Response of antioxidative enzymes to stress induced by aminoglycoside class of antibiotics in a fresh water cyanobacterium *Synechococcus* sp. PCC 7942

Dissertation submitted in partial fulfillment of

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Master of Philosophy in Biotechnology

By

Thingnam Surendrakumar Singh

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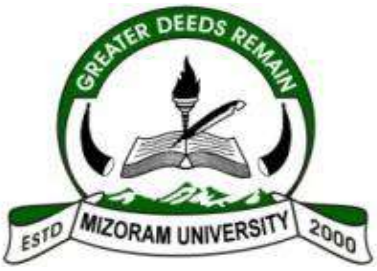

Under the supervision of

Dr. Jyotirmoy Bhattacharya
Associate Professor, Department of Biotechnology
School of Life Sciences, Mizoram University,
Aizawl, Mizoram

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ABBREVAITIONS

APS	Ammonium persulphate
^{o}C	Degree Celsius
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$Co(NO_3)_2.6H_2O$	Cobalt (II) nitrate hexahydrate
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
EDTA	Ethylenediaminetetraacetic acid
gm	gram
Gm	Gentamicin sulphat
hrs	hour
H_3BO_3	Boric Acid
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid
K_2HPO_4	Dipotassium phosphate
M	Molar
Min	Minute (s)
ml	Millilitre
mM	Millimolar
$MgCl_2$	Magnesium chloride
$MnCl_2.4H_2O$	Magnesium chloride tetrahydrate
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
Na_2CO_3	Sodium carbonate (anhydrous)
NaCl	Sodium chloride
$Na_2MoO_4.2H_2O$	Sodium molybdate
NaOH	Sodium hydroxide
N_2	Dinitrogen
ng	Nanogram

nm	Nanometer
OD	Optical density
PAGE	Polyacralamide gel electrophoresis
PMSF	Phenylmethylsulfonyl fluoride
ROS	Reative oxyzen species
rpm	Revolution per minute
Str/Sm	Streptomycine sulphate
sec	Second (s)
TEMED	N,N,N′,N′-tetramethyl ethylenediamine
Tris (free base)	2-amino-2-hydroxymethyl propane-1,3-diol
Tris-HCL	Tris (hydroxymethyl) aminomethane hydrochloride
TAE	Tris-Acetate EDTA buffer
U	Unit
UV	Ultraviolet
w/v	Weight-to-volume ratio
X	Concentration index
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate
%	Percent
µg	Microgram
µl	Microlitre
µmol	Micromole
µM	Micromolar

LIST OF TABLE AND FIGURES

Declaration of the Candidate

I, Thingnam Surendrakumar Singh, hereby declare that the subject matter of this dissertation entitled **"*Response of antioxidative enzymes to stress induced by aminoglycoside class of antibiotics in a fresh water cyanobacterium Synechococcus sp. PCC 7942*"** is a record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and the dissertation had not been submitted by me for any research degree in any other University/Institute.

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

Dr. Joyotrimony Bhattacharya
Head
(Department of Biotechnology)

Thingnam Surendrakumar Singh
(Candidate)

(Dr. Jyotirmoy Bhattacharya)

(Supervisor)

CERTIFICATE

This is to certify that the dissertation entitled **"*Response of antioxidative enzymes to stress induced by aminoglycoside class of antibiotics in a fresh water cyanobacterium Synechococcus sp. PCC 7942"*** submitted to Mizoram University for the award of Master of Philosophy in Biotechnology by **Thingnam Surendrakumar Singh** Registration No. ***MZU/M.Phil/357*** *of 26.05.2017*, Research scholar in the Department of Biotechnology, is a record of research, based on the results of the experiments and investigations carried out independently by him during the period from 2016 to 2017 of study, under my guidance and supervision and has not been previously submitted for the award of any Indian or foreign University.

It is further certified that the scholar fulfilled all the requirements as laid down by the University for the purpose of submission of M.Phil dissertation.

Place: **Dr. Jyotirmoy Bhattacharya**

Associate Professor,

Date: **(Signature of the supervisor)**

.....Earnestly dedicated to my parents Th. Ranjit Singh and Th. Ibechaobi Devi and my family whose hand always rise for me for their spiritual inspiration and motivation for the highest ideals of life carrying out my work to achieved my goal.....

CHAPTER 1

Introduction

1.1 Cyanobacteria: Cyanobacteria are one of the oldest photosynthetic organisms on earth. They are Gram-negative prokaryotes, and their species include unicellular, filamentous, branched-filamentous and non-filamentous colonial forms. Many of them, the filamentous forms exhibit multiple cellular differentiation alternatives, including formation of vegetative cells (sites for photosynthesis), heterocysts (sites for nitrogen-fixation), akinetes/spores (perennating bodies) and motile trichomes called hormogonia. Because of their ability to perform multiple ecologically important functions (photosynthesis and nitrogen-fixation), they have occupied almost all conceivable niches on earth (Whitton and Potts, 2000). However, like other organisms, they also encounter various kinds of stress conditions in their natural habitats. These conditions may include nutrient limitation, exposure to antibiotics, high light, herbicides, extreme temperatures and high salinity. Although each one of these types of stressors produces distinct effects on cyanobacteria, they also trigger a common response in the form of oxidative stress (Latifi et al., 2009). Oxidative stress is a condition in which cells over accumulate reactive oxygen species (ROS), mainly superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$). Unless destroyed promptly and efficiently, ROS can be deleterious to cells. The following sections describe the intracellular sources of ROS, and the mechanisms that have evolved in cyanobacteria to cope with ROS toxicity.

1.2 Sources and targets of ROS in cyanobacteria: In cyanobacteria, the transfer of electrons from reduced iron-sulphur centers of photosystem I (PSI) to O_2 mainly contributes to generation of $O_2^{\cdot -}$ (Mehler reaction). This radical can also be formed at the acceptor side of PSII because of imbalanced electron transport between PSII and PSI. However, mobility of $O_2^{\cdot -}$ is limited across membranes, and therefore its action is restricted to destruction of iron-sulphur clusters of photosynthetic apparatus. The production of intracellular H_2O_2 occurs from dismutation of $O_2^{\cdot -}$ and/or incomplete oxidation of water at the donor side of PSII. Unlike $O_2^{\cdot -}$, H_2O_2 can freely cross membranes and together with ferrous ion gives rise to $HO^{\bullet}$ (Fenton reaction), the most toxic of all ROS (Banerjee et al., 2013).

Cyanobacteria are more susceptible to ROS compared with other organisms because the photosynthetic and respiratory apparatus both contribute to ROS generation in cyanobacteria, and there is little compartmentation, if any between photosynthetic and respiratory apparatus, which eases the movement of ROS with in cells (Bernroitner et al., 2009). High concentrations of ROS have been implicated in inhibition of photosynthesis in cyanobacteria (Nishiyama et al., 2006). Since cyanobacterial membranes are rich in polyunsaturated fatty acids (PUFA), they are also sensitive to lipid peroxidation initiated by $HO^{\bullet}$ leading to destruction of membrane structure and function. Nucleic acids are also vulnerable to $HO^{\bullet}$, as purine, pyrimidine and deoxyribose backbone are all targets of this radical (Latifi et al., 2009).

1.3 Mechanisms of protection against ROS: Cyanobacteria employ various strategies to protect itself from ROS toxicity. Such strategies include prevention of ROS formation and/or detoxification of ROS. The orange carotenoid proteins, high-light inducible proteins, and iron-starvation-induced protein A contribute to prevention of

ROS formation by nonphotochemical quenching (NPQ), a mechanism to dissipate excess light energy incident on photosynthetic apparatus safely as heat. ROS detoxification in cyanobacteria is carried out both by non-enzymatic and enzymatic antioxidants. Whereas the non-enzymatic molecules include α-tocopherol, carotenoids and glutathione, the enzymatic antioxidants are Superoxide dismutase (SOD), Catalase and Peroxiredoxins (Latifi et al., 2009).

SOD catalyze dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2 (Imlay, 2003). Two types of SODs are mainly found in cyanobacteria: those that contain iron in its active sites (FeSOD) and those that contain manganese (MnSOD). Ni- and Cu/Zn-containing SODs are rare in cyanobacteria, and their presence is limited only to marine forms. Both unicellular (e.g., *Synechocystis* PCC 6803, *Synechococcus* PCC 7942) and filamentous cyanobacteria (e.g., *Anabaena* PCC 7120, *Nostoc punctiforme*) possess FeSOD (Bhattacharya et al., 2004; Moirangthem et al., 2014, 2015). A critical role of FeSOD in stress tolerance was proved by oxidative stress- and cold stress-sensitive nature of a FeSOD mutant of *Synechococcus* sp. PCC 7942 (Thomas et al., 1999). MnSOD are mostly found in filamentous cyanobacteria, and its role in mitigating oxidative stress have been highlighted in *Anabaena* PCC 7120 (Priya et al., 2007; Raghavan et al., 2011).

Catalase dismutates H_2O_2 to water and oxygen. They are classified into monofunctional haem-containing catalase, bifunctional haem containing catalase-peroxidase and monofunctional manganese-containing catalase (Bernroitner et al., 2009; Latifi et al., 2009; Perelman et al., 2003; Tichy and Vermaas, 1999). The bifunctional catalase-peroxidase (KatG) is commonly found in cyanobacteria. The physiological function of catalase-peroxidase has been unearthed in *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942, and the protein was necessary for

conferring protection against H_2O_2 (Perelman et al., 2003; Tichy and Vermaas, 1999). The monofunctional haem-containing catalases and manganese-catalases are not as widespread as catalase-peroxidase in cyanobacteria, and the latter is restricted only to nitrogen-fixing cyanobacteria (Banerjee et al., 2009).

The peroxiredoxins (Prx) are present in multiple numbers in cyanobacteria. For instance, five Prx proteins are present in *Synechocystis* PCC 6803, six in *Synechococcus* PCC 7942 and seven in *Anabaena* PCC 7120. These Prx proteins can be further classified into 1-Cys Prx, 2-Cys Prx, Type II Prx, and PrxQ depending on the number and location of the conserved cysteine residues and in subunit composition (Bernroitner et al., 2009). H_2O_2, peroxynitrite and alkyl hydroperoxides serves as substrates for Prx proteins (Latifi et al., 2009). Although both catalase and Prx detoxify H_2O_2, they vary in terms of their substrate affinity and dependence on reducing equivalents. Catalase possesses low affinity to H_2O_2, whereas Prx show high affinity to H_2O_2. Unlike catalase, a continuous supply of electrons from photosynthetic electron transport is required by Prx for catalysis. A Prx defective mutant of *Synechococcus* sp. PCC 7942 was found to be highly sensitive to oxidative stress, supporting the crucial role of Prx proteins in combating oxidative stress in cyanobacteria (Perelman et al., 2003).

1.4 Present study: As discussed above, antioxidative enzymes play a critical role in survival of cyanobacteria under oxidative stress induced by various abiotic stress conditions. In recent years, there has been a rising concern regarding pollution caused by clinically relevant antibiotics in natural environments. Due to extensive and indiscriminate use of antibiotics in human and animal health practices, mostly in developing countries, a large amount of these chemicals have been discharged in water

and soil ecosystems (Baquero et al., 2008; Martinez, 2009). Antibiotic pollution in such habitats has led to selection of antibiotic-resistant pathogenic microorganisms, and further dissemination of antibiotic-resistant genes through horizontal gene transfer in bacterial populations (Martinez, 2009). The antibiotic-resistant bacteria efficiently perform exclusion and export of antibiotics, enzyme-mediated modification of antibiotics to less-toxic forms, and remodeling of core metabolic pathways. The effects of antibiotic pollution on non-pathogenic and ecologically useful microorganisms, however, have received less attention. Cyanobacteria offers an excellent model system to study ecological risks of antibiotic pollution due to their dominance in aquatic ecosystems (70% of total phytoplankton mass) and ability to produce more than a third of total free oxygen by carbon dioxide-fixation. Dias and colleagues (2015) have recently shown that many species of aquatic cyanobacteria are sensitive to antibiotics. However, the reasons of sensitivity are not known. Studies with bacteria have pointed to the fact that various classes of antibiotics (β-lactams; cell-wall synthesis inhibitor, fluoroquinolones; DNA-replication inhibitors and aminoglycosides; protein synthesis inhibitors) with different mode of action share a common mechanism of killing cells by generating oxidative stress (Kohanski et al., 2007, 2008, 2010). Based on this recent observation, it is reasonable to assume involvement of oxidative stress in sensitivity of cyanobacteria to antibiotics. Thus, the present study was designed to probe whether oxidative stress is one of the causes of antibiotic-induced lethality in a fresh water unicellular cyanobacterium *Synechococcus* sp. PCC 7942. The specific objectives of this study are to determine: i) the growth response of *Synechococcus* to streptomycin and gentamicin (aminoglycosides), and ii) the responses of major antioxidative enzyme activities (superoxide dismutase and catalase-peroxidase) to streptomycin and gentamicin.

CHAPTER 2

Materials and Methods

2.1 Organism and culture methods: The unicellular, non-nitrogen-fixing cyanobacterium *Synechococcus* sp. PCC 7942 (hereafter referred to as *Synechococcus*) was obtained from Prof. Rakefet Schwarz's Laboratory (Bar-Ilan University, Israel). *Synechococcus* was routinely grown at 25 °C in liquid $BG11_0$-medium supplemented with 17.6 mM $NaNO_3$ and buffered (pH 7.5) with equimolar concentration of HEPES (BG11-medium; Rippka *et al*., 1979). Axenic culture of *Synechococcus* was regularly maintained on solid BG11-medium (slants) containing 1.5% (w/v) agar. Continuous illumination was provided to the cultures at a photon fluence rate of 20 µmol m^{-2} s^{-1}. The concentrations (mM) of macronutrients in BG11-medium were: $K_2HPO_4.3H_2O$, 0.18; Na_2CO_3, 0.19; $MgSO_4.7H_2O$, 0.30; $CaCl_2.2H_2O$, 0.25; EDTA (disodium salt), 0.003; Citric acid, 0.029; Ferric ammonium citrate, 0.030. The concentrations (µM) of micronutrients in $BG11_0$-medium were: H_3BO_3, 46; $MnCl_2.4H_2O$, 9.2; $ZnSO_4.7H_2O$, 0.77; $Na_2MoO_4.2H_2O$, 1.6; $CuSO_4.5H_2O$, 0.32; $Co(NO_3)_2.6H_2O$, 0.17.

High quality Millipore water was used for preparation of BG11-medium. All glasswares were soaked with 6 M HCl overnight and then extensively rinsed with water (Millipore) prior to use. Sterilization of BG11-medium was achieved by autoclaving at 121 °C (15 pounds per square inch) for 15 min before use.

2.2 Preparation of drug solutions: Streptomycin sulphate (Str) and Gentamicin sulphate (Gm) belonging to aminoglycoside class of antibiotics, Methyl viologen (MV; a

oxidative stress generating agent) and Hydrogen peroxide (H_2O_2) were used during the present study. The stock solutions of antibiotics and MV were prepared by dissolving appropriate amounts in sterile Millipore water and stored at -20 °C. These solutions were further sterilized by ultrafiltration using membrane filters of pore size 0.45 μm (Millipore), immediately before use. The H_2O_2 work solutions were prepared by dilution with sterile Millipore water from a 30 % (w/v) stock solution immediately before use.

2.3 Growth determination of *Synechococcus*: The exponentially growing cultures of *Synechococcus* were harvested by centrifugation (2300x*g* for 5 min), washed twice with sterile BG11-medium and transferred to 40 ml of the same medium at equal cell density (Optical Density of approximately 0.04 at 750 nm) in 100 ml Erlenmeyer flasks. After addition of required concentrations of antibiotics, the cultures were incubated as described in section 2.1 with occasional manual shaking. The growth of cultures was monitored periodically by measuring optical density (OD) of cell suspensions at 750 nm using a Cary 60 spectrophotometer (Agilent Technologies, USA). A blank was prepared using BG11-medium. Growth of *Synechococcus* was also measured in terms of Chlorophyll *a* (Chl *a*) using the method described by Mackinney (1941). In brief, 1 ml of culture was harvested by centrifugation (2300x*g* for 5 min). An equal volume of methanol (100 %) was added to the cell pellet and the mixture incubated for 10 min. The chlorophyll *a* concentration was determined in the supernatant of centrifuged extract from absorbance value at 663 nm (Cary 60 spectrophotometer) and calculated using the formula: Chlorophyll *a* (μg ml^{-1}) = Absorbance at 663 nm x13.43. A 100 % methanol served as a blank for Chl *a* determination.

2.4 Determination of intracellular hydroperoxide level: The concentration of hydroperoxide was determined by ferrous oxidation-xylenol orange method as described by Nomura et al., (2006). The *Synechococcus* cells equivalent to 0.5 µg Chl *a* ml^{-1} were inoculated in 80 ml of BG11-media in the presence and absence of different concentrations of antibiotics. Cultures were also exposed to MV (3 µM) for hydroperoxide level measurement (positive control). After a treatment period of 1 day, cultures were centrifuged (2300x*g* for 5 min) and cell pellets washed twice with fresh BG11-medium. The pellets (approximately 1.5 µg Chl *a*) were then resuspended in 0.8 ml of methanol containing 0.01% butylated hydroxytoluene (BHT), 0.1 ml of Reagent A (2.5 mM ammonium iron (II) sulphate, 0.25 M sulphuric acid) and 0.1 ml of Reagent B (40 mM BHT, 1.25 mM xylenol orange in methanol). The mixture was incubated for 30 min at room temperature and then centrifuged at 10000x*g* to remove any cell debris. The absorbance of supernatant fraction was measured at 560 nm and the concentration of hydroperoxides was determined using the extinction coefficient (E_{560} = 4.3x10^4 M^{-1} cm^{-1}).

2.5 Extraction of total protein and determination of total protein concentration: The cultures of *Synechococcus* were grown in the presence and absence of different concentrations of antibiotics as described in section 2.4. After a day of treatment, cells of *Synechococcus* were centrifuged at 2300x*g* for 5 min at room temperature and the cell pellet washed twice with buffer (36 mM potassium phosphate, pH 7.4). After resuspension in the same buffer supplemented with protease inhibitors (phenylmethylsulphonyl fluoride, 1 mM and 5 µM each of leupeptin and pepstatin) the cells were broken by ultrasonication (Soniprep 150, MSE) at 4 °C. The cell extracts were then centrifuged at 10000x*g* for 30 min at 4 °C and the supernatant was

used for protein determination. The total protein concentration of cell extracts was measured using the method described by Bradford (1976). 20 µl of the protein sample was mixed with 1 ml of Bradford reagent (Quick Start protein assay kit, BioRad) and the mixture incubated in dark for 10 min followed by measurement of absorbance at 595 nm. A calibration curve was prepared by using Bovine serum albumin (BSA) solution as standard.

2.6 Determination of Superoxide dismutase (SOD; EC 1.15.1.1) activity by non-denaturing (native) Polyacrylamide gel electrophoresis (PAGE): SOD activity assay by non-denaturing PAGE utilizes the ability of SOD to inhibit riboflavin-mediated photochemical reduction of nitrobluetetrazolium by superoxide radicals (Beauchamp and Fridovich 1971). SOD activity assays were performed on 12 % non-denaturing polyacrylamide gels containing 10 % glycerol and no SDS in BioRad mini gel apparatus (BioRad Laboratories, California, USA). The gels, sample loading buffer and electrophoresis buffer were prepared according to the protocol described by Sambrook and Russel (2001). A 12% resolving gel (5 ml gel volume) contained 1.65 ml of Millipore water, 2 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 5 % stacking gel (2 ml gel volume) was as follows: 1.42 ml of Millipore water, 0.33 ml of 30% acrylamide and N,N'-methylenebisacrylamide mix (1:29), 0.25 ml of 1 M Tris buffer (pH 6.8), 0.02 ml of 10% ammonium persulphate, and 0.002 ml of TEMED. SDS was also omitted from sample loading dye (50 mM Tris, pH 6.8; 0.1% Bromophenol blue; 10% Glycerol) and electrophoresis buffer (25 mM Tris; 250 mM Glycine, pH 8.3). Total protein equivalent to 25 µg was loaded on gels and

electrophoresis was carried out for 3 h under constant amperage at 4 °C. were separated by electrophoresis at , and then the gel was soaked in 28 µM TEMED (N, N, N', N'–tetramethyl ethylenediamine) and 28 µM riboflavin in 36 mM potassium phosphate buffer (pH 7.8) for 30 min. The gel was further soaked for 10 min in 2.5 mM nitroblue tetrazolium and illuminated with cool fluorescent white light at an intensity of 25 µmol m^{-2} s^{-1} for colour development. The SOD activities were observed as an achromatic zone on a purple-blue background of the gel. Individual SOD isoforms were identified by 5 min of treatment with 5 mM H_2O_2 before staining the gel. Finally, the gel images were captured using a gel documentation system (Syngene, UK).

2.7 Determination of Catalase activity (EC 1.11.1.6): Catalase activity (H_2O_2 dismutation activity) was determined in extracts of drug-treated and untreated cultures of *Synechococcus* as described in section 2.6. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and 50 µg of total protein. H_2O_2 dismutation was monitored by decrease in absorbance at 240 nm with time at 25 °C. The activity was calculated using an extinction coefficient of 43.6 M^{-1} cm^{-1} (Beers and Sizer 1952). One unit of enzyme was defined as the quantity that catalyzes the dissociation of 1 µmol of H_2O_2 in 1 min.

2.8 Statistical analysis: All experiments were repeated with two biological and technical replicates, and the results are presented as mean±standard deviation for all quantitative assays. Significant differences between control and test samples were tested by one-way analysis of variance (ANOVA) using Graph Pad Instat software. The probability (*P*) values less than 0.05 were considered significantly different.

2.9 Chemicals: The chemicals used during the present study were obtained from Himedia, SRL, Merck India, BioRad and Sigma.

CHAPTER 3

Results

3.1 Effects of aminoglycosides on growth of *Synechococcus*: The growth response of *Synechococcus* was assessed in the presence of increasing concentrations of Str and Gm (aminoglycosides) in BG11-medium. Compared with untreated control (100 %), the optical density (OD at 750 nm) values of cultures treated with 50, 100 and 750 µg L^{-1} of Str reduced by 51, 82 and 86 %, respectively, after 6 days of incubation (Fig. 1A). Similar results were obtained when growth was measured in terms of Chl *a*; *Synechococcus* treated with 50, 100 and 750 µg L^{-1} of Str for similar time period showed a growth decline of 64, 92 and 94 %, respectively (Fig. 1B), as can also be seen from Fig. 1C. Incubation in the presence of 50, 100 and 750 µg L^{-1} of Gm for a period of 6 days reduced the OD values of *Synechococcus* cultures by 5, 25 and 88 %, respectively, compared with the control (Fig. 2A). Also, Chl *a* measurements indicated a growth reduction of 7, 44 and 94 % with increasing concentrations of Gm as above (Fig. 2B). These results indicate that *Synechococcus* is sensitive to both Str and Gm. Further, the sensitivity is dose-dependent and Str appears to be a more potent growth inhibitor than Gm.

