

मृदा के जैविक परत से प्राप्त हुआ नाइट्रोजन स्थिरीकारक
नील हरित शैवाल में शुष्कता सहनशीलता की कार्यात्मक
विशेषताएँ

**PHYSIOLOGICAL ATTRIBUTES OF
DESICCATION TOLERANCE IN A NITROGEN
FIXING CYANOBACTERIUM ISOLATED FROM
SOIL BIOLOGICAL CRUSTS**

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NEW DELHI – 110012**

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TOLERANCE IN A NITROGEN FIXING
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CRUSTS**

By

Mr. ANNAYYA

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Approved by:

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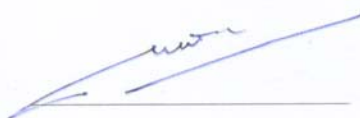
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CERTIFICATE

This is to certify that the thesis entitled “**Physiological attributes of desiccation tolerance in a nitrogen fixing cyanobacterium isolated from soil biological crusts**” submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfilment of the requirements for the award of **MASTER OF SCIENCE IN MICROBIOLOGY**, embodies the results of bona fide research work carried out by **Mr. ANNAYYA, Roll No: 21144** under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help availed during the course of investigation as well as source of information have been duly acknowledged by him.

Date:

Place: New Delhi

(**Dr. G. Abraham**)

Chairperson
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“I accept that it is not always moonshine and roses, and you can't expect things must always go your way.”

- A. B. de Villiers

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The cyanobacteria or the blue green algae are gram-negative photoautotrophic prokaryotic organisms which appeared on earth billion years ago and due to their oxygen evolving potential, made the earth oxygenic by allowing the other organisms on earth to develop. They are cosmopolitan in distribution and have been found to occur in almost every ecological niche and contribute significantly to carbon and nitrogen economy of tropical paddy fields. They may be used as model organisms due to similarity of photosynthesis and respiration with higher plants. Stanier (1970) introduced the term cyanobacteria for the Gram-negative prokaryotic autotrophic organisms. They are one of the oldest life forms evolved on Earth in the Pre-Cambrian times around 2.4 to 3.2 billion years ago (Sheridan et al., 2003). The cellular organization of the cyanobacteria ranges from unicellular, colonial, filamentous to branched filamentous forms (Thajuddin and Subramanian 2005). Stanier and Van Niel (1962) advocated the inclusion and classification of cyanobacteria under photosynthetic bacteria due to prokaryotic nature of cells and oxygenic photosynthesis. The cyanobacteria have wide distribution across the ecosystems, aquatic to terrestrial environments as well as extreme habitats such as deserts, hot thermal vents and springs, hypersaline water and salt pans, and ice caps etc. (Whitton and Potts, 2000). Recently, Gaysina et al., (2019) reviewed the diversity of cyanobacteria from different climatic zones such as temperate, tropical as well as polar regions. In fact, during the course of evolution the cyanobacteria have been subjected to extreme selection pressures which accounts for the competitive success of cyanobacteria. Because of this reason the cyanobacteria have an inherent ability to tolerate environmental extremes such as high temperature, UV radiation, desiccation, water and saline stresses (Groniger et al., 2000; Herrero and Flores, 2008). Rai et al., (2000) observed that the ability to carry out oxygenic photosynthetic nitrogen fixation either alone or in symbiotic association with other organisms is important from an agricultural perspective. Cyanobacteria develop special type of cells known as ‘heterocysts’ helps in the fixation of atmospheric nitrogen (Elhai and Wolk, 1990). The heterocysts are known as “nitrogen fixing factories” and house the enzyme nitrogenase responsible for the nitrogen fixation. Certain non-heterocystous forms capable of N₂-fixation have also been reported (Rippka et al., 1979). Because of the nitrogen fixing potential they are able to grow in environments where little or no combined nitrogen is available. Besides enriching the nutrient content

of the soil, the cyanobacteria have been reported to release various kinds of biologically active compounds such as proteins, vitamins, carbohydrates, amino acids, polysaccharides and phytohormones (Hussain et al., 2013; Delattre et al., 2016; Singh et al., 2017). These compounds help in plant growth promotion and are therefore important in exploiting them as bio-inoculants. Further, these properties make them potential microorganisms as bio-inoculants as well as “ecosystem engineers”. In the recent times, the cyanobacteria have been exploited because of their potential to produce novel and biologically active natural compounds which are important for biotechnological applications.

Plant growth and productivity are greatly affected by environmental stresses such as drought, high salinity, and high and/low temperature (Zhang et al., 2010). Drought stress is a major agricultural problem leading to reduction in crop yield in arid and semi-arid regions of the globe. The climatic variations and anthropogenic activities have also resulted in the degradation of land in arid, semiarid and dry sub-humid areas leading to desertification (MEA, 2005). The recurrent drought conditions can have serious issues regarding the food supply of the ever increasing population (Somerville and Briscoe, 2001). Reduced availability of water is a serious threat to the proliferation of the cyanobacteria in the field conditions. However, fields they grow are constantly exposed to the continuous dehydration, desiccation, and rewetting. A number of reports are available regarding the diversity and occurrence of cyanobacteria in hostile environments such as deserts, thermal springs, hot brines, frigid lakes, thermal springs, nutrient poor ponds and salt brines (Fogg, 1982; Orcutt et al., 1986; Ward et al., 1989; de Chazal et al., 1992). Cyanobacteria exhibit considerable ability to tolerate desiccation and several terrestrial cyanobacteria are often considered to be desiccation tolerant (Potts, 1994). Apte (2001) reported induction of physiological drought in cyanobacteria due to high growth under osmolarity. Drought stress exerts its effect on the morphological (Lan et al., 2010; Cuddy et al., 2011), physiological and biochemical attributes (Lin and Wu, 2014; Sen et al., 2017). Water stress decreases the nitrogen fixation by adversely affecting the nitrogenase activity in free-living forms (Stewart, 1974; Jones, 1977; Whitton et al., 1979). The sum total of the several simple and complex interactions taking place at the structural, physiological and molecular levels are important in determining the desiccation tolerance.

Cyanobacteria are usually the pioneer colonizers in bare soils and they form biological soil crusts (BSC) and exert crucial influences on the development of soil-

ecosystems (Belnap, 2003). Biological soil crusts may increase soil aggregation and stability thereby reducing wind and water erosion (Mazor et al., 1996; Eldridge and Kinnell, 1997). BSCs would increase soil fertility by nitrogen and carbon fixation (Starks et al., 1981; Eldridge and Greene, 1994; Lange et al., 1994). Morphologically tangled filamentous cyanobacteria improve soil aggregate stability by adding organic matter and secreting extracellular polymeric substances (Rossi et al., 2012). Numerous strains in biological soil crusts are also capable of tolerance or resistance to desiccation by maintaining a steady balance between the internal water content and external water availability. Establishment and use of biological soil crusts to ameliorate desertification and to restore arid lands and improve soil texture has been studied (Yang et al., 2001; Jusu et al., 2004; Guo et al., 2008). Desiccation affects microbial dynamics in three fundamental ways: as a resource, as solvent and as a transport medium (Tecon and Or, 2017). The cyanobacteria have been reported in bare soils where they form biological soil crusts and help in the development of soil ecosystems as pioneer colonizers (Belnap, 2003). Desiccation tolerance is required to survive the stressful conditions in the biological soil crusts (Lange, 2003). The biological soil crusts are important in combating desertification and to restore the soil texture by increasing the soil fertility through nitrogen and carbon fixation (Lange et al., 1994; Guo et al., 2008; Lin and Wu, 2014).

Desertification is a serious environmental issue and it leads to loss of biodiversity and low soil productivity (Park et al., 2017). It has reportedly affected 10 to 20% of these lands, on which 250 million people live, while another 1 billion people live in areas at risk of undergoing further desertification (Reynolds et al., 2004). Desiccation tolerant cyanobacteria have been considered as one of the eco-friendly and viable solution for the restoration of degraded soil. Artificial cyanobacterial inoculation technology is considered as an effective and desirable method for controlling desertification (Lan et al., 2014). For the restoration of soil fertility, identification and characterization of cyanobacteria having tolerance to desiccation stress is required. Very few studies have addressed the problem of drought/moisture deficit stress in cyanobacteria. There are a number of strategies to enhance the ability of plants to tolerate drought stress. This involves water saving in irrigation, traditional breeding approaches and genetic engineering to develop transgenic plants which are drought tolerant. However, these approaches are not cost effective but highly technical and labor intensive. The plant growth promoting rhizobacteria are found in the rhizosphere and

also as endophytes and has been reported to facilitate plant growth in optimal and abiotic stress conditions (Cassan et al., 2009; Niu et al., 2018). With the increase in drought stress conditions, understanding the drought stress tolerance in cyanobacteria may provide useful information on their exploitation as biofertilizer.

As compared with plants, very little is known about drought-tolerance mechanisms in cyanobacteria. Systematic information is however, not available regarding the desiccation tolerance of cyanobacteria. Therefore, in the present study, attempts have been made to characterize the desiccation tolerance potential of cyanobacteria. Hence, the present study has been proposed with the following objectives.

- To characterize the desiccation tolerance in selected cyanobacterial isolate from soil biological crusts
- To study the impact of desiccation on the defense, nitrogen metabolism and protein profile of the selected cyanobacterium.

The cyanobacteria, one of the most primitive group of microorganisms evolved primarily from anoxygenic phototrophs and made the Earth's atmosphere oxygenic. Cyanobacteria have a gram-negative structure and have a thicker peptidoglycan layer compared to other gram-negative bacteria. Hamilton et al. (2014) reported that they are the only unique photosynthetic prokaryotes to evolve O₂. According to the theory proposed by Margulis (1970), the ability to conduct oxygenic photosynthesis in cyanobacteria was later transmitted this ability to eukaryotes via the endosymbiosis 2.5–3.5 billion years ago. Similarities exist between chloroplasts of eukaryotic cells and cyanobacteria in relation to genomic organization, photosynthetic pigments and their structures, carbon fixation and photorespiration (Raven and Allen, 2003; Douglas et al., 2003). The diverse nature of cyanobacteria and their presence in various ecological habitats have been reported by Fogg et al. (1973). These organisms are important in the biogeochemical cycle of nitrogen, carbon and oxygen (De Ruyter and Fromme 2008). Further, the ability to carry out oxygenic photosynthesis makes them primary producers and CO₂ sink. The unicellular, filamentous and non-filamentous cyanobacteria are placed in different orders on the basis of the nature of filaments and the structures involved in reproduction. The presence of chlorophyll is one of the characteristics identified in cyanobacteria. They also have other accessory pigments that include allophycocyanin, phycoerythrin and phycocyanin which play an important role in effectively utilizing the light spectrum in a given environment. Because of their ability to fix atmospheric nitrogen, the heterocystous cyanobacteria develop symbiotic relationships with a variety of other plant species such as fungi, bryophyte, pteridophyte, gymnosperm and angiosperm (Bergman, 2002).

Cyanobacteria show great structural diversity which forms the basis of its classification. These are spread across about 150 genera and classification of cyanobacteria takes into account the Bacteriological factor. The cyanobacteria that divide by binary division are assigned to the order Chroococcales. Members of this order aggregate to form colonies that are held together by a slimy matrix. The members of the order Pleurocapsales reproduce through multiple fission in addition to releasing endospores. The order is also composed of a wide range of organisms that can be found in an array of habitats ranging from terrestrial and marine environments. The order Oscillatoriales is largely composed of filamentous

cyanobacteria having uniseriate filaments. However, members of this group lack true branching, akinetes and heterocysts. Members of this Order can be found in a variety of environments from fresh water and saline water bodies to terrestrial habitats. Members of Order Nostocales have heterogeneous cellular composition in their trichomes. The vegetative cells in this order are also divided into heterocysts that have a thick hyaline protoplast and are involved in nitrogen fixation. Nostocales consist of filamentous cyanobacteria having one-seriate trichomes/filaments or trichomes sometimes having false branches. Members of Order Stigonematales share several similar traits to Nostocales. This includes such traits as trichomes with heterogeneous cellular composition as well as heterocysts and akinetes vegetative cells. Members of the order exhibit multiserial filaments with true branching. Table 2.1 depicts the taxonomic classification of the cyanobacteria according to Castenholz and Waterbury (1989).

The cyanobacteria are generally identified on the basis of morphological attributes such as the trichome size and shape, position and location of the heterocysts as well as akinetes in the filament and also the division of the cells (Rippka et al., 1979). Anand (1979) used biometric parameters and observed that the proximity of the akinetes to the heterocyst is an important criterion in the identification of the taxa. However, the absence of these characters coupled with the phenotypic changes that occur due to growth under various environmental conditions, makes the identification cumbersome and erroneous. Holton et al., (1968) have observed a significant correlation between the morphological nature of the cyanobacteria and the fatty acid composition. Distinct subgroups within the genera of *Anabaena* and *Nostoc* could be identified due to the biochemical diversity (Rippka et al., 1979). Flores and Herrero (1994) observed that variability exists with respect to the expression of enzymes related to nitrogen assimilation which is under the global control mechanism involving the *ntcA* genes. They further opined that this can be employed as a tool in the screening of the genotypes using molecular tools. The relationship between the morphological, physiological and biochemical characters have been investigated using phylogenetic tools based on 16S rRNA gene sequences (Ezhilarasi and Ananad, 2010). Li et al. (2000) carried out DNA based comparison of 22 different morphological species of *Anabaena* species. Molecular tools such as 16S rRNA, ITS-I were employed in the cyanobacterial strains such as *Prochlorococcus* and *Synechococcus* isolates (Rocap et al., 2002; Ernst et al., 2003).

Table 2.1: The classification of cyanobacteria showing taxonomically important features (Castenholz and Waterbury 1989)

A. Non-Filamentous cyanobacteria	
I. Unicellular/non-filamentous aggregates of cells within a cell wall/ gel-like matrix	
1. Order- Chroococcales	Binary division in one, two or three planes, symmetric or asymmetric, reproduction through budding; akinete formation rare
2. Order- Pleurocapsales	Reproduction through internal multiple fission, or by mixture of multiple- and binary fission; daughter cells are smaller than parent; akinetes are formed rarely
B. Filamentous cyanobacteria	
II. Binary division in one plane which give rise to one-seriate trichomes/filaments; trichomes sometime have false branches	
3. Order- Oscillatoriales	Absence of heterocysts and akinetes
4. Order- Nostocales	Presence of heterocysts; some forms may also produce akinetes
III. Binary division periodically or commonly giving rise to multi-seriate trichomes/filaments	
5. Order- Stigonematales	Multi-seriate trichomes or trichomes with true branches, or both; formation of heterocysts; some may also form akinetes with true branches

A genomic finger printing technique, restricted fragment length polymorphism (RFLP) involving *psbA* genes was used in the freshwater species of *Synechococcus* (Postius et al., 1996; Ernst et al., 2003). Prasanna et al. (2006) used morphological as well as molecular markers to characterize *Anabaena* strains from various geographical regions. Therefore, it is important to use the molecular tools in the cyanobacteria especially when they are used for industrial exploitation for value added products.

Since some cyanobacteria are able to fix atmospheric nitrogen, they act as an important source of nitrogen in the ecosystem (Scanlan et al., 2009). The members of the cyanobacteria fix nitrogen are endowed with the specialized cells known as heterocyst (thick-walled modified cells) which house the enzyme nitrogenase which

converts molecular nitrogen into ammonia. Nitrogen fixation has also been carried out by many members of the non-heterocystous cyanobacteria. Table 2.2 shows the list of unicellular, filamentous heterocystous and filamentous non-heterocystous cyanobacteria. Cyanobacterial species such as *Anabaena variabilis*, *Nostoc muscorum*, *Aulosira fertissima* and *Tolypothrix tenuis* are found to be effective biofertilizers. On an average the cyanobacteria can contribute 20-30 Kg N ha⁻¹ season⁻¹ to the paddy fields (Vaishampayan et al., 2001). Algalization of rice fields for the improvement and maintenance of soil fertility has been suggested by Venkataramanan (1972). Asian countries such as India, China and Vietnam are using cyanobacteria as biofertilizer for paddy cultivation (Lumpkin and Plucknett, 1982). The ability of cyanobacteria to live on the minimum level of inputs such as solar energy, carbon dioxide and water has an advantage (Woese, 1987; Castenholz, 2001). Cyanobacterial biofertilizers can be conveniently used by small scale farmers to harvest higher and sustainable yields, maintain healthier soils for future use. Amount of nitrogen fixed by the cyanobacteria is quite substantial and could be exploited by the farmers from the economically weaker sections of the society (Issa et al., 2014). The most suitable environment for the growth of diazotrophic cyanobacteria are the rice fields. Majority of the studies conducted on the rice fields in India showed that *Anabaena*, *Nostoc*, *Aulosira*, *Aphanothece*, *Gleotrichia* and *Tolypothrix* are dominant cyanobacterial genera in the Indian rice field soils (Dash et al., 2017). Investigations have been conducted in the past to enumerate the abundance and genetic diversity of cyanobacteria from diverse habitats. Srivastava et al. (2009) conducted extensive studies to understand the diversity of cyanobacteria from the rice fields of Eastern Uttar Pradesh. Prasanna et al. (2009) observed that the diversity of cyanobacteria in the rice rhizosphere showed the 80% of the isolates comprised of *Anabaena* and *Nostoc*. The most commonly occurring rice field cyanobacterium *Anabaena* was found to show tremendous diversity (Nayak et al., 2009).

Cyanobacteria improve the bioavailability of phosphorus to the plants by solubilizing and mobilizing the insoluble organic phosphates. The phosphatase enzyme present in the cyanobacteria has the ability to solubilize the insoluble form of (Ca)₃(PO₄)₂, FePO₄, AlPO₄ and hydroxyapatite [Ca₅(PO₄)₃OH] in soils and sediments (Bose et al., 1971; Dorich et al., 1985; Wolf et al., 1985; Cameron and Julian, 1988). The phosphate is released either as exudates or through autolysis and decomposition of

the cells. Cyanobacteria also show plant growth promoting activity as they release extracellular plant growth promoting substances (Singh and Trehan, 1973; Rodgers et al., 1979; Marsalek et al., 1992). A major characteristic of cyanobacteria is their ability to secrete phytohormones (Yang et al., 2009). The phytohormone production has been reported mostly from cyanobacterium isolated from crop fields. It has been observed that several strains of cyanobacteria produce vitamins (Grieco and Desrochers., 1978) and amino acids (Vorontsova et al., 1988). Secretion of the growth hormone, auxin was reported in cyanobacteria such as *Anabaena*, *Calothrix*, *Cylindrospermum*, *Glactothece*, *Nostoc*, *Plactonema* and *Synechocystis* (Selykh and Semenova, 2000; Sergeeva et al., 2002). Production of gibberellins was reported in *Anabaenopsis*, *Cylindromum*, etc. (Mohan and Mukherji, 1978). Interestingly, the cytokinin production was also reported in *Anabaena*, *Chlorogloeopsis*, *Calothrix*, etc. (Rodgers et al., 1979; Selykh and Semenova, 2000). Cyanobacteria can be used for the reclamation of salt affected soils (Singh, 1961). Amendment of saline and alkali soils and improvement in the physico-chemical quality of the soil was reported by Kaushik and Subhashini (1985). Cyanobacterial species such as *Anabaena oscillarioides*, *A. aphanizomenoides* and *Microcystis aeruginosa* exhibited considerable salt tolerance ability (Moisander et al., 2002). The cyanobacteria are also extensively used in bioremediation. Removal of pesticides using cyanobacteria has been reported by Al-Hasan et al. (2001). Singh et al. (2011) reported that heavy metals can be removed from the ecosystem using cyanobacteria. Cyanobacteria have energy applications and it was observed that the energy rich compounds produced by cyanobacteria can be used for biofuel production (Rosgaard et al., 2012). Nozzi et al. (2013) observed hydrogen production by cyanobacteria. Cyanobacteria are now used as excellent candidates for human consumption.

Table 2.2. List of various forms of cyanobacteria

Cyanobacterial forms	Cyanobacterial members
Unicellular	<i>Synechococcus</i> , <i>Anacysts</i> , <i>Aphanaothece</i> , <i>Gleothece</i> , <i>Dermocapsa</i>
Filamentous non-heterocystous	<i>Plectonema</i> , <i>Lyngbya</i> , <i>Oscillatoria</i> , <i>Trichodesmium</i> , <i>Calothrix</i>
Filamentous heterocystous	<i>Anabaena</i> , <i>Nostoc</i> , <i>Aulosira</i> , <i>Westilliopsis</i> , <i>Scytonema</i> , <i>Tolypothrix</i>

Liang et al. (2004) observed that the incorporation of cyanobacteria improved the nutritive value of pastas, snack foods, candy bars or gums, and beverages. *Spirulina* is rich in proteins (more than 60%), beta-carotene, thiamine and riboflavin (Soletto et al., 2005). Kulshreshtha et al. (2008) reported that besides a number of unexplored bioactive compounds, *Spirulina* contain B-complex vitamins, minerals, proteins γ -linolenic acid and super antioxidants such as β -carotene, Vitamin E and trace elements. Recently, Singh et al. (2017) reviewed the importance of cyanobacterial metabolites from industrial and pharmaceutical perspective. A carrier based cyanobacterial biofertilizer comprising of four cyanobacteria such as *Anabaena*, *Nostoc*, *Aulosira* and *Tolypothrix* was developed at Centre for Conservation and utilization of BGA, ICAR-Indian Agricultural Research Institute, New Delhi.

Increasing abiotic stress conditions is posing a serious threat to the exploitation of cyanobacteria as bio-inoculants. Cyanobacteria have the capacity to endure desiccation and they are always exposed to continuous dehydration, desiccation and rewetting. Since the cyanobacteria are used as bio-inoculants for crops such as vegetables, wheat, sorghum, maize, cotton and sugarcane it is imperative to understand the desiccation tolerance of the cyanobacteria. The primary target of abiotic stresses in cyanobacteria is the biological membrane (Kurdrid et al., 2011; Li et al., 2012). Morphological changes in response to desiccation have been reported by Peat and Potts (1987). Further, it has been reported that increasing levels of desiccation leads to physiological and biochemical changes in the cyanobacteria (Coxson and Karshaw, 1983; Olie and Potts; 1986; Sen et al., 2017). Liang et al. (2012) suggested that dehydration and rehydration in the diazotrophic filamentous cyanobacterium *Nostoc flagelliforme* leads to metabolic shifts and changes in the physiology and proteome profile. Desiccation induced changes in the gene expression of cyanobacteria has been observed (Higo et al., 2007; Katoh., 2012). Scherer and Potts (1989) reported accumulation of water stress proteins in the desiccation tolerant cyanobacterium. Apel and Hirt (2004) reported that desiccation leads to the production of reactive oxygen species (ROS), enzyme inhibition, chlorophyll degradation, DNA damage and lipid peroxidation. It was observed that this will lead to dysfunction of the cellular machinery ultimately leading to cell death. However, the cells have developed efficient defense systems against the production of reactive oxygen species. Increased activity of enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT),

ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione reductase (GR) and peroxiredoxin (PR) leads to scavenging of free radicals (Mittler et al., 2004). Elevated levels of non-enzymatic compounds such as cellular proline, carotenoids, tocopherols, ascorbic acid and glutathione also contribute to defense. However, the defense system has been found to vary from organism to organism (Takeda et al., 1995; Abd El-Baky et al., 2004). Morgan (1984) reported the accumulation of specific metabolites for osmoregulation. These compounds in turn help the cells to retain water even at very low water potentials to maintain the turgor pressure, metabolic activity, and growth (Hanson and Hits 1982).

Among the different types of abiotic stress conditions, drought is a serious problem for the agricultural lands worldwide. Climatic variations and anthropogenic activities in arid, semiarid and dry sub humid areas lead to desertification. The desertification in turn leads to loss of biodiversity, low soil productivity and severe economic losses. It has been reported by (Lan et al., 2014) that the dry lands cover nearly 41.3% of all land. Desertification has seriously affected the livelihood of more than 250 million people (Reynolds et al., 2007). In this context, combating the problem of desertification needs top priority by the researchers and policy makers to save the humanity. The stabilization of the desertified land has been attempted using various means. Application of chemicals such as gypsum and planting trees has been undertaken by several workers (Yang et al., 2001). Since, the desert areas receive very less precipitation and without adequate water supply it is very difficult to manage the situation. In this context, the cyanobacteria offer effective solution for the restoration of degraded soils. In arid and semiarid regions, cyanobacterial crusts are commonly found which are dominated by cyanobacteria but other organisms such as green algae, fungi and bacteria are also found (West, 1990). Morphologically tangled filamentous cyanobacteria improve soil aggregate stability by adding organic matter and secreting extracellular polymeric substances (Rossi et al., 2012). Cyanobacteria can also survive extreme environmental conditions such as low precipitation and desiccation. In addition to this, the cyanobacteria have the ability to improve soil fertility through nitrogen fixation and nutrient mobilization (Belnap et al., 2001). Nisha et al. (2007) has reported the induction of cyanobacterial crusts to improve soil stability, productivity and nutrition. Establishment and use of biological soil crusts to ameliorate desertification and to restore arid lands and improve soil texture has been studied (Yang et al., 2001; Jusu et al., 2004; Guo et al., 2008).

Understanding the drought tolerance of agriculturally important microbes is important for achieving the global food security. However, in the natural environment, the cyanobacteria are constantly exposed to the continuous dehydration, desiccation, and rewetting. This is important from the agronomic perspective as it is used as biofertilizer for crops such as wheat, sugar cane, cotton, sorghum and vegetables (Abd-Alla et al., 1994). Reports are available on the desiccation tolerance of a number of cyanobacteria (Potts, 1994). Cameron and Blank (1966) observed the revival of spores from a 107-year-old sample of *Nostoc commune*. Cyanobacteria have been reported to exhibit considerable resilience to osmotic/water stress (Apte 2001; Fernandes et al., 1993). It was observed that the organisms which undergo desiccation are able to resume their metabolic activities due to rehydration. This remarkable ability is referred to as anhydrobiosis (Crowe, 2002). However, it has been observed that vital metabolic activities of cyanobacteria such as photosynthesis, respiration and nitrogen fixation revive within minutes of re-wetting (Scherer et al., 1984). Growth and productivity are greatly affected by environmental stresses such as drought, high salinity, and high and/low temperature (Zhang et al., 2010). Karnieli (1997) reported that the cyanobacteria are flexible organisms and have the capability to endure desiccation tolerance. Moreover, the cyanobacteria which are applied in the fields are very often subjected to repeated dehydration, desiccation, and rewetting. The remarkable capacity of *Nostoc commune* to endure very high levels of desiccation and longevity of more than 107 years has been reported by Cameron (1962).

Plants can be grown under desiccated and dry environment with the assistance of plant growth promoting rhizobacteria (PGPR). Generally, the plant growth promoting rhizobacteria are found in the rhizosphere in association with the plant roots. The well-established activities of the plant growth promoting rhizobacteria consist of nitrogen fixation, phytohormone production, phosphate solubilisation, ammonia production and the production of siderophores (Glick et al., 1999). It has been reported that the plant growth promoting rhizobacteria are potential candidates for the promotion of plant growth and alleviation of drought stress (Sandhya et al., 2009). However, there have been very few reports on the desiccation tolerant cyanobacteria from arid habitats having plant growth promoting activities which can be further exploited as bio-inoculants for crops that do not require standing water. Plants growing in natural drought conditions may harbor drought tolerant cyanobacteria with plant growth promoting attributes. It is therefore important to screen the cyanobacteria having

desiccation tolerance for plant growth promoting attributes. Karthikeyan et al. (2007) evaluated the potential of plant growth promoting cyanobacteria as inoculants for wheat. In a study, Prasanna et al. (2009) studied the rhizosphere dynamics of the inoculated cyanobacteria and their role as growth promoters in rice crop. Such studies have provided base line information on the exploitation of cyanobacteria with plant growth promoting attributes for developing carrier based biofertilizers for integrated nutrient management.

Study of proteins especially, cytosolic and membrane proteins in response to dehydration is also essential to elucidate the molecular mechanisms involved in drought stress management by cyanobacteria. Therefore, understanding the proteome dynamics of the cyanobacteria under stress conditions is also important. In this context, 2DE coupled with mass spectroscopic techniques is emerging as an important technique to understand the protein dynamics (Yadav et al., 2016; Sen et al., 2017). Dehydration induced up regulation and down regulation of several proteins in the cyanobacterium *Anabaena* PCC 7120 has been reported by Higo et al. (2007). Desiccation stress has resulted in alterations and composition of photosynthetic apparatus of cyanobacteria in response to water stress (Scherer and Potts, 1989). Proteomic studies revealed information on the proteins responsible for the stimulation electron transfer from phycobilisomes to PSI in *Synechocystis* sp. PCC 6803 (Ashby and Mullineaux, 1999). Plant growth and development is impeded due to the reactive oxygen species (ROS) generated during drought stress. The reactive oxygen species generated in the cells may result in the differential expression of proteins related to oxidative stress defense, cellular process and DNA repair. Raghavan et al. (2015) identified the role of proteins related to superoxide dismutase in oxidative stress management in the cyanobacteria. Proteins involved in the protection of DNA during oxidative damage have been identified through proteomic studies (Hernandez et al., 2007). Clegg (2001) suggested that stress proteins which function as molecular chaperones protect the proteins from denaturation. Cyanobacteria subjected to dehydration treatment showed decreased abundance of proteins related to amino acid biosynthesis and ammonia assimilation (Wolk et al., 1994). Zhang et al. (2015) observed that the genes related to nucleic acid metabolism were up regulated in response to dehydration. Inhibition of RNA polymerization in desiccation tolerant *Nostoc commune* resulted in decreased carbon and nitrogen utilization. Differential regulation of nitrate and nitrite binding protein ABC transporter and consequent inhibition in the nitrogen metabolism was observed in

cyanobacteria (Flores and Herrero, 1994; Maeda and Omata, 1997). Response of the cyanobacterium *Synechocystis* sp. PCC 6803 to phosphate acclimation has been observed (Fuszard et al., 2013). The role of ATP is important in survival of the cyanobacteria under desiccated conditions. Therefore, phosphate deficiency may affect DNA, RNA and ATP. Ion homeostasis involving ions such as Na⁺ and K⁺ play an important role in environmental stress conditions as they are important in turgor acclimation, membrane energetics, regulation of pH, enzyme activities and gene expression. Using proteomic tools, Ballal et al. (2007) identified transport proteins in the cyanobacteria. Increased abundance of cell envelope proteins such as porins due to dehydration might alter the membrane permeability (Sen et al., 2017). Proteomic studies have been conducted in cyanobacteria to identify the outer membrane proteins (Ekman et al., 2013; Hahn et al., 2015). During prolonged dehydration, increased abundance of proteins involved in lipid transport, fission, protein translocation and membrane modification has been observed in cyanobacteria (Kobayashi et al., 2007). Proteomic studies will also lead to the presence of several hypothetical or unknown proteins in any system. These proteins can be further identified through *in silico* studies. Therefore, hypothetical and unknown proteins in response to dehydration can be further explored for better identification of their role in desiccation stress management in cyanobacteria. Sen et al. (2015) identified several hypothetical and unknown membrane proteins in the cyanobacterium *Anabaena* PCC 7120 through *in silico* approaches. Therefore, the proteomic approaches will serve as an important interface to understand the responses to environmental stress and adaptation as well as possibilities for genetic manipulation. Adaptation of the cyanobacteria to desiccation stress is associated with elaborate changes in the proteome profile. Therefore, the target of proteome analysis is to get a holistic view of the differential expressed proteins as well as the hypothetical and unidentified proteins to work out the proteome dynamics in response to particular abiotic stress in question. The identification of the differentially expressed proteins will be further helpful in deciphering the precise proteins responsible for the desiccation stress in cyanobacteria. It will ultimately help in imparting desiccation stress tolerance in cyanobacteria and also to develop transgenic cyanobacteria with enhanced desiccation tolerance which can be used as bio-inoculant or for the restoration and management of drought affected ecosystems. These efforts may further strengthen the exploitation of cyanobacteria as an eco-friendly addition to enhance the productivity of the soils and their management.

Identification of cyanobacteria with plant growth promoting activity is important in the management and alleviation of desiccation stress. A number of mechanisms have been proposed for plant growth promoting rhizobacteria mediated desiccation stress. Yang et al. (2009) introduced the term Induced Systemic Tolerance (IST) for the physical and chemical changes in plants due to the action of microbes. There have been reports on the modification of phytohormonal activity by the microbes to impart drought tolerance to the plants (Fahad et al., 2015). The phytohormones play a key role in plants to survive the abiotic stress conditions (Skirycz and Inze, 2010). In addition to the phytohormones, the volatiles also play a lead role in drought tolerance in the plants (Loreto and Schnitzler, 2010). Cho et al. (2008) highlighted the role of bacterial volatiles with respect to stress tolerance. The plant growth promoting rhizobacteria also alter the root architecture and morphology of plants in relation to desiccation stress. Gusain et al. (2015) reported that the plant growth promoting rhizobacteria improves the drought tolerance in plants through the stabilization of cell membrane and induction of antioxidant enzymes. Sharma and Khanna (2013) observed that co-inoculation with a strain of *Mesorhizobium* was effective in mitigating the drought stress through the accumulation of more proline. Stress conditions lead to the accumulation of several compatible solutes in plants through metabolic adjustments. Plant growth promoting rhizobacteria inoculation in drought stress plants enhanced the production of proline levels (Bano et al., 2013). Yang et al. (2010) observe the accumulation of trehalose in response to plant growth promoting rhizobacteria inoculation. Soil microbial activity is hampered due to drought owing to disturbed physico-chemical and biological properties. Bensalim et al. (1998) observed that plants treated with exopolysaccharide (EPS) producing bacteria showed improvement in growth. Drought stress leads to the generation of free radicals which is dangerous for the proteins, lipids and DNA. In order to remove the free radicals plants have developed enzymatic and non-enzymatic antioxidants which lead to minimizing the oxidative damage (Miller et al., 2010). Vurukonda et al. (2016) observed enhancement in the antioxidant activity of plants due to plant growth promoting rhizobacteria. Alteration of the antioxidant activity is therefore important in enhancing the drought tolerance in plants. It is in this context that the identification of potential cyanobacteria strains with plant growth promoting activity is important in the management and mitigation of desiccation stress. Further, the cyanobacteria are now used as bio-inoculants for plants

that do not require standing water. Therefore, we may identify potential desiccation tolerant strains of cyanobacteria for drought stress management and eco-restoration.

Agriculture is highly vulnerable to global climatic change. To sustain the productivity in view of the climate change, exploitation of plant-microbe interaction is relevant. Efficient and promising cyanobacterial strains offer solution to reduce the dependence on chemical nitrogen fertilizers. Focused research on native drought tolerant cyanobacteria with plant growth promoting rhizobacteria traits could be explored for maintaining the sustainability of the ecosystem. For safer, healthier and balanced living of human beings and animals, a balanced and conserved ecosystem is important. Cyanobacteria enrich the soil organic nitrogen and carbon and also enhance the availability of phosphorous to the plants. Hence, they are important in managing the health of the ecosystem through degradation of harmful chemicals and pesticides. Investigations on the desiccation stress adaptability of the cyanobacteria are therefore important to exploit them in sustainable agriculture. Therefore, research efforts are required to identify and characterize the promising cyanobacteria with plant growth promoting attributes for the management of desiccation stress.

3.1 Sampling site

The BSCs (Biological Soil Crusts) samples collected from the arid regions of Rajasthan (Jaisalmer, Barmer, Jodhpur and Bikaner) were used for further isolation and characterization of desiccation tolerant cyanobacteria.

3.2 Sample processing and isolation of cyanobacteria

The crusts of cyanobacteria were brought to the laboratory and dried under shade for a week. The samples were subsequently ground with the help of mortar and pestle and the entire samples were passed through stainless sieve (2 mm). Land residue and gravel etc. were discarded. Soil samples (0.1 g) were inoculated in 100 ml BG-11 medium supplemented with Turk Island (TI) Salts and polyethylene glycol (5-20%) as described by Rippka et al. 1979 and Codd et al. 1979. A separate set of conical flasks containing BG-11 medium as mentioned above but with added nitrate source was also incubated for isolation of non-nitrogen fixers. The flasks were incubated at 30 ± 2 °C illuminated with white fluorescent tubes ($72 \text{ photon } \mu\text{mol m}^{-2} \text{ s}^{-1}$ for 30 days). Adequate growth was observed after 30 days and the biomass was examined under a light microscope (Phase contrast microscope 0.90 dry, Nikon Eclipse Ci) for the presence of cyanobacteria. The mixed cultures were homogenized and 1ml of the suspension was added to 9 ml of appropriate BG-11 media and further diluted by 10^{-4} fold. From each dilution, 100 μl was spread onto BG-11 medium (supplemented with TI salts with and without nitrogen and polyethylene glycol) plates for the selection of cyanobacteria. Out of the several cyanobacterial strains, one fast growing cyanobacterium was selected based on visual observations. Based on the cell length, width, position of heterocyst and pattern of branching identification of the cyanobacterial strain was carried out (Desikachari., 1959).

3.3 Experimental organism, polyethylene glycol treatment and Growth conditions

These isolates were exposed to artificial desiccation induced by various levels of polyethylene glycol-6000 (Polyethylene Glycol) ranging from 0-15 % and the one promising nitrogen fixing cyanobacterium among them which could tolerate maximum concentration of polyethylene glycol-6000 was selected for present studies based on chlorophyll content and dry weight. The cultures were maintained in BG-11 medium

without combined nitrogen sources with the pH of the medium maintained at 7.5. Light intensity and temperature were maintained at $72 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $30 \pm 2^{\circ}\text{C}$ respectively for 16/8 hr light and dark period. To minimize the contamination, the glassware was immersed in chromic sulfuric acid mixture for 24 hr followed by washing in running tap water for 15 min. Subsequently washed with detergent and tap water and then rinsed using double distilled water and dried in a hot air oven. Non-absorbent cotton wool was used to plug the culture flasks.

3.4 Chemicals and glassware

Analytical grade chemicals were used in present study, purchased from SRL, Hi-media and Sigma Chemicals. Glassware of Borosil grade were used.

3.5 Sterilization

The glassware and the nutrient solutions were sterilized by moist heat in an autoclave at 121°C , 15 lb/inch² pressure for 20 minutes.

3.6 Growth medium

BG 11 basal medium (Stanier et al., 1971) with $\frac{1}{4}$ th strength of Turks Island Salts (Garlick et al., 1977; Codd et al., 1979; The Merck Index, 9954) was used to grow and maintain cyanobacterial cultures. The composition of the growth medium is as in Appendix 1.

3.7 DNA extraction and PCR

DNA isolation

Cyanobacterial suspension (2 ml) was taken in an eppendorf tube and centrifuged at 14,000 rpm for 10 min. The pellet obtained was used for DNA extraction. DNASure Plant Mini Kit Manufacturer's Protocol (Nucleo-poreTM, NP-79105) was used and the protocol was modified slightly at particular steps to improve the efficiency of recovery of DNA. The amount of extracted DNA was determined by recording the absorbance at 260 nm. The value of 1 absorbance at A_{260} is equal to 50 $\mu\text{g/mL}$ for standard DNA. The integrity of isolated genomic DNA was checked by performing gel electrophoresis using 0.8% agarose (Pronadisa, Europe) against standard DNA of 20 ng concentration (λ DNA) at 70 volts for 30 min. The DNA bands were visualized in a gel documentation unit (Alpha innotec). Purity of DNA was checked by determining the ratio of absorbance at 260 nm and 280 nm.

16S rRNA gene amplification

16S rRNA gene fragment was amplified with universal primers FD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and RP2 (5'-ACGGCTACCTTGTTACGAC TT-3') (Weisburg et al., 1991) by modified PCR protocol (Lyra et al., 1997). The reaction was performed in a total volume of 25 µl having 1XTAE buffer containing 2 mM MgCl₂, 10 mM of deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP), 16S primers (FD1 and RP2) of 2.5 pmoles each, 3 µL DNA sample and 1U of Taq Polymerase (Fermentas, USA). Amplification was achieved in a Thermal Cycler (Prima-duo™, HiMedia) for initial denaturation (95°C for 5 min.) followed by 35 cycles composed of denaturation (94°C for 1 min.), annealing temperature (57°C for 1 min.) and extension (72°C for 1:30 min) followed by a final extension of 7 min at 72°C temperature. The PCR products were kept on hold at 4°C for 24 hrs. Gel electrophoresis of PCR product was undertaken.

Electrophoresis

10 µl of the amplified PCR product were loaded on horizontal 1% (w/v) agarose (Pronadisa, Europe) gel in 1XTAE buffer (0.5 M EDTA, 1M Tris-acetate, pH 8.0) stained with ethidium bromide solution (0.5 µg/ml) and electrophoresed at 75 volts for about 1 hr. In all, 1 kb molecular size standard (Fermentas) was run along with the amplified products to determine the approximate band size. The amplified products were visualized on UV transilluminator and were preserved in the form of pictures using Gel Doc System (AlphaImager EC). Then the amplified PCR product (1.5 kb) was outsourced for sequencing (Eurofins Genomics, New Delhi).

3.8 Desiccation treatment

Drought stress was achieved through polyethylene glycol-6000 (PEG) treatment in the culture media (BG-11 without combined nitrogen). The cyanobacterial isolates from BSC samples were exposed to various levels of polyethylene glycol-6000 (PEG) ranging from 0-20% to determine lethal concentration value (LC₅₀). Based on this, treatments were decided and proceeded with BG 11 basal medium supplemented with specific concentrations of polyethylene glycol-6000. The cultures which did not receive any desiccation treatment served as control.

3.9 Dry weight

Suspension of cyanobacterial culture was homogenized using homogenizer and a known volume of suspension (10 ml) was filtered through Whatman No. 42 filter paper. The filter papers were pre-weighed after oven-drying for 1 hour which was accounted for initial weight. The filtrate was oven dried at 60°C for 2-3 hours and cooled in desiccator to achieve a constant weight (final weight). Dry weight was then calculated by taking differences between initial and final weights according to Sorokin (1973) and it was expressed as µg/ml culture.

3.10 Relative water content (RWC)

Relative water content (RWC) is used to determine the water retention capacity of cells. Relative water content was determined according to Lin and Wu (2014). 50 mL of cyanobacterial culture was filtered through a cellulose acetate filter (pore size of 0.45 µm; Sartorius, Gottingen, Germany). The filtrate was placed in hot air oven at 60°C to dry. The water loss (WL) of cells was determined using the following equation:

$$WL (\%) = 100 \times [(W_0 - W_t)/(W_0 - W_d)], \text{ Where}$$

W_0 - initial wet weight after removing water drops by lightly blotting with tissue paper;

W_t - instantaneous weight measured after every 30 min;

W_d - dry weight measured after the culture had been dried at 60°C for 48 h and cooled in a desiccator.

RWC was then calculated as following:

$$RWC (\%) = 100 - WL$$

3.11 Average filament length

Average filament length was determined according to the procedure given by Mishra et al., (2003). A small volume of the finely homogenized cyanobacterial suspension was pipetted and mounted on a clear grease free slide. The filaments were focused under low power of the microscope and the filaments present in a field were all counted for their number of cells (vegetative cells as well as heterocysts). The average length of filament in a sample was obtained by adding up the number of cells in an individual filament counted and dividing it by their total number.

3.12 Heterocyst frequency

Heterocyst frequency was estimated according to Mishra et al., (2003). It is defined as the number of heterocyst present per hundred vegetative cells. Heterocyst frequency was calculated as follows.

$$\text{Heterocyst frequency (\%)} = \frac{\text{Total number of heterocysts}}{\text{Total number of vegetative cells}}$$

3.13 Pigment profile

3.13.1 Chlorophyll

The cold extraction method developed by McKinney (1941) was used to determine total chlorophyll content.

Reagent: Acetone (90%)

(i) Procedure

A known volume of Cyanobacterial culture (10 ml) was homogenized and centrifuged at 3000 rpm for 10 min using Falcon tubes (15 ml). Then the pellet was washed with double distilled water (10 ml) to remove polyethylene glycol content by centrifugation. Supernatant was discarded and an equal volume (10 ml) of 90% acetone was added to the pellet. The tubes were incubated at 4°C overnight. The suspension was centrifuged once again and the O.D. was read at 650 nm and 665 nm using spectrophotometer using 90% acetone as blank. The amount of chlorophyll (µg/mg dry weight) was calculated using the following formula.

$$\text{Total chlorophyll (\mu g/mg dry weight)} = [(20.2 \times A_{650}) + (8.02 \times A_{665})] \times \frac{\text{Total amount of cell suspension}}{1000 \times \text{original volume of suspension}}$$

3.13.2 Carotenoids

The carotenoid content of the cyanobacterium was estimated according to the protocol given by Jensen (1978).

Reagent: Acetone (90%)

(ii) Procedure

A known amount of cyanobacterial suspension (10 ml) was homogenized and centrifuged at 3000 rpm for 10 min and the pellet was washed with double distilled

water to remove adhering polyethylene glycol content. Acetone (90%, 10 ml) was added to the pellet and was incubated overnight at 4°C. The total carotenoid content (µg/mg dry weight) was estimated from the absorbance measured at 480 and 510 nm against 90% acetone as blank.

$$\text{Carotenoids} = [(7.6 \times A_{480}) - (1.49 \times A_{510})] \times \frac{\text{Total amount of cell suspension}}{1000 \times \text{original volume of suspension}}$$

(µg/mg dry weight)

3.13.3 Phycobilins

The protocol of Bennett and Bogorad (1973) was used to calculate the contents of phycoerythrin, phycocyanin and allophycocyanin in cyanobacteria.

Reagent: phosphate buffer (0.05 M, pH 7.5)

(iii) Procedure

After extracting the chlorophyll and carotenoid content by cold extraction method, the supernatant was discarded and pellet was added with equal volume (10 ml) of 0.05 M phosphate buffer, pH 7.5 (prepared by mixing equal volumes of 0.1 M KH₂PO₄ and 0.1 M K₂HPO₄). Repeated freezing and thawing of the pellet were done for few days till the pellet became colorless and the pigment was completely released into the supernatant. Absorbance of the samples was read at 562, 615 and 652 nm. Blank was prepared using 0.05 M phosphate buffer. The contents of phycobilins were calculated using the following equation.

$$\text{Phycocyanin (PC))} = \frac{A_{615} - (0.474 \times A_{652})}{5.34}$$

(µg/mg dry weight)

$$\text{Allophycocyanin (APC)} = \frac{A_{652} - (0.208 \times A_{615})}{5.06}$$

(µg/mg dry weight)

$$\text{Phycoerythrin (PE)} = \frac{A_{562} - [(2.41 \times \text{PC}) - (0.649 \times \text{APC})]}{9.62}$$

(µg/mg dry weight)

3.14 Total Carbohydrate content

The total carbohydrate content was estimated according to the method developed by Spiro (1966).

Reagents:

- (i) Standard glucose solution 10-100 $\mu\text{g ml}^{-1}$ prepared in distilled water
- (ii) Anthrone reagent: Prepared by mixing 0.1 g Anthrone reagent and 1 g thiourea in 100 ml of 75% H_2SO_4 and incubated at 85 $^{\circ}\text{C}$ for 20 minutes in water bath.

Procedure:

1 ml of homogenized cyanobacterial suspension was taken in Falcon tubes (15 ml) and 4 ml Anthrone reagent was added to it. The sample was then incubated at 100 $^{\circ}\text{C}$ for 15 minutes in water bath. After the incubation period, the sample was cooled under tap water. Absorbance was measured using spectrophotometer at 620 nm and a standard curve was prepared using glucose as standard. Total sugar content was expressed in $\mu\text{g/mg}$ dry weight.

3.15 Estimation of Protein

Protein content of was estimated according to the method of Lowry et al. (1951).

Reagents:

- I. Sodium hydroxide (1 N)
- II. (i) Sodium carbonate solution (5%)
 - (ii) Copper sulphate 0.5% in 1% of Sodium potassium tartarate.
 - 2ml (ii) was mixed with 48 ml of (i) prepared immediately before use.
- III Folin-ciocalteau reagent: The commercial reagent was diluted with an equal volume of water just before use.
- IV. Standard protein: Bovine serum albumin solution was prepared in the range of 10 to 100 $\mu\text{g ml}^{-1}$.

Procedure:

0.5 ml of homogenized cyanobacterial suspension was taken and an equal volume of 1 N NaOH was added to it and the samples were kept in a boiling water bath for 5 min. After cooling, reagent II (2.5 ml) was added and allowed to stand for 10 min

at room temperature. Then reagent III (0.5 ml) was added rapidly and mixed thoroughly. After 15 min incubation, the absorbance of the blue solution was read in a spectrophotometer at 650 nm. A calibration curve of protein was prepared by using bovine serum albumin as standard. The soluble protein content was expressed in $\mu\text{g}/\text{mg}$ dry weight.

3.16 Photosynthetic efficiency (F_v/F_m)

The chlorophyll fluorescence for photosynthetic activity will be studied according to Franklin et al. (1992) and Krause and Weis (1988). The chlorophyll a fluorescence measurement provides information on the photochemistry of photosystem II and quantum yield which is estimated as F_v/F_m . This measurement involves the size and number of active reaction centers of the photosynthetic apparatus and efficiency of the water splitting complex. The cyanobacterium *Anabaena sp.* was exposed to different concentrations of polyethylene glycol (0%, 5% and 10%). Further, the chlorophyll fluorescence was measured using pulse-amplitude modulation fluorometer (PAM-2500 Heinz Walz GmbH, Germany).

3.17 Estimation of lipid peroxidation

Determination of the total thiobarbituric acid reactive substances (TBARS) as equivalent of malondialdehyde (MDA) is the principle behind estimation of lipid peroxidation as according to Cakmak and Horst (1991).

Reagents

1. Trichloroacetic acid (20% w/v)
2. Thiobarbituric acid (0.5% w/v)

Homogenized cyanobacterial suspension (20 ml) was centrifuged and washed with phosphate buffer (50 mM, pH 7.0) at 3000 rpm for 5 min. The pellet was collected and ground with the help of mortar and pestle by using TCA (5% w/v). Then the extract was centrifuged at 10000 rpm for 10 min. Supernatant (0.5 ml) was taken and 2 ml of 20% TCA (containing 0.5% TBA) was added to it and incubated for 20 min at 90 °C in water bath. The reaction mixture was kept on ice for 10 min to stop the reaction. It was again centrifuged at 10000 rpm for 10 min and the supernatant was taken for spectrophotometry. The absorbance was read at 532 nm and 600 nm. Extinction coefficient of $155 \text{ mM}^{-1}\text{cm}^{-1}$ was used to determine the amount of Malondialdehyde (MDA) and lipid peroxidation was expressed as nmol MDA mg^{-1} dry weight.

3.18 Estimation of reactive oxygen species

3.18.1. Estimation of superoxide radical (O_2^-)

Amount of superoxide radical was determined according to the method developed by Elstner and Heupal (1976) with few modifications suggested by Jiang and Zhang (2001). The principle is to monitor the formation of nitrite from hydroxylamine in the presence of superoxide in each homogenized sample.

Reagents

1. Phosphate buffer (pH 7.8) : 65 mM
2. Hydroxylamine (NH_2OH) : 10 mM
3. Sulfanilamide : 17 mM
4. α -N(N-1)-naphthyl ethylenediamine dihydrochloride (NEDD) : 7 mM
5. Diethyl ether : 100%

A known volume of cyanobacterial cells (40 ml) were taken and centrifuged and washed several times with 1 mM EDTA in 65 mM phosphate buffer (pH 7.8). Then centrifuged at 10000 g for 10 min and supernatant was collected. The reaction mixture consisted of 0.9 ml 65 mM phosphate buffer (pH 7.8), 0.1 mM hydroxylamine and 1ml of supernatant. After incubating the reaction mixture for 20 min at 25⁰ C, 17 mM sulfanilamide was added followed by 7 mM NEDD and mixed thoroughly and the reaction was allowed for 20 min. An equal volume of diethyl ether was mixed well with each sample after the completion of reaction. Then the samples were left for separation of diazo-compound in aqueous phase. Absorbance of pink color was measured at 530 nm. Superoxide radical formed was quantified with the help of standard curve drawn using NO_2^- and expressed as nmol/mg dry weight.

