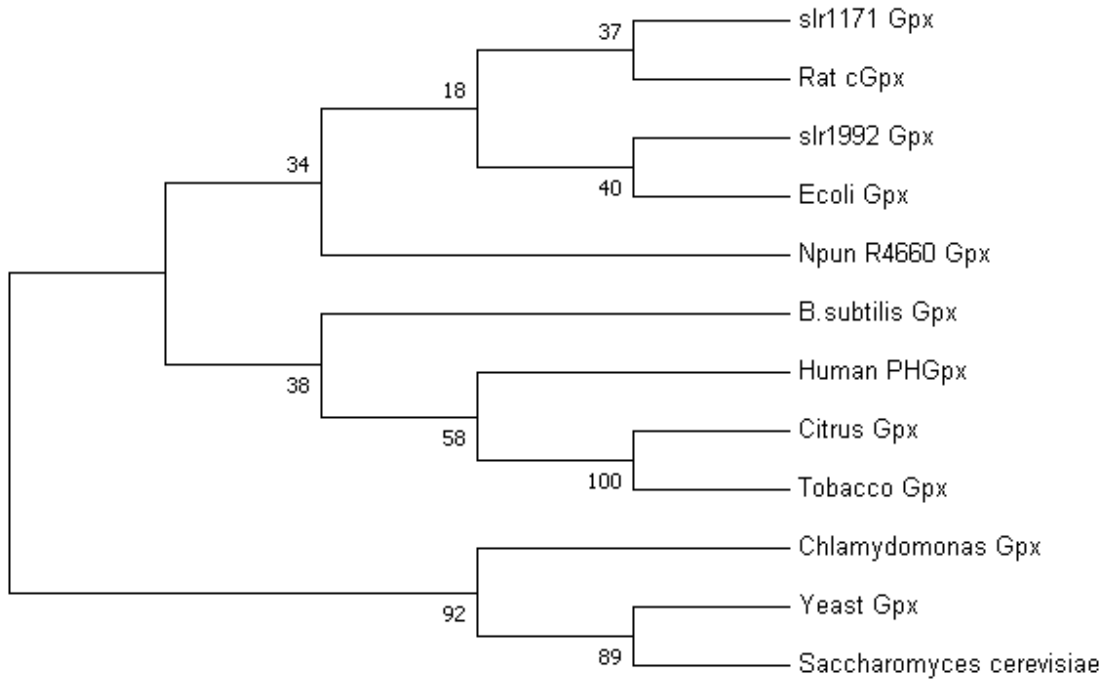


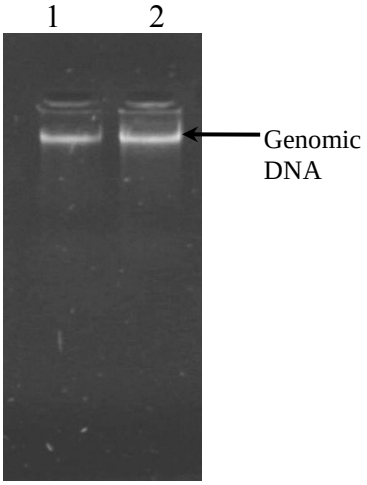
Fig 3.2.9 (C) - Phylogenetic tree showing the distance i.e. identity of the protein coded by Npun_R4660 to other queries using Maximum parsimony method.

Fig 3.2.9 (A, B, C): Phylogenetic trees constructed using Neighbor-joining, Maximum likelihood and maximum parsimony methods to show the distance between Npun_R4660 and other query sequences.

3.3 Cloning and overexpression of the glutathione peroxidase-like protein (Npun_R4660) from *Nostoc punctiforme* ATCC 29133.

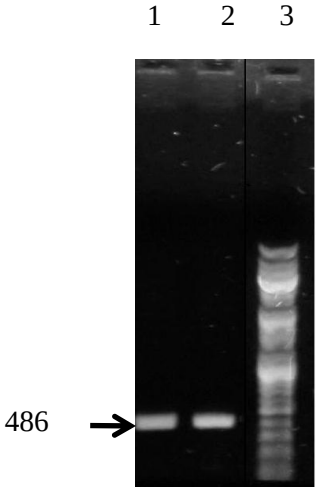
The presence of a glutathione peroxidase-like protein was deduced using activity analysis and bioinformatics approach. But in order to characterize it further, it has to be cloned and overexpressed. An open reading frame (ORF) encoding for a putative glutathione peroxidase in *Nostoc punctiforme* ATCC 29133 was identified as Npun_R4660 by bioinformatics approach. From the genomic DNA of *N. punctiforme,* the 486 bp ORF was amplified by polymerase chain reaction. *Nde*I and *Xho*I sites were present at the 5' and 3'respectively at the end of the forward and reverse primers used for cloning. The PCR amplified product was sub-cloned into pJET cloning vector. The ORF was further introduced into pET-20B vector for overexpression of the corresponding protein in *Escherichia coli* BL21 DE3 strain. Induction of *E.coli* cultures was done by Isopropylthiogalactoside (IPTG) and, Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of cell-free extracts, after which an approximately 18 kDa protein was detected in the cytoplasm. This matched with the theoretical molecular weight of the glutathione peroxidase-like protein. This protein was not present in control cultures transformed with pET-20B without the cyanobacterial ORF. These results suggest that foreign enzymes such as glutathione peroxidase can be produced in large amounts for biotechnological purposes successfully even in cells of a different origin like *E.coli*. Details of the methods used and steps performed are as written in Chapter 2.

Genomic DNA was successfully isolated (Fig.3.3.3.1). The desired gene (Npun_R4660) was amplified using PCR-based techniques (fig. 3.3.3. 2). Digestion was successfully done using restriction enzymes *Nde*I and then with *Xho*I (Fig. 3.3.3. 3). The fragment was retrieved by gel purification after the *Nde*I/*Xho*I digestion (Fig 3.3.3.4). Digestion was done with another restriction enzyme (*Eco*RV) in order to test our results (Fig 3.3.3.4). The presence and overexpression of the protein was studied and viewed using SDS-PAGE (Fig. 3.3.3.5). With the exception of the two glutathione peroxidase-like proteins of *Synechocystis* PCC 6803, no other glutathione peroxidase-like protein has been characterized from cyanobacteria till date thus proving this study to be of great significance. The attempt to clone the glutathione peroxidase-like protein from *Nostoc punctiforme* ATCC29133 was done successfully. Futhermore, the glutathione peroxidase-like protein was also produced in bulk in *E.coli*. The biochemical characterization of the protein can now be comfortably carried out.



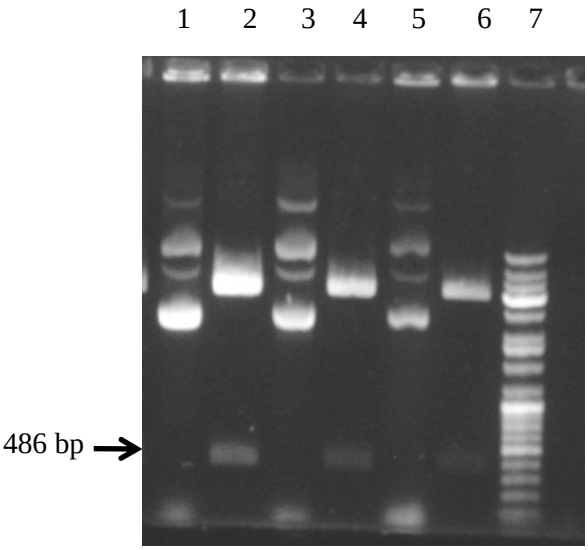
Lane 1: Genomic DNA
Lane 2: Genomic DNA

Fig 3.3.1: Genomic DNA isolated from *N.punctiforme*.