3.2 Impact of aminoglycosides on hydroperoxide levels in *Synechococcus* cells: The intracellular hydroperoxide levels (an indicator of oxidative stress) was determined in cultures of *Synechococcus* exposed to increasing concentrations of Str and Gm for 1 day in BG11-medium. As shown in Table 1, treatment with 50, 100 and 750 µg L^{-1} of Str enhanced levels by 113 %, 139 %, and 174 %, respectively, similar concentrations

of Gm increased levels by 102 %, 131 %, and 148 %, respectively, compared with control (100 %). These results suggest that exposure to Str and Gm led to a significant increase (P<0.05) in hydroperoxide levels, except in case of 50 µg L^{-1} treated Gm. For comparative purpose, hydroperoxide level was also measured in parallel in cells treated with a redox-cycling pro-oxidant Methyl viologen (MV; 3 µM) for one day. An increase of 179 % (P<0.05) in hydroperoxide level (20.42±0.37 nmol µg Chl a^{-1}) was noted in MV-treated cells compared to control cultures (11.42±0.85 nmol µg Chl a^{-1}). These results suggest that Str and Gm exposures generate oxidative stress in *Synechococcus* similar to the levels generated by MV, a known oxidative stress generating herbicide.

3.3 Impact of aminoglycosides on Superoxide dismutase (SOD) activity in *Synechococcus*: SOD activities were evaluated in cellular extracts of *Synechococcus* exposed to increasing concentrations of Str and Gm for 1 day in BG11-medium by non-denaturing gel electrophoresis followed by SOD activity staining. A total of five achromatic SOD activity bands (I-V) were detected in cellular extracts of *Synechococcus* (Fig. 3A) of which three SOD activity bands (I, IV and V) were H_2O_2-sensitive and the other two bands (II and III) were H_2O_2-insensitive (Fig. 3B). The SOD activity band I was barely detectable in the control, 50 and 100 µg L^{-1} Str-treated cultures, and the intensity of the remaining four SOD activity bands (II-V) were similar. Although intensity of bands (IV and V) in cultures treated with 750 µg L^{-1} Str were similar to the control and the cultures treated with low concentrations of Str, a clear SOD activity arising from band I was observed, and the intensity of bands II and III were comaparatively much stronger. As shown in Fig. 4, similar SOD activity profiles as seen in Str-treated cultures were also obtained in cultures treated

with Gm. These results suggest that exposure to high concentration of antibiotics (750 µg L^{-1}) leads to increase in SOD activity, particularly H_2O_2-insensitive SOD activities (bands II and III) and a H_2O_2-sensitive band I in *Synechococcus*.

To assess whether Str- and Gm-induced SOD response is similar to that produced by other oxidative stress generating agents, SOD activity analysis was conducted in cultures treated with 3 µM MV and 2 mM H_2O_2 for 1 day. Unlike in control cultures, band I was produced in response to MV, and band II appeared to be stronger in intensity. The other bands (III-V) were similar in intensity to that of the control (Fig. 5A; compare lanes 1 and 2). In H_2O_2-treated cultures, however, the intensity of band III was stronger compared with the control, and band V decreased considerably in its intensity. The SOD activity band I was barely detectable and the bands III and IV were similar in intensity to that of the control (Fig. 5A; compare lanes 1 and 3). The H_2O_2-sensitivity of all the five SOD activity bands in MV- and H_2O_2-treated cultures was similar to that shown earlier (Fig. 5B; compare with Fig. 3A). These results suggest that there are both similarities and differences in the response of SOD activities to oxidative stress generated by antibiotics and those generated by MV and H_2O_2.

3.4 Impact of aminoglycosides on Catalase activity in *Synechococcus*: Catalase protect cells from toxic effects of H_2O_2. The Catalase activity was determined in cultures of *Synechococcus* exposed to increasing concentrations of Str and Gm for 1 day in BG11-medium. As shown in Table 2, Catalase activity decreased significantly ($P<0.05$) in cells exposed to both Str and Gm compared with control cultures. 50, 100 and 750 µg L^{-1} of Str-treated cultures exhibited an inhibition of 19 %, 28 %, and 15 %, respectively. Cultures treated with similar concentrations of Gm inhibited the

Catalase activity by 16 %, 34 %, and 32 %, respectively. These results indicate that dismutation of H_2O_2 (substrate of Catalase) may be negatively impacted in *Synechococcus* exposed to antibiotics of aminoglycoside class.

CHAPTER 4

Discussion

Widespread antibiotic pollution in natural environments is a matter of serious clinical and environmental concern. Antibiotics in concentrations ranging from ng L^{-1} to µg L^{-1} have been detected in sewage, surface, ground, and drinking waters worldwide (Baquero, 2008; Martinez, 2009). Even concentrations up to 31 mg L^{-1} of fluoroquinolone antibiotics have been reported (much above human therapeutic blood plasma concentrations) in water bodies around a drug-manufacturing industry in India (Larsson et al., 2007). Since cyanobacteria are one of the most dominant groups of photosynthetic microorganisms and major primary producers in many aquatic ecosystems, the detrimental effects of antibiotic pollution on cyanobacteria merits attention (Baquero, 2008). Hence, the present study was designed to characterize the impacts of aminoglycoside class of antibiotics (Str and Gm) on a unicellular, non-nitrogen-fixing model aquatic cyanobacterium *Synechococcus* sp. PCC 7942. The growth studies revealed that *Synechococcus* is sensitive to aminoglycosides in µg L^{-1} range, an environmentally relevant range of concentration. Further, Str was a more effective growth inhibitor at lower concentrations than Gm. A previous study has reported a much higher range of concentrations of kanamycin (Kan) and Gm (0.1-1.6 mg L^{-1}) as growth inhibitory to other freshwater cyanobacteria, including *Microcystis aeruginosa*, *Aphanizomenon gracile*, *Chrisosporum bergii* and *Planktothrix agardhii* (Dias et al., 2015). Also, a concentration range of Gm as high as 1-10 mg L^{-1} has been reported to inhibit growth in the unicellular cyanobacterium *Synechocystis* PCC 6803

(Cameron and Pakrasi, 2011). Possibly, *Synechococcus* is more sensitive to aminoglycosides compared to other cyanobacteria.

The primary mode of action of antibiotics and their secondary effects have been extensively studied in bacteria due to their clinical importance (Kohanski et al., 2010). However, there is a paucity of such studies in ecologically important organisms like cyanobacteria. Aminoglycosides have been shown to exert bactericidal effects by inhibiting multiple functions. These antibiotics primarily target the 30S subunit of the ribosome resulting in misincorporation of aminoacids into elongating polypeptide chains. Some of these mistranslated proteins are targeted to bacterial membranes and affect membrane integrity. Recent studies in *Escherichia coli* and species of *Pseudomonas* have shown that aminoglycosides as well as antibiotics of other classes (ß-lactams and fluoroquinolones) induce a common mode of killing by increased production of ROS within cells, particularly $HO^{\bullet}$ (Dwyer et al., 2009; Kohanski et al., 2007, 2008, 2010; Wang and Zhao, 2009; Yeom et al., 2010). The increased generation of $HO^{\bullet}$ has been ascribed to increased oxidation of NADH (reduced nicotinamide adenine dinucleotide) produced through TCA (Tricarboxylic acid) cycle leading to increase in respiratory activity of bacterial cells. As a consequence, rate of $O_2^{\bullet-}$ generation is enhanced. These $O_2^{\bullet-}$ interact and damage iron-sulphur proteins. The ferrous iron released from iron-sulphur proteins reacts further with H_2O_2 forming $HO^{\bullet}$ which causes cell death (Kohanski et al., 2007). Prompted by such findings in bacteria, an attempt was made to determine the contribution of oxidative stress in aminoglycoside lethality in *Synechococcus*. The measurements of intracellular concentrations of hydroperoxide (an indicator of oxidative stress and precursor to $HO^{\bullet}$) showed that its level in cells exposed to Str and Gm was considerably higher than that in untreated cultures, and similar to that found in

cultures treated with MV, a redox-cycling drug that accepts electrons from iron-sulphur clusters of PSI and generates $O_2^{\cdot-}$ and $HO^{\bullet}$ in cyanobacteria (Latifi et al, 2009; Moirangthem et al., 2014). These observations suggest that aminoglycosides indeed generate oxidative stress in *Synechococcus*. Similar to this finding, oxidative stress has also been implicated in Gm lethality in *Synechocystis* PCC 6803. The PSI electron transport chain has been suggested as a source of ROS in Gm-treated *Synechocystis* PCC 6803 (Cameron and Pakrasi, 2011).