3.18.2. Estimation of hydrogen peroxide (H_2O_2)

The amount of hydrogen peroxide was measured according to Velikova et al., (2000)

Reagents

1. Trichloroacetic acid (TCA) : 0.1% (w/v)
2. Potassium phosphate buffer (pH 7.0) : 10 mM
3. Potassium iodide : 1 M

Cyanobacterial suspension was centrifuged at 6000 g for 10 min and the pellets were homogenized in 0.1% TCA. Homogenate was again centrifuged at 8000 g for 15 min. the reaction mixture composed of supernatant, 10 mM Potassium phosphate buffer (pH 7.0) and 1M Potassium iodide. Absorbance of solution was recorded at 390 nm and the amount of hydrogen peroxide was calculated from a standard graph prepared by using graded H₂O₂ solution and expressed as nmol mg⁻¹ dry weight.

3.19 Assay of antioxidants (enzymatic and non-enzymatic)

3.19.1 Superoxide dismutase

Preparation of samples

Cyanobacterial suspension (30 ml) was homogenized and washed with distilled water by centrifugation (3000 rpm for 5 min) to remove traces of polyethylene glycol-6000. Pellet was collected in a clean mortar and grinded with pestle using liquid nitrogen. After tissue became powder, grinding continued with cell lysis buffer (7 ml, added intermittently). Finally, the ground suspension was put in an oak ridge tube and centrifuged for 20 min at 10000 rpm, 4°C. Then the supernatant containing enzymes was stored at 4°C. The same extract was used for assay of superoxide dismutase, ascorbate peroxidase and catalase enzyme activity.

Reagents

1. Methionine (200 mM): L-Methionine 0.298 g was dissolved in water and the volume was made up to 10 ml with double distilled water.
2. Nitro-blue tetrazolium chloride (NBT) (2.25 mM): NBT 0.0184 g was dissolved in water and the volume was made up to 10 ml with double distilled water.
3. EDTA (3.0 mM): EDTA 0.0558 g was dissolved in water and the volume was made up to 50 ml with double distilled water.
4. Riboflavin (60 µM): Riboflavin 0.0023 g was dissolved in water and the volume was made up to 100 ml with double distilled water.
5. Sodium carbonate (1.5 M): Sodium carbonate 7.95 g was dissolved in double distilled water and the volume was made up to 50 ml with double distilled water.
6. Phosphate buffer (100 mM, pH 7.8):

Sol A: Potassium dihydrogen phosphate 6.8 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Buffer was prepared by mixing 8.5 ml of sol. A and 91.5 ml of sol. B and final pH was adjusted with the help of pH meter.

Enzyme assay

The superoxide dismutase enzyme activity (SOD) was assayed by the method according to Giannopolitus and Ries (1977) and Dhindsa et al. (1981). The basis for estimation is the decrease in optical density of formazone caused by superoxide radical and nitro-blue tetrazolium dye, by superoxide dismutase. The reaction mixture (3 ml) consisted of 13.33 mM methionine (0.2 ml of 200 mM), 75 μ M nitro- blue tetrazolium chloride (NBT) (0.1 ml of 2.25 mM), 0.1 mM EDTA (0.1 ml of 3 mM), 50 mM phosphate buffer (pH 7.8) (1.5 ml of 100 mM), 50 mM sodium carbonate (0.1 ml of 1.5 M), 0.5 ml enzyme, 0.5 ml of water (final volume of 3.0 ml). Riboflavin (2 mM, 0.1 ml) was added to the above reaction mixture and placed under two fluorescent lamps (15 W) for 15 min to start the reaction. A control was set which had the complete reaction mixture without enzyme that gave the maximal color. Then tube containing the reaction mixture was kept in dark conditions to stop the reaction. A non-illuminated complete reaction mixture was labeled as a blank. The absorbance was recorded at 560 nm. The amount of enzyme, which reduced the absorbance to 50% compared to tube lacking enzyme, was considered as one unit of enzyme activity and was expressed in U/mg dry weight.

3.19.2 Ascorbate peroxidase

Ascorbate peroxidase (APX) was quantified by finding the decrease in absorbance due to ascorbic acid at 290 nm (Nakano and Asada, 1981).

Reagents

1. Ascorbic acid (3.0 mM): Ascorbic acid (0.0265 g) was dissolved in water and the volume was made up to 50 ml with double distilled water.
2. EDTA (3.0 mM): EDTA (0.0558 g) was dissolved in water and the volume was made up to 50 ml with double distilled water.

3. Hydrogen peroxide (1.5 mM): 16 μ l of standard hydrogen peroxide (30%) was dissolved in water and the volume was made up to 100 ml with double distilled water.

4. Phosphate buffer (100 mM, pH 7.0)

Sol A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Buffer solution was prepared by mixing 39 ml of sol. A and 61 ml of sol. B, and final pH was adjusted with the help of pH meter.

Enzyme assay

Reaction mixture (3ml) contained 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 0.5 mM ascorbic acid (0.5 ml of 3.0 mM), 0.1 mM EDTA (0.1 ml of 3.0 mM), 0.5 ml enzyme. The reaction was started by addition of 0.4 ml of 1.5 mM H_2O_2 . Decrease in absorbance was measured at 290 nm in an UV-visible spectrophotometer (Halo DB 20, USA) for every 30s up to 180s. The amount of enzyme consumed to degrade 1 μ mol ascorbate per mg protein was recorded as one enzyme unit and was expressed in U/mg dry weight.

3.19.3 Catalase

Catalase activity was determined by measuring the disappearance of H_2O_2 according to the method of Aebi (1984).

Reagents

1. Phosphate buffer (0.1 M, pH 7.0)

Sol A: Potassium dihydrogen phosphate 6.8 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Buffer was prepared by mixing 39 ml of solution A and 61 ml of solution B, and final pH was adjusted.

2. Hydrogen Peroxide (75 mM): A solution of 75 mM H₂O₂ was prepared by dissolving 775 µl of 30% H₂O₂ in 100 ml of double distilled water.

Enzyme assay

Reaction mixture (3 ml) composed of 0.5 ml of 75 mM H₂O₂, 1.5 ml of 0.1 M phosphate buffer (pH 7) in cuvette, 0.5 ml of enzyme extract and 0.5ml of distilled water. The reaction was started by adding 0.5 ml enzyme extract into the cuvette. Decrease in absorbance was recorded at 240 nm after 30s and 60s in a UV-visible spectrophotometer (Halo DB 20, USA). The initial and final amounts of H₂O₂ were measured and the catalase activity was determined by calculating the quantity of H₂O₂ degraded. The amount of enzyme required to decompose 1 µmole H₂O₂ per milligram protein was calculated and expressed in U/mg dry weight.

3.19.4 Proline content

The proline is an important non-enzymatic antioxidant. Proline content in cyanobacterial cell was quantified according to Bates et al. (1973).

Reagents

1. Sulfosalicylic acid: 3% aqueous solution.
2. Acid ninhydrin: 2.48 g ninhydrin was dissolved in 60 ml glacial acetic acid and 40 ml of 6N phosphoric acid, kept in 4 °C.
3. Toluene
4. Glacial acetic acid

Assay

Homogenized cyanobacterial culture (20 ml) was washed with distilled water by centrifugation. The pellet was ground using 3% sulfosalicylic acid (10 ml) and the extract was filtered using Whatman #2 paper. Filtrate (2 ml) was mixed with 2 ml acid ninhydrin and 2 ml of glacial acetic acid. The mixture was then heated in a water bath for 1 hr at 100 °C. After heating, tubes were quickly placed in ice to stop the reaction. Toluene (4 ml) was added to the mixture and vortexed for 15-20 sec. Absorbance of the chromophore from the toluene phase was measured at 520 nm in UV-visible spectrophotometer (Halo DB 20, USA). A reagent blank and a standard curve were prepared using L-proline. The total proline content was expressed in nmol mg⁻¹ dry weight.

3.20 Assay of N- assimilation enzymes

3.20.1 Nitrate reductase

Majority of the plants take up N in the form of nitrate. Subsequently, in the nitrogen assimilation pathway, nitrate reductase is the first enzyme involved and it catalyzes the reduction of nitrate to nitrite which is then used in synthesis of different amino acids. Nitrate reductase (NR) assay was performed according to the protocol given by Herrero et al. (1981).

Reagents:

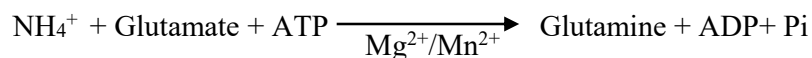
1. Sodium nitrate (NaNO_3)
2. Sulphanilamide (1g in 100 ml of 1:4 HCl and water)
3. α – (N-1)) naphthyl ethylene diaminedihydrochloride (NEDD), (0.2% W/V)

Enzyme assay

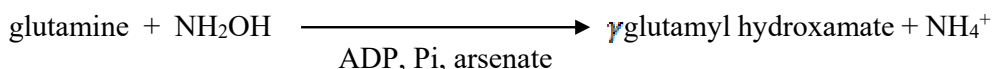
A known amount of homogenized algal suspension was centrifuged and washed with distilled water. The pellet was then dissolved in basal medium containing NaNO_3 (10 mM, pH 7.0) and incubated at 30°C overnight. Next day, a known proportion of cyanobacterial cell suspension and distilled water (so as to make total 1ml) was taken and Sulphanilamide (2 ml) was added and mixed thoroughly. 2 ml α – (N-1) naphthyl ethylene diaminedihydrochloride (NEDD) was added subsequently and the sample was incubated at room temperature for 30 minutes. Pink color appeared and the absorbance was measured at 540 nm. Activity was expressed in $\mu\text{mol NO}_2 \text{ mg}^{-1}$ of protein. Standard curve was prepared using sodium nitrite. Nitrite was calculated by the method of Snell and Snell (1949).

3.20.2 Glutamine Synthetase activity

Glutamine synthetase catalyzes the synthesis of glutamine from glutamate and ammonia which is an ATP requiring reaction.



Besides this, cyanobacterial glutamine synthetase also catalyzes the synthesis of γ - glutamyl hydroxamate by the transferase reaction.



The amount of γ -glutamyl hydroxamate synthesized through the transferase activity was quantified according to Shapiro and Stadtman (1979).

Reagents

1. Glutamine : 0.1 M, pH 7.0
2. Sodium arsenate : 0.1 M, pH 7.0
3. Sodium ADP : 0.01M, pH 7.0
4. Mn Cl₂ : 0.1 M
5. Hydroxylamine – HCl : 2.0 M
6. Imidazole HCl buffer : 100 mM, pH 7.0
7. γ - glutamyl hydroxamate : 10 mM standard
8. Stop mixture: Ferric chloride (10% w/v), Trichloroacetic acid (24% w/v), 6 N HCl and double distilled water in the ratio of 8:2:1:13

Reaction mixture was prepared by mixing the following volumes of the above solutions.

- I. Imidazole buffer : 2.0 ml
- II. Glutamine : 1.5 ml
- III. MnCl₂ : 1.5 ml
- IV. Sodium ADP : 2.0 ml
- V. Sodium arsenate : 1.0 ml
- VI. Hydroxylamine- HCl : 1.5 ml
- VII. Water : 2.0 ml

Hydroxylamine hydrochloride was neutralized with 15 ml of 2 N NaOH before adding other components of the mixture.

Enzyme assay

Homogenized cyanobacterial suspension (5 ml) was added with 0.25 ml toluene and mixed thoroughly with vortex and incubated overnight at 4°C. Next day, the suspension was centrifuged at 3000 rpm for 5 min. The upper toluene layer was removed using a pipette. The pellet was dissolved in 5 ml distilled water and mixed properly. Then 0.5 ml from the mixture was taken in a separate tube and 0.5 ml imidazole buffer was added to it (vortex was done). Reaction mixture (0.5 ml) was added to start the reaction and incubated at 37°C for 30 min in water bath. Stop mixture

(3 ml) was added to stop the reaction after incubation and absorbance was read at 540 nm. The glutamine synthetase transferase activity is expressed as $\mu\text{mol } \gamma\text{-glutamyl hydroxamate formed mg protein}^{-1}$.

3.20.3 Estimation of Nitrogenase Activity

The atmospheric nitrogen is fixed and reduced to ammonia by nitrogenase enzyme complex (EC 1.18.2.1). This is dependent on reduced ferredoxin and is also coupled to reduction of protons resulting in the formation of molecular hydrogen:



Besides N_2 , several compounds with a triple bond like nitrile, cyanide, acetylene are reduced by nitrogenase. Hence, the principle behind present assay is, the reduction of acetylene (C_2H_2) to ethylene (C_2H_4). Acetylene reduction assay was done according to Stewart et al. (1968). The assay was done in glass rimless tubes of 20 ml capacity. A known volume of cyanobacterial cell suspension (5 ml) was collected in tube and tubes were sealed with suba seal rubber stoppers and paraffin strips. With help of injection, 10% acetylene gas was injected after drawing same amount of air. The tubes were then incubated at $28 \pm 1^\circ\text{C}$ at 2500 lux intensity with intermittent shaking for 3 hours. Reaction was stopped by injecting 0.8 ml of 15% (w/v) trichloroacetic acid. Amount of ethylene produced in tubes was analyzed by a gas chromatograph (Nucon model GLC 5500) fitted with a poropak R column and a hydrogen flame ionization detector. The activity of nitrogenase enzyme was expressed as $\text{nmol C}_2\text{H}_4 \text{ formed mg}^{-1} \text{ chlorophyll h}^{-1}$.

3.21. Plant Growth Promoting Activities

Many cyanobacteria are known to possess plant growth promoting activities. The selected isolate was qualitatively evaluated for four important PGP activities viz., IAA production, ammonia production, phosphate solubilization and siderophore production.

3.21.1. IAA production

Production of IAA by cyanobacteria was evaluated according to Gordon and Weber (1951).

Reagents

1. Tryptophan

2. Salkowski reagent (3 ml of 0.5 M FeCl_3 + 70 ml of Concentrated sulfuric acid + 100 ml distilled water)

The cyanobacteria were grown in BG-11 medium supplemented with filter sterilized tryptophan (100 $\mu\text{g/ml}$). cyanobacterial suspension (1 ml) was taken in an eppendorf tube and centrifuged at 10000 rpm for 10 min. Then the supernatant was transferred to a glass tube and 4 ml salkowski reagent was added to it. The samples were incubated at room temperature for 30 min to allow the development of color.

3.21.2. Production of ammonia

Ammonia release by cyanobacterial cells was evaluated according to Dye (1962). Cyanobacterial cell suspension (10 ml) was taken and centrifuged at 3000 rpm for 5 min. Nessler's reagent (2 ml) was added to the suspension and observed for any color change.

3.21.3. Phosphate solubilization

Phosphate solubilization attribute was determined by the method of Pikovskaya (1948).

Reagents

1. Potassium chloride (equivalent in amount to K_2HPO_4)
2. Tricalcium phosphate (1 mg/ml of medium)
3. Tris-HCl buffer (0.4 M, pH 8.5)
4. Phenolphthalein diphosphate (16.6 M)

The cyanobacterial culture was grown in BG-11 medium supplemented with tricalcium phosphate (as phosphorous source) and potassium chloride (as substitute for K_2HPO_4). The reaction mixture (3 ml) consisted of tris-HCl buffer (1.5 ml), phenolphthalein diphosphate (0.1 ml) and supernatant of cyanobacterial cell suspension. Reaction mixture was kept in dark condition at room temperature for 30 min. Then reaction was terminated by adding 1 M K_2HPO_4 (0.1 ml) and observed for color change.

3.21.4. Siderophore production

Siderophore production by cyanobacterial cells was qualitatively evaluated according to Schwyn and Neilands (1987).

Reagents

1. CAS dye (121 mg dissolved in 100 ml distilled water)
2. $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1 mM) prepared in 10 mM HCl
3. HDTMA solution (145.8 mg in 80 ml distilled water)

Solution A (200ml) : CAS dye was mixed with 20 ml $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and then the mixture was slowly added to HDTMA solution along the wall of container.

Solution B : BG-1 medium (900 ml) added with 2% agar

Both the solutions were prepared and autoclaved separately. Finally, CAS-Blue agar was prepared by adding solution A to solution B along the wall of container to achieve maximum mixing without formation of foam. CAS-Blue agar was (25-30 ml) poured into each petriplate. Cyanobacterial culture was inoculated by point inoculation and plates were observed for appearance of pale yellow zone around the colonies after incubation.

3.22 Fatty acid profiling

Fatty acid profile was determined according to Bligh and Dyer (1959) and Ichihara and Fukubayashi (2010) followed by GC-MS analysis.

Reagents and solvents

1. Solvent mixture : chloroform + methanol in (2:1) ratio
2. Hexane
3. Stock solution : HCl (9.7 ml) + methanol (40.3 ml)
4. Toluene

Lipid Extraction

A known volume of homogenized cyanobacterial suspension (50 ml) was taken, centrifuged and washed with double distilled water at 10000 g for 15 min and pellet was dried in hot air oven. Dried pellet (1 g) was mixed with solvent mixture (7 ml) and vortexed for 30 sec. Then the solution was centrifuged at 7000 rpm for 2 min and the supernatant was collected in a glass vial. The bottom biomass left over after transferring supernatant was added with 7 ml solvent mixture and vortex, centrifugation was repeated and the supernatant was collected in the same previous vial. Again, lipid in

bottom biomass was recovered again with 6 ml solvent mixture. Finally, the glass vial contained lipid extracted in 20 ml solvent mixture.

Purification of the extracted lipid

The solvent in glass vial was allowed to evaporate in hot air oven leaving unrefined lipid in vial. Hexane was added to unrefined lipid in 10:1 ratio. Mixture was then centrifuged at 10000 rpm for 2 min at room temperature. Supernatant was collected in pre-weighed glass vial. Hexane was allowed to evaporate in hot air oven. After visible disappearance of hexane, the vial was heated at 90°C for 1 hr, cooled and weighed.

$$\text{Lipid content (\%)} = \frac{\text{Wt. of lipid} \times 100}{\text{Wt. of sample}}$$

Preparation of Fatty acid methyl esters (FAME)

Extracted lipid (0.1 ml) was taken in screw cap glass tubes (30 ml). It was added with toluene (0.2 ml), methanol (1.5 ml) and stock solution (0.3 ml) and kept on incubator shaker at 45°C overnight. Then hexane (1 ml) and double distilled water (1 ml) were added and kept on shaker for 5min. Top 1 ml supernatant containing FAMES was taken in glass vial (5 ml).

GC/MS (gas chromatography/mass spectrometry) analysis

Fatty acid profile for each of the experimental conditions was determined by Gas Chromatography (Shimadzu GC-2010) using Flame Ionization Detector (FID). 2.5 µl of esterified sample was injected and analyzed through FID (detector temperature, 280 °C; injection temperature, 270°C; carrier gas, nitrogen at a flow rate of 1.21 ml/min) fitted with a 100 m long, (0.25 mm i.d.) SP-2560 column with a 0.20 mm film thickness and a polyethylene glycol modified nitroterephthalic acid stationary phase. Chromatography conditions used were: initial column temperature 140°C with equilibration time of 0.5 min which was held for 5 min, a 4°C/min rise to 240°C, held for 15 min (total 45 min). Fatty acids were identified by comparing the retention times with FAME standards (Sigma) and quantified by normalization of the area under relevant peaks using Varian Star software version 4.51.

3.23 Proteomic profiling of the test cyanobacterium exposed to Polyethylene glycol

Poly ethylene glycol treatment

The test cyanobacterium was grown in BG 11 medium containing TI salts. Three sets of the given cyanobacteria were maintained among which two sets received 5% and 10% polyethylene glycol treatment and one set did not receive any polyethylene glycol treatment (0% polyethylene glycol).

Protein extraction

Banding pattern and the efficacy of the protocol in extraction and solubilization of the proteins was detected by performing initial protein profiling. Protein extraction was performed according to the method developed by Issacson et al. (2006). A known volume of cyanobacterial suspension (1 L) was centrifuged at 10000 rpm for 10 min. The pellet (2 g) was ground into powder using liquid nitrogen and suspended in 10 ml extraction buffer containing 50 mM Hepes (pH 7.5), 40% Sucrose and 0.1% β -Mercapto ethanol and saturated phenol (Sigma Aldrich, USA) in the ratio of 1:4. Cells were kept on a rocker for 30 minutes in an ice bucket and then centrifuged (5000 X g, 10 min). The upper layer of the supernatant was taken and added with chilled precipitation buffer (0.1M ammonium acetate in methanol) and incubated for 12 hr at -20°C. Centrifugation was repeated again (15000 X g, 15 min) and the pellet was washed 3 times with acetone (80% v/v) and dried in vacuum evaporator. Then the precipitated protein was solubilized in buffer (200 μ l) containing 7 M urea, 2 M thiourea and 4% CHAPS. The Concentration of protein was measured using the method developed by Bradford (1976). Composition of extraction buffer, precipitation buffer, washing buffer, solubilization buffer, are mentioned in Appendix 2.

Second Dimensional Gel Electrophoresis

Two-dimensional electrophoresis (2-DE) of proteins was carried out according to the method of O'Farrel (1975) with some modifications. Protein samples were rehydrated by following steps. 50 mM DTT and 1 % IPG buffer (pH 3-10) were added to the protein samples and centrifuged at 15000 X g for 15 min. Solubilization buffer (300 μ l) containing protein (600 μ g) was loaded to immobilized pH gradient (IPG) strips (pH 4-7; 17 cm; Biorad, USA). Then strips were covered with mineral oil for 16 h at 20°C. Isoelectric focusing (IEF) was conducted at 20°C with IEF cell (PROTEAN i12, Biorad, USA). Focusing was performed in six steps (200 volts 3 hrs rapid, 500 volts 30 min rapid, 1000 volts 30 min rapid, 8000 volts 1h linear, 8000 volts/h rapid

48000 volts, 500 volts, 20 h rapid). The strips were equilibrated after IEF for 12 min by incubating them first in an equilibration solution (6 M urea; 30% w/v glycerol; 2% SDS; 50 mM Tris-HCl, pH 8.8) containing 2% w/v DTT and the strips were incubated for 12 min in the same equilibration solution containing iodoacetamide (3.9% w/v) instead of DTT (2%). Equilibrated strips were then transferred to 12% SDS polyacrylamide gels and separated in the second dimension at a constant voltage of 100 V (PROTEAN II xi, Biorad, USA). The gels were stained with colloidal Coomassie Brilliant Blue (CBB) and subsequently destained by rinsing with milli-Q water several times. Composition of equilibration buffer, IEF protocol buffer, SDS-PAGE composition, composition of electrophoresis buffer, solubilization staining solution etc are mentioned in Appendix 3.

Analysis of the Gel

Subsequently the stained gels were scanned with an Image Scanner (GE Healthcare; Life Sciences, USA) with a resolution 300 dpi and by pooling the gels (using three replicates of each control and treatment). For analyzing images, the gels were scanned and protein spots were analyzed using the software PDQuestTM basic version (Biorad, USA). Spots were matched after back ground subtraction and detection of spots. Spot quantification in control and treated gels was done by measuring the spot volumes (intensity x mm²). Treatment-responsive proteins were the spots exhibiting more than two fold change in their abundance during the treatment or a significant variation between the control and other treatments, as determined by the paired Student's t-test ($p \leq 0.05$).

3.24 Statistical analysis

All the experiments were carried out in triplicate with six replications for each of the three treatments (0%, 5% and 10% polyethylene glycol concentration). The data obtained on various parameters were subjected to statistical analysis using one way analysis of variance (ANOVA). Duncan multiple range test (DMRT) was employed to compare the mean performances of different treatments for the parameters studied. The data was also analyzed using Pearson correlation.

A number of isolates were obtained from the soil samples collected from the soil biological crusts from Rajasthan. The isolated cyanobacteria were found to grow in polyethylene glycol concentrations in the range of 5 – 15% (Table 4.1) In the present study, among the ten isolates, the isolate S-1(2) showed better dry weight and chlorophyll accumulation (Table 4.2) at different levels of polyethylene glycol treatment compared to all other isolates purified from biological soil crust samples. The selected cyanobacterium was grown in BG-11 medium supplemented with Turk Island (TI) salts and in the absence of combined nitrogen. The organism was grown under the following conditions such as control (without polyethylene glycol) and exposed to 5% and 10% polyethylene glycol (polyethylene glycol). Various physiological and biochemical characteristics of the cyanobacterium was estimated on the 12th day of incubation.

4.1 Identification of the cyanobacterium

The cyanobacterial isolates were maintained in BG-11 medium supplemented with TI salts and varying levels of polyethylene glycol (5 and 10%). One set of cultures was also maintained in the same medium without polyethylene glycol (0%). The selected strain was free-floating and formed suspension in the liquid medium and the color of the cells was blue-green (Plate 4.1). Microscopic analysis revealed that cells grow as straight filaments (Plate 4.2). On an average, the number of cells in a filament range from 25 to 50 cells. The vegetative cells were cylindrical in shape and the diameter varied from 5.4 to 5.8 μm . The heterocyst was oval in shape and position was intercalary and the strain formed akinetes during late exponential phase of the growth. Akinete was formed away from the heterocyst and was slightly longer than the vegetative cell. Based on these morphological observations, the strain was identified as *Anabaena* sp.

4.2 Isolation of DNA and PCR

The DNA from the cyanobacterium *Anabaena* sp. was extracted in 30 μL solvent from DNASure Plant Mini Kit. Extracted DNA had a concentration of 12ng/ μL ($A_{260} = 0.24$) and was pure ($A_{260} / A_{280} = 1.74$). Agarose gel electrophoresis revealed that the isolated was intact and showed a clear band as shown in (Plate 4.3). Then the DNA was subjected to 16S rRNA gene amplification with the primers FD1 and RP2. Single amplified PCR product of 1.5kb (Plate 4.4) was observed on performing electrophoresis and documenting on gel documentation unit.

Table 4.1. Selection of cyanobacteria from soil biological crusts based on growth in medium supplemented with polyethylene glycol (0-20%)

Sample	Treatment				
	PEG (0%)	PEG (5%)	PEG (10%)	PEG (15%)	PEG (20%)
Sample 1	+	+	+	+	-
Sample 2	+	+	+	+	-
Sample 3	+	+	+	-	-
Sample 4	+	+	+	+	-
Sample 5	+	+	+	+	-
Sample 6	+	+	+	+	-
Sample 7	+	+	+	+	-
Sample 8	+	+	+	+	-
Sample 9	+	+	+	+	-
Sample 10	+	+	+	+	-

Table 4.2. Dry weight and chlorophyll content of cyanobacterial isolates from the soil biological crusts. Dry weight and chlorophyll content were estimated on 12th day of polyethylene glycol treatment

Samples	Total chlorophyll (µg/mg dry weight)				Dry weight (µg/ml)			
	PEG (0%)	PEG (5%)	PEG (10%)	PEG (15%)	PEG (0%)	PEG (5%)	PEG (10%)	PEG (15%)
S-1	12.33	19.51	8.49	5.60	121.1	200.3	83.50	50.8
S-2	11.41	15.82	7.44	3.60	110.35	191	78.52	47.89
S-3	8.75	10.58	5.01	3.32	85.82	150.11	62.89	38.53
S-4	7.62	12.70	4.00	2.16	65.12	135.73	55.38	23.41
S-5	9.70	13.36	7.45	3.95	97.67	161.59	65.79	39.53
S-6	10.47	17.32	6.47	4.14	100.01	175.5	70.17	44.01
S-7	11.23	18.09	6.75	3.89	101.90	186.00	73.90	43.27
S-8	8.25	15.27	5.40	2.52	79.31	143.63	60.05	34.95
S-9	9.17	11.75	8.04	1.55	98.01	156	66.70	39.97
S-10	9.91	10.01	4.35	3.48	98.37	170.46	70.05	40.35

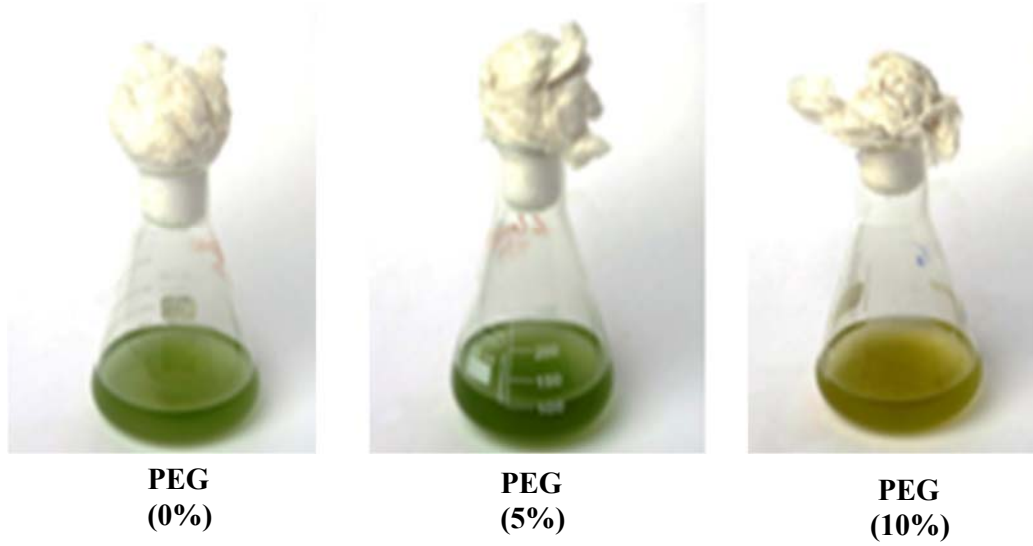


Plate 4.1. The selected cyanobacterium *Anabaena* sp. grown in BG-11 medium without nitrogen and supplemented with TI salts



Plate 4.2. The cyanobacterium *Anabaena* sp. observed under phase contrast microscope

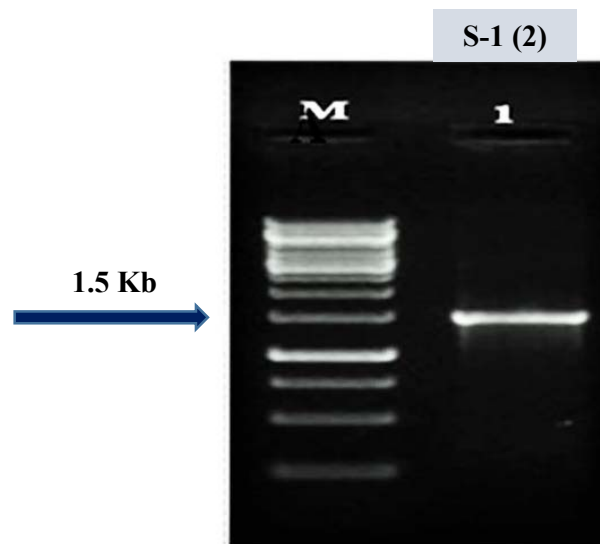


Plate 4.3. Gel picture showing the genomic DNA of *Anabaena* sp.

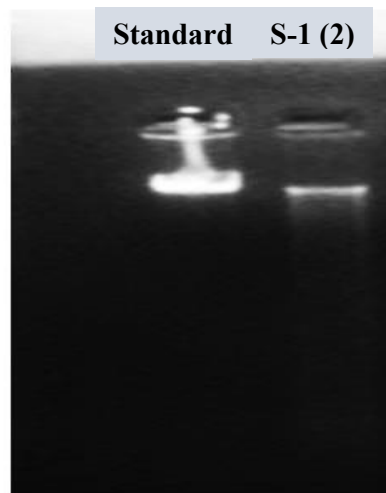


Plate 4.4. PCR amplification of 16S rRNA gene of the cyanobacterium *Anabaena* sp.

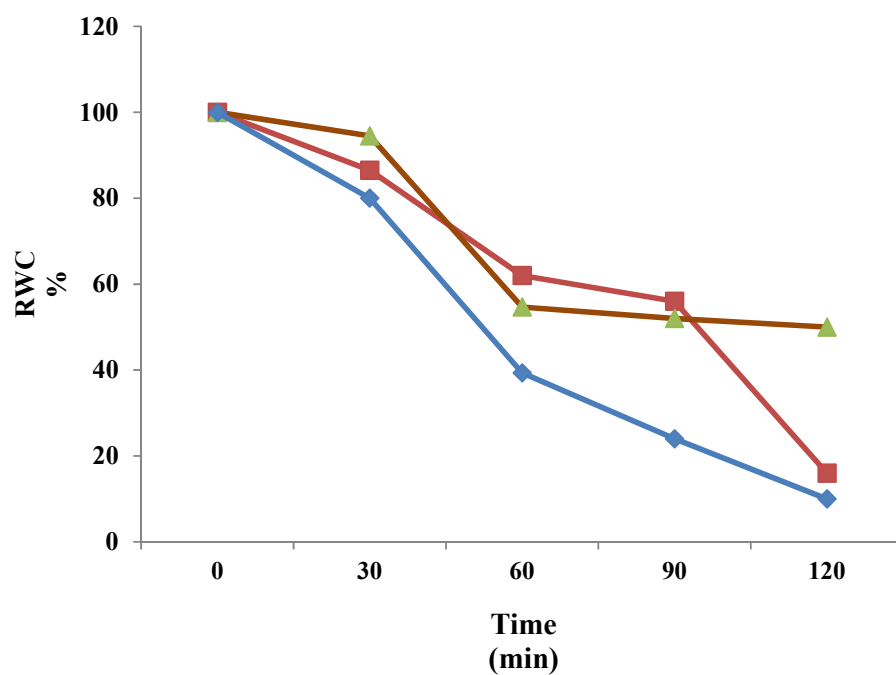
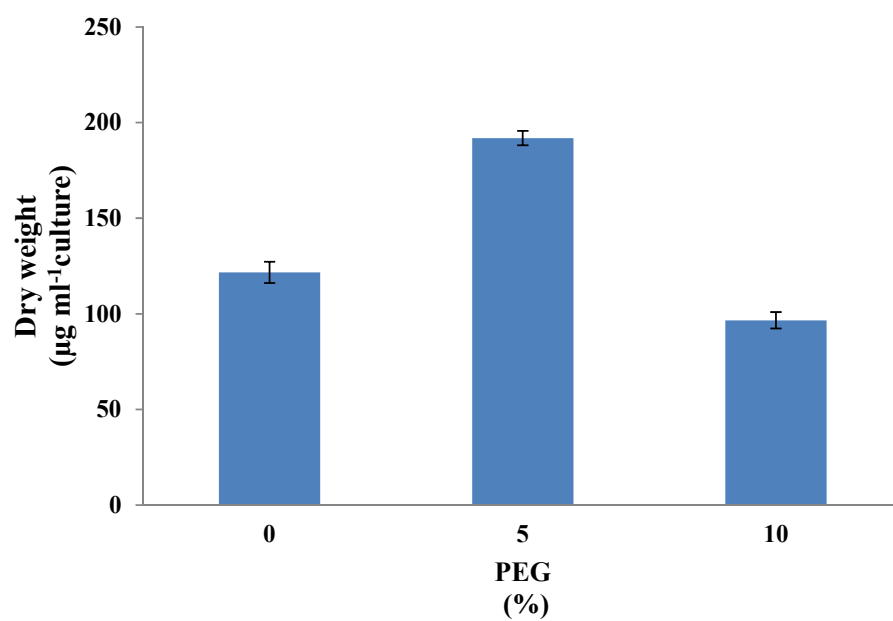


Fig.4.2. Changes in the Relative water content of cells of the cyanobacterium *Anabaena* sp. in response to various levels of polyethylene glycol induced desiccation stress

4.3 Effect of various concentrations of polyethylene glycol on the cellular constituents of the cyanobacterium *Anabaena* sp. grown in BG-11 medium supplemented with TI salts.

4.3.1. Dry Weight

The growth of *Anabaena* sp. grown in various concentrations of polyethylene glycol was estimated in terms of dry weight (Fig.4.1). It was observed that polyethylene glycol influenced the growth in a differential manner. While the cells exposed to 5% showed optimal growth, cells exposed to 10% polyethylene glycol showed significant inhibition in the growth. However, the cells not exposed to polyethylene glycol showed a better response as compared to the cells grown at 10% polyethylene glycol. An increase of 57.8% in dry weight was observed in *Anabaena* sp exposed to 5% polyethylene glycol, whereas the cells grown at 10% polyethylene glycol showed decrease in the dry weight accumulation by 23%. From the data obtained on growth, it was evident that the cyanobacterium *Anabaena* sp. was able to grow optimally at 5% polyethylene glycol.

4.3.2. Relative water content (RWC)

Differences in relative water content (RWC) were observed in the cyanobacterium *Anabaena* sp. exposed to different concentrations of polyethylene glycol. Reduction in water content from 100% to 0% was estimated during the time interval of 30 min to 120 min. it was observed that the order of resistance to dehydration was 5% > 0% > 10% (Fig.4.2)

4.3.3. Chlorophyll

The chlorophyll content was estimated as an index of growth in the test cyanobacterium exposed to desiccation. The chlorophyll content observed in the cells exposed to 5% polyethylene glycol was 16.9 ± 1.28 $\mu\text{g}/\text{mg}$ dry weight (Fig.4.3). However, at a higher concentration of polyethylene glycol i. e, 10% the chlorophyll content showed a value of 6.6 ± 0.71 $\mu\text{g}/\text{mg}$ dry weight. The cells grown in absence of polyethylene glycol recorded chlorophyll content of 11.2 ± 0.88 $\mu\text{g}/\text{mg}$ dry weight. Enhanced levels of chlorophyll content at 5% polyethylene glycol, shows the ability of cyanobacterium *Anabaena* sp. to cope with desiccation. The cells grown at 10% polyethylene glycol and without polyethylene glycol however failed to grow optimally.

Table 4.3. Effect of various concentrations of polyethylene glycol on growth, cellular constituents and photosynthetic efficiency of the cyanobacterium *Anabaena* sp.

Treatments	Dry weight ($\mu\text{g/ml}$ culture)	Sugars ($\mu\text{g/mg}$ dry weight)	Protein ($\mu\text{g/mg}$ dry weight)	Fv/Fm
PEG (0%)	121.67 $\pm$ 5.56	80.38 $\pm$ 2.37	44.73 $\pm$ 4.63	0.57 $\pm$ 0
PEG (5%)	191.92 $\pm$ 3.78	119.42 $\pm$ 3.13	66.23 $\pm$ 2.39	0.60 $\pm$ 0.01
PEG (10%)	96.60 $\pm$ 4.29	103.35 $\pm$ 2.86	44.33 $\pm$ 1.91	0.47 $\pm$ 0.01
F value	106.531	77.102	12.025	147.473
SE(m)	6.77	3.159	5.11	0.007
C.D.	19.7684	9.22428	14.9212	0.02044

Data are means $\pm$ standard error of six replicates (n=6).

Table 4.4. Variation in the pigment profile of the cyanobacterium *Anabaena* sp. in response to desiccation induced by polyethylene glycol

Treatment	Chlorophyll a (µg/mg dry weight)	Carotenoids (µg/mg dry weight)	Phycocyanin (µg/mg dry weight)	Phycoerythrin (µg/mg dry weight)	Allophycocyanin (µg/mg dry weight)
PEG (0%)	11.17±0.88	6.96±0.92	48.02±4.35	7.82±1.24	8.46±1.05
PEG (5%)	16.85±1.28	10.30±1.88	63.20±1.85	9.69±1.29	13.43±1.39
PEG (10%)	6.58±0.71	3.64±0.57	41.08±3.48	5.53±0.70	5.06±0.34
F value	34.798	7.727	10.616	3.54	20.17
SE(m)	1.233	1.695	4.909	1.566	1.325
C.D.	3.60036	4.9494	14.33428	4.57272	3.869

Data are means±standard error of six replicates (n=6).

Table 4.5. Effect of desiccation stress induced by polyethylene glycol on heterocyst frequency, average filament length and the activities of nitrate reductase, glutamine synthetase and nitrogenase enzymes of *Anabaena* sp

Treatments	Nitrate Reductase ($\mu\text{mol NO}_2$ mg⁻¹ of protein)	Glutamine Synthetase (U/mg protein)	Superoxide (nmol/mg DW)	Nitrogenase nmol C₂H₄ formed mg⁻¹ chlorophyll h⁻¹	Heterocyst frequency (%)	Average Filament Length
0% PEG	36.02±1.85	30.82±1.39	5.88±0.71	99.28±4.49	7.95±0.35	56.45±3.4
5% PEG	53.50±2.36	26.60±1.6	11.93±1.06	126.13±4.88	11.53±0.68	73.63±3.03
10% PEG	42.02±4.56	19.98±1.16	21.80±1.56	87.78±3.67	6.80±0.77	45.30±2.58
F value	7.057	13.536	49.705	23.727	11.364	18.649
SE(m)	4.73	2.099	1.611	5.713	1.035	4.674
C.D.	13.81	6.13	4.70	16.68	3.02	13.65

Data are means±standard error of six replicates (n=6).

Table 4.6. Differential accumulation of reactive oxygen species, malondialdehyde, proline and differential activity of antioxidant enzymes in response to varying concentrations of polyethylene glycol in *Anabaena* sp

Treatments	H ₂ O ₂ (nmol/ mg dry weight)	MDA (nmol/mg dry weight)	SOD (U/mg dry weight)	APX (U/mg dry weight)	Catalase (U/mg dry weight)	Proline (nmol/mg dry weight)
PEG (0%)	13.85±0.93	1.70±0.15	2.82±0.21	5.23±0.34	3.73±0.27	9.98±0.64
PEG (5%)	20.40±1.08	3.70±0.25	5.07±0.26	7.80±0.29	8.17±0.51	23.23±1.58
PEG (10%)	31.45±1.48	6.67±0.26	6.83±0.33	13.22±1.38	12.20±1.1	13.75±0.81
F value	49.879	130.301	70.439	19.752	31.028	33.426
SE(m)	1.78	0.31	0.339	1.296	1.075	1.67
C.D.	5.20	0.90	0.99	3.78	3.14	4.88

Data are means±standard error of six replicates (n=6).

H₂O₂ – Hydrogen peroxide, SOD – Superoxide dismutase, APX – Ascorbate peroxidase

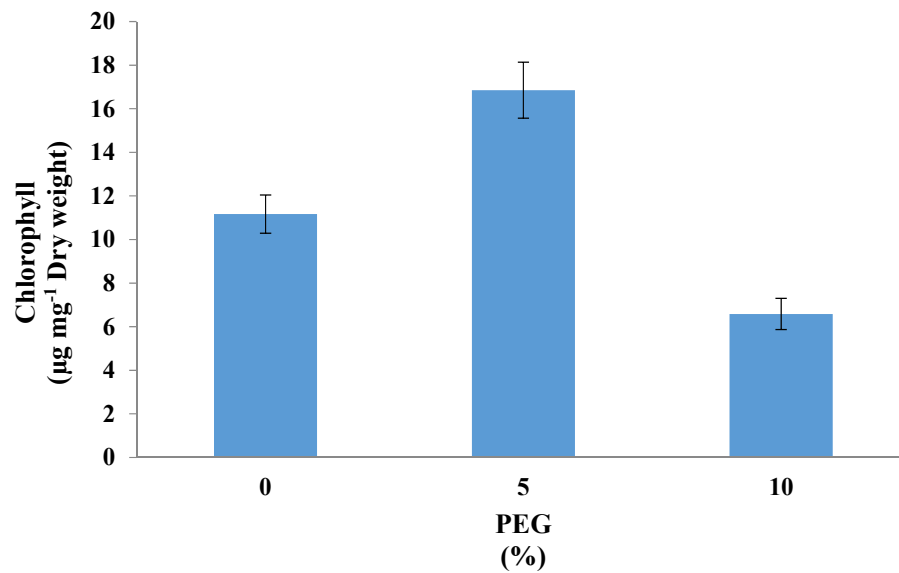


Fig.4.3. Differential accumulation of chlorophyll in the cyanobacterium *Anabaena* sp. in response to different levels of polyethylene glycol induced desiccation stress

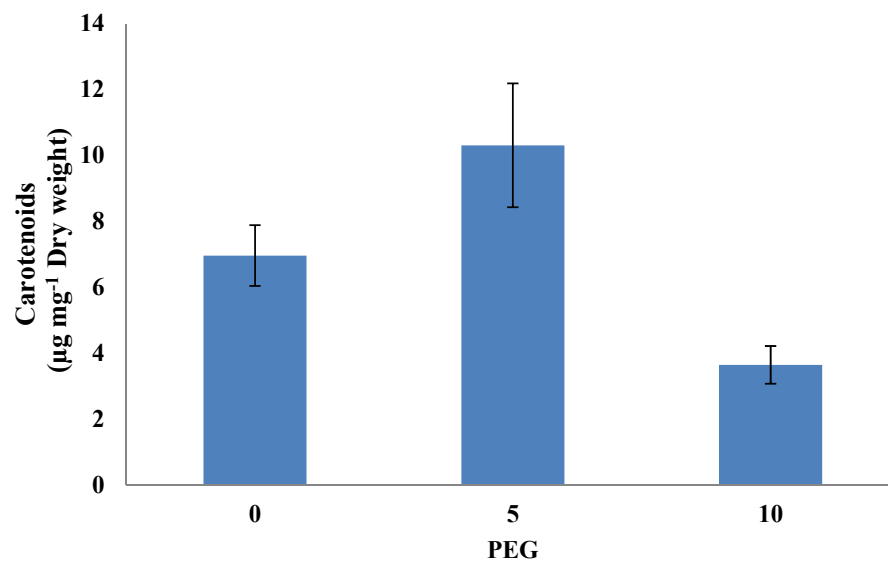


Fig.4.4. Effect of polyethylene glycol induced desiccation stress on carotenoid content of the cyanobacterium *Anabaena* sp.

4.3.4. Carotenoids

The non-enzymatic antioxidant, carotenoids were estimated in response to growth under varying levels of desiccation. Maximum content of carotenoids was observed when the cyanobacterium *Anabaena* sp. was grown at 5% (Fig.4.4). The lowest carotenoid content was recorded 10% polyethylene glycol and the cells not receiving any polyethylene glycol treatment were found to contain intermediate levels. As compared to cells not exposed to polyethylene glycol, the cyanobacteria grown at 5% recorded an increase of 48% in the carotenoid content. However, the cells grown at 10% polyethylene glycol showed a decrease of 64.6% in carotenoid content as compared to the cells grown at 5% polyethylene glycol level. Therefore, the cyanobacterium *Anabaena* sp. showed significant increase in the carotenoid content at 5% polyethylene glycol treatment.

4.3.5. Phycobiliproteins (phycocyanin, phycoerythrin, allophycocyanin)

Accessory pigments such as phycocyanin, phycoerythrin and allophycocyanin in the cyanobacteria were estimated following exposure to different levels of polyethylene glycol for 12 days. Increase in the phycobiliprotein content was pronounced when the cyanobacterium was grown in medium supplemented with 5% polyethylene glycol. Higher concentration polyethylene glycol resulted in reduced accumulation of phycobiliproteins (Fig.4.5). As compared to 0% polyethylene glycol exposure, phycocyanin and phycoerythrin content of cyanobacterium was enhanced by 31.6% and 24% respectively, in case of 5% polyethylene glycol treatment. On the other hand, phycocyanin and phycoerythrin content was diminished by 14.5% and 29% respectively in cells exposed to 10% polyethylene glycol when compared to control. Both phycocyanin and phycoerythrin contents were of intermediate levels in cyanobacteria grown without any polyethylene glycol treatment. Another component of phycobiliproteins viz., allophycocyanin however recorded 58.7% increase in cells treated with 5% polyethylene glycol whereas those grown at 10% polyethylene glycol concentration showed more than 50% decrease as compared to control. The results indicate the sensitivity of phycobiliproteins at higher concentration of polyethylene glycol. Further it was observed that the cells not receiving any polyethylene glycol treatment also recorded low quantity of phycobiliproteins.

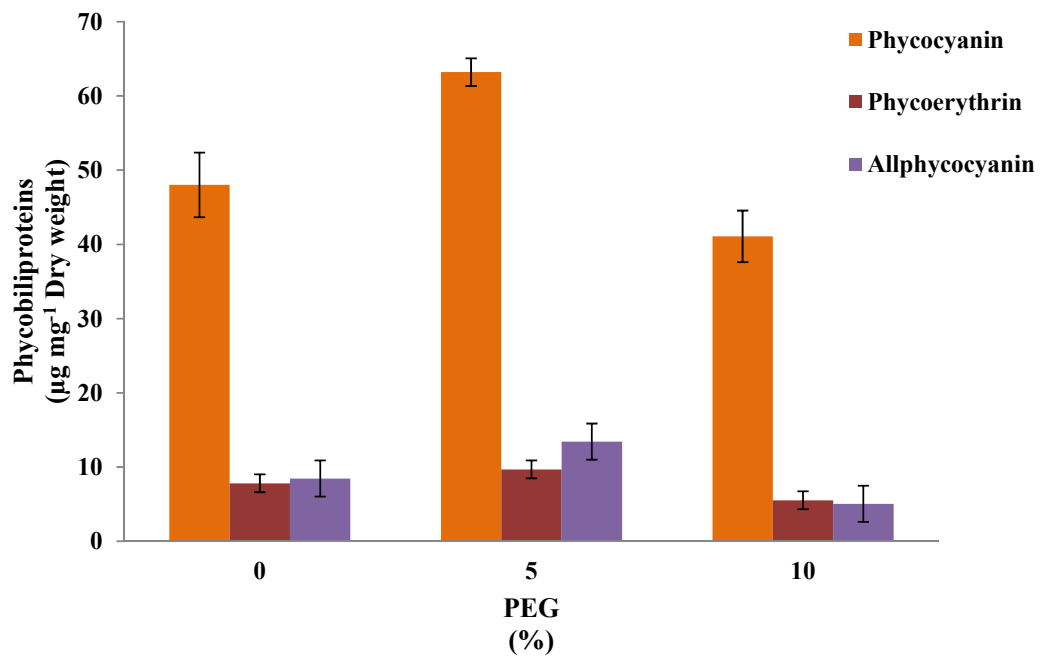


Fig.4.5. Effect of different concentrations of polyethylene glycol on phycobiliproteins content in the cyanobacterium *Anabaena* sp.

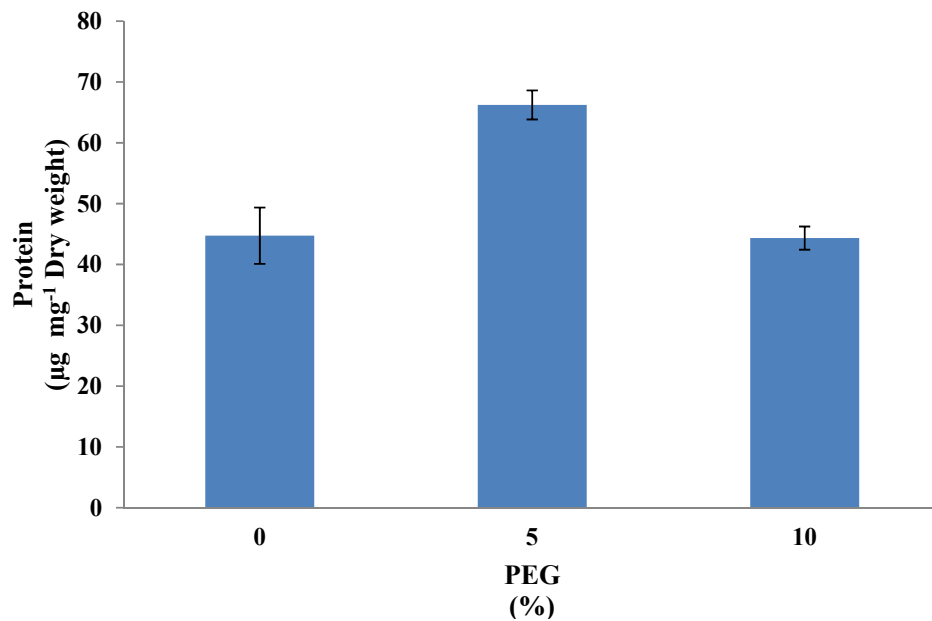


Fig.4.6 Variation in protein content of the cyanobacterium *Anabaena* sp. in response to desiccation stress induced by different levels of polyethylene glycol

4.3.6. Proteins

The soluble protein content of the cyanobacterium *Anabaena* sp. grown under varying levels of polyethylene glycol was studied (Fig.4.6). The cells exposed to polyethylene glycol concentration of 0% and 10% recorded decrease in the protein content by 32.5% and 33% when compared to the cells grown at 5% polyethylene glycol treatment. It was observed that there is no significant difference in protein content between the cells grown at 10% polyethylene glycol and the cells not exposed to any polyethylene glycol treatment. From the data, it appears that the protein content is optimal at 5% polyethylene glycol. On the other hand, higher concentration of polyethylene glycol resulted in significant reduction in protein content.

4.3.7. Sugars

The sugar content of cyanobacterium *Anabaena* sp. exposed to different polyethylene glycol levels was analyzed. Higher sugar content was noticed in the cells grown at 5% and 10% polyethylene glycol (Fig.4.7). The lowest sugar content was observed in cells which were not exposed to polyethylene glycol. The cells grown at 10% polyethylene glycol showed marginal increase in the sugar content by 28.5% as compared to those grown without any polyethylene glycol treatment. However, the cyanobacteria showed optimal growth at 5% polyethylene glycol and exhibited significant increase in the sugar content by 48.5%.

4.4 Impact of polyethylene glycol induced desiccation on photosynthesis of the cyanobacterium *Anabaena* sp.

In general, the photosynthetic process is observed to be sensitive to desiccation. Photochemical efficiency (F_v/F_m) was measured in the cyanobacterium *Anabaena* sp. exposed to polyethylene glycol. Significant reduction in the photosynthetic activity was observed at 10% polyethylene glycol (Fig.4.8). However, the cells exposed to 5% polyethylene glycol maintained optimal photosynthetic efficiency (0.602) followed by 0% polyethylene glycol (0.273) and 10% (0.472). It was observed that the photochemical efficiency of the cyanobacteria was inhibited maximally at 10% polyethylene glycol ($p < 0.05$).

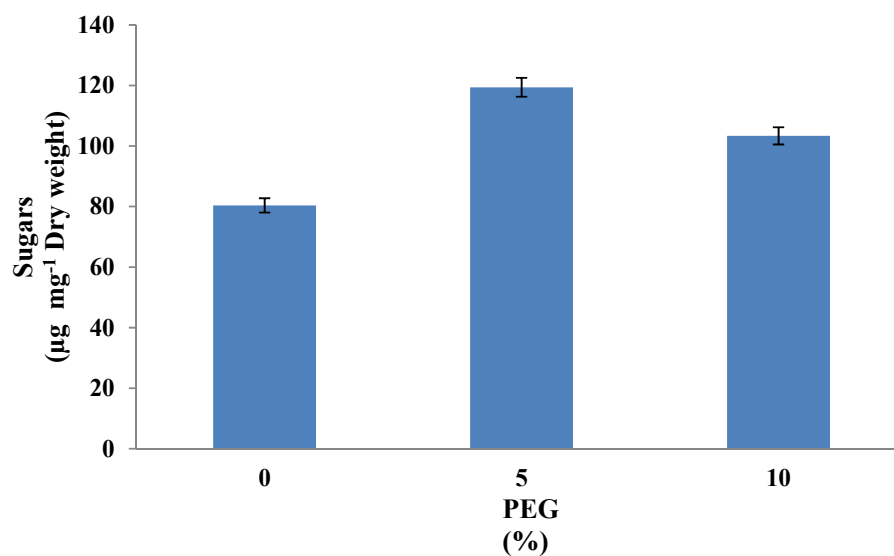


Fig.4.7. Effect of various concentrations of polyethylene glycol on sugar content of the cyanobacterium *Anabaena* sp. in response to polyethylene glycol induced desiccation stress

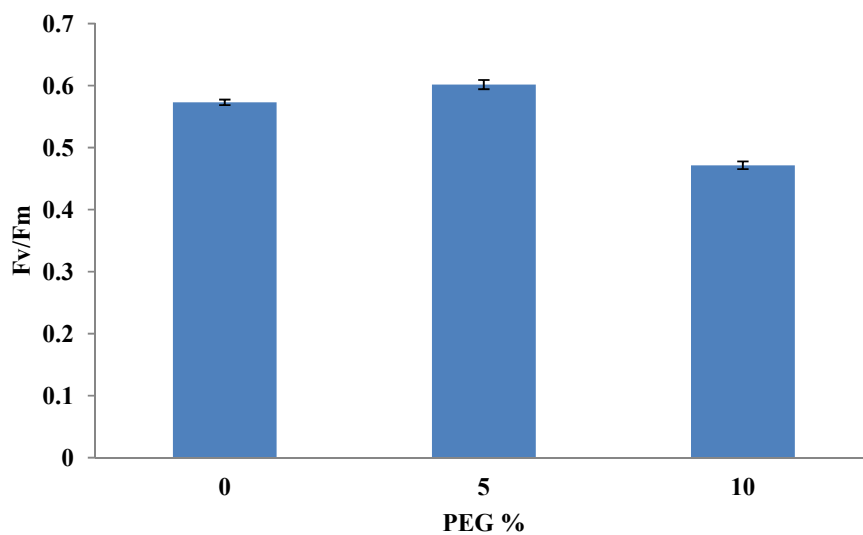


Fig.4.8. Impact of polyethylene glycol induced desiccation on the differential chlorophyll fluorescence in the cyanobacterium *Anabaena* sp.

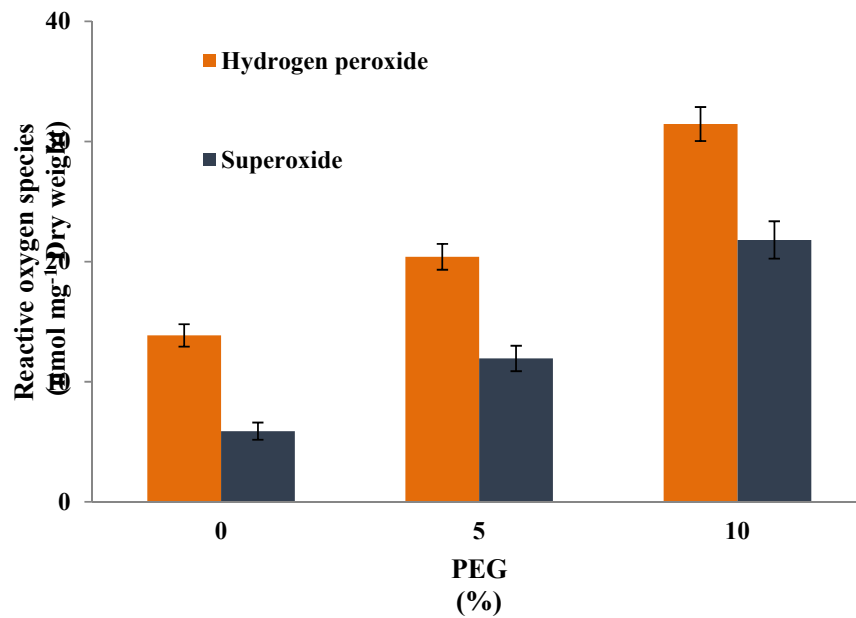
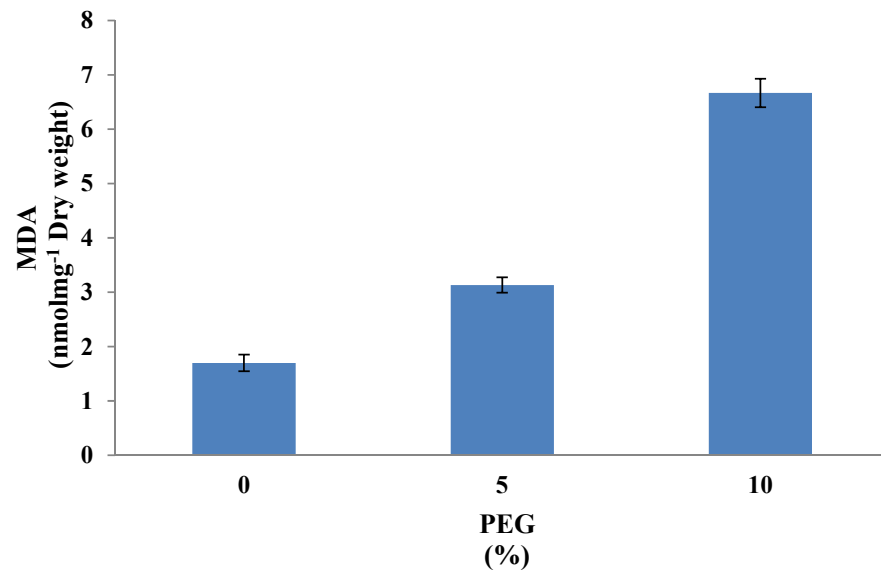


Fig.4.10. Differential accumulation of reactive oxygen species in *Anabaena* sp. in response to desiccation stress induced by polyethylene glycol

4.5 Differential accumulation of lipid peroxidation products and reactive oxygen species (ROS) in the cyanobacterium *Anabaena* sp. in response to different concentrations of polyethylene glycol.

4.5.1 MDA

Exposure of *Anabaena* sp. to polyethylene glycol resulted in the accumulation of lipid peroxidation products such as malondialdehyde (MDA) in the cells (Fig.4.9). Significant ($p < 0.05$) levels of MDA accumulation was observed at 10% polyethylene glycol. However, the cells grown at 5% polyethylene glycol showed the lowest increase in the lipid peroxidation products. Nearly 3 fold increase in MDA accumulation was observed when the cyanobacterium was grown at 10% polyethylene glycol and the results showed the marked increase in the lipid peroxidation at higher concentration of polyethylene glycol and induction of higher levels of lipid peroxidation.

4.5.2 Reactive oxygen species (H_2O_2 and O_2^-)

The response of cyanobacterium *Anabaena* sp. to varying levels of polyethylene glycol resulted in significant accumulation of H_2O_2 and O_2^- (Fig.4.10). The H_2O_2 production enhanced significantly ($p < 0.05$) in the cells exposed to 10% polyethylene glycol. Similarly, the cells exposed to 10% polyethylene glycol treatment also showed significant enhancement in the accumulation of superoxide ($p < 0.05$). Generation of ROS such as superoxides and peroxides may lead to oxidative stress and decreased growth potential under the influence of polyethylene glycol induced desiccation stress. Since the increased levels of superoxides and peroxides lead in turn to increased lipid peroxidation, their products such as MDA also accumulate in higher quantity.

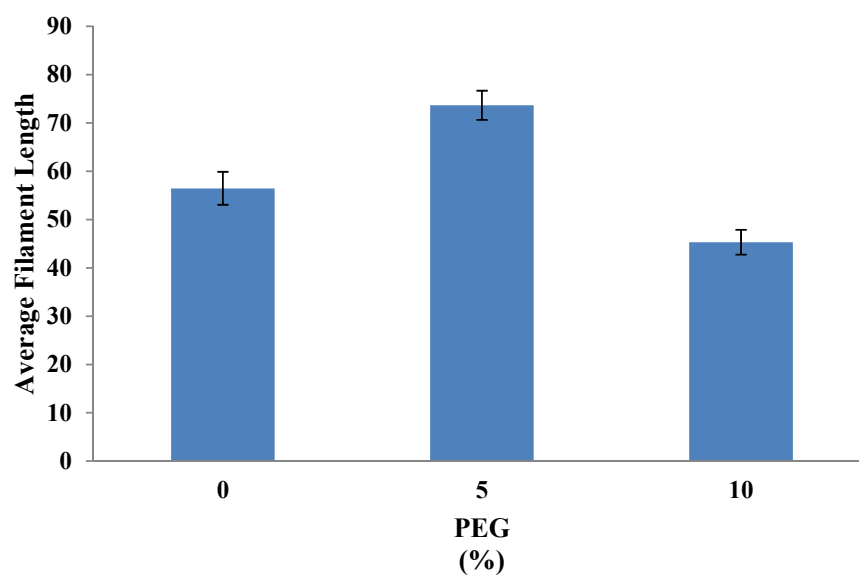


Fig.4.11. Variation in average filament length of the cyanobacterium *Anabaena* sp. exposed to different levels of polyethylene glycol

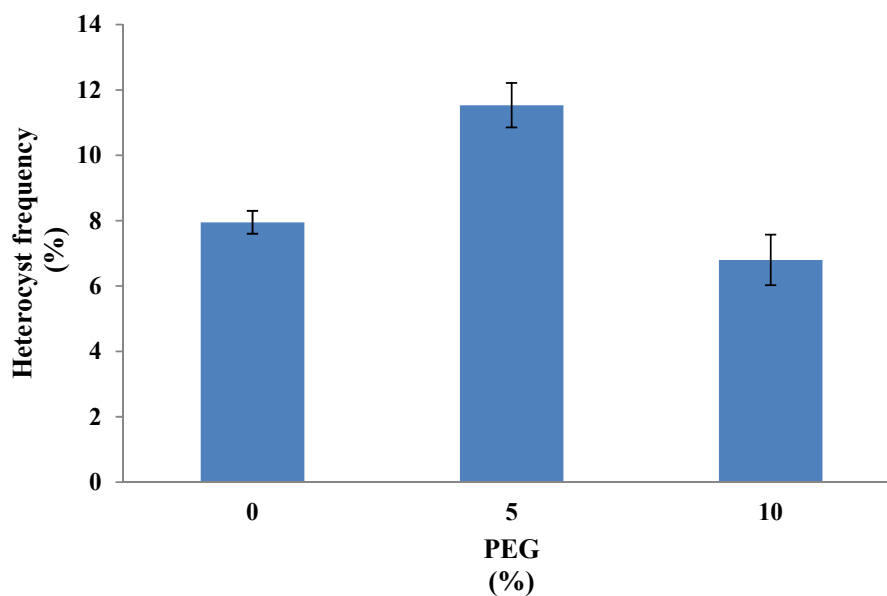


Fig.4.12. Effect of different concentrations of polyethylene glycol on heterocyst frequency of the cyanobacterium *Anabaena* sp.

4.6 Effect of varying concentrations of polyethylene glycol (0, 5 and 10%) on the average filament length, heterocyst frequency and nitrogen assimilation enzymes of the cyanobacterium *Anabaena* sp.

4.6.1. Average filament length

Significant differences in the average filament length of the experimental organism was observed in response to polyethylene glycol treatment ($p < 0.05$). *Anabaena* sp. grown at polyethylene glycol concentration of 5% polyethylene glycol recorded the highest filament length. The lowest filament length was observed in cells exposed to 10% polyethylene glycol (Fig.4.11). At 5% treatment, the cells showed an increase in filament length by 30.4%. However, at 10% polyethylene glycol treatment a decrease by 19.8% was noticed as compared to the cells grown without any added polyethylene glycol.

4.6.2. Heterocyst frequency

Heterocysts are the sites of nitrogen fixation and in the present study, we estimated the heterocyst frequency of cyanobacterium *Anabaena* sp. grown under various levels of polyethylene glycol (Fig.4.12). Maximum frequency of heterocyst was observed in cells exposed to 5% polyethylene glycol (11.5%) followed by 0% polyethylene glycol (7.9%) and the lowest frequency was noticed in cells grown at 10% polyethylene glycol (6.8%). These results showed significant changes in heterocyst frequency of the cyanobacterium due to desiccation stress induced by polyethylene glycol ($p < 0.05$).

4.6.3. Nitrate reductase

Nitrate reductase is the first enzyme involved in assimilation of nitrogen and it converts nitrate into nitrite. Significantly higher levels of nitrate reductase activity was shown by the cyanobacterium exposed to desiccation ($p < 0.05$). Nearly 1.5 fold increase in the nitrate reductase activity was shown by cells exposed to 5% polyethylene glycol compared to 0% polyethylene glycol treatment. However, in case of the cells receiving 10% polyethylene glycol treatment, its activity increased by 1.2 fold (Fig.4.13).

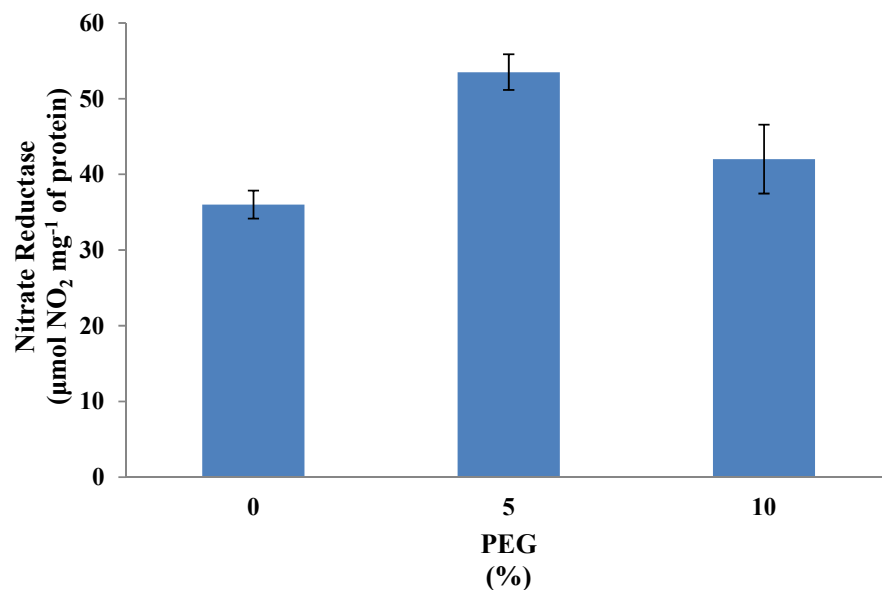


Fig.4.13. Activity of nitrate reductase enzyme in the cyanobacterium *Anabaena* sp. in response to different levels of polyethylene glycol induced desiccation stress

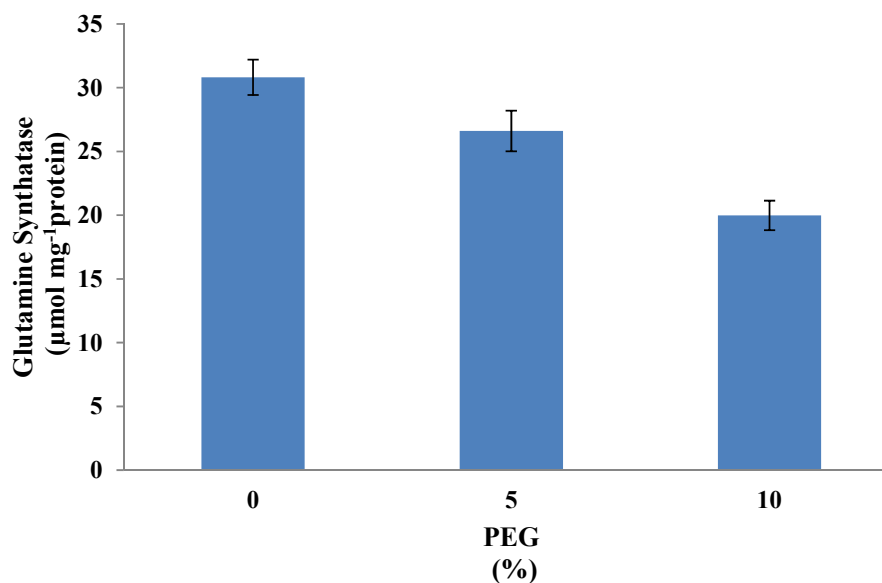


Fig.4.14. Activity of glutamine synthetase enzyme due to desiccation induced by different concentrations of polyethylene glycol on the cyanobacterium *Anabaena* sp.

4.6.4. Glutamine synthetase

The enzyme glutamine synthetase helps in incorporation of ammonia into glutamate to produce glutamine. It was observed that the glutamine synthetase activity decreased by 13.7% when the test cyanobacterium was exposed to 5% polyethylene glycol (Fig.4.14). Further reduction by 25% was at a higher concentration of 10% polyethylene glycol.

4.6.5. Nitrogenase

The nitrogenase enzyme converts molecular nitrogen into ammonia and its activity is important for the cyanobacteria. Significant reduction in the nitrogenase activity was noticed at 10% polyethylene glycol treatment as well as cells were not exposed to polyethylene glycol ($p < 0.05$). Interestingly, its activity increased by 27% in the cyanobacterium *Anabaena* sp. exposed to 5% polyethylene glycol (Fig.4.15). The results showed potential of *Anabaena* sp. grown at 5% polyethylene glycol to maintain nitrogen fixation at a moderate level of desiccation.

4.7 Impact of desiccation induced by polyethylene glycol on defense mechanism (enzymatic and non-enzymatic antioxidants) of the cyanobacterium *Anabaena* sp.

4.7.1. Superoxide dismutase

Superoxide dismutase activity in response to polyethylene glycol treatment is shown in Fig.4.16. Superoxide dismutase activity increased significantly ($p < 0.05$) due to increase in the concentration of polyethylene glycol in the BG-11 medium. The cells which were exposed to 5% polyethylene glycol showed 5.07 ± 0.26 U mg^{-1} dry weight whereas the cells exposed to 10% polyethylene glycol showed 6.83 ± 0.33 U mg^{-1} dry weight. The lowest activity was recorded in cells grown in the absence of polyethylene glycol 2.82 ± 0.21 U mg^{-1} dry weight.

4.7.2. Ascorbate peroxidase

The activity of ascorbate peroxidase enzyme which decomposes H_2O_2 , increased in response to increasing desiccation levels induced by polyethylene glycol (Fig.4.17). Significant enhancement in the ascorbate peroxidase activity was observed at 5% and 10% polyethylene glycol treatment ($p < 0.05$). Increment in ascorbate peroxidase activity was 49% at 5% polyethylene glycol whereas at 10% polyethylene glycol, the activity increased further by 152%. The cells grown without polyethylene glycol showed the lowest ascorbate peroxidase activity.

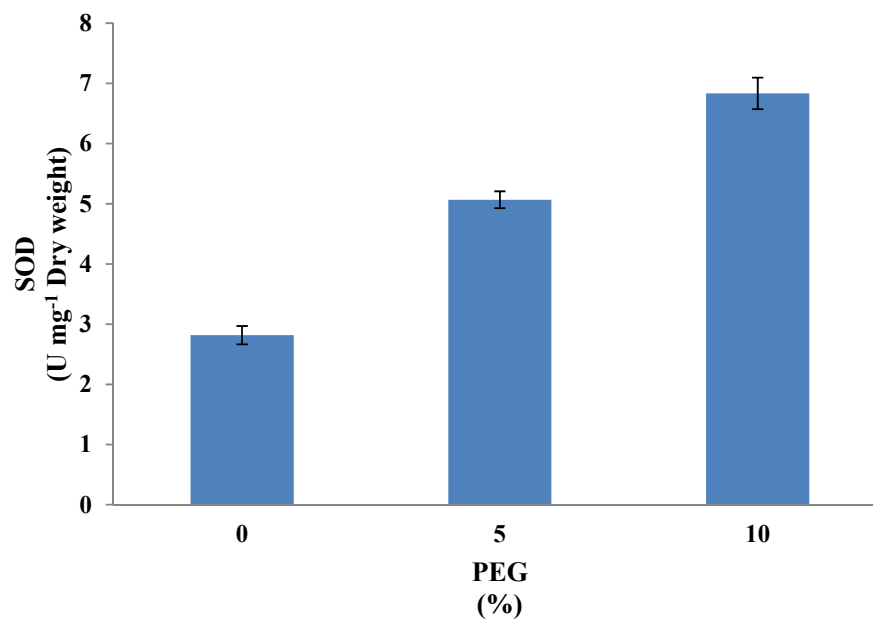
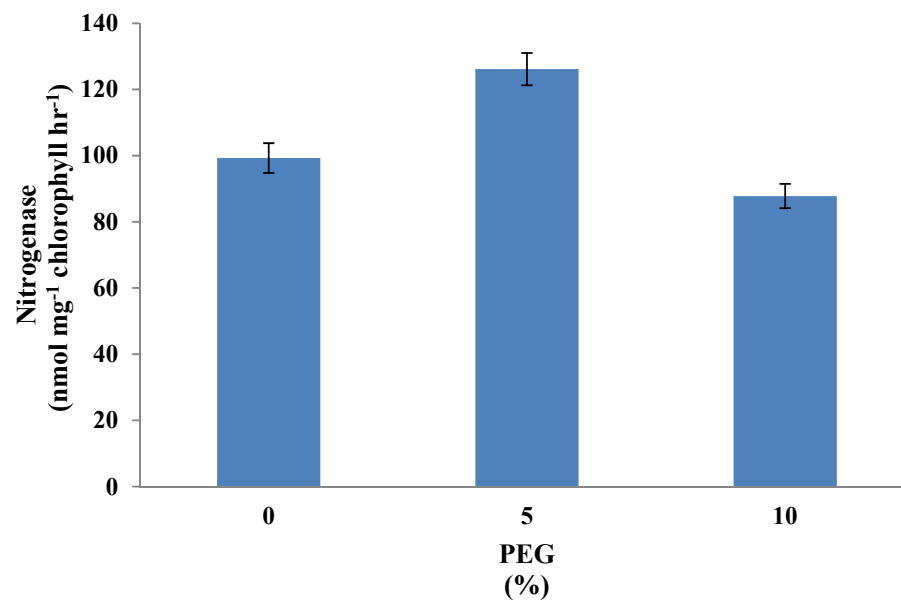


Fig.4.16. Differential activity of the enzyme superoxide dismutase at varying concentrations of polyethylene glycol in the cyanobacterium *Anabaena* sp.

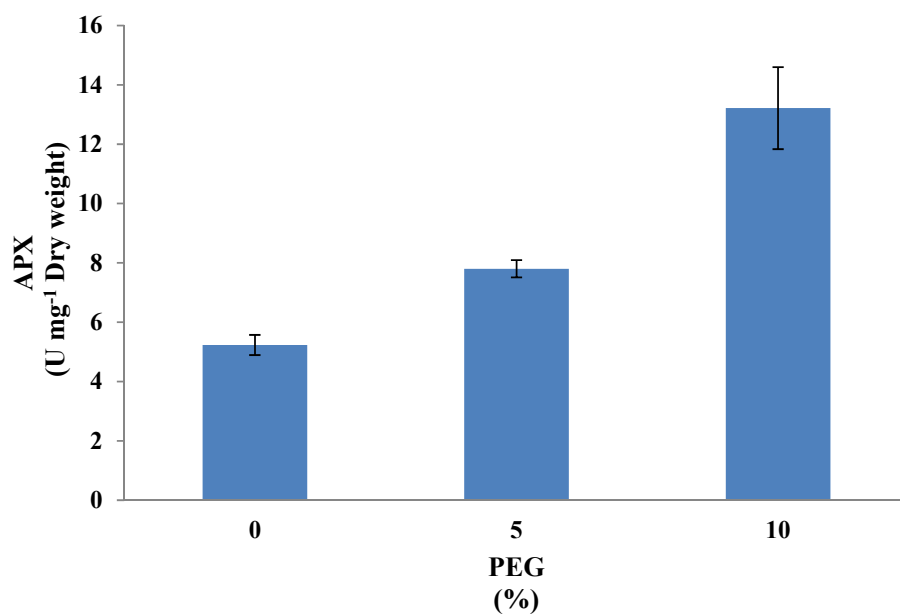


Fig.4.17. Activity of the enzyme ascorbate peroxidase in response to polyethylene glycol induced desiccation stress in the cyanobacterium *Anabaena* sp.

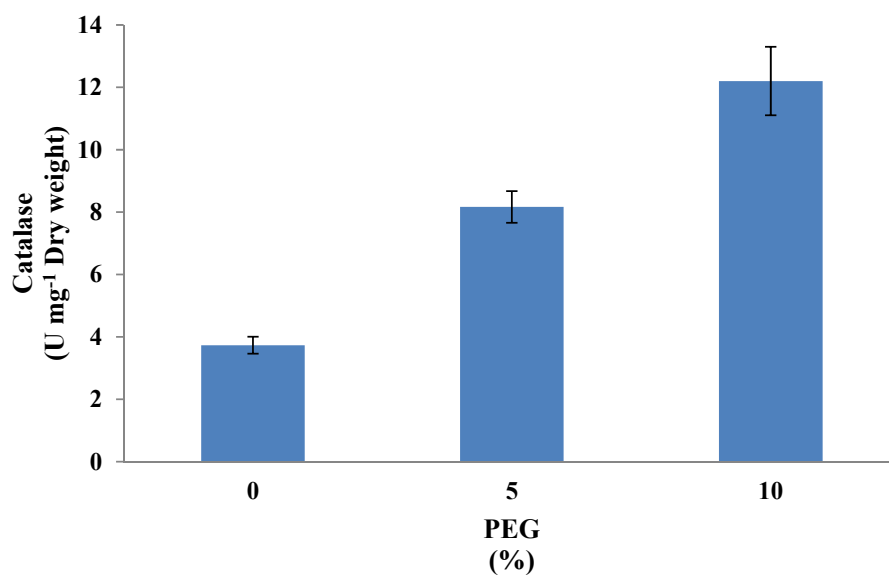


Fig.4.18. Response of the enzyme catalase enzyme activity in the cyanobacterium *Anabaena* sp. exposed to different concentrations of polyethylene glycol

4.7.3. Catalase

Catalase is another enzyme involved in the scavenging of H_2O_2 . Its activity also showed significant ($p < 0.05$) enhancement in response to desiccation induced by polyethylene glycol (Fig.4.18). The cells which were not exposed to polyethylene glycol showed the lowest catalase activity (3.73 U mg^{-1} dry weight). Upon exposure to 5% polyethylene glycol, the catalase activity increased to 8.17 U mg^{-1} dry weight. Further increase in the activity was noticed at 10% polyethylene glycol (12.2 U mg^{-1} dry weight). From the results it was quite evident that the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase, catalase enhanced significantly in response to stress imposed by desiccation. Enhancement in the activity of these enzymes shows efficient functioning of antioxidant machinery of *Anabaena* sp.

4.7.4. Proline content

Fig.4.19 shows the accumulation of proline in the cyanobacterium *Anabaena* sp. exposed to polyethylene glycol. Significant levels of proline accumulation were noticed in response to polyethylene glycol treatment ($p < 0.05$). More than 2 folds enhancement in the proline content was observed in the cells exposed to a polyethylene glycol concentration of 5%. On the other hand, cells exposed to 10% polyethylene glycol showed increase in the proline content by nearly 1.5 fold. This indicates the involvement of non-enzymatic antioxidant, proline in combating stress conditions induced by desiccation.

4.8 Qualitative evaluation of plant growth promoting activities of the cyanobacterium *Anabaena* sp. and the effect of polyethylene glycol treatment on them.

Plant growth promoting attributes

Plant growth promoting activities of cyanobacteria in the presence of polyethylene glycol were determined and it is depicted in Table 4.2. *Anabaena* sp. did not exhibit IAA activity and the same was observed for cultures grown at 5% and 10% polyethylene glycol. The ammonia production was estimated by ammonia release method using Nessler's reagent. Maximum release was observed at 5% followed by 0% as it was evident by intensity of yellow color produced on reaction of Nessler's reagent with ammonia in medium of growth. However, the cells incubated at 10% polyethylene glycol showed the lowest ammonia production. Similarly, the cells incubated at higher

concentration of polyethylene glycol showed the lowest phosphate solubilization activity as compared to the cyanobacterium grown at 0% and 5% polyethylene glycol. *Anabaena* sp. showed siderophore production as showed by formation of pale yellow zone around the colony. At 5% polyethylene glycol treatment, *Anabaena* sp. produced the largest zone and higher intensity of color followed by 10%. The cultures grown at 10% polyethylene glycol showed the lowest zone of all and lower intensity of color.

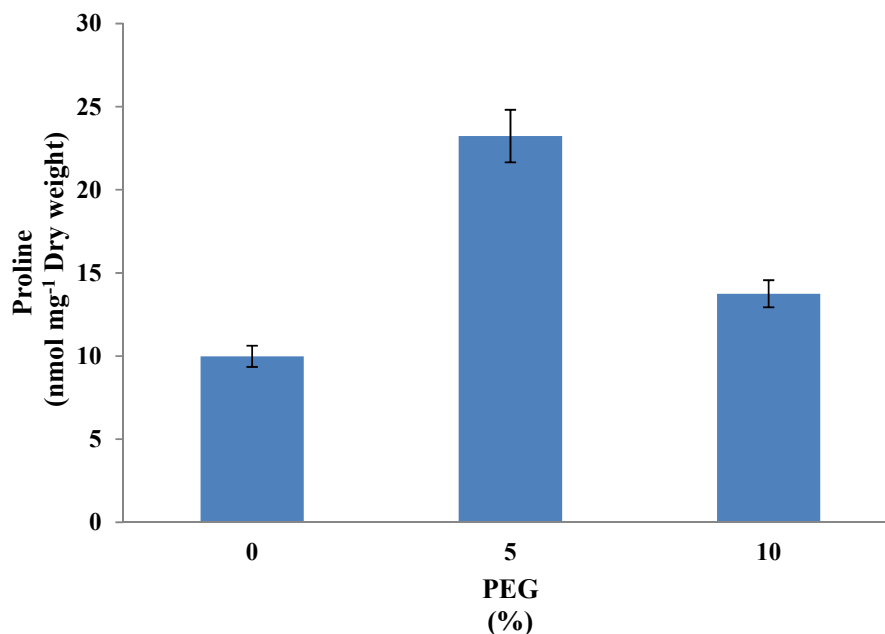


Fig.4.19. Accumulation of proline in the cyanobacterium *Anabaena* sp. in response to varying levels of polyethylene glycol

Table 4.7. Plant growth promoting attributes of the cyanobacterium *Anabaena* sp.

Sl. No.	PGPR attributes	0% PEG	5% PEG	10% PEG
1	Ammonia Production	+++	+++++	++
2	Siderophores	++++	++	+
3	IAA production	-	-	-
4	Phosphate solubilization	++++	++	+

Very low : +
Low : ++
Medium : +++

High : ++++
Very high : +++++
Absent : -----

Table 4.8. Fatty acid profile of the cyanobacterium *Anabaena* sp. in response to polyethylene glycol

Sl. No.	Fatty acids	Common name	Fatty acids (%)		
			PEG (0%)	PEG (5%)	PEG (10%)
1	C4:1	Butendioic acid	0.24	0.28	0.26
2	C8:0	Caprylic acid	1.16	1.4	1.2
3	C13:0	Tridecanoic acid	0.16	0.21	0.19
4	C14:0	Myristic acid	2.06	3.08	2.02
5	C14:1	Myristoleic acid	3.28	2.26	3.21
6	C15:0	Pentadecanoic acid	0.14	0.24	0.16
7	C16:0	Palmitic acid	26.01	30.6	28.1
8	C16:1	Palmitoleic acid	30.4	34.6	32.5
9	C16:2	Hexadecanoic acid	0.16	0.18	0.14
10	C17:0	Margaric acid	0.14	0.16	0.12
11	C17:1	Heptadecanoic acid	6.2	10.4	8.6
12	C18:0	Stearic acid	3.02	5.3	1.76
13	C18:1	Oleic acid	4.06	7.06	4.16
14	C18:2	Linoleic acid	1.06	3.14	1.42
ΣSFA			32.69	40.99	33.55
ΣMUFA			44.18	54.6	48.73
ΣPUFA			1.22	3.32	1.56
undetected			21.91	1.09	16.16

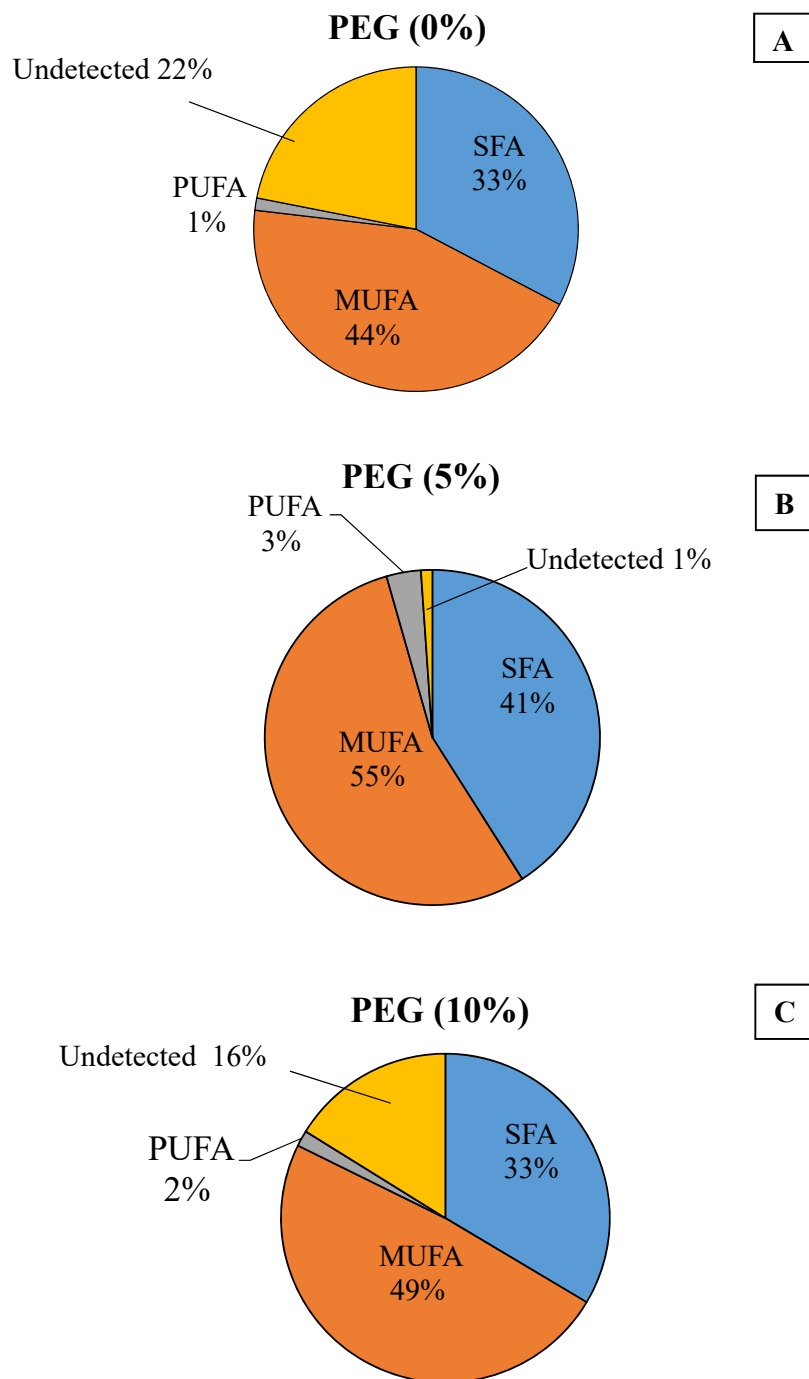


Fig.4.20 (a-c). The relative percent of monounsaturated fatty acids, saturated fatty acids and polyunsaturated fatty acids contents of the cyanobacterium *Anabaena* sp. in response to desiccation

4.9 Differential expression of different fatty acids in response to polyethylene glycol induced desiccation in the cyanobacterium *Anabaena* sp.

Fatty acid composition

Fatty acid composition of the cyanobacterium *Anabaena* sp. was studied. Table 4.3 shows the identified FAME profile of the test cyanobacteria. Optimal level of fatty acid was observed in *Anabaena* sp. growing at polyethylene glycol concentration of 5%. The major fatty acids identified in the present cyanobacteria were palmitic and palmitoleic acids. Fatty acids such as tridecanoic acid (C-13:0), pentadecanoic acid (C-15:0) and margaric acid (C-17:0) were found in trace amounts. Fig.4.20 (a-c) shows the relative percent of monounsaturated fatty acids, saturated fatty acids and polyunsaturated fatty acids contents of the cyanobacterium *Anabaena* sp. in response to desiccation. The cultures exposed to polyethylene glycol showed increased levels of saturated fatty acids. The contents of saturated fatty acids were 32.69, 40.99 and 33.55 at 0, 5 and 10% respectively. The percent of monounsaturated fatty acids was highest at 5% polyethylene glycol treatment followed by 10 and 0%. polyunsaturated fatty acids content of the cells however did not show significant changes irrespective of the treatment. However, cultures grown in the absence of polyethylene glycol showed lower levels of saturated fatty acids. Significant increase in the levels of mono unsaturated fatty acids was observed in the cyanobacterium *Anabaena* sp. exposed to 5% polyethylene glycol and 10% polyethylene glycol. The present study also revealed significant changes in the poly unsaturated fatty acids content

4.10 Effect of polyethylene glycol (5%) on differential expression (Up regulation / down regulation) of proteins in the cyanobacterium *Anabaena* sp. grown in BG-11 medium supplemented with T.I. salts.

2-DE

Proteome profiling of *Anabaena* sp. in response to desiccation stress was analyzed by conducting 2-DE in the cyanobacterium *Anabaena* sp. exposed to polyethylene glycol (5%) for 12 days. Master gel was obtained (Plate 4.5) after PDQuest analysis showing differentially expressed proteins of the cyanobacterium *Anabaena* sp. exposed to PEG (5%). Plate 4.6. shows the comparative protein profile of *Anabaena* sp. 2-DE analysis revealed that the proteins are distributed in acidic pH range of 4-7 and molecular range of 170 to 10KDa approximately. 265 protein spots were

detected through PD-Quest analysis. Protein spots showing significant changes are shown in Table 4.4. Out of 265 spots, 45 spots showed significant changes in response to desiccation stress induced by polyethylene glycol. Up regulation of 28 protein spots was observed at 5% whereas 17 spots were down regulated (Fig.4.21. Scatter plot). The differentially expressed proteins in *Anabaena* sp. based on pI (Isoelectric point) and molecular weight is depicted in Table 4.5.

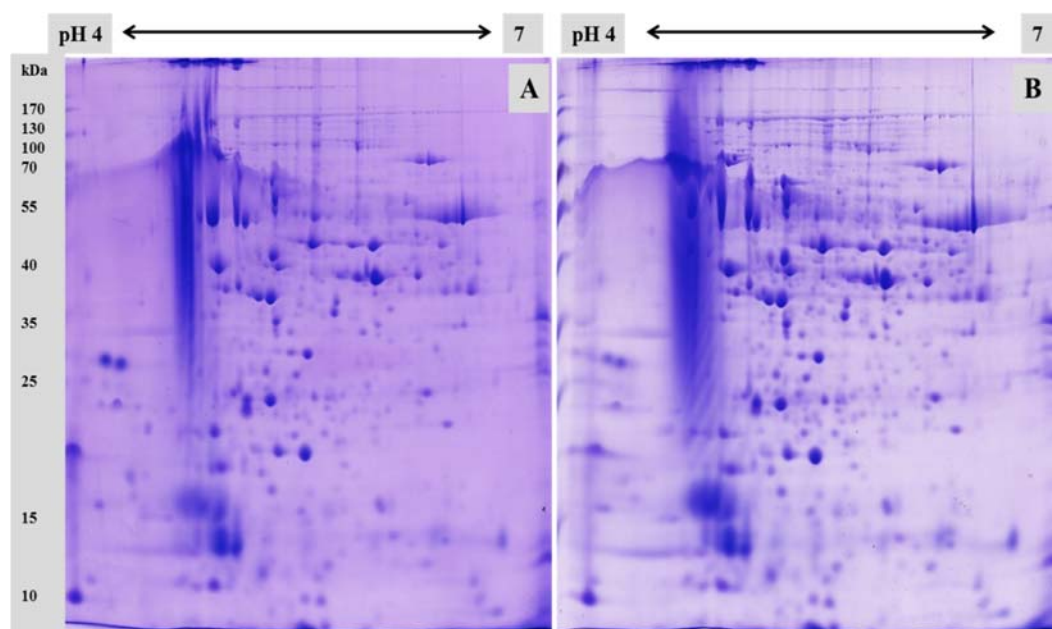


Plate 4.5. The 2DE image showing proteins of *Anabaena* sp. from control (A) and cells treated with 5% PEG (B). Gels were run in triplicate and proteins (600 µg) were loaded onto 17cm pH strip (4-7) with 12% vertical SDS PAGE for the second dimension

Table 4.9. Differentially expressed proteins observed in *Anabaena* sp. in response to polyethylene glycol (5%) treatment

Treatments	No. of variable spots	Quantitative changes	
		Up regulated	Down regulated
Control (0%)	0	0	0
PEG (5%)	45	28	17

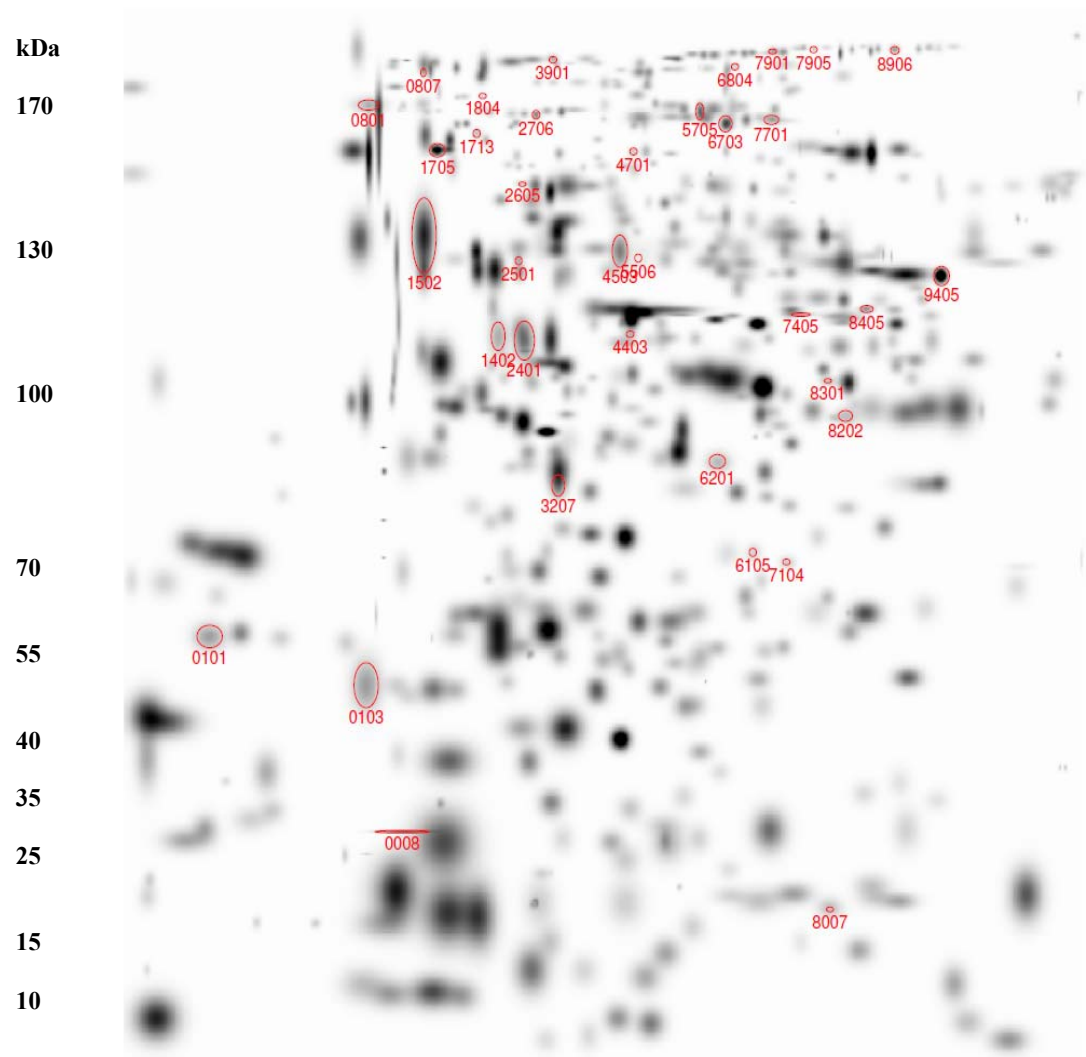
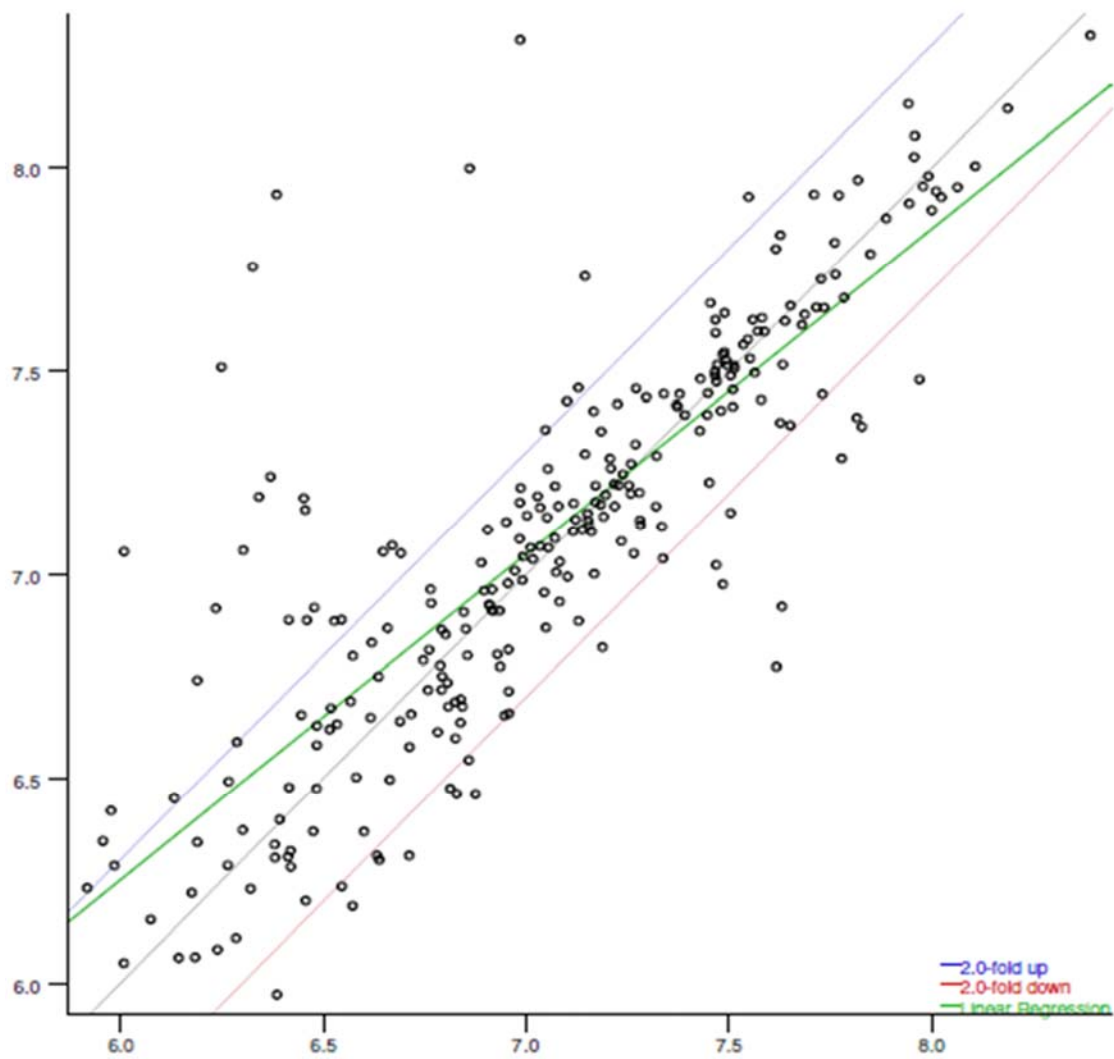


Plate 4.6. Master gel obtained after PDQuest analysis showing differentially expressed proteins of the cyanobacterium *Anabaena* sp. exposed to PEG (5%)



Polyethylene glycol (0 v/s 5 %) treatment

Fig.4.21. Scatter plot showing intensity of protein spots between polyethylene glycol treatments (0 and 5%) in the cyanobacterium *Anabaena* sp.