Lane 1: PCR product
Lane 2: PCR product
Lane 3: Low Range ruler

Fig 3.3.2: PCR amplification of Npun_R4660 gene



Lane 1: Np4660-pJET putative #1
Lane 2: Np4660-pJET putative #1: *Xho*I digest
Lane 3: Np4660-pJET putative #2
Lane 4: Np4660-pJET putative #2: *Xho*I digest
Lane 5: Np4660-pJET putative #3
Lane 6: Np4660-pJET putative #3: *Xho*I digest
Lane 7 : Low range ruler

Fig3.3.3: Restriction digestion of 4660-pJET clones with *Xho*I

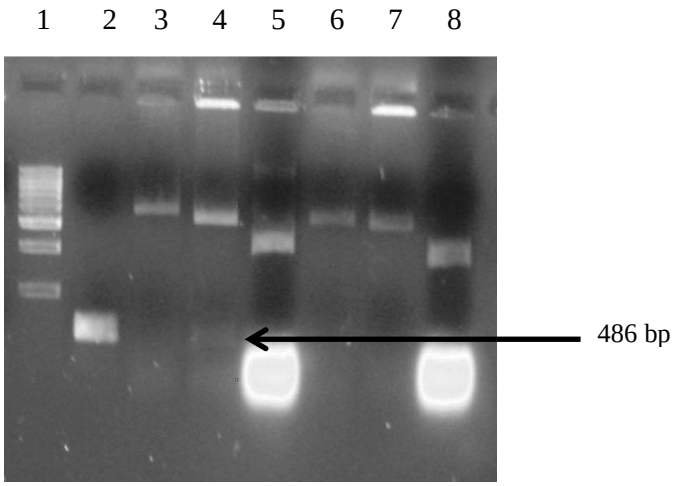


Fig 3.3.4: Restriction digestion of Np4660-pET20b clone.Lane 1- I kb ladder; Lane 2-500 bp fragment; Lane 3- Np4660-pET20b/*Eco*RV digest; Lane 4- Np4660-pET20b/*Nde*I, *Xho*I digest; Lane 5- Np4660-pET20b (undigested); Lane 6- pET20b/ *Eco*RV digest; Lane 7- pET20b/ *Nde*I, *Xho*I digest; Lane 8- pET20b (undigested)

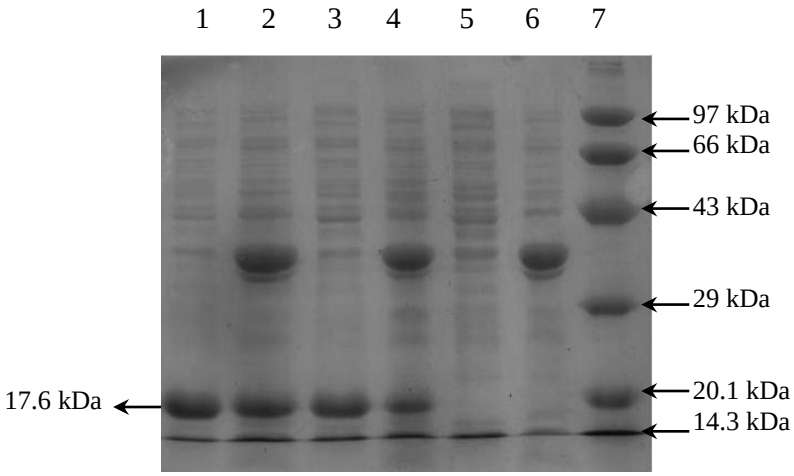


Fig 3.3.5: Overexpression analysis of Np4660-pET20b by SDS-PAGE. 12% gel was run and protein induced at 29°C and 37°C. Lane 1- Np4660-pET20b (supernatant fraction) 29°C; Lane 2- Np4660-pET20b (pellet fraction) 29°C; Lane 3- Np4660-pET20b (supernatant fraction) 37°C; Lane 4- Np4660-pET20b (pellet fraction) 37°C; Lane 5- pET20B (supernatant fraction) 37°C; Lane 6:-pET20b (pellet fraction) 37°C; Lane 7- Marker

CHAPTER 4

DISCUSSION

Cyanobacteria have been endowed with the ability to detoxify various reactive oxygen species. The enzymatic line of defense against ROS - superoxide dismutases, catalases and peroxidases work in concert with each other to remove these harmful ROS. However, no enzymatic scavengers are known that can detoxify hydroxyl radicals. SODs catalyze the dismutation of superoxide anion ($O_2^{\cdot -}$) into hydrogen peroxide and oxygen and catalases dismutate the H_2O_2 produced from SOD activity to water and oxygen. Peroxidases also possess the ability to detoxify H_2O_2. Peroxidases scavenge endogenously generated low levels of H_2O_2 while catalases mainly detoxify exogenously added H_2O_2 (high levels). Peroxidases also have a broad substrate specificity that includes alkyl hydroperoxides and peroxynitrites unlike catalases.

A gene coding for a glutathione peroxidase-like protein (Npun_R4660) was isolated from *Nostoc punctiforme* ATCC 29133. The glutathione peroxidase-like protein consisted of a 486bp encoding 161 amino acids with a calculated molecular mass of approximately 18 KDa. The deduced amino acid shared 43.48% identity with that of slr1992, a glutathione peroxidase-like protein from *Synechocystic* PCC 6803. The deduced amino acid sequence for Npun_R4660 showed 29 - 43 % identity to the glutathione peroxidase-like proteins from yeast, eukaryotic algae, higher plants and mammals. However, the selenocysteine residue of mammalian glutathione peroxidase-like proteins was replaced by cysteine in Npun_R4660 at the catalytic site. Npun_R4660 was also found to have two highly conserved amino acid residues Gln(Q) at position 71 and Trp(W) at position 123. This coincided with the glutathione peroxidase-like proteins of the other query sequences.

Ascorbate peroxidase is the major enzyme responsible for scavenging H_2O_2 while glutathione peroxidase (GPX) is the major H_2O_2 scavenger in animals (Ursini *et al.*, 1995; Shigeoka *et al*., 2002). For protection against lipid peroxidation due to alkyl hydroperoxides and lipid hydroperoxides, GPX also plays an important role (Gaber *et al.*, 2004). In the cDNA of higher plants, several of them have been found to code for GPX similar to those found in animals (Criqui *et al.*, 1992; Holland *et al*., 1993; Churin *et al*., 1999; Li and Sherman, 2000). Similarly, these genes coding for GPX were also found in *Saccharomyces cerevisiae* (Inoue *et al*., 1999) as well as in *Chlamydomonas* (Yokota *et al*., 1988). Even though plant genes carry Cys residue instead of Sec residue as compared to mammalian GPXs, the GPX-like proteins of plants still show highest similarity to them. However, the replacement of Sec by Cys in plant systems render the GPX-like enzyme activity to be lower than those in mammalian systems (Maiorino *et al*.,1995).

Two NADPH-dependent glutathione peroxidase-like proteins (slr1171 and slr1992) had been characterized from *Synechosystis* sp. PCC 6803 by Gaber *et al.*, 2001. These two proteins have been found to be unable to utilize glutathione, alkyl hydroperoxides or unsaturated fatty acid hydroperoxides. Catalase-peroxidase and thioredoxin peroxidase has also been identified as H_2O_2 and/or alkyl hydroperoxide scavengers in *Synechosystis* sp. PCC 6803 (Jakopitsch *et al*., 1999; Yamamoto *et al*., 1999). Increase in response to *tert*- butyl hydroperoxide, H_2O_2, methyl viologen , high osmolarity, high light or high salinity been reported in photosynthetic organisms including algae with respect to the steady-state levels of glutathione peroxidase mRNA and/or GPX protein (Sugimoto and Sakamoto,1997; Roeckel-Drevet *et al.*, 1998; Leisinger *et al*., 1999).