In many organisms, including cyanobacteria adaptation to oxidative stress is facilitated by enzymatic and non-enzymatic antioxidants (Latifi et al., 2009). Indeed, Glutathione, a major non-enzymatic antioxidant in cyanobacteria, has been shown to promote resistance to Gm-induced oxidative stress in *Synechocystis* PCC 6803 (Cameron and Pakrasi, 2011). In *Synechococcus*, a plethora of antioxidative enzymes participate in mitigation of the harmful effects of ROS. These include FeSOD, catalase-peroxidase and peroxiredoxins (Latifi et al., 2009). Since SOD acts as a primary defence against ROS, its activity was determined in *Synechococcus* cultures. A total of five SOD activity bands were detected by native PAGE analysis in this cyanobacterium. Of the five SOD activity bands three were found to be sensitive to H_2O_2 and two of them were insensitive to H_2O_2. H_2O_2-sensitivity is a hallmark of FeSODs, and MnSODs are usually H_2O_2-insensitive (Moirangthem et al., 2014, 2015). Hence, it is likely that the activity bands I, IV and V are FeSODs; the band V being the major FeSOD activity and the bands I and IV are likely to be higher oligomers of FeSOD. The remaining activity bands II and III possibly represent MnSOD activity. In agreement with this finding, a previous study has also reported the presence of MnSOD activity in *Synechococcus* (Thomas et al., 1999). The total SOD activity in *Synechococcus* increased following exposure to lethal concentrations

of Str and Gm (750 µg L^{-1}) compared with control. As judged by band intensities, the activities of MnSODs (particularly band III) and an FeSOD activity represented by band I (oligomer of highest molecular weight) increased substantially in cultures treated with 750 µg L^{-1} Str and Gm, whereas the FeSOD activities arising from bands IV and V remained more or less unchanged. Further, SOD activity determinations in *Synechococcus* cultures exposed to known oxidative stress generating agents (MV and H_2O_2) suggested both drug-specific responses (H_2O_2-mediated inhibition of the major FeSOD activity represented by band V) and shared SOD responses with aminoglycosides. For instance, both MV and H_2O_2 stimulated MnSOD activities; band II and band III activity was promoted by MV, and H_2O_2, respectively, similar to that observed in cells treated with aminoglycosides. Overall, the antibiotics-induced increase in SOD activity in *Synechococcus* is consistent with an earlier report of SOD activity enhancement in *Microcystis aeruginosa* in response to spiramycin (Liu et al., 2012). However, further in-depth studies may be needed to decipher the link between oxidative stress and formation of functional protein aggregates, as observed during this study for FeSOD (band I).

Intracellular generation of H_2O_2 gets a boost with enhanced SOD activity. Unless dissociated at a faster rate, H_2O_2 can initiate $HO^{\bullet}$ production dramatically (Imlay, 2003). Although cells do not possess a mechanism to detoxify $HO^{\bullet}$, detoxification of H_2O_2 may be achieved by catalase, catalase-peroxidase and/or peroxiredoxins (Latifi et al., 2009). In *Microcystis aeruginosa*, the catalase activity has been shown to decrease after exposure to lethal concentrations of antibiotics (Liu et al., 2012; Wan et al., 2014). Similarly, aminoglycoside treated cultures of *Synechococcus* also exhibited a decrease in catalase activity. A decrease in catalase activity concomitant with an increase in SOD activity is conducive to massive $HO^{\bullet}$

accumulation in cells. Hence, it is likely that ROS and resultant oxidative stress may be involved in aminoglycoside-mediated cell death pathway in *Synechococcus*.

Taken together, this study reveals that the antibiotics Str and Gm (aminoglycosides) are growth inhibitory to *Synechococcus*. This growth sensitivity was coupled to enhanced intracellular levels of hydroperoxides, indicating that cyanobacterial cells exposed to such antibiotics undergo oxidative stress. The SOD and catalase-peroxidase are two major enzymatic antioxidants present in *Synechococcus*. Oxidative stress induced by antibiotics led to increase in the activities of two MnSOD and a FeSOD, particularly when high concentrations of the antibiotics (750 µg L^{-1}) were used. However, the catalase activity was found to decrease in response to all the antibiotic concentrations tested. As a consequence of differential response of SODs and catalase, H_2O_2 may over-accumulate and stimulate $HO^{\bullet}$ formation leading to the observed lethality in *Synechococcus*. Antibiotic-sensitive nature of cyanobacteria may have huge ecological implications in terms of loss of primary productivity in aquatic ecosystems polluted with antibiotics.

CHAPTER 5

Summary

Over the years, human health improvement practices, animal farming and aquaculture have all derived benefits from the use of clinically relevant antibiotics. However, large scale usage of antibiotics and their improper release into water bodies by drug-manufacturing industries, hospitals, and wastewater treatment plants are not only raising serious clinical, but also ecological concerns worldwide, including in India. Antibiotics in concentrations ranging from ng L^{-1} to µg L^{-1} have been detected in many aquatic ecosystems. Therefore, the purpose of this study was to probe growth impacts and involvement of oxidative stress in response to antibiotic exposure in an aquatic photosynthetic cyanobacterium *Synechococcus* sp. PCC 7942. The aminoglycoside class of antibiotics, streptomycin and gentamicin, which exert their bactericidal effects by targeting 30S ribosome subunit and stimulate mistranslation of proteins were chosen for the present study. The growth of *Synechococcus* was determined at increasing concentrations (50-750 µg L^{-1}) of streptomycin and gentamicin. Both the antibiotics inhibited the growth of *Synechococcus* in a dose-dependent manner. Further, an enhanced accumulation of hydroperoxides (causative agent of oxidative stress) was observed in cells exposed to antibiotics. The major enzymatic antioxidants that counter the effects of oxidative stress in *Synechococcus* are Superoxide dismutase (SOD) and Catalase-peroxidase. Multiple SOD activities were found to be present in this cyanobacterium. Oxidative stress induced by antibiotics led to increase in the activities of two MnSOD and a FeSOD at high concentrations of antibiotics (750 µg L^{-1}). However, a decrease in cellular catalase

activity was detected at all concentrations of antibiotics tested. A reduction in the catalase activity concomitant with an increase in SOD activity is conducive to intracellular accumulation of hydroxyl radicals, a form of highly reactive oxygen species which cannot be detoxified by cells and can damage vital cellular components, including nucleic acids, lipids and proteins. Based on these observations, it is proposed that the sensitivity of *Synechococcus* to aminoglycosides may be linked to oxidative stress. Given the role of cyanobacteria as one of the major primary producers in aquatic ecosystems, its sensitivity to antibiotics may have huge detrimental consequences for not only primary production but entire food web in antibiotic-polluted ecosystems.

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Table 5.1. Effects of antibiotics on the hydroperoxide level in *Synechococcus* sp. PCC 7942.

Antibiotics	Concentrations (µg L^{-1})	Hydroperoxide content (nmol µg Chl a^{-1})
Streptomycin	0	11.48±0.85
	50	13.01±0.24
	100	16.00±0.78
	750	20.35±1.08
Gentamicin	50	11.76±0.28
	100	15.08±0.61
	750	16.95±0.48

The cultures of *Synechococcus* were grown in the absence and in the presence of increasing concentrations of antibiotics for 1 day in BG11-medium. Such cultures were then harvested and cell pellets washed twice with fresh BG11-medium. The cells equivalent to approximately 1.5 µg Chl *a* were used for determination of hydroperoxide levels. The hydroperoxide concentrations are from means of two independent experiments ±standard deviation.

Table 5.2. Effects of antibiotics on Catalase activity in *Synechococcus* sp. PCC 7942.

Antibiotics	Concentrations (µg L^{-1})	Catalase activity (Unit mg $protein^{-1}$)
Streptomycin	0	17.60±2.81
	50	14.31±0.62
	100	12.69±1.21
	750	15.05±0.88
Gentamicin	50	13.60±2.95
	100	11.62±2.00
	750	12.05±1.66

The cultures of *Synechococcus* were grown in the absence and in the presence of increasing concentrations of antibiotics for 1 day in BG11-medium. Such cultures were then harvested, washed with Potassium phosphate buffer (pH 7.4), and broken by ultrasonication in the same buffer at 4 °C. The cell-free extracts equivalent to 50 µg of total protein was used for determination of Catalase activity. Values of Catalase activities are means of two independent experiments ±standard deviation.

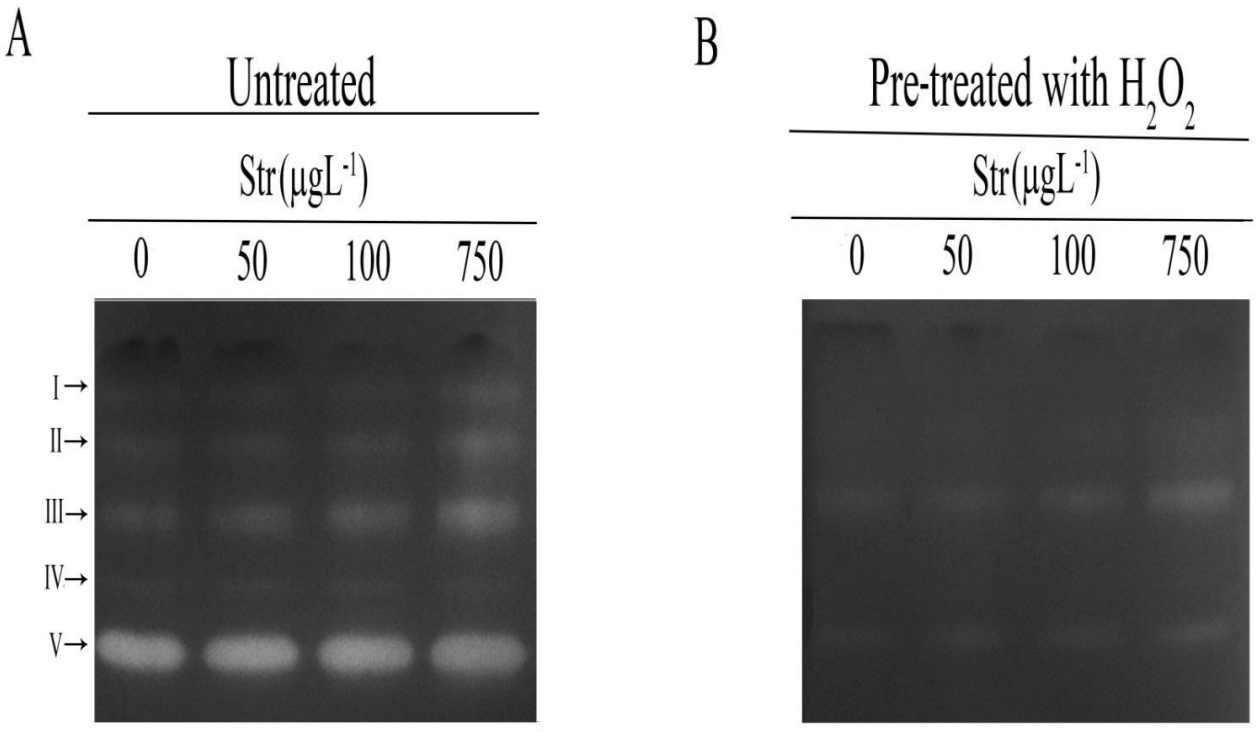

Fig. 5.3 Effect of Streptomycin (Str) on Superoxide dismutase (SOD) activity in *Synechococcus* sp. PCC 7924. The log-phase cultures of *Synechococcus* exposed to increasing concentrations of Str for a day were washed with 36 mM Potassium phosphate buffer (pH 7.4), and broken by ultrasonication. Protein extracts from the cultures (25 µg total protein) were loaded on a 12 % native gel, separated by electrophoresis, and stained for SOD activity (A). The SOD metalloforms (bands I-V) were identified in cellular extracts of Str-treated cultures by incubating separate lanes of the same gel in 5 mM H_2O_2 for 5 min prior to staining (B). The SOD assay was repeated three times with similar results and a representative gel picture is shown.

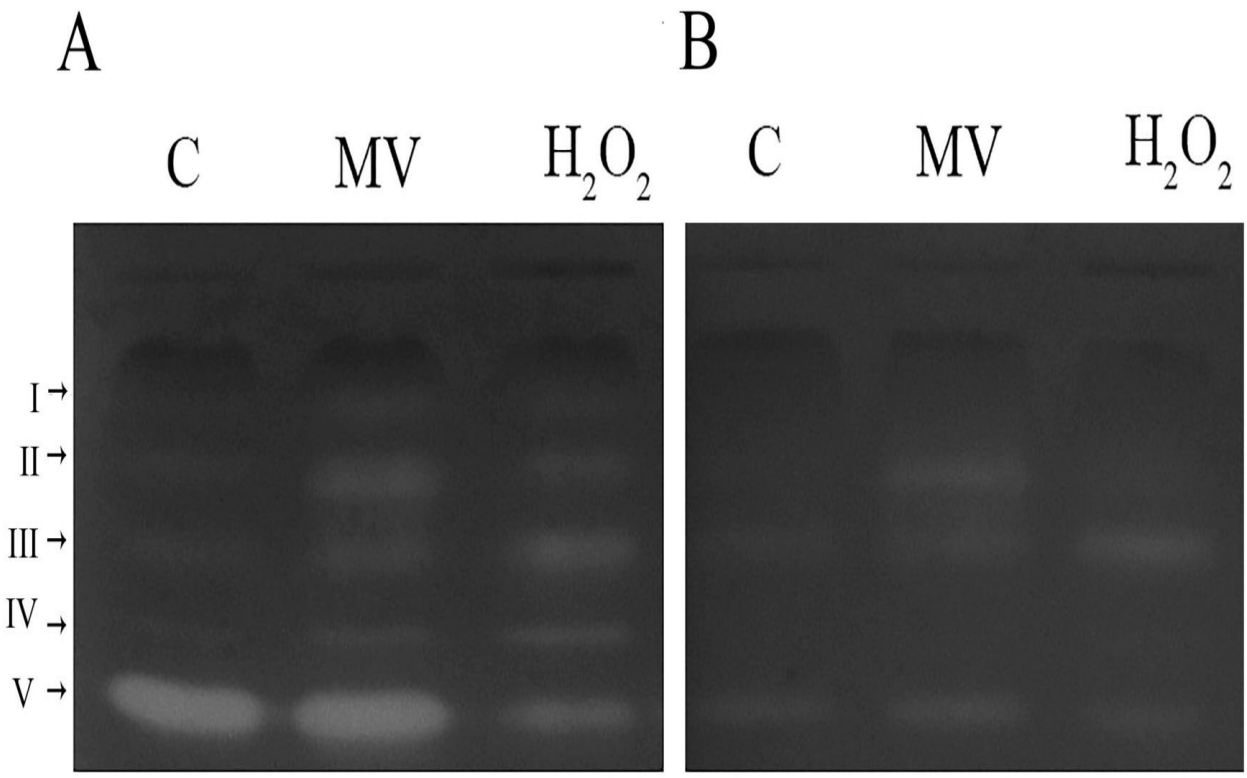

Fig. 5.5 Effects of Methyl viologen (MV) and Hydrogen peroxide (H_2O_2) on Superoxide dismutase (SOD) activity in *Synechococcus* sp. PCC 7924. The log-phase cultures of *Synechococcus* were exposed to 3 µM MV and 2 mM H_2O_2 for a day. Such cultures were washed with 36 mM Potassium phosphate buffer (pH 7.4), and broken by ultrasonication. Protein extracts from the cultures (25 µg total protein) were loaded on a 12 % native gel, separated by electrophoresis, and stained for SOD activity (A). The SOD metalloforms (bands I-V) were identified in cellular extracts of MV- and H_2O_2-treated cultures by incubating separate lanes of the same gel in 5 mM H_2O_2 for 5 min prior to staining (B). The SOD assay was repeated two times with similar results and a representative gel picture is shown.

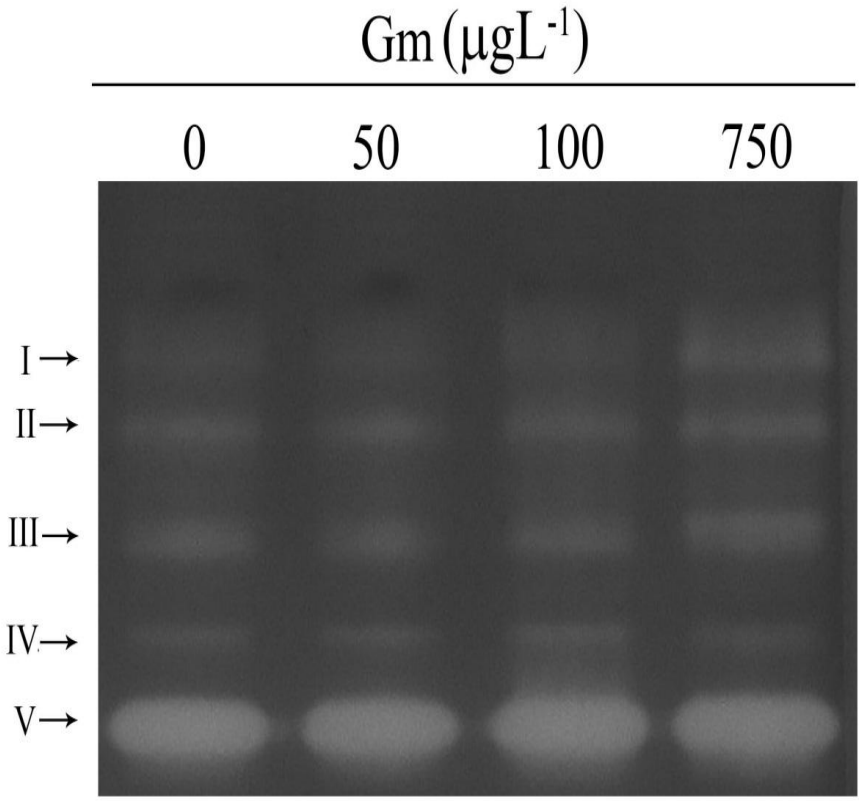

Fig. 5.4 Effect of Gentamicin (Gm) on Superoxide dismutase (SOD) activity in *Synechococcus* sp. PCC 7924. The log-phase cultures of *Synechococcus* exposed to increasing concentrations of Gm for a day were washed with 36 mM Potassium phosphate buffer (pH 7.4), and broken by ultrasonication. Protein extracts from the cultures (25 µg total protein) were loaded on a 12 % native gel, separated by electrophoresis, and stained for SOD activity. Bands I-V represents different SOD metalloforms. The SOD assay was repeated three times with similar results and a representative gel picture is shown.

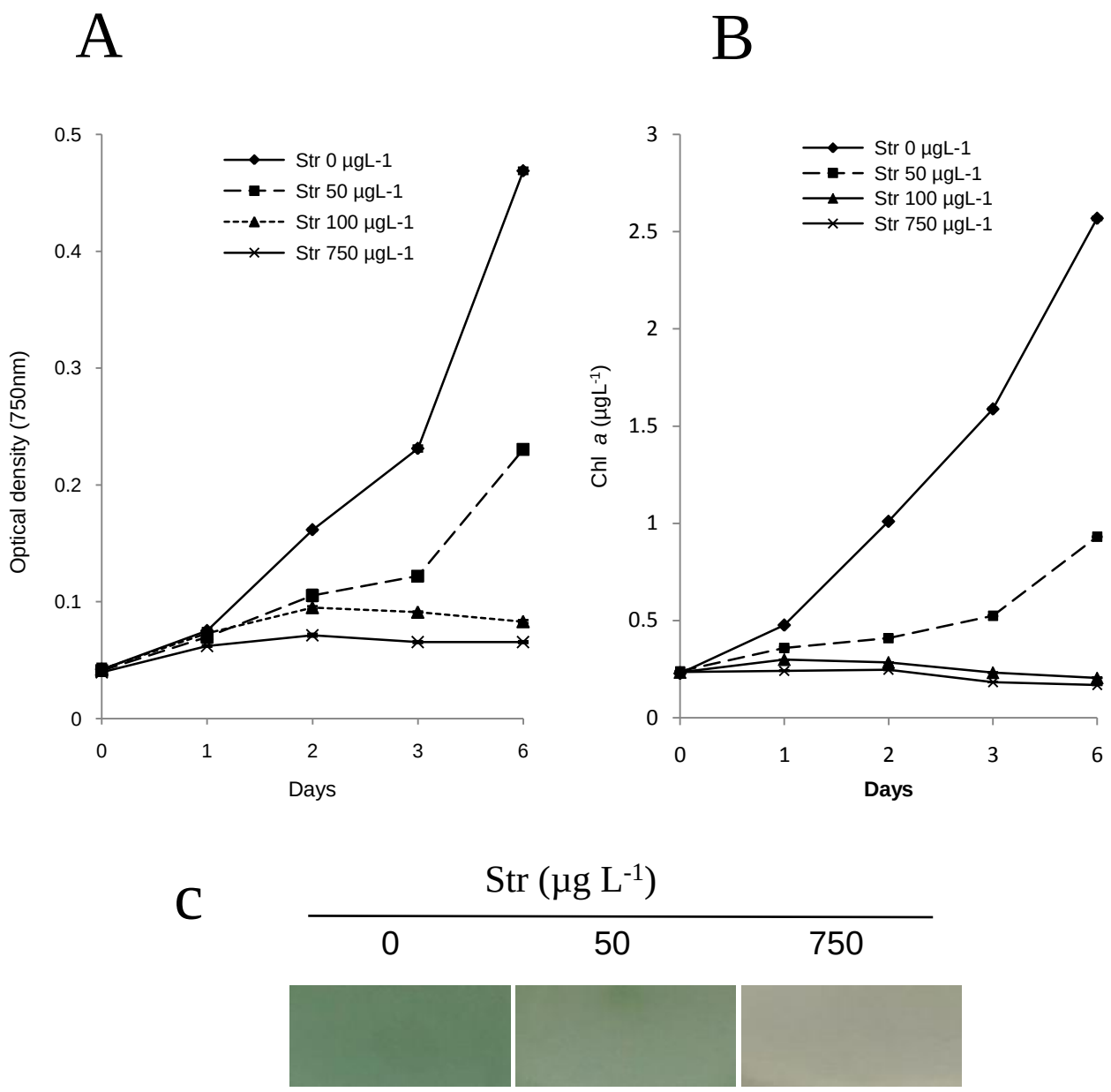

Fig. 5.1 Effects of Streptomycin (Str) on growth of *Synechococcus* sp. PCC 7924. The log-phase cultures of *Synechococcus* were inoculated at equal cell density in BG11-medium containing increasing concentrations of Str. Growth was determined in such cultures by measuring optical density (OD) at 750 nm (A) and Chlorophyll *a* (Chl *a*; B) for 6 days, and photographed (C). Error bars, sometimes smaller than the symbols, represent standard deviation of two independent experiments with two technical repeats.

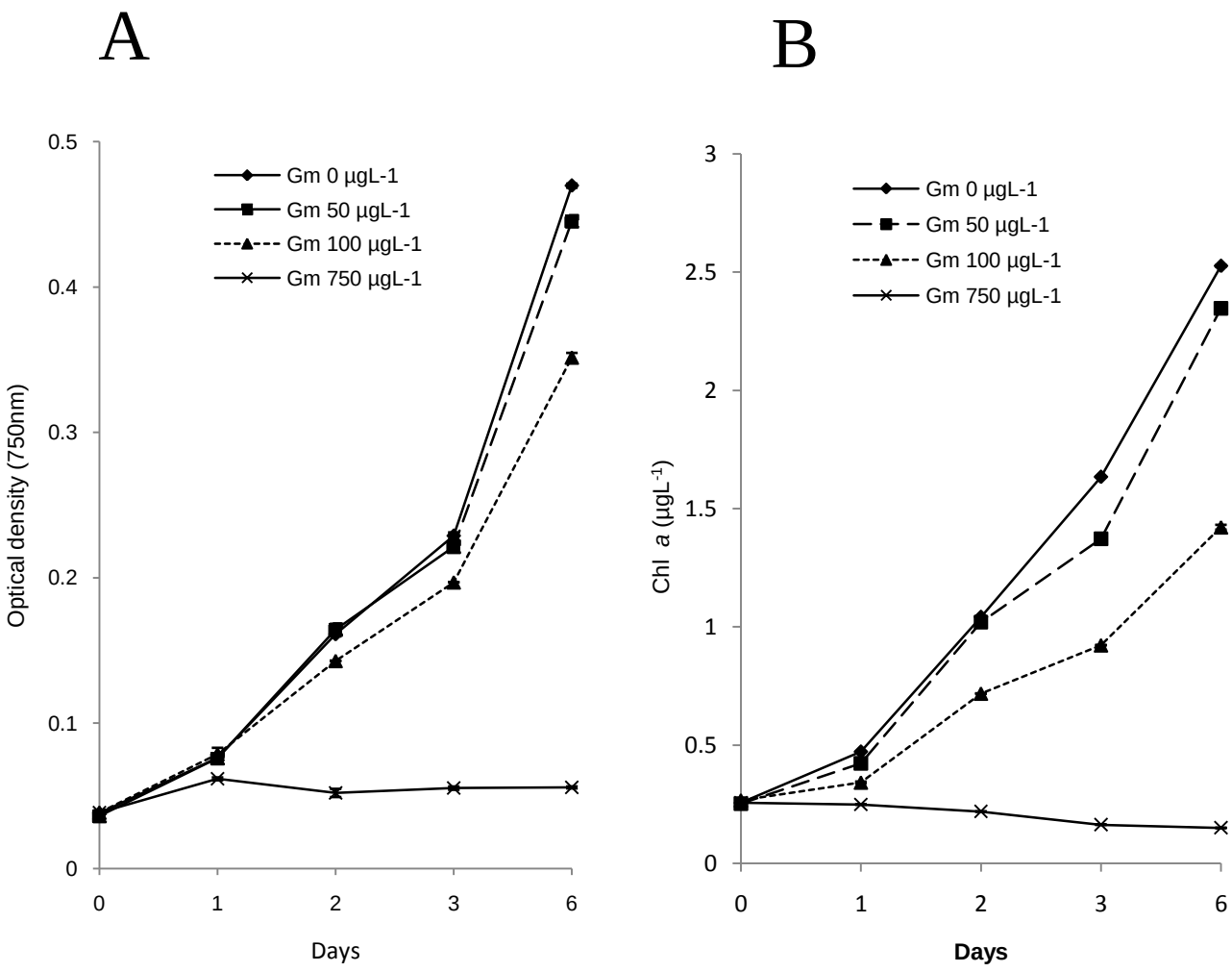

Fig. 5.2 Effects of Gentamicin (Gm) on growth of *Synechococcus* sp. PCC 7924. The log-phase cultures of *Synechococcus* were inoculated at equal cell density in BG11-medium containing increasing concentrations of Gm. Growth was determined in such cultures by measuring optical density (OD) at 633 nm (A) and Chlorophyll *a* (Chl *a*; B) for 6 days. Error bars, sometimes smaller than the symbols, represent standard deviation of two independent experiments with two technical repeats.

Response of antioxidative enzymes to stress induced by aminoglycoside class of antibiotics in a fresh water cyanobacterium*Synechococcus* sp. PCC 7942

Dissertation submitted in partial fulfillment of

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By

Thingnam Surendrakumar Singh

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Under the supervision of

Dr. Jyotirmoy Bhattacharya

Associate Professor, Department of Biotechnology

School of Life Sciences, Mizoram University,

Aizawl, Mizoram

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Abstract

Over the years, human health improvement practices, animal farming and aquaculture have all derived benefits from the use of clinically relevant antibiotics. However, large scale usage of antibiotics and their improper release into water bodies by drug-manufacturing industries, hospitals, and wastewater treatment plants are not only raising serious clinical, but also ecological concerns worldwide, including in India. Antibiotics in concentrations ranging from ng L^{-1} to µg L^{-1} have been detected in many aquatic ecosystems. Therefore, the purpose of this study was to probe growth impacts and involvement of oxidative stress in response to antibiotic exposure in an aquatic photosynthetic cyanobacterium *Synechococcus* sp. PCC 7942. The aminoglycoside class of antibiotics, streptomycin and gentamicin, which exert their bactericidal effects by targeting 30S ribosome subunit and stimulate mistranslation of proteins were chosen for the present study. The growth of *Synechococcus* was determined at increasing concentrations (50-750 µg L^{-1})of streptomycin and gentamicin. Both the antibiotics inhibited the growth of *Synechococcus* in a dose-dependent manner. Further, an enhanced accumulation of hydroperoxides (causative agent of oxidative stress) was observed in cells exposed to antibiotics. The major enzymatic antioxidants that counter the effects of oxidative stress in *Synechococcus* are Superoxide dismutase (SOD) and Catalase-peroxidase. Multiple SOD activities were found to be present in this cyanobacterium. Oxidative stress induced by antibiotics led to increase in the activities of two MnSOD and a FeSOD at high concentrations of antibiotics (750 µg L^{-1}). However, a decrease in cellular catalase activity was detected at all concentrations of antibiotics tested. A reduction in the catalase activity concomitant with an increase in SOD activity is conducive to

intracellular accumulation of hydroxyl radicals, a form of highly reactive oxygen species which cannot be detoxified by cells and can damage vital cellular components, including nucleic acids, lipids and proteins. Based on these observations, it is proposed that the sensitivity of *Synechococcus* to aminoglycosides may be linked to oxidative stress. Given the role of cyanobacteria as one of the major primary producers in aquatic ecosystems, its sensitivity to antibiotics may have huge detrimental consequences for not only primary production but entire food web in antibiotic-polluted ecosystems.