Table 4.10. Correlation analysis of the parameters (Linear distributed) in response to polyethylene glycol

	Polyethylene glycol
Polyethylene glycol	1.0000
Hydrogen Peroxide	0.9893
Malondialdehyde	0.9937
Superoxide dismutase	0.9976
Ascorbate peroxidase	0.9794
Catalase	0.9996
Glutamine Synthetase	-0.9919
Superoxide	0.9906

Table 4.11. Correlation analysis among different physiological and biochemical parameters without polyethylene glycol

	DW	CHLO	CARO	PC	PE	APC	Sugars	Protein	H2O2	MDA	SOD	APX	CAT	Proline	NR	GS	O ₂ ⁻²	NITR	HET	FIL	Fv/Fm
DW	1.000																				
CHLO	-0.478	1.000																			
CARO	-0.574	0.636	1.000																		
PC	0.349	0.087	0.087	1.000																	
PE	-0.636	-0.036	0.621	-0.389	1.000																
APC	0.026	-0.700	-0.465	0.249	0.034	1.000															
Sugars	0.146	-0.858	-0.605	0.011	0.095	0.923	1.000														
Protein	-0.519	-0.140	-0.177	-0.331	0.103	0.492	0.337	1.000													
H2O2	-0.428	-0.450	-0.110	-0.798	0.597	0.305	0.443	0.624	1.000												
MDA	-0.749	0.089	0.619	-0.105	0.897	0.217	0.179	0.147	0.388	1.000											
SOD	0.788	-0.281	-0.560	0.553	-0.849	0.203	0.097	-0.070	-0.506	-0.819	1.000										
APX	0.201	0.599	0.097	0.682	-0.581	-0.309	-0.448	-0.499	-0.941	-0.295	0.334	1.000									
CAT	0.133	-0.552	-0.636	-0.706	0.069	0.173	0.507	0.096	0.606	-0.096	-0.218	-0.515	1.000								
Proline	-0.318	-0.062	-0.473	0.030	-0.247	0.594	0.502	0.583	0.134	0.072	0.010	0.120	0.237	1.000							
NR	-0.592	0.141	0.780	-0.144	0.948	-0.078	-0.062	-0.120	0.310	0.913	-0.809	-0.311	-0.160	-0.336	1.000						
GS	-0.810	0.219	0.214	-0.589	0.597	0.017	0.110	0.320	0.549	0.675	-0.884	-0.270	0.406	0.433	0.498	1.000					
O ₂ ⁻²	0.349	-0.451	-0.727	-0.592	-0.208	0.006	0.345	-0.084	0.350	-0.370	-0.006	-0.277	0.946	0.176	-0.388	0.210	1.000				
NITR	0.496	-0.691	-0.267	0.624	-0.085	0.725	0.641	-0.049	-0.130	-0.004	0.520	-0.089	-0.177	-0.014	-0.053	-0.563	-0.225	1.000			
HET	0.890	-0.138	-0.214	0.402	-0.497	-0.326	-0.200	-0.831	-0.639	-0.605	0.573	0.423	-0.056	-0.555	-0.343	-0.733	0.193	0.284	1.000		
FIL	-0.323	-0.557	-0.042	-0.685	0.668	0.350	0.483	0.523	0.967	0.429	-0.452	-0.986	0.519	-0.034	0.406	0.404	0.251	0.060	-0.524	1.000	
Fv/Fm	-0.645	0.076	-0.015	-0.843	0.336	-0.032	0.013	0.745	0.798	0.203	-0.501	-0.611	0.423	0.306	0.085	0.652	0.261	-0.597	-0.777	0.641	1.000

Table 4.12. Correlation analysis among different physiological and biochemical parameters (5% polyethylene glycol)

	DW	CHLO	CARO	PC	PE	APC	Sugars	Protein	H2O2	MDA	SOD	APX	CAT	Proline	NR	GS	O ₂ ⁻²	NITR	HET	FIL	Fv/Fm
DW	1.000																				
CHLO	0.589	1.000																			
CARO	0.864	0.378	1.000																		
PC	-0.814	-0.142	-0.693	1.000																	
PE	-0.350	-0.646	-0.187	-0.213	1.000																
APC	-0.672	-0.753	-0.228	0.433	0.562	1.000															
Sugars	-0.745	-0.208	-0.949	0.695	-0.044	0.035	1.000														
Protein	-0.219	-0.138	0.204	0.418	-0.097	0.634	-0.330	1.000													
H2O2	0.026	-0.524	0.476	-0.199	0.431	0.700	-0.576	0.512	1.000												
MDA	0.485	0.372	0.378	-0.588	0.243	-0.331	-0.528	-0.072	-0.162	1.000											
SOD	-0.012	-0.500	-0.145	-0.102	-0.151	-0.060	0.226	-0.279	0.048	-0.516	1.000										
APX	0.065	-0.533	0.314	-0.516	0.828	0.475	-0.453	-0.042	0.757	0.163	-0.036	1.000									
CAT	0.406	0.224	0.268	-0.567	0.267	-0.366	-0.143	-0.709	0.033	0.110	-0.120	0.477	1.000								
Proline	-0.057	-0.508	-0.150	-0.127	0.028	0.002	0.280	-0.460	0.168	-0.657	0.879	0.205	0.275	1.000							
NR	0.358	0.789	0.425	-0.033	-0.239	-0.242	-0.404	0.272	-0.121	0.508	-0.895	-0.176	0.126	-0.840	1.000						
GS	-0.663	-0.046	-0.684	0.479	0.459	0.248	0.569	-0.114	-0.336	0.108	-0.577	-0.010	0.068	-0.406	0.223	1.000					
O ₂ ⁻²	-0.304	0.143	-0.099	0.376	0.155	0.302	0.170	0.078	0.193	-0.324	-0.562	0.185	0.416	-0.153	0.426	0.521	1.000				
NITR	0.198	-0.243	0.141	-0.354	0.103	-0.106	0.031	-0.559	0.279	-0.496	0.548	0.423	0.691	0.857	-0.485	-0.346	0.187	1.000			
HET	0.507	-0.173	0.763	-0.454	-0.087	0.189	-0.805	0.495	0.699	0.093	0.279	0.334	-0.221	0.103	-0.079	-0.832	-0.415	0.081	1.000		
FIL	-0.828	-0.448	-0.542	0.865	0.063	0.752	0.410	0.705	0.163	-0.426	-0.074	-0.216	-0.768	-0.194	-0.135	0.357	0.176	-0.485	-0.098	1.000	
Fv/Fm	-0.684	-0.694	-0.387	0.662	0.031	0.761	0.323	0.580	0.415	-0.719	0.410	-0.031	-0.640	0.322	-0.532	-0.065	0.024	-0.017	0.202	0.834	1.000

Table 4.13. Correlation analysis among different physiological and biochemical parameters (10% polyethylene glycol)

	DW	CHLO	CARO	PC	PE	APC	Sugars	Protein	H2O2	MDA	SOD	APX	CAT	Proline	NR	GS	O ₂ ⁻²	NITR	HET	FIL	Fv/Fm
DW	1.000																				
CHLO	0.745	1.000																			
CARO	-0.314	0.202	1.000																		
PC	0.346	0.250	-0.650	1.000																	
PE	-0.174	0.476	0.828	-0.264	1.000																
APC	0.581	0.535	0.171	0.093	-0.033	1.000															
Sugars	0.514	0.635	-0.350	0.457	0.136	-0.135	1.000														
Protein	-0.503	0.099	0.879	-0.303	0.848	0.033	-0.342	1.000													
H2O2	0.147	0.732	0.636	-0.208	0.839	0.169	0.479	0.528	1.000												
MDA	-0.661	-0.245	0.781	-0.787	0.496	-0.095	-0.426	0.654	0.411	1.000											
SOD	-0.674	-0.786	-0.356	-0.127	-0.365	-0.805	-0.059	-0.252	-0.409	0.210	1.000										
APX	-0.616	-0.766	-0.311	-0.252	-0.332	-0.857	-0.054	-0.281	-0.392	0.208	0.974	1.000									
CAT	-0.351	-0.097	0.556	-0.851	0.444	-0.420	-0.001	0.272	0.460	0.722	0.303	0.432	1.000								
Proline	-0.153	0.319	0.924	-0.682	0.862	0.005	-0.109	0.728	0.715	0.654	-0.336	-0.223	0.715	1.000							
NR	0.370	0.160	-0.457	0.562	-0.520	0.669	0.042	-0.362	-0.238	-0.367	-0.242	-0.406	-0.749	-0.677	1.000						
GS	-0.507	0.041	0.343	0.327	0.556	-0.101	-0.023	0.728	0.327	0.270	0.000	-0.149	-0.194	0.142	0.042	1.000					
O ₂ ⁻²	-0.113	0.352	0.553	-0.398	0.795	-0.430	0.407	0.444	0.748	0.388	-0.010	0.107	0.751	0.787	-0.798	0.147	1.000				
NITR	-0.045	0.071	-0.375	0.713	-0.195	0.219	0.235	-0.053	-0.034	-0.208	0.073	-0.145	-0.637	-0.598	0.767	0.570	-0.479	1.000			
HET	0.063	0.570	0.307	0.418	0.751	-0.074	0.436	0.562	0.590	-0.122	-0.389	-0.416	-0.130	0.359	-0.223	0.691	0.527	0.192	1.000		
FIL	0.337	0.013	0.078	-0.570	-0.360	0.513	-0.276	-0.351	-0.082	0.208	-0.189	-0.115	0.257	0.073	0.213	-0.754	-0.257	-0.367	-0.770	1.000	
Fv/Fm	0.632	0.448	-0.656	0.649	-0.189	-0.179	0.845	-0.595	0.025	-0.814	-0.062	-0.027	-0.303	-0.388	0.071	-0.227	0.122	0.123	0.320	-0.312	1.000

4.11 Correlation analysis

The Pearson product-moment correlation coefficient (r) was computed ($p < 0.05$) to assess the relationship among various physiological and biochemical parameters of the cyanobacterium *Anabaena* sp. exposed to various levels of polyethylene glycol. In case of most of the parameters, proportionate increase or decrease in variables was not found with increasing polyethylene glycol concentration from 0% to 5% and then 10%. To obey the assumptions of Pearson's correlation coefficient, variables must have linear relationship. Some parameters had proportionate changes with polyethylene glycol concentration such as accumulation of reactive oxygen species and lipid peroxidation products; production of antioxidant enzymes (directly proportional) and glutamine synthetase activity (reversely proportional). Therefore, only those parameters which obey the assumption were correlated with polyethylene glycol concentration. However, rest of the parameters was correlated among themselves separately at 0, 5 and 10% polyethylene glycol.

Table 4.6. shows the correlation analysis of the parameters with polyethylene glycol concentrations. It revealed that polyethylene glycol is most significantly and positively correlated with catalase ($r=0.999$) followed by superoxide dismutase ($r=0.997$), malondialdehyde content ($r=0.993$), superoxide radical ($r = 0.99$), hydrogen peroxide ($r = 0.989$) and ascorbate peroxidase ($r=0.979$). However, the glutamine synthetase activity was negatively correlated with polyethylene glycol concentration ($r = -0.991$). However, no linear relationship was observed between the rests of the parameters with respect to polyethylene glycol concentration. A correlation was worked out for different polyethylene glycol concentrations (0, 5 and 10%) individually (Table 4.7 – 4.9). At 0% polyethylene glycol treatment, the maximum positive correlation was observed between average filament length and hydrogen peroxide accumulation ($r=0.967$) followed by superoxide accumulation and catalase production ($r=0.965$). The maximum negative correlation at 0% polyethylene glycol was found between average filament length and production of ascorbate peroxidase ($r=-0.986$). Similarly, in case of treatment with 5% polyethylene glycol, the maximum positive correlation was recorded between proline accumulation and superoxide dismutase production with $r=0.879$ and the maximum negative correlation was between sugar and carotenoid synthesis with $r= -0.949$. At 10% concentration of polyethylene glycol,

production of superoxide dismutase showed maximum positive correlation with ascorbate peroxidase production ($r= 0.974$) and allophycocyanin showed the maximum negative correlation with production of ascorbate peroxidase ($r= -0.857$). All the abbreviations used in correlation table are listed in their expanded forms in Appendix 4.

Duncan multiple range test (DMRT) - post hoc test

A one way ANOVA (Table 4.10.) was conducted using SPSS software to compare the effect of different polyethylene glycol concentrations on various physiological and biochemical parameters of the cyanobacterium *Anabaena* sp. One way ANOVA was used to determine the significance ($p<0.05$ or $p<0.01$) on the effect of different concentrations of polyethylene glycol on various parameters. It was observed that the effect of various levels of polyethylene glycol was significant ($p<0.05$) for all the parameters except nitrate reductase and phycoerythrin.

Further, post hoc test was conducted and each requested post hoc test provided homogenous subset results and the groups were listed in order of their ascending means. The means that were listed under each subset comprised a set of means that were not significantly different from each other. Duncan multiple range test (DMRT) was conducted and the results are summarized as follows. Out of 21 parameters analyzed with DMRT, in 12 parameters, the mean score for treatments 0, 5 and 10% polyethylene glycol concentration were significantly different from each other which suggested that the effect of polyethylene glycol was significant on these parameters at all three treatments. Those parameters were dry weight, chlorophyll content, allophycocyanin, sugars, hydrogen peroxide, superoxide radical, malondialdehyde content, superoxide dismutase, catalase, proline accumulation, average filament length and chlorophyll fluorescence (F_v/F_m).

Further, the five parameters viz., phycocyanin, protein, nitrate reductase, nitrogenase and heterocyst frequency showed no significant differences in means between 0% and 10% polyethylene glycol treatments but the mean at 5% polyethylene glycol concentration was statistically significant from both 0% and 10% polyethylene glycol levels. This suggested that the effect of polyethylene glycol at 0% and 10% concentration on these parameters was statistically insignificant. However, in case of

ascorbate peroxidase and glutamine synthetase, it was found that the means at 0% and 5% polyethylene glycol concentration were grouped under the same subset implying statistical insignificance between these two treatments. But the mean score for 10% concentration was significantly different from the mean scores at 0 and 5% polyethylene glycol levels for ascorbate peroxidase and glutamine synthetase.

However, post hoc analysis of means of carotenoid production at various polyethylene glycol levels showed that the mean score at 0% polyethylene glycol was neither significantly different from 5% nor from 10%, but there was a significance difference in means at 5% and 10% polyethylene glycol concentration. It is noteworthy that the result of post hoc analysis for phycoerythrin production was altogether different from all other parameters as expected. Because one way ANOVA showed that there was no significant effect of polyethylene glycol on phycoerythrin. Therefore, DMRT grouped all the means for 0, 5 and 10% polyethylene glycol levels in the same subset implying there was no significant difference among the treatments (0, 5 and 10% polyethylene glycol).

In the Duncan Multiple Range Test results shown in Table 4.10.1- 4.10.u,

TRT = Treatment

1 = Without Polyethylene glycol

2= Polyethylene glycol (5%)

3= Polyethylene glycol (10%)

Table 4.14.a – 4.14.u. Analysis of various parameters of the cyanobacterium *Anabaena* sp. using Duncan multiple range test

4.10.a Dry weight

TRT		N	Subset		
			1	2	3
Duncan ^a	3	6	96.6000		
	1	6		1.2167E2	
	2	6			1.9192E2
	Sig.		1.000	1.000	1.000

4.10.b. Chlorophyll

TRT		N	Subset		
			1	2	3
Duncan ^a	3	6	6.5833		
	1	6		11.1667	
	2	6			16.8500
	Sig.		1.000	1.000	1.000

4.10.c. Carotenoids

TRT		N	Subset	
			1	2
Duncan ^a	3	6	3.6383	
	1	6	6.9550	6.9550
	2	6		10.3033
	Sig.		.079	.077

4.10.d. Phycocyanin

TRT		N	Subset	
			1	2
Duncan ^a	3	6	41.0833	63.2000
	1	6	48.0167	
	2	6		
	Sig.		.188	1.000

4.10.e. Phycoerythrin

TRT		N	Subset	
			1	2
Duncan ^a	3	6	5.5267	
	1	6	7.8217	
	2	6	9.6867	
	Sig.		.057	

4.10.f. Allophycocyanin

TRT		N	Subset		
			1	2	3
Duncan ^a	3	6	5.0567	8.4567	13.4250
	1	6			
	2	6			
	Sig.		1.000	1.000	1.000

4.10.g. Sugars

TRT		N	Subset		
			1	2	3
Duncan ^a	1	6	80.3833	1.0335E2	1.1942E2
	3	6			
	2	6			
	Sig.		1.000	1.000	1.000

4.10.h. Protein

TRT		N	Subset	
			1	2
Duncan ^a	3	6	44.3333	66.2333
	1	6	44.7333	
	2	6		
	Sig.		.939	1.000

4.10.i. H2O2

TRT		N	Subset		
			1	2	3
Duncan ^a	1	6	13.8500	20.4000	31.4500
	2	6			
	3	6			
	Sig.		1.000	1.000	1.000

4.10.j. MDA

		N	Subset		
			1	2	3
Duncan ^a	1	6	1.7000		
	2	6		3.7000	
	3	6			6.6667
	Sig.		1.000	1.000	1.000

4.10.k. superoxide dismutase

		N	Subset		
			1	2	3
Duncan ^a	1	6	2.8167		
	2	6		5.0667	
	3	6			6.8333
	Sig.		1.000	1.000	1.000

4.10.l. ascorbate peroxidase

		N	Subset	
			1	2
Duncan ^a	1	6	5.2333	
	2	6	7.8000	
	3	6		13.2167
	Sig.		.076	1.000

4.10.m. Catalase

TRT		N	Subset		
			1	2	3
Duncan ^a	1	6	3.7333		
	2	6		8.1667	
	3	6			12.2000
	Sig.		1.000	1.000	1.000

4.10.n. Proline

TRT		N	Subset		
			1	2	3
Duncan ^a	1	6	9.9833		
	3	6		13.7500	
	2	6			23.2333
	Sig.		1.000	1.000	1.000

4.10.o. Nitrate Reductase

TRT		N	Subset	
			1	2
Duncan ^a	1	6	36.0167	
	3	6	42.0167	
	2	6		53.5000
	Sig.		.233	1.000

4.10.p. Glutamine Synthetase

TRT		N	Subset	
			1	2
Duncan ^a	3	6	19.9833	
	2	6		26.6000
	1	6		30.8167
	Sig.		1.000	.072

4.10.q. Superoxide

TRT		N	Subset		
			1	2	3
Duncan ^a	1	6	5.8833		
	2	6		11.9333	
	3	6			21.8000
	Sig.		1.000	1.000	1.000

4.10.r. Nitrogenase

TRT		N	Subset	
			1	2
Duncan ^a	3	6	87.7833	
	1	6	99.2833	
	2	6		1.2613E2
	Sig.		.072	1.000

4.10.s. Heterocyst frequency

		N	Subset	
			1	2
Duncan ^a	3	6	6.8000	11.5333
	1	6	7.9500	
	2	6		
	Sig.		.293	1.000

4.10.t. Average Filament Length

		N	Subset		
			1	2	3
Duncan ^a	3	6	45.3000	56.4500	73.6333
	1	6			
	2	6			
	Sig.		1.000	1.000	1.000

4.10.u. Fv/Fm

		N	Subset		
			1	2	3
Duncan ^a	3	6	.4715	.5730	.6017
	1	6			
	2	6			
	Sig.		1.000	1.000	1.000

Several cyanobacterial strains have been isolated in the present study. In general, the isolated strains grew luxuriantly in the medium supplemented with or without polyethylene glycol. The strain S-1(2) formed evenly distributed suspension with blue color. Microscopic analysis revealed that the cells grew as straight filaments. Approximately 25 – 50 cells were found the filament. Cylindrical shaped vegetative cells (5.4-5.8 μm), intercalary and oval heterocysts were also observed in this strain. Akinete formation was observed and the position of the akinetes was away from the heterocyst. The morphological features of the strain resembled *Anabaena* sp. (Desikachari, 1959). Further, 16S rDNA amplification was carried out to support the morphological identification of the test cyanobacteria. Variable regions present in the 16S rDNA are highly conserved and allow identification of species (Fox et al., 1992). Nubel et al. (2000) observed characterization of cyanobacteria on the basis of structures such as hormogonia, heterocysts and akinetes. Therefore, homology in terms of percent similarity revealed through BLAST analysis provide additional information for identification (Prasanna, 2007)

In the present study, it was observed that the growth of cyanobacterium was influenced in a differential manner due to polyethylene glycol treatment. It was evident from the results that that maximum growth was supported by polyethylene glycol (5%). Cells grown in the absence of polyethylene glycol also showed decrease in the dry weight. In general, cyanobacteria exposed to desiccation showed decrease in growth as reported by Katoh et al. (2012). Inhibition in growth of cyanobacteria due to desiccation is also reported by Joset et al. (1996) and Apte et al. (1997). However, poor growth of cyanobacteria in the absence of polyethylene glycol may be attributed to lack of acclimation to desiccation free environment. Interestingly, cells were able to grow properly in case of treatment with 5% polyethylene glycol, probably due to the maintenance of optimal growth conditions. Mauch-Mani et al. (2017) reported that growth in already stressed conditions enabled cyanobacteria to grow optimally at lower levels of stress conditions. The differences in relative water content were observed in the test cyanobacteria exposed to polyethylene glycol. In response to polyethylene glycol treatment, *Anabaena* sp. displayed higher levels of resistance at 5% level. Relative water content is employed as an index of water retention capacity of the cells. Lin and Wu (2014) reported tolerance of algae and cyanobacteria to drought stress

through the estimation of relative water content. In the present study, we observed that the cyanobacterium *Anabaena* sp. exposed to 5% poly ethylene glycol exhibited comparatively better levels of relative water content as compared to other concentrations of polyethylene glycol.

Growth of the cyanobacterium *Anabaena* sp. under different treatments of polyethylene glycol showed inhibition in photosynthetic pigments such as chlorophyll and phycobiliproteins. Optimal chlorophyll content was observed at 5% polyethylene glycol whereas at 10% polyethylene glycol the chlorophyll content decreased. Cells grown in the absence polyethylene glycol also showed decrease in chlorophyll content. Chlorophyll content varies from sp. to sp. though it is considered as an index of biomass production (Boyer et al., 2009). Todd et al. (2008) reported that chlorophyll content is highly susceptible to changes caused by environment. There are also reports on the decrease in chlorophyll content of the cyanobacterium *Anabaena* sp. in response to osmotic stress (Sharma et al., 2012). The test cyanobacterium maintained at 5% polyethylene glycol level showed the highest carotenoid content whereas the cells exposed to 10% polyethylene glycol showed the lowest carotenoid content. Enhanced accumulation of carotenoid content was observed in cyanobacteria exposed to desiccation stress (Lin and Wu., 2014). Accumulation of carotenoids was found to increase in cyanobacteria exposed to stress conditions as reported by Joshi et al. (2018). It is therefore speculated that due to increase in the carotenoid content, the cyanobacteria, *Anabaena* sp. grown at 5% polyethylene glycol exhibited better growth response to stress induced by polyethylene glycol. The phycobiliproteins such as phycocyanin, phycoerythrin, allophycocyanin serve as accessory pigments in photosynthesis. Therefore, damage of phycobiliproteins caused due to desiccation induced stress may also result in reduction in photosynthetic efficiency. Prasad et al. (2005) observed that the pigment phycocyanin is sensitive to stress due to its localization on the intracellular thylakoid membrane. Lin and Wu (2014) also observed inhibition in the pigment synthesis of cyanobacteria due to desiccation induced by polyethylene glycol. Further, Sundaram and Soumya (2011) also observed reduction in pigment composition of cyanobacteria due to osmotic stress conditions. Therefore, the observed decrease in the pigment content in the cyanobacteria may lead to decrease in photosynthesis and consequently reduction in growth. However, the optimum levels of pigment content resulted in better growth probably due to maintenance of optimum levels of photosynthesis.

The soluble protein content of the test cyanobacteria, *Anabaena* sp. showed decreased protein content at 10% polyethylene glycol and also in the cells not receiving any polyethylene glycol treatment. Conflicting reports are available on the protein content in organisms exposed to stress (Assche et al., 1988; Jusu et al., 2004). Accumulation of stress proteins lead to increase in the protein content. Lan et al. (2010) observed increase in general protein content of organisms due to accumulation of specific stress proteins. Daly (2012) observed death of cells due to protein damage. A significant correlation was observed between desiccation and protein content in the cyanobacterium *Chroococcidiopsis* (Fagliarone et al., 2017). Desiccation stress induced by polyethylene glycol resulted in an increase in sugar content of the cyanobacteria, *Anabaena* sp. significant increase in the sugar content was observed at polyethylene glycol concentration of 5%. Accumulation of sugars is a consequence in response to desiccation (Sakamoto et al., 2009). In the cyanobacterium *Anabaena* sp. PCC 7120, desiccation resulted in enhanced sugar content. There are reports on accumulation of sugars especially, trehalose which prevent the *in vivo* aggregation of proteins (Fisher, 2006). In the present study, increased accumulation of proteins may be due to water deficit condition and it probably helps the cyanobacteria to overcome the desiccation stress.

The photosynthetic activity was measured in terms of photochemical efficiency (Fv/Fm). It was observed that the photosynthetic activity in the cyanobacterium *Anabaena* sp. was affected by desiccation. Bartak et al. (2016) observed that the primary photosynthetic process in the cyanobacteria, *Nostoc commune* was inhibited by dehydration. However, the cells exposed to 5% polyethylene glycol level maintained optimal photosynthetic activity. These results are in agreement with the observations of Kviderova et al. (2011). Normal photosynthesis exhibited by these cells grown at 5% polyethylene glycol indicates optimal rates of respiration and conservation of energy for growth and essential metabolic processes. Normal photosynthesis was observed in the cyanobacteria *Anabaena fertilissima* adapted to salt (Swapnil and Rai, 2018).

Increased lipid peroxidation was observed in the cells exposed to 5% and 10% polyethylene glycol. Increased content of MDA following lipid peroxidation also indicates oxidative stress in *Anabaena* sp. due to desiccation. Accumulation of lipid peroxidation of products has been reported in cyanobacteria exposed to various types of stresses including desiccation (Prasad et al., 2005; Sen et al., 2017). Exposing the

cyanobacterium *Anabaena* sp. to different concentrations of polyethylene glycol resulted in oxidative stress due to generation of reactive oxygen species such as superoxide radicals. We have also observed the formation of hydrogen peroxide in cells subjected to desiccation. The highest accumulation of superoxides and hydrogen peroxide was observed at higher polyethylene glycol levels of 10%. The generation of reactive oxygen species poses threat to cells due to oxidative damage. Generation of reactive oxygen species and oxidative damage was reported in cyanobacterium *Anabaena* sp. PCC 7120 subjected to desiccation (Sen et al., 2017). Therefore, in the present study, the peroxides and superoxide may inhibit metabolic activities due to disruption in electron transfer. Prasad et al (2005) reported that stress conditions disrupt electron transport in the cyanobacterium *Plectonema coryanum* under stress conditions.

Superoxide dismutase activity increased in cells exposed to 5 and 10% polyethylene glycol but not in cells which are not exposed to polyethylene glycol. In fact, the first line of defense against toxicity induced by reactive oxygen species is achieved by dismutation of superoxide radicals to hydrogen peroxide by the enzyme superoxide dismutase. Increase in superoxide dismutase activity therefore might be considered as part of the response mechanism in response to desiccation stress. Increase in the superoxide dismutase activity in the cyanobacterium *Anabaena* sp. thus indicates a role for superoxide dismutase as an enzyme involved in frontline defense. Enhanced activity of superoxide dismutase in response to stress conditions have been reported (Abd El- Baky et al., 2004). Yadav et al. (2016) observed up regulation of superoxide dismutase activity in the cyanobacterium *Anabaena* sp. exhibiting high levels of halotolerance. The activity of ascorbate peroxidase was also increased significantly due to polyethylene glycol treatment. The enzyme, ascorbate peroxidase is a potent scavenger of hydrogen peroxide, which reduces the accumulation and diffusion of peroxides (Sen et al., 2017). Swapnil and Rai (2018) observed increase in the activity of ascorbate peroxidase in the cyanobacterium *Anabaena fertilissima* exposed to salt. Our results indicate the role of ascorbate peroxidase in the cyanobacterium *Anabaena* sp. in response to desiccation. Though, Mutsuda et al. (1996) reported that the presence of ascorbate peroxidase is limited in cyanobacteria, its activity was observed by Rozen et al. (1992). Similar to superoxide dismutase and ascorbate peroxidase enzyme activity, the cyanobacterium *Anabaena* sp. also showed higher levels of catalase activity. The enzyme catalase prevents peroxidative damage to the cellular constituents

by preventing the diffusion of peroxides across biological membranes. Therefore, in the present study, enhancement in the catalase activity probably attributed to substantial accumulation of peroxides in the cells. Induction of the enzyme catalase in response to osmotic stress was observed (Chakravarty et al., 2016). Further the presence of genes encoding catalase enzyme was identified in the cyanobacterium *Anabaena* sp. PCC 7120 through gene analysis. Sen et al. (2017) also showed the prominent role played by catalase in the cyanobacterium *Anabaena* sp. PCC 7120 under desiccation. Significant increase in cellular content was observed in the cyanobacterium *Anabaena* sp. in response to desiccation. Intracellular accumulation of proline in response to stress has been reported to provide protection against stress conditions (Wu et al., 1998). Molinari et al. (2007) observed that the drought tolerant plants accumulated more proline than normal plants. Lin and Wu (2014) also observed higher levels of proline accumulation in soil algae and cyanobacteria in response to drought. Accumulation of proline was found to be triggered by the production of reactive oxygen species (Tripathi and Gaur, 2004). Increase in the intracellular proline content was also observed in the cyanobacterium *Cylindrospermum* sp. exposed to salt stress (Chris et al., 2006). Based on the results, it may be concluded that the accumulation of proline has an adaptive significance in the cyanobacterium *Anabaena* sp. exposed to desiccation as it may reduce lipid peroxidation through free radical accumulation and consequently minimize lipid peroxidation. Defense mechanisms that operate in cells may vary but it appears in the cyanobacterium *Anabaena* sp. exposed to desiccation, both enzymatic and non-enzymatic antioxidants operate in a coordinated fashion to adapt to the stress conditions.

The activity of enzymes related to nitrogen assimilation was also studied. It was observed that the activity of nitrate reductase, the primary enzyme involved in nitrogen assimilation increased in response to desiccation induced by polyethylene glycol. However, the key enzyme involved in assimilation of ammonia decreased in response to desiccation. Enhanced accumulation of nitrate was observed in response to osmotic stress conditions induced by salinity (Rai and Tiwari, 1999). Swapnil et al. (2015) reported increase in the nitrate reductase activity in the genetically transformed cyanobacterium *Anabaena* sp. PCC 7120 due to osmotic stress. There is an increase in demand for nitrogen in cyanobacteria exposed to salinity (Reddy et al., 1989). Supplementation of nitrogen enhanced the growth of *Anabaena* sp. exposed to salt

stress. Increase in nitrate reductase activity in the test cyanobacterium may be therefore due to increase in the demand for nitrogen to cope with metabolic activities. Atmospheric nitrogen is the major source of nitrogen for all nitrogen fixing organisms. However, the glutamine synthetase activity decreased due to desiccation in the test cyanobacteria. Reduction in glutamine synthetase activity was observed in wild type cyanobacterium of *Anabaena* sp. PCC 7120 exposed to salt (Swapnil et al., 2015). Inhibition of glutamine synthetase activity was reported in the cyanobacterium *Synechocystis* sp. DUN52 (Warr et al., 1984). Reduction in the glutamine synthetase activity is expected to slow down the accumulation of ammonia and may hamper the growth. The nitrogen fixing enzyme was optimal at 5% polyethylene glycol whereas it was inhibited significantly at treatment with 10% polyethylene glycol. Inhibition of nitrogenase activity was reported in cyanobacteria due to osmotic stress conditions (Fernandez and Apte, 2000; Moisander et al., 2002). Decrease in the filament length and heterocyst frequency was observed in *Anabaena* sp. at higher concentration of polyethylene glycol. Heterocysts are the sites of nitrogen fixation and inhibition of nitrogen fixation due to damage by stress conditions have been reported in cyanobacteria (Tel-Or and Melhamed-Harel., 1981; Blumwald et al., 1983). Disturbed electron transport and loss of protective ability of heterocysts to prevent oxygen from entering them are the reasons for decrease in nitrogen fixation. There is also a possibility of the presence of heterocysts which appear normal under the microscope but have defective physiology. This may be one of the reasons for reduction in nitrogenase activity of the cyanobacteria, *Anabaena* sp. exposed to desiccation.

The isolated cyanobacterium *Anabaena* sp. was tested for plant growth promoting activities and it showed phosphate solubilization, ammonia production and siderophore production. However, the isolate did not show the production of IAA. The high levels of plant growth promoting activities may be due to the occurrence in the stress habitat. Karthikeyan et al. (2007) evaluated plant growth promoting attributes of cyanobacteria for wheat crop. Similarly, Prasanna et al. (2009) conducted studies on the plant growth promoting cyanobacteria which can be used as inoculants for rice crop. One of the known mechanisms of plant growth promoting cyanobacteria is nitrogen fixation and consequent release of ammonia into soil. In addition, plant growth promotion is also linked to phytohormone and siderophore production. Several plant growth promoting rhizobacteria have been shown to minimize the drought stress in

plants through these activities (Niu et al., 2018). Although the results confirmed that the cyanobacterium *Anabaena* sp. showed plant growth promoting activities, further research is needed to elucidate the mechanisms involved. Drought tolerant cyanobacteria with plant growth promoting potential are the potential candidates in restoration of desertified habitats. Therefore, the findings of our research can be useful in developing cyanobacterial bio-inoculants for desiccation stress management in plants.

Differential levels of fatty acid accumulation were observed in the cyanobacterium *Anabaena* sp. exposed to desiccation. Pal et al (2011) observed differential accumulation of saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids in the cyanobacterium *Nannochloropsis* sp. in response to stress conditions. Increase in the unsaturation of fatty acids in membrane lipids was found to be associated with stress tolerance to salinity (Allakhverdiev et al., 2001). In the cyanobacterium *Synechococcus* sp. PCC 7942, the composition of fatty acid changed significantly in response to oxidative stress (Verma et al., 2018). Maintaining the integrity of membrane is a key factor associated with desiccation tolerance (Carpenter and Crowe, 1989). Therefore, the observed changes in fatty acid composition of the cyanobacterium *Anabaena* sp. exposed to desiccation indicate the role of fatty acid in stabilization of cell membrane. Modulation of fatty acid composition thus plays an important role in desiccation tolerance of the test cyanobacteria.

2-DE analysis showed the differential expression of proteins was observed in the cyanobacterium *Anabaena* sp. At 5% polyethylene glycol treatment, several proteins were found to be up regulated. Further, it was observed that elaborate changes occurred in the proteome profile of the cyanobacteria due to desiccation. Yadav et al. (2016) observed differential expression of proteins in the cyanobacterium *Anabaena* sp. due to osmotic stress. Stress proteins of the cyanobacteria can be evaluated using proteomic approach (Basu and Apte, 2012). The up regulated protein may help in countering the stress induced due to desiccation in the test cyanobacteria. The proteins may belong to photosynthesis, energy metabolism, chaperonins, DNA damage repair, etc. Sen et al. (2017) also identified proteins belonging to the above-mentioned category in *Anabaena* sp. PCC 7120 exposed to desiccation. Further, detailed analysis of the proteins may lead to identification of differentially expressed proteins involved in desiccation tolerance of the cyanobacterium *Anabaena* sp.

Therefore, in the present study, we have been able to decipher the role of polyethylene glycol on the growth of the cyanobacterium, *Anabaena* sp. the findings of the present study is in agreement with the findings of previous workers. Results show the ability of the cyanobacterium to tolerate desiccation level upto 5% due to polyethylene glycol. The results also show that the plant growth promoting potential of the identified cyanobacterium. This finding is important for the development of bio-inoculants for the management of abiotic stress in plants. Further the observation that several proteins are differentially regulated in response to desiccation may help us to decipher the epigenetic and metabolic changes induced by polyethylene glycol. Isolation and identification of desiccation tolerant cyanobacterium may help in improving the nitrogen economy of desiccated habitats. Further, promising and potential cyanobacterial strains could also be used to enhance the plant growth under desiccation stress conditions.

Desiccation stress is one of the most deleterious abiotic stresses that have increased in its severity in the recent times. This has a serious bonding on food security of the world population. In the coming years, drought is expected to cause serious problems for growth of the plants as well as microorganisms. A number of microorganisms inhabit the rhizosphere forming a complex community and influencing growth of the plants and productivity through a range of metabolic activities and plant interactions. Some of the microbial communities play a significant role in the mitigation of abiotic stresses on plants. In this context, the cyanobacteria or blue green algae, the Gram –ve prokaryotes which can grow under photoautotrophic conditions are important. Although the cyanobacteria are not dominant microorganisms in soil, they have a significant effect on soil fertility and productivity. Very few attempts have been made to understand the impact of desiccation on growth and physiological attributes of cyanobacteria exposed to desiccation. Identification of desiccation tolerant cyanobacteria helps in designing strategies to exploit them as bio-inoculants in ecological restoration programs. Therefore, in the present study, attempts were made to isolate, screen and decipher the mechanisms in response to desiccation.

The isolated cyanobacterium was identified as *Anabaena* sp. based on the morphological attributes and 16 S rDNA analysis. *Anabaena* sp. was maintained in BG-11 medium supplemented with Turk Island salt and in the presence or absence of polyethylene glycol. The filament length of cyanobacteria was maximum in the cells grown under 5% polyethylene glycol treatment whereas the filaments of cells expose to 10% polyethylene glycol showed the lowest length. Similarly, the heterocyst frequency was highest at 5% polyethylene glycol treatment followed by 0% and 10%. The growth of cyanobacteria measured as increment in the dry weight showed differential response and the cells grown at 5% polyethylene glycol showed the best growth response. Significant differences in the relative water content (RWC) were observed in the test cyanobacterium exposed to various levels of polyethylene glycol. The chlorophyll content decreased significantly in the cells exposed to 10% polyethylene glycol whereas at 5% polyethylene glycol the cells showed optimal chlorophyll content. The pigments such as phycocyanin, phycoerythrin and allophycocyanin contents also showed a differential response to polyethylene glycol treatment. The carotenoid content was also found to increase in response to

polyethylene glycol treatment (5%). Exposure to higher concentration of polyethylene glycol (10%) resulted in significant reduction in the protein content of the cyanobacterium *Anabaena* sp. the cyanobacteria showed optimal growth at 5% polyethylene glycol and showed a significant increase in the sugar content. The photosynthetic activity was measured and significant inhibition in the photosynthetic activity was observed at 10% polyethylene glycol treatment. Cells exposed to 5% polyethylene glycol maintained optimal photosynthetic efficiency.

Exposure of the cyanobacterium *Anabaena* sp. to polyethylene glycol resulted in the accumulation of lipid peroxidation products such as malondialdehyde (MDA). Nearly 3 fold increases in the MDA content was observed in the cells exposed to 10% polyethylene glycol as compared to lower levels of MDA accumulation in cells grown at 5%. Enhanced levels of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) was also observed in cells exposed to various levels of polyethylene glycol. Significant enhancement in the H_2O_2 and O_2^- accumulation was noticed in case of the cells exposed to 10% polyethylene glycol. The activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) also showed significant enhancement due to exposure to polyethylene glycol. The accumulation of proline was found to enhance significantly in response to polyethylene glycol exposure in the cyanobacterium *Anabaena* sp.

Nitrogen assimilation parameters such as nitrate reductase, glutamine synthetase and nitrogenase were studied. While increase in the nitrate reductase activity was observed in *Anabaena* sp. at 5% polyethylene glycol, the cells exposed to 10% also showed enhancement in the nitrate reductase activity probably due to increase in demand for nitrogen. However, the activity of primary enzyme responsible for ammonia assimilation (glutamine synthetase) decreased. Significant decrease in the nitrogenase activity was observed in the cells treated with 10% polyethylene glycol but the cells maintained at 5% polyethylene glycol showed increase in the nitrogenase activity. A correlation could not be made between the heterocyst frequency and nitrogenase activity and in turn the nitrogen fixation due to physiologically defective heterocysts.

The isolated cyanobacteria maintained at different levels of polyethylene glycol showed plant growth promoting activities such as ammonia production, siderophore production and phosphate solubilisation. Differential levels of these activities were

observed at various concentrations of polyethylene glycol. However, the cyanobacterium *Anabaena* sp. did not show any IAA activity. These results showed potential of the isolate *Anabaena* sp. as a candidate with plant growth promoting activities. Significant changes in the fatty acid profile were observed in the test cyanobacterium upon exposing to various levels of polyethylene glycol. Major fatty acids identified were palmitic acid, palmitoleic acid.

Differential expression of proteins was observed in the cyanobacterium *Anabaena* sp. exposed to polyethylene glycol (5%). Out of the 265 protein spots, 45 spots showed significant changes in response to desiccation stress induced by polyethylene glycol. Further, 28 protein spots were found to be up regulated due to polyethylene glycol (5%) whereas 14 spots were down regulated. Further, analysis of these proteins will review the precise role of these differentially expressed proteins.

The experimental organism *Anabaena* sp. displayed differential levels of tolerance to desiccation induced by polyethylene glycol. Desiccation stress influenced growth, cellular constituents, photosynthetic activity, accumulation of lipid peroxidation products, antioxidant enzymes, enzymes related to nitrogen metabolism, plant growth promoting activities and fatty acid profile. The study also revealed the differential expression of proteins in response to stress induced by desiccation. Understanding of the physiological mechanisms in response to drought in cyanobacteria will help in dissecting the tolerance mechanisms at the molecular level. Better understanding of the tolerance mechanisms as well as the identification of promising drought tolerant cyanobacteria will help in the restoration of decertified soils.

**PHYSIOLOGICAL ATTRIBUTES OF DESICCATION TOLERANCE IN A
NITROGEN FIXING CYANOBACTERIUM ISOLATED FROM SOIL
BIOLOGICAL CRUSTS**

In the present study, soil samples have been collected from soil biological crusts from different localities of Rajasthan, India. From these soil samples, cyanobacterial strains able to grow at desiccation levels of 5 and 10% induced by polyethylene glycol were isolated. Based on the dry weight accumulation and chlorophyll content, a potential strain was selected for further investigations. Based on the morphological attributes, the isolated cyanobacterium was identified as *Anabaena* sp. The 16S rRNA gene analysis showed amplification product of 1500 bp. Further, the isolate was maintained at varying polyethylene glycol concentrations of 0, 5 and 10%. Differential growth response, relative water content, pigments (phycocyanin, phycoerythrin, allophycocyanin), protein and sugar contents were observed in response to polyethylene glycol treatment. The photosynthetic efficiency of the cyanobacterium also showed differential response to desiccation. Differences in the filament length and heterocyst frequency were also observed in *Anabaena* sp. in response to polyethylene glycol. Nitrate reductase activity increased significantly in the cyanobacterium grown at 5% polyethylene glycol whereas glutamine synthetase activity showed decrease upon increasing the polyethylene glycol concentration. Increase in the nitrogenase activity was observed at 5% polyethylene glycol. Exposure to polyethylene glycol increased the accumulation of lipid peroxidation products, superoxides and hydrogen peroxide in the cyanobacterium. The isolated cyanobacterium *Anabaena* sp. exhibited plant growth promoting attributes such as ammonia production, phosphorus solubilization and siderophore production. However, the isolate did not exhibit IAA production. The isolate *Anabaena* sp. also showed significantly higher levels of antioxidant enzyme activity such as superoxide dismutase, ascorbate peroxidase, catalase and non-enzymatic antioxidant proline in response to polyethylene glycol treatment. *Anabaena* sp. maintained optimal levels of fatty acid content at polyethylene glycol concentration of 5%. Polyethylene glycol treatment (5 and 10%) increased the level of monounsaturated fatty acids. 2-DE analysis of *Anabaena* sp. exposed to 5% polyethylene glycol exhibited significant changes in the protein profile and differential expression of proteins were observed. The results showed the ability of the cyanobacterium *Anabaena* sp. to grow at higher desiccation levels induced by polyethylene glycol. Further, characterization of the organism is required to understand its potential in mitigating the desiccation stress.

मृदा के जैविक परत से प्राप्त हुआ नाइट्रोजन स्थिरीकारक नील हरित शैवाल में शुष्कता सहनशीलता की कार्याकीय विशेषताएँ

प्रस्तुत अध्ययन में, राजस्थान के विभिन्न शुष्क स्थानों से मृदा के जैविक परत से मृदा के नमूने एकत्र किए गए थे। इन मृदा के नमूनों से, पॉलीइथिलीन ग्लाइकोल द्वारा उपचारित 5 और 10% के शुष्कता के स्तर पर संवृद्धि करने में सक्षम नील हरित शैवाल उपभेदों को पृथक्करण किया गया। तदुपरांत, शुष्क वजन संचय और पर्णहरित परिमाण के आधार पर, आगे की अध्ययन के लिए एक सर्वश्रेष्ठ उपजाति का चयन किया गया। रूपात्मक विशेषताओं के आधार पर, पृथक नील हरित शैवाल की पहचान एनाबिना उपजाति के रूप में की गई। 16S rRNA जीन विश्लेषण ने 1500 बेसपेयर का प्रवर्धन उत्पाद दिखाया। आगे, 0, 5 और 10% की पॉलीइथिलीन ग्लाइकोल सांद्रता में उपजाति को बनाए रखा गया था। पॉलीइथाइलीन ग्लाइकोल उपचार की वजह से वृद्धि, सापेक्ष जल परिमाण, वर्णक (फाइकोसाइनिन, फाइकोरोथ्रिन, एलोफाइकोसायनिन), प्रोटीन और कुल घुलनशील शर्करा की परिमाण में भिन्नता देखी गई। नील हरित शैवाल की प्रकाश संश्लेषक दक्षता ने भी शुष्कता के लिए अंतर प्रतिक्रिया दिखाई। एनाबिना उपजाति में सूत्र की लंबाई और हेटेरोसीस्ट आवृत्ति में अंतर भी देखा गया। 5% पॉलीइथाइलीन ग्लाइकोल में उगाए गए नील हरित शैवाल में नाइट्रेट रिडक्टेस गतिविधि में काफी वृद्धि पाई गई जबकि पॉलीइथाइलीन ग्लाइकोल की सांद्रता में वृद्धि होने पर ग्लूटामाइन सिंथेटेज़ गतिविधि में कमी होने लगी। 5% पॉलीइथाइलीन ग्लाइकोल की सांद्रता पर नाइट्रोजनेज गतिविधि वृद्धि दर्ज की गई। पॉलीइथिलीन ग्लाइकोल की सांद्रता ने नील हरित शैवाल में लिपिड परॉक्सीडेशन उत्पादों, सुपरऑक्साइड और हाइड्रोजन परॉक्साइड में वृद्धि दर्ज की गई। नील हरित शैवाल एनाबिना उपजाति ने अमोनिया उत्पादन, फास्फोरस घुलनशीलता और साइडरोफोर उत्पादन पाया गया जो पौधे की वृद्धि के लिए आवश्यक हैं। हालांकि, उपजाति ने IAA उत्पादन का प्रदर्शन नहीं किया। पॉलीइथिलीन ग्लाइकोल उदभासन से प्रति -आक्सीकारक एंजाइम जैसे कि सुपरऑक्साइड डिस्म्यूटेस, परॉक्सीडेस तथा कैटालेस की क्रियाशील मात्रा और प्रोलीन परिमाण में सार्थक रूप से उच्च वृद्धि पाई गयी। एनाबिना उपजाति ने पॉलीथीन ग्लाइकोल 5% पर फैटी एसिड परिमाण का उच्चतम स्तर पाया गया। पॉलीइथिलीन ग्लाइकोल के द्वारा उपचारित करने पर (5 और 10%) मोनोअनसैचुरेटेड फैटी एसिड की मात्रा बढ़ गई। 2-DE विश्लेषण से पता चला कि 5% पॉलीइथाइलीन ग्लाइकोल की सांद्रता पर एनाबिना उपजाति का प्रोटीन परिच्छेदिका में महत्वपूर्ण बदलाव हुए और प्रोटीन में अंतर अभिव्यक्ति देखी गई। प्रस्तुत अध्ययन में पॉलीइथाइलीन ग्लाइकोल की सांद्रता में वृद्धि होने पर नील हरित शैवाल एनाबिना उपजाति पर पड़ने वाले प्रभाव को कम करने का अपनी क्षमता को दिखाया गया। एनाबिना उपजाति के द्वारा शुष्कता तनाव को कम करने की क्षमता को समझने के लिए उन्नत अध्ययन की आवश्यकता है।

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APPENDICES

Appendix 1

Composition of BG11 basal medium

Constituents	Concentration (g/L)
Sodium nitrate	1.50
Di potassium hydrogen phosphate	0.04
Magnesium sulphate	0.075
Calcium chloride	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA (di sodium magnesium salt)	0.001
Sodium carbonate	0.02
Micronutrient solution (A5 solution)	1 ml
pH	7.1- 7.3

Composition of T.I. Salts

Constituents	Concentration (g/L)	Concentration ($\frac{1}{4}$ th strength)(g/L)
Potassium chloride	0.1675	0.04188
Magnesium chloride	1.375	0.34375
Calcium chloride	0.3675	0.09188
Sodium chloride	7.04	1.76
Magnesium sulphate	1.73	0.4325

APPENDIX 2

Extraction Buffer

HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulphonic acid)

(pH 7.5) 50 mM

Sucrose (w/v) 40%

β -mercaptoethanol (v/v) 1%

Double distilled water

Precipitation buffer

Ammonium acetate (w/v) 0.1M

Methanol 100%

Washing Buffer

Acetone (v/v) 80%

Solubilization Buffer

Urea (w/v) 7M

Thiourea (w/v) 2M

CHAPS (w/v) 2%

Quantification (Bradford, 1976)

Bovine serum albumin Stock (BSA) 1mg/ml

APPENDIX 3

IPG Strips

Immobilized pH gradient Strips 17 cm 4-7 pH linear

Rehydration

Dithiothreitol (DTT) (w/v)	50mM
Ampholine (Bio-lyte) (v/v)	0.2% (3-10 pH)

IEF (Iso-electric focusing)

IEF Program

Step	Voltage	Gradient	Current (μAmp)	Value	Unit
1	200	Rapid	50	02:30	HH:MM
2	500	Rapid	50	00:30	HH:MM
3	1000	Rapid	50	00:30	HH:MM
4	8000	Linear	50	01:00	HH:MM
5	8000	Rapid	50	48000	V: HH
6	500	Rapid	50	20:00	HH:MM

Equilibration Buffer:-

Reduction Buffer

Composition	Quantity
Tris-HCl (pH 8.8) (w/v)	50 Mm
Urea (w/v)	6 M
Sodium dodecyl sulfate (SDS) (w/v)	2%
Glycerol (v/v)	30%

Dithiothreitol (DDT) (w/v)	130 mM
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Alkylation Buffer

Composition	Quantity
Tris-HCl (pH 8.8) (w/v)	50 Mm
Urea (w/v)	6 M
Sodium dodecyl sulfate (SDS) (w/v)	2%
Glycerol (v/v)	30%
2-Iodoacetamide (w/v)	135 mM

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Preparation

Stock Solution

S No.	Composition	Quantity
1	Tris-HCl (pH 8.8) (w/v)	1.5 M
2	Acrylamide/Bis acrylamide (w/v)	30%
3	SDS* (w/v)	10%
4	Ammonium per sulfate (APS)* (w/v)	10%
5	TEMED (v/v)	0.040%
6	Double distilled water (v/v)	

*Freshly prepared

pH maintained 8.8 using 1N HCl and final volume make up 100 ml and store at 4°C

Preparation of acrylamide/bis acrylamide

Acrylamide (w/v)	29.10 g
Bis acrylamide (w/v)	0.8 g
Double distilled water	

Final volumes make up 100 ml and store at 4°C

Preparation of Agarose

Composition

Agarose (w/v)	0.5%
Bromophenol blue (w/v)	0.01%
Double distilled water	

Running Buffer (Electrophoresis Buffer) pH 8.3

Composition (10X)	Quantity
Tris (w/v)	250 mM
Glycine (w/v)	1920 mM
SDS (w/v)	1%
Double distilled water	

pH maintained by 1N HCl and finally volume make up 1000 ml and store in room temperature

Staining Solution

Composition

Coomassie Brilliant Blue (CBB) (w/v)	0.1%
Methanol (v/v)	40%
Glacial acetic acid (v/v)	10%
Double distilled water (v/v)	50%

Distaining Solution

Composition

Methanol (v/v)	20%
Glacial acetic acid (v/v)	05%

Double distilled water (v/v)

75%

APPENDIX 4

DW	- Dry weight
CHLO	- Chlorophyll
CARO	- Carotenoid
PC	- Phycocyanin
PE	- Phycoerythrin
APC	- Allophycocyanin
H ₂ O ₂	- Hydrogen Peroxide
MDA	- Malondialdehyde
SOD	- Superoxide dismutase
APX	- Ascorbate peroxidase
CAT	- Catalase
NR	- Nitrate reductase
GS	- Glutamine synthetase
O ₂ ⁻²	- Superoxide
NITR	- Nitrogenase
HET	- Heterocyst frequency
FIL	- Filament length
Fv/Fm	- Chlorophyll fluorescence
PEG	- Polyethylene glycol
TRT	- Treatment