It was shown that slr1171 and slr1992 proteins seem to respond to the oxidative stress caused by high light, MV treatment, and high salinity by scavenging the lipid hydroperoxide generated in the process (Gaber *et al*., 2004). *Tert*- butyl hydroperoxide, an alkyl hydroperoxide has been found to readily react with transition-metal reductants or catalysts to form radicals thereby causing oxidative damage over long distances (Asada, 1994). Thus, it becomes essential for the cell to possess defense mechanisms by which the peroxide intermediates can be degraded. Alkyl hydroperoxides can be utilized by both slr1171 and slr1992 proteins (Gaber *et a*l., 2001). However in slr1992, the levels of transcript and protein were found to be unaffected by treatment with *tert*-butyl hydroperoxide (Gaber *et* al., 2004). This suggested that slr1992 may have some different functions as compared to slr1171 i.e. slr1171 may play more role than slr1992 for protection against ROS created by this *tert*- butyl hydroperoxide. Npun_R4660 was also found to be unaffected by different levels of *tert*- butyl hydroperoxide. When Npun_R4660 was subjected to different concentrations of *tert*- butyl hydroperoxide, the level of activity was seen to be almost the same as the control cultures suggesting that the enzyme is not sensitive to *tert*-butyl hydroperoxide and therefore may be involved in the detoxification of the same along with other antioxidative enzymes. However, when the same was subjected to treatment with different concentrations of H_2O_2, we could see from the activity band that there was loss of transcript activity indicating sensitivity to H_2O_2. When Npun_R4660 was compared to slr1171 and slr1992 using several bioinformatics software, it showed more similarity to slr1992. On the basis of this, we identified the protein Npun_R4660 from *Nostoc punctiforme* as as encoding for a glutathione peroxidase-like protein.

The glutathione peroxidase-like protein (Npun_R4660) was then modelled using the sequence retrieved from Cyanobase. The three dimensional structure was constructed using SWISS-Model and Phyre. The model was evaluated using Rampage (Lovell *et al*. 2003) and viewed using PyMOL (Delano, 2005) and Swiss PDB Viewer (Guex and Peitsch, 1997). While validating the modelled structure by using VERIFY3D, at least 80% of the amino acids have scored >= 0.2 in the 3D/1D profile, which is an indication of good quality of the modelled structure The Ramachandran plot of the modelled protein when analysed as well which showed that ~97.5% of the amino acids fall in the favourable region thus, confirming the stability of the modelled protein. The secondary structure and the topology was also deduced using PDBsum which revealed the presence of 2 beta sheets, 1 beta α beta unit, 2 beta hairpins, 1 psi loop, 4 beta bulges, 6 beta strands, eight helices, 5 helix-helix interacts, 11 beta turns and 1 gamma turn. Homology modelling was performed on the basis of significant sequence similarity using the templates identified. The evolutionary trees deduced using Mega7 confirmed the likelihood that Npun_R4660 and slr1992 are more closely related and similar than with slr1171.

The genomic DNA of *Nostoc punctiforme* was isolated. PCR was performed with specific primers to amplify Npun_R4660. It was then cloned and overexpressed successfully in the cytosol of *E.*coli, an organism of non-photosynthetic source. The protein may be further characterized in future with regard to its glutathione peroxidase-like activity. Overexpression of this gene in cyanobacteria or generation of mutants would allow us to understand its physiological roles in a more precise manner.

CHAPTER 5

SUMMARY

Glutathione peroxidases are constitutive enzymes that detoxify lipid hydroperoxides and thus play a significant role in the antioxidant machinery of aerobic organisms. It has been known to convert endogenously produced H_2O_2 to simpler molecules which are less harmful. An *in silico* based approach revealed that the genome of the diazotrophic, oxygenic, heterocystous cyanobacterium *Nostoc punctiforme* ATCC 29133 possesses one open reading frame(ORF) encoding for a putative glutathione peroxidase-like gene (Npun_R4660). The present study aims to characterize the protein using methods like molecular cloning and heterologous overexpression. An attempt has also been made to analyze the activity of the enzyme in stress conditions created by exogenously incorporating prooxidants into its environment. Analysis of the properties of the protein using bioinformatics was also performed. The findings are summarized below:

1. The presence of the glutathione peroxidase-like protein was established in *Nostoc punctiforme* ATCC 29133 using in-gel assays that would detect its activity. The assay confirmed the presence of an active glutathione peroxidase in the organism. Prooxidants like H_2O_2 and *tert*-butyl hydroperoxide were used to subject the organism to abiotic stress in the form of ROS. As glutathione peroxidase enzyme is believed to play an important role in the defence against ROS in aerobic organisms, the level up to which the enzyme actively takes part in the detoxification of ROS produced by hydrogen peroxide and *tert*-butyl hydroperoxide were also monitored. Glutathione peroxidase was actively engaged in the removal of ROS when H_2O_2 was added to its environment to a concentration of 1mM. In case of the stress produced as a result of the addition of *tert*-butyl hydroperoxide, glutathione peroxidase activity was actively seen in all the concentrations used. It can thus be concluded that glutathione peroxidase is

present in the cyanobacterium *Nostoc punctiforme* ATCC 29133 and that it plays an active part in the detoxification of ROS produced in its system.

2. A gene corresponding to the glutathione peroxidase-like protein was identified in *Nostoc punctiforme* ATCC29133 using *Synechocystis* PCC 6803 as the query sequence. The physico-chemical properties of the glutathione peroxidase-like protein showed a certain level of similarities in terms of molecular weight, pI, stability index etc. from the previously characterized glutathione peroxidase-like proteins from *Synechocystis* PCC 6803. Multiple sequence alignment of the protein with several other known glutathione peroxidase proteins, both from Eukaryota and Prokaryota showed a highly conserved cysteine at position 65. A phylogenetic tree drawn from the sequences also showed that the glutathione peroxidase-like protein of *Nostoc punctiforme* ATCC 29133 is closely related with the glutathione peroxidase-like proteins of *Synechocystis* PCC 6803 by clustering together. Phobius software did not detect any signal peptide in the protein and classified it as a non- cytoplasmic protein. The protein was successfully modelled and the Ramachandran plot of the modelled protein confirmed the its stability.

3. The glutathione peroxidase-like protein from *Nostoc punctiforme* ATCC 29133 was cloned and overexpressed successfully in *E.coli*. After successfully isolating genomic DNA, the DNA fragment containing open reading frame Npun_R4660 was amplifed using specific primers. Amplified DNA fragments were cloned and plasmids were constructed to express *Npun*_R4660. The plasmids were digested with appropriate restriction enzymes and cloned into *E.coli* systems. A protein of approximately 18KDa

was produced in the soluble phase of the cytosol of *E.coli* in bulk quantity. Therefore, this strategy of recombinant protein production in *E.coli* may be used in producing protein in bulk quantity for characterization or for any other uses.

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1) Lalnunmawii E, Bhattacharya J (2018) Cloning and overexpression studies on the glutathione peroxidase-like protein from the cyanobacterium Nostoc punctiforme ATCC 29133. Science and Technology Journal Vol.6 Issue:I January 2018 pp 57-61 .

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6) Ibrahim KS, Laishram A, Lalnunmawii E, Kumar NS (2015) In silico characterization of human cytochrome P450 monooxygenases. Science Vision Vol 15 No 4 pp178 – 188.

List of papers presented:

1) Oral presentation on ' Response of glutathione peroxidase in Nostoc punctiforme ATCC 29133 to oxidative stress created by hydrogen peroxide, methyl viologen and tert-butyl hydroperoxide' in 4th Asian PGPR Conference held on 11th & 12th May, 2018 at Mizoram University. (Appreciation award was received)

2) Poster presentation on ' Isolation, amplification, cloning and overexpression of the glutathione peroxidase-like protein from the cyanobacterium Nostoc punctiforme ATCC 29133' in 4th Asian PGPR conference on 11th & 12th May, 2018 at Mizoram University.

Characterization of a glutathione peroxidase-like protein from a diazotrophic cyanobacterium *Nostoc punctiforme* ATCC 29133 and its role in stress survival

Abstract of the thesis submitted in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Biotechnology

by

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ABSTRACT

Cyanobacteria photosynthetic prokaryotes which has the capacity to produce oxygen . Many of them possess the ability to fix atmospheric nitrogen which makes them a potential source of biofertilizers in paddy fields. Cyanobacteria are found in a wide range of ecological habitats - water, soil as well as air. They have been found to inhabit places like hot springs, deserts and brackish water. Cyanobacterial habitats also include those that are considered extreme, such as frozen lakes, hot springs and salt works. They are often subjected to abiotic stress in the form of environmental factors such nutrient limitation, different intensities of light, herbicides, extreme temperatures and high salinity. The growth of cyanobacteria and also its ability to fix atmospheric nitrogen is often inhibited by these factors The stress produced by different factors can lead to the formation reactive oxygen species which at high levels are detrimental to the organism and can cause cell death. Cyanobacteria, at the same time possess a wide range of enzymatic antioxidants as well as non-enzymatic antioxidants to tackle oxidative stress if and when it arises in cells. The non-enzymatic antioxidants include tocopherols, carotenoids etc. while the enzymatic antioxidants comprises of superoxide dismutase (SOD), catalases and peroxidases.

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet-}$), H_2O_2, hydroxyl free radicals ($HO^{\bullet}$) and singlet oxygen ($^1O_2$) are amongst the most notorious ROS which can cause harm if not removed. The respiratory electron transport pathway contributes to the generation of toxic oxygen species in cyanobacteria. But the major source of ROS in higher plants, oxygen-evolving photosynthetic cyanobacteria and green algae is the photosynthetic machinery. ROS have multiple effects on the cellular functions in aerobic organisms. They cause damage to nucleic acids, oxidize proteins and also cause lipid peroxidation. Numerous strategies have been developed by cyanobacteria in order to avoid ROS production and also to scavenge them once they are produced. The enzymatic antioxidants work in concert with each other to detoxify these ROS. The superoxide dismutases (SOD) acts initially as the first line of defence against oxidative stress by catalyzing dismutation of $O_2^{\bullet-}$ into H_2O_2 and O_2. The catalases, peroxiredoxins, and Dps proteins protect cells from H_2O_2 toxicity. Catalases dismutate H_2O_2 to water and oxygen, and free intracellular iron is scavenged by DPS proteins, which can otherwise interact with H_2O_2 to form $HO^{\bullet}$. The peroxiredoxins (Prx) catalyze detoxification of a broad range of substrates, including H_2O_2, peroxynitrite and alkyl hydroperoxides to water or nitrate or corresponding alcohols, respectively. Unlike catalases, which mainly detoxify H_2O_2 (high levels) and exhibit photosynthetic electron transport independent catalysis, peroxiredoxins mainly scavenge endogenously generated H_2O_2 (low levels) by coupling catalysis to photosynthetic electron transport.

Although, catalase and peroxidase perform the same function they differ in their mechanism of action. Peroxidases are believed to play a crucial role in the detoxification of endogenous hydrogen peroxide, whereas catalases detoxify exogenous hydrogen peroxide. The enzymes of the former group rely on electrons donated by ascorbate, glutathione or thioredoxin. Peroxidases can also detoxify other peroxides, such as alkyl hydroperoxides and peroxynitrite. In general, catalases exhibit lower affinity for hydrogen peroxide and higher K_{cat} compared to peroxidases.

Peroxidase detoxifies cell-toxic molecules into simpler and less toxic molecules, thus it represents an important part of the antioxidant machinery of cyanobacteria. In chloroplasts of higher plants, ascorbate-dependent peroxidase is the principle scavenger of hydrogen peroxide, and ascorbate concentrations are high (millimolar range) (Pérez-Pérez *et al.,* 2009). In contrast, ascorbate concentration in cyanobacteria is low (micromolar range), and genes which code for ascorbate peroxidase is missing from the already sequenced cyanobacterial genomes. The concentration of glutathione in cyanobacteria range from 2-4 mM, and two glutathione peroxidase-like proteins have been identified in *Synechocystis* sp. PCC 6803 and characterized. However, glutathione peroxidase like genes/ proteins has not been characterized in other cyanobacteria.

Nostoc punctiforme ATCC 29133 (hereafter *Nostoc punctiforme*) is a filamentous, heterocystous, nitrogen-fixing cyanobacterium the genome of which has been completely sequenced. Therefore, the present study becomes of critical importance. The findings of the study are summarized below:

1) The presence of the glutathione peroxidase-like protein was established in *Nostoc punctiforme* ATCC 29133 using in-gel assays that would detect its activity. The assay confirmed the presence of an active glutathione peroxidase in the organism. Prooxidants like H_2O_2 and *tert*-butyl hydroperoxide were used to subject the organism to abiotic stress in the form of ROS. As glutathione peroxidase enzyme is believed to play an important role in the defence against ROS in aerobic organisms, the level up to which the enzyme actively takes part in the detoxification of ROS produced by hydrogen peroxide and *tert*-butyl hydroperoxide were also monitored. Glutathione peroxidase was actively engaged in the removal of ROS when H_2O_2 was added to its environment to a concentration of 1mM. In case of the stress produced as a result of the addition of *tert*-butyl hydroperoxide, glutathione peroxidase activity was actively seen in all the concentrations used. It can thus be concluded that glutathione peroxidase is present in the cyanobacterium *Nostoc punctiforme* ATCC 29133 and that it plays an active part in the detoxification of ROS produced in its system.

2) A gene corresponding to the glutathione peroxidase-like protein was identified in *Nostoc punctiforme* ATCC29133 using *Synechocystis* PCC 6803 as the query sequence. The physico-chemical properties of the glutathione peroxidase-like

protein showed a certain level of similarities in terms of molecular weight, pI, stability index etc. from the previously characterized glutathione peroxidase-like proteins from *Synechocystis* PCC 6803. Multiple sequence alignment of the protein with several other known glutathione peroxidase proteins, both from Eukaryota and Prokaryota showed a highly conserved cysteine at position 65. A phylogenetic tree drawn from the sequences also showed that the glutathione peroxidase-like protein of *Nostoc punctiforme* ATCC 29133 is closely related with the glutathione peroxidase-like proteins of *Synechocystis* PCC 6803 by clustering together. Phobius software did not detect any signal peptide in the protein and classified it as a non- cytoplasmic protein. The protein was successfully modelled and the Ramachandran plot of the modelled protein confirmed the its stability.

3) The glutathione peroxidase-like protein from *Nostoc punctiforme* ATCC 29133 was cloned and overexpressed successfully in *E.coli*. After successfully isolating genomic DNA, the DNA fragment containing open reading frame Npun_R4660 was amplifed using specific primers. Amplified DNA fragments were cloned and plasmids were constructed to express *Npun*_R4660. The plasmids were digested with appropriate restriction enzymes and cloned into *E.coli* systems. A protein of approximately 18KDa was produced in the soluble phase of the cytosol of *E.coli* in bulk quantity. Therefore, this strategy of recombinant protein production in *E.coli* may be used in producing protein in bulk quantity for characterization or for any other uses.