A DISSERTATION ON

Effect Of Chromium Uptake and Differential Cr Tolerance In *Spirulina* Strains To Combat Abiotic Stress And Its Potential As A Fertilizer

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
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IN PARTIAL FULFILMENT
FOR THE
DEGREE OF MASTER OF TECHNOLOGY
IN BIOTECHNOLOGY

BY
SIMRA NAEEM KHAN
B. Tech-M. Tech Biotechnology (X Semester)
Roll No: 1801101005

UNDER THE SUPERVISION OF

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering

INTEGRAL UNIVERSITY, DASAULI, KURSI ROAD
LUCKNOW- 226026

DECLARATION FORM

I, **Simra Naeem Khan**, a student of **B.Tech.- M.Tech (Dual Degree) Biotechnology** (5^{th}Year/ 10^{th} Semester), Integral University have completed my six months dissertation work entitled **"Effect of chromium uptake and differential chromium tolerance in *Spirulina* strains to combat abiotic stress and its potential as a biofertilizer"** successfully from **Algal biochemistry and stress biology laboratory, Integral information and research Centre (IIRC-1) Integral University Lucknow,** under the able guidance of **Dr. Alvina Farooqui Professor and Head.**

I, hereby, affirm that the work She been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Name and Signature of Student with Date

Name and Signature of Course Coordinator with Date

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE

Certificate that Ms. **Simra Naeem Khan** (Enrollment Number **1800101895**) has carried out the research work presented in this thesis entitled **"Effect of chromium uptake and differential chromium tolerance in Spirulina strains to combat abiotic stress and its potential as a biofertilizer"** for the award of **B.Tech - M.Tech (Dual Degree) Biotechnology** from Integral University, Lucknow under our supervision. The thesis embodies results of original work and studies carried out by the student himself and the contents of the thesis do not form the basis for the award of any other degree to the candidate or to anybody else from this or any other University/Institution. The dissertation was a compulsory part of her **B.Tech- M.Tech (Dual Degree) Biotechnology.**

I wish her good luck and bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Ms. Simra Naeem Khan,** a student of **B.Tech-M.Tech (Dual Degree) Biotechnology** (5th Year/10th Semester), Integral University has completed her six months dissertation work entitled "Effect of chromium uptake and differential chromium tolerance in Spirulina strains to combat abiotic stress and its potential as a biofertilizer" successfully. She has completed this work from **Algal biochemistry and stress biology laboratory, Integral information and research Centre (IIRC-1) Integral University Lucknow**, under the guidance of **Dr. Alvina Farooqui** Professor and Head. The dissertation was a compulsory part of her **B.Tech- M.Tech (Dual Degree) Biotechnology.**

I wish her good luck and bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering & Information Technology

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Ms. Simra Naeem Khan** a student of **B.Tech-M.Tech Dual Degree Biotechnology (5th year/10th semester)**, has completed her six months dissertation work entitled **"Effect of chromium uptake and differential chromium tolerance in *Spirulina* strains to combat abiotic stress and its potential as a biofertilizer"** successfully. She has completed this work from (Department of Bioengineering, Integral University, Lucknow) under the guidance **of Dr. Alvina Farooqui**. This dissertation was a compulsory part of her **B.Tech.-M.tech Dual Degree Biotechnology** degree.

I wish her good luck and bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering
Integral University, Lucknow

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Simra Naeem Khan

Table of Content

List of Tables

List of figures

ABBREVIATIONS

GHG	Greenhouse gas emissions
SPR	Spirulina
Cr	Chromium
Sp.	Species
Chl-a	Chlorophyll -a
Chl-b	Chlorophyll-b
HS	Hydrogen Sulphide
ROS	Reactive oxygen species
MDA	Malonaldehyde
OD	Optical density
PBS	Phosphate buffer

1. Introduction

Cyanobacteria are filamentous and non-heterocyst gram-negative prokaryotic bacteria that are able to produce their own food with the help of photosynthesis. They have aerobic mode of respiration and possess photoautotrophic mode of nutrition. They are found in a variety of agroecosystems such as paddy fields, as well as from Antarctica to the Arctic poles (Pandey et al., 2004). They meet their own nitrogen needs through nitrogen (N_2) fixation and create certain bioactive substances that boost crop development, protect crops from diseases, and enhance soil nutrient status. Its size ranges from 1 to 100µm. It possesses a primordial nucleus and lacks membrane-bound organelles. Cyanobacteria were once thought of as algae, hence its widespread name is "blue-green algae," and share many traits with eukaryotic algae, such as physical traits and ecological niches (Stanier, R. Y., & Cohen-Bazire, G. (1977). Cyanobacteria lack a membrane-bound nucleus, mitochondria, Golgi apparatus, chloroplasts, and endoplasmic reticulum, much like all other prokaryotes. Many have sheaths that help form colonies of cells or filaments. Chlorophyll a, and b are the two types of photosynthetic green pigment found in cyanobacteria. Phycobilin (blue pigment), phycoerythrin (a red pigment), carotenoids (yellow pigment) (Pagels et al., 2021). The distinctive blue-green hue that gives these creatures their well-known moniker is created when phycobilin and chlorophyll combine. The largest prokaryotic organisms are cyanobacteria, which have a size range of 0.5 to 60 micrometres. They are quite widespread and widely distributed in fresh water, where they can be found as both plankton and benthos members. They are also widely distributed in settings like coral reefs, tidal spray zones, and tide pools; a few species are even found in the ocean plankton. On land, cyanobacteria are widespread in soil which is at least one metre (39 inches) deep (Castenholz et al., 2015). They are also known to thrive on damp surfaces of rocks and trees, where they take the form of cushions or layers. It splits the water molecule to liberate oxygen while using water as an electron-donating substrate. They are also known as nitrogen fixer as they are able to fix the environmental free nitrogen into the plant roots.

Cyanobacteria possess a specialised structure known as heterocyst. These are pale yellow in colour and significantly expanded in size. Heterocysts can form individually or in chains, with terminal or intercalary positions. Under anaerobic conditions, it works as a nitrogen fixation site (converts N_2 to NH_3). They are typically found in filamentous cyanobacteria such as *Nostoc*, *Oscillatoria*, *Spirulina*, *Microcystis*, and *Anabaena*. Cyanobacteria are important in rice

farming because of their nitrogen-fixing abilities and other beneficial impacts on soil and plants. Plants are subjected to two forms of stress: biotic and abiotic. Abiotic stress is an environmental element that is imposed on plants as a consequence of physical or chemical stress, whereas biotic stress happens with the mean of biological mean exposed to crop plants, such as bacteria, virus, fungus, insects, and pest. It has a potential to improve agricultural production and reduce GHG emissions (Singh et al., 2011). It was recently postulated that cyanobacteria might be important bio-agents in the ecological regeneration of damaged area. Cyanobacteria are especially beneficial for wastewater treatment since they can breakdown a variety of hazardous chemicals, including pesticides. Cyanobacterial biomass is an efficient bio-fertilizer source for improving deteriorated soil physicochemical features such as water-holding capacity and mineral nutrient status. Cyanobacteria are distinguished by their widespread distribution, quick generation period, and capacity to fix atmospheric N_2. Cyanobacteria, like other prokaryotic bacteria, are being used as bio-inoculants to improve soil fertility and environmental quality. Genetically modified cyanobacteria have been designed with unique genes for the production of a variety of bio-fuels such as bio-diesel, bio-hydrogen, bio-methane, and syngas, and therefore provide new pathways for the economically sustainable creation of bio-fuels. The present study was designed in a way to evaluate the stress tolerance efficiency of *Spirulina* strains under Chromium (Cr (VI)) stress. For this we have calculated the enzymatic and non-enzymatic activity of spirulina under (VI) stress. Owing to the plausible results, *Spirulina* strains have emerged as a potential biofertilizer for paddy crops grown in Cr (VI) contaminated areas.

2. Objectives

1) Maintenance and Growth study of selected *Spirulina* strains.
2) Study of the extent of Chromium (Cr (VI)) tolerance by selected *Spirulina* strains.
3) Estimation of Free radicals' generation under Cr (VI) stress by checking enzymatic and non- enzymatic activity.
4) To check the extent of immobilized Cr (VI) tolerant Spirulina strain as a biofertilizer on paddy crop growth in pot experiment.

3. Review of literature

3.1. Cyanobacteria

Cyanobacteria are the most primitive, gram-negative photosynthetic organisms of the kingdom Monera and division Cyanophyta. They are widely distributed and feature a basic prokaryotic cellular structure. They are the world's first oxygenic phototrophs, with only bacteria outnumbering them in terms of dispersion (Whitton, B. A., & Potts, M. (2012). Cyanobacteria were most likely the earliest creatures to produce oxygenic photosynthesis, and its putative fossil genesis dates back 3.46 billion years. However, the earliest clear fossil cyanobacteria are around 2 billion years old and developed in tidal-flat sedimentary rocks. The old earth's oxygen-free atmosphere suffered a major influence around 2.32 billion years ago with the sudden increase of oxygen via the commencement of oxygenic photosynthesis (Rasmussen *et al*., 2008). Prior to the formation of respiration, oxygen was exceedingly hazardous to life, and as a result, the first worldwide calamity for most living things on Earth occurred. Cyanobacteria may now be found in practically all biomes and ecosystems on the planet (Whitton and Potts, 2000). However, in order to properly colonise terrestrial ecosystems, biological creatures must be able to resist significant desiccation. Most living forms on Earth are killed by air-drying because it severely damages membrane structure, proteins, and nucleic acids (Whitton, B. A., & Potts M. 2012) Throughout their long evolutionary history, cyanobacteria have developed a marvel known as anhydrobiosis, the ability of their cells to experience complete dehydration during air-drying without being killed; this is one of the drought resistance systems also known as "desiccation tolerance" (Alpert et al., 2005). As a result, cyanobacteria colonised an increasing proportion of the accessible terrestrial environments. When the importance of the microclimate for tiny creatures' living circumstances was discovered, it became clear that, in terms of temperature, sunlight-exposed soils or rock surfaces are among the harshest terrestrial environments for photoautotrophic organisms. Cyanobacteria may be found on almost all exposed rock surfaces on Earth, either epilithically (on the rock surface) or endolithically (within the photic zone of the rock). Cyanobacteria can be found as biofilm (thin, smooth layers of pro- and eukaryotic algae only) or biological crusts (thick, uneven layers, often including lichens and sometimes bryophytes) on rocks in hot deserts (Beudel and Wessels, 1991) polar deserts (Broady 1981). Many extremophiles have developed to be able to survive at pH extremes. At low pH, cyanobacteria predominate and are even seen in acid lakes (pH 4.1-5). A

study conducted by Steinberg in 1998 (Steinberg et al., 1998) confirmed the presence of filamentous cyanobacteria, Chroococcus turgidus, Limnothrix sp., Mastigocladus sp., Oscillatoria sp., Spirulina sp. (pH 2.9), and Synechococcus sp. (pH 4) in acid lakes in Germany.

3.2. Habitats

When the importance of the microclimate for tiny creatures living circumstances was discovered, it became clear that, in terms of temperature, sunlight-exposed soils or rock surfaces are among the harshest terrestrial environments for photoautotrophic organisms. Cyanobacteria may be found on almost all exposed rock surfaces on Earth. Cyanobacteria can be found on inselbergs in savannas as biofilms (thin, smooth layers of pro- and eukaryotic algae only) or biological crusts. (Boison et al., 2004) detected it in temperate locations, (Horath and Bachofen 2009) in the alpine zone, and even on the surfaces of man-made stone buildings (Karsten et al., 2007). Cyanobacteria are widely distributed and may be found in virtually all environmental settings on Earth, revealing their lengthy evolutionary history. It has been stated that they create thick mats near the brine's bottom. These mats contain Microcoleuschthonoplastes and Oscillatoria species, as well as additional cyanobacterial components such as Aphanocapsa marina, Lyngbya aestuarii, and Spirulina subsalsa as minor partners (Newman et al., 2017). Cyanobacteria have been characterised as covering thermal waters all over the planet, with a projected top limit of 65°C to 68°C (Setchell, 1903). In hot springs, frequent species include Mastigocoleuslaminosus, Phormidiumtenue, and Synechococcus elongates var. amphigranulatus. Cyanobacteria may also resist cold temperatures; Phormidiumsp. has been found in ice layers in Antarctic lakes. Calothrix and Rivularia are frequent cyanobacteria found in maritime Arctic locations but Gloeocapsa and Nostoc are numerous in freshwaters. (Broady et al., 1996) discovered over 700 taxa of nonmarine algae in Antarctica, with the flora was dominated by species of Anabaena, Aphanocapsa, Calothrix, Chroococcidiopsis, Gloeocapsa, Lyngbya, Mastigocladus, Microchaete, Microcoleus, and Oscillatoria. Phormidium, Plectonema, Pseudoanabaena, Nodularia, Nostoc, Schizothrix, Scytonema, Stigonema, Synechococcus, and Tolypothrix are some of the more common species. Many extremophiles have developed to be able to survive at pH extremes. At low pH, cyanobacteria predominate and are even seen in acid lakes (pH 4.1-5). The above remark was later corroborated by Steinberg's 1998 study (Steinberg et al., 1998) which established the presence of filamentous cyanobacteria. In acid lakes in Germany, Chroococcus turgidus, Limnothrix sp., Mastigocladus sp., Oscillatoria sp., Spirulina sp. (pH 2.9), and Synechococcus sp. (pH 4) were found. Extreme alkaliphiles live in soda lakes or soils rich with soda (natron), where the pH can increase to 12,

yet these organisms develop poorly at neutral pH (Grant and Tindall, 1986). Soda lakes are occasionally monospecific, inhabited by Spirulina platensis, which provides as human food (single cell protein) of excellent nutritional value. Thermophilic cyanobacteria are found as mat colonies in neutral/alkaline pH geothermal springs and can withstand temperatures of up to 74°C. The makeup of mat communities is mostly temperature dependent, and mats have been clearly separated based on cyanobacterial species (Castenholz, 1996).

3.3. Ecology of cyanobacteria

Based on the earliest deoxy ribose Nucleic Acid (DNA)-biomarker data, it is hypothesised that cyanobacteria appeared around 2600 million years ago (Hedges et al., 2001). It is widely believed that ancient cyanobacteria played a significant role in the evolution of an oxygenic atmosphere from an oxygenless one. Furthermore, it is thought that the oxygen-rich gas composition of Earth's atmosphere is the result of a significant contribution from cyanobacteria (Dismukes 2001) and cyanobacterial photosynthesis continues to contribute to the production of oxygen and the maintenance of our atmosphere's balance. Cyanobacteria's extensive evolutionary history can be linked to their effective survival in a variety of settings and their broad ecological tolerance (Budel et al., 2011). Furthermore, cyanobacteria have evolved a broad ecological tolerance to a variety of abiotic factors like as light, temperature, moisture, salt, and alkalinity, and they exhibit adaption characteristics owing to their widespread dispersion and success. Cyanobacterial distribution is widespread on the planet, with populations found in freshwater, marine, and terrestrial settings. They are abundant in aquatic ecosystems as plankton, with individuals found loosely or securely clinging to the surfaces of plants, rocks, and sediments, and some can be found in acidic and hot springs, deserts, salt lakes, and ice shelves and the Arctic (Rastogi and Sinha, 2009). Cyanobacteria play an important role in many terrestrial habitats as well, and they may dwell in rocks or soils and form mutual connections with plants, fungi, and animals (Thajuddin and Subramanian, 2005). Aside from form and habitat range, members of this phylum display enormous heterogeneity in the level of natural product synthesis. Cyanobacteria have evolved to create a wide range of secondary metabolites that have aided species survival in these diverse and highly competitive ecological niches (Kalaitzis et al., 2009). Cyanobacteria are well recognised for their ability to create a variety of hepatotoxic, neurotoxic, and tumour-promoting secondary metabolites, and they are frequently connected with toxic blooms found in many eutrophic fresh and brackish waterways (Codd et al., 1999) Cyanobacteria are a distinct phylum that thrives in competitive habitats and, as a result, produce natural bioactive compounds (Clardy and Walsh, 2004)

3.4. Cyanobacterial physiology and morphology:

Cyanobacteria are the most self-sufficient photosynthetic organisms known. As a result, they can live in every ecological situation and on any substrate. As a result, they are among the most rudimentary colonisers of desolate places. Many of them can fix nitrogen from the atmosphere. Cyanobacterial cells are larger and more complicated than bacteria cells, and their cell structure is distinctively prokaryotic. It has a single envelope with a peptidoglycan wall, bare DNA, and 70S ribosomes, but no membrane-bound structures like as endoplasmic reticulum, Golgi bodies, mitochondria, lysosomes, plastids, or sap vacuoles (Bullerjahn et al., 2014). The cell wall is four layers thick, having peptidoglycan in the second layer. The outer protoplast includes chromoplasm, which is made up of photosynthetic thylakoids. The thylakoids are free in the cytoplasm, and their membranes contain chlorophyll a, carotenes, and xanthophyll, but not chlorophyll b (Liberton et al., 2013). Phycobilisomes are microscopic granules that are linked to thylakoid membranes and contain phycobilin, which are photosynthetic pigments (Douglas, 1994). Each gas vacuole is made up of a number of sub microscopic particles known as gas vesicles. Gas vacuoles serve as a light screen, a buoyancy controlling mechanism, and a source of pneumatic strength (Reynolds, 1987) A naked, circular, double-stranded DNA is coiled in the central section of the cytoplasm known as centroplasm, and the coiled-up DNA is comparable to a single chromosome in higher animals and is commonly referred to as a nucleoid (Alvarenga et al., 2017). Small circular DNA pieces, similar to bacterium genetic material, are also present in addition to nucleoid and are known as plasmids or transposons. The presence of the 70S ribosomal unit can be found in numerous locations (Doolittle, 1980). The plasma membrane is frequently connected to the nucleoid by a semicircular collection of coiled membranes. The cells include four types of inclusions: a- granules (cyanophycean starch), B-granules (lipid droplets), volutin granules, and polyhedral bodies (ribulose biphosphate carboxylase).

Cyanobacteria photosynthesize utilizing water as the electron contributor and deliver oxygen as in algae, in spite of the fact that few strains can likewise utilize hydrogen sulphide (HS) and convert it to essential sulphur (Madigan et al., 2003). Usually, cyanobacteria can endure low oxygen conditions and concentrations of HS that are poisonous to eukaryotic algae. This resistance may add to their capacity to get by in anoxic, eutrophic lake dregs and in addition in certain tangle conditions. Cyanobacteria show impressive morphological variation. They may be unicellular, colonial or filamentous. Every filament comprises a sheath of adhesive and at least one cell strands called trichomes. Single trichome fibres may further be of two kinds,

homocystous (undifferentiated, e.g., Oscillatoria) and heterocystous (separated, having heterocysts, e.g., Nostoc). Spirulina has a spirally snaked filament. Colonies develop in some, e.g., Nostoc. Flagella are missing, yet coasting developments are known in various cyanobacteria. The name Oscillatoria has been given to a typical blue-green alga based on pendulum-like wavering developments of its foremost region. Some cyanobacteria can deliver two sorts of particular cells: (1) heterocysts, which give the site to nitrogen obsession and in this way balance nitrogen request under states of nitrogen insufficiency, and (2) akinetes which are resting cells that enable the species to endure negative development conditions. Asexual reproduction of cyanobacteria occurs by the formation of hormogonia or endospores (exospores are modified endospores) or by fragmentation of colonies (Lee, 1999).

3.5. Heterocyst of Cyanobacteria

In addition to the capacity to perform oxygen-producing photosynthesis, certain cyanobacterial strains have gained the ability to fix molecular dinitrogen (N^2). Nitrogenase, an enzyme responsible for nitrogen fixation, is highly sensitive to oxygen, hence oxygenic photosynthesis and nitrogen fixation cannot occur concurrently in cyanobacterial cells (Fay, 1992). To counter this challenge, certain filamentous cyanobacterial strains can restrict N-fixation to specialised cells known as heterocysts. Heterocysts are specialised, morphologically distinct, terminally developed cells that proliferate in a semiregular configuration along the filament in the absence of other sources of combined nitrogen. Heterocysts form from vegetative cells, which are oxygenic photosynthetic sites and provide a microoxic environment for the oxygen-sensitive nitrogenase. Nitrogenase is the enzyme responsible for N-fixation, which allows cyanobacterial strains to survive on sunshine, air (CO_2 and N_2), and certain minerals. Both cell types rely on each other and exchange metabolites and signalling molecules under nitrogen-fixing circumstances. This exchange is most likely accomplished by protein-complex-mediated cell-to-cell contact or the continuous periplasmic space that surrounds all cells in the multicellular filament. Global nitrogen control factor regulates the stimulation of several genes involved in heterocyst differentiation and function. NtcA regulates HetR, the activator of heterocyst development, as well as the recognised heterocyst differentiation inhibitors PatS and HetN. Inhibitor gradients that promote the decay of HetR govern the pattern of spaced heterocysts, verifying mathematical predictions of two-dimensional pattern development in heterocystous cells of cyanobacteria.

3.6. Classification

Microalgae examined by phycologists were classified as both prokaryotic and eukaryotic species under the International Code of Botanical Nomenclature (ICBN). The blue-green algae were the most numerous in the former category. Because of their close proximity to eubacteria and prokaryotic nature, work under the International Code of Nomenclature of Bacteria (ICNB) was more suitable (Rippika et al., 1979) offered a taxonomy revision based on morphological, biochemical, and molecular characteristics most recently. In the newest edition of Bergey's Manual (Boone and Castenholz, 2001), the oxygenic photosynthetic prokaryotes are classified as 'Cyanobacteria' rather than the previously published designation Oxyphotobacteria, and the cyanobacteria are classified as "form genera." The Botanical Code and the Bacteriological Code do not recognise this word, which was coined by (Castenholz 1992).

The conventional categorization method of cyanobacteria needed skill to identify the organism based on physical characteristics Because of variations in environmental conditions, more than half of the strains in culture collections are misdiagnosed; even diagnostic features may be lost during cultivation (Komarek and Anagnostidis, 1989). These constraints have highlighted the importance of approaches in cyanobacteria taxonomy such as gene sequencing using 16 s rRNA (Nubel et al., 1997), rbc-L gene nucleotide base composition (Fahrenkrug et al., 1992) and DNA-DNA hybridization (Wilmotte, 2004). DNA fingerprinting technologies such as randomly amplified polymorphic DNA (RAPD) (Neilan, 1995) and restricted fragment length polymorphism (RFLP) are presently commonly utilised.

3.7. Cyanobacteria as a biofertilizer

Nitrogen is a crucial ingredient for plant development. It exists in the form of elemental nitrogen and accounts for more than 79% of total gases. Crop plants, on the other hand, cannot use elemental nitrogen. On a worldwide scale, industrial fertiliser nitrogen contributes for just one-third of the 180 million tonnes of nitrogen deposited to the earth's surface each year, with the other two-thirds coming through biological processes, mostly microbial activity. Biofertilizers, also known as microbial inoculants, are made up of bacteria (Azotobacter), algae (Blue-green algae), and mycorrhizal fungi; they are natural, beneficial, and environmentally friendly, and they offer nutrients to plants while also maintaining soil structure (Board, 2004). Cyanobacteria may exist as either unicellular or multicellular filaments. Some forms contain heterocysts, which are terminally specialised structures. Heterocysts are found in all aerobic photo diazotrophic cyanobacteria. Aerobic filamentous heterocystous as well as colonial forms are

found in the common flora of arable areas and are thought to be significant in the nitrogen economy of rice farming. Cyanobacteria fix atmospheric nitrogen only when nitrogen is scarce, and the enzyme nitrogenase stays inhibited in the presence of mixed nitrogen sources, a reversible suppression similar to the oxygen effect. According to, the activity of the enzyme nitrogenase was not reduced in the soil-rice-algae combination. The presence of less than 40 ppm ammoniacal-N. Publications have been published throughout the last six decades on the use of cyanobacterial inoculants (algalization) to improve biological N2-fixation in paddy rice fields. It has been claimed that cyanobacteria may add 15-18 Kg N through their activity, and hence nitrogen-fixing strains are utilised as bio-fertilizers in tropical areas (Watanabe & Cholitkul, 1979) Diazotrophic cyanobacteria improve the nitrogen economy in rice soils. Organic phosphates in the soil and water are additionally mobilised by cyanobacteria into inorganic orthophosphates, which are then added to agriculture fields (Choudhary and Bimal, 2010). As these elements have an ecologically determining role in the field, cyanobacteria abundance and nitrogen-fixing capabilities can be modified by many environmental circumstances such as moisture, soil pH, combined nitrogen levels, and temperature (Pereira et al. 2009).

3.8. Metal requirements by Cyanobacteria

Metals are required by most organisms as macronutrients, micronutrients, or enzyme cofactors. Fe, Mg, Cu, and Mn are important micronutrients that function as cofactors in the oxygenic photosynthetic electron transport mechanism. Toxic elements of high or low relevance as trace elements include zinc (Zn), nickel (Ni), copper (Cu), vanadium (V), cobalt (Co), tungsten (W), and chromium (Cr). Aluminium (Al), Arsenic (As), Mercury (Hg), Silver (Ag), Cadmium (Cd), Lead (Pb), and Uranium (U) appear to be more or less harmful to plants, algae, and microorganisms as nutrient (Nies, 2003). Photosynthetic species require a higher total metal requirement than non-photosynthetic ones. The photosynthetic machinery is composed of several membrane-embedded protein super complexes including numerous cofactors. Fe cofactors such as iron-sulphur (Fe-S) clusters, cytochromes, and non-hemeFe, the Mn cluster of PSII, Cu in plastocyanin, and Mg at the core of each chlorophyll (chl) ring are among them. As a result, the demand for these metals much exceeds that of the oxygenic photosynthetic cyanobacterium Synechocystis sp. PCC 6803, which has a cellular Mn quota two times more than that of the nonoxygenic purple bacterium Rhodobactercapsulatus. Many cyanobacteria can fix atmospheric nitrogen via an enzyme multiprotein complex called nitrogenase, which is made up of two proteins: di nitrogenase and di nitrogenase reductase. State that the former requires a

Fe-Mo co-factor, whereas the latter requires a Fe co-factor. Other cyanobacteria that cannot fix nitrogen (or occasionally even if they can) absorb nitrate, which is possibly the most abundant source of combined nitrogen for cyanobacterial nutrition after entering the cell, nitrate is converted to ammonium in two steps by nitrate reductase and nitrite reductase, respectively. A Mo co-factor is required by the enzyme nitrate reductase. A molybdate storage system has been discovered in the filamentous heterocyst-forming cyanobacterium Anabaena variabilis ATCC 29413 (which requires Mo as a component of two essential co-factors, both for the enzymes nitrate reductase and nitrogenase) and the closely related strain Anabaena sp. PCC 7120. Photosynthetic organisms ranging from cyanobacteria to vascular plants have developed a variety of effective metal absorption and accumulation methods to provide an adequate supply of metals (Shcolnick and Keren, 2006). According to Cavet et al. (2003), cyanobacteria have metal requirements that other bacteria do not have: copper in thylakoidal plastocyanin, zinc in carboxysomal carbonic anhydrase, cobalt in cobalamin, magnesium in chlorophyll, molybdenum in heterocystous nitrogenase, and manganese in thylakoidal water-splitting oxygen-evolving complex. The photosynthetic apparatus, on the other hand, has a significant impact on the stability of metal homeostasis. Metals serve an important function as cofactors in oxygenic photosynthesis, but they also represent a considerable oxidative risk factor due to their harmful interaction with oxygen. Because of the intense redox chemistry conducted by the two photosystems, reactive oxygen species (ROS) can be created at many places. Cyanobacteria that develop in metal-polluted settings may survive high concentrations of harmful metals such as Cu, Cd, and Zn (Mallick et al., 1990). As a result, strict supervision of metal transit and storage is required to assure enough supply and prevent against oxidative damage. The multiplication of photosynthetic organisms is dependent on this precise balance between metal needs and oxidative damage

4. Materials and methods

Table 1: Chemicals used

S.No.	Chemical Name	Chemical formula	Company
1.	Diphenylamine (DPA)	$C_{12}H_{11}N$	HIMEDIA
2.	Acetone	C_3H_6O	HIMEDIA
3.	Potassium dichromate	$K_2 Cr_2O_7$	MERCK
4.	Sulfuric acid	H_2SO_4	HIMEDIA
5.	Phosphoric acid	H_3PO_4	HIMEDIA
6.	Sodium hydroxide	NaOH	HIMEDIA
7.	Hydrochloric Acid	HCl	HIMEDIA

4.1. Media preparation

The Zarrouk's media with Ph of 9.5 was prepared with the following composition

$NaHCO_3$	33.6 gm
$NaNO_3$	5.0 gm
K_2SO_4	2.0 gm
K_2HPO_4	1.0 gm
NaCl	2.0 gm
$MgSO_4.7H_2O$	0.4 gm
$CaCl_2.7H_2O$	0.08 gm
$FeSO_4.7H_2O$	0.02 gm
EDTA	0.16 gm

Then the sterilization of Zarrouk's media was done by autoclaving it at 15 psi pressure and 121^oC temperature for 15 minutes.

Table 2: Instruments Used

Instruments	Model	Uses
Electronic analytical balance	ME204	Measure the mass of chemicals
Hot air oven	HAC-405	For sample drying
Incubator shaker	OLSC-109-3	For growth of bacteria, yeast and tissue culture To provide aeration To evenly distribute nutrients in the culture media For the proper agitation at a specific speed of 120 rpm
Magnetic stirrer	REMI 5MLH	Mixing fluid sample
pH meter	EI-111	Measure activity of hydrogen ions in solution
Refrigerator	GL-P292KDSR	To preserve specimen
UV-vis spectrophotometer	CARY 100	Quantitative determination of absorber's concentration in the solution

Table 3: Glassware Used

Glassware	Capacity	Quantity	Company
Erlenmeyer Flasks	100-250 mL	15	Borosil, India
round bottom flasks	1.0-2.0 L	10	Borosil, India
Test Tubes	10 mL	25	Borosil, India
Petri Plates		12	Borosil, India
Measuring Cylinder	10mL-100mL	3	Borosil, India

4.4. Experimental organisms

Two cyanobacterial strains of *Spirulina* namely *Limnospora Fusiformis* TICT-01 and *Limnospora Fusiformis* IUAF-001 were taken from the Plant tissue culture laboratory, IIRC (Integral Information and Research Centre). Integral university, Lucknow.

4.5. Growth conditions

The selected strains of *Spirulina* were maintained in the culture room at 25± 5 ^{0}C under 2400 Lux intensity with a photoperiod of Light: dark 14:10h. For the routine experiments, cultures were grown in sterilized Zarrouk's media with pH 9 (Huges et al., 1958). The cultures were manually shaken thrice a day.

4.6. Metal treatment

The preparation of Chromium (Cr) stock solution, Potassium dichromate ($K_2Cr_2O_7$), make MERCK was dissolved in Zarrouk's media. For the preparation of 1000mM, By diluting this stock solution, working solutions of strength 2, 4, 8,16 and 32µM were prepared. For 24 hours, cultures were cultured in metal enhanced media. 14 hours of light and 10 hours of darkness were followed by a 20-minutes centrifugation at 8000g. Following centrifugation, the pellet was washed with distilled water and then washed twice with EDTA solution. Following that, pellets were employed in the determination of chl-a and metal absorption. All tests were carried out in triplicate with the use of exponentially developed cultures (OD_{750}-0.3) to ensure the repeatability of the results.

$K_2Cr_2O_7$ (MERCK):

Molecular formula	-	$K_2Cr_2O_7$
Molecular weight	-	294.185
Physical state	-	Powder

4.8. Microalgal growth determination

The microalgal growth was determined by the colorimetric method. Cell density was estimated by taking absorbance at 680 nm through an ultraviolet/visible spectrophotometer model Kinetic, Eppendorf make Hamburg, Germany. A calibration curve was drawn to convert optical density (OD) 680nm to dry microalgae biomass (Daneshvar et al., 2018). In this, 10 different

microalgal samples were diluted appropriately, leading to the calibration curve described by Eq. (1) in the OD 680 range of 0.1 to 1.0:

Dry algal biomass (gL^{-1}) = $0.2284OD_{680}$-0.0041(R_2=0.977) (1)

The gravimetrical method was used for the determination of microalgal dry weight. A specific microalgae volume was dried at 60 ˚C until it reached a constant weight (Hu et al., 2021). The estimation of biomass productivity (P) and growth rate (μ) was done by using Eq. (2) & (3):

$M = \ln (C_e/C_0) (t_e-t_0)^{-1}$ (2)

$P = (C_e-C_0) (t_e-t_0)^{-1}$ (3)

Where, C_e is Concentration of microalgae on the final,

C_0 is Concentration of microalgae on the first day,

t_e is End time of the experiment and

t_o is Onset time of the experiments

4.9. Determination of pigment content

The determination of chlorophyll (a and b) and carotenoids was done according to the protocol given by Naaz et al., 2021. The 10 mL microalgal culture was centrifuged at 500 rpm for 10 minutes. The pellet was separated and mixed with distilled water (1 mL) and acetone (4 mL). This solution was kept in the fridge for overnight. Thereafter it was centrifuged at 500 rpm for 5 minutes and OD of supernatant was taken at 645 nm for chlorophyll (a), 663 nm for chlorophyll (b) and 470 nm for Carotenoids. 80% acetone was used as blank. The chlorophyll and carotenoid concentrations were finally estimated by equations 5-7.

Chlorophyll (a) = $15.65A_{663}$ - $7.34A_{645}$ (5)

Chlorophyll (b) = $27.05A_{645}$ -$11.21A_{663}$ (6)

The cellular content was expressed in mg L^{-1}.

4.10. Metal Uptake

For detection of heavy metals in biological as well as environmental samples, few spectrophotometry methods have been developed. Here in our study, we are using the detection of Cr (VI) by using Diphenyl carbazide Method (Ahmadi F et al., 2009).

4.11. Standard and Reagent preparation

- First, prepare the stock solution of heavy metals.
- Prepare the stock metal solution at different ppm.
- Here we take 5 ppm i.e., 50ppm, 100ppm, 150ppm, 200ppm and 250ppm.

4.12. Stock solution of heavy metals

- The chromium stock solution is made by Potassium dichromate ($K_2Cr_2O_7$). All the required solutions are prepared with analytical grade salt and double-Distilled water. 2.835g of 99 % $K_2Cr_2O_7$ is dissolved in 1L distilled water in a volumetric flask to obtain 1000 ppm (mg/L) of Cr (VI) stock solution.

$$\text{Cr equivalent to 1gm} = \frac{\text{Molecular weight of K2 Cr 2O7}\times 100}{\text{Atomic weight of Cr} \times 2 \times \text{purit}}$$

4.13. Preparation of Calibration curve

The solution required to make a standard calibration curve is a reference solution and on the other side there is a sample solution whose concentration supposed to be analysed under UV-vis spectrophotometer. The solutions at different concentration before the test were analysed by UV-vis spectrophotometer and different concentration provides different absorbance. A plot drawn between absorbance as well as known concentration is calibration curve. From these calibration curves, the unknown concentration of the supernatant after the tests were found out that is our final concentration. For the detection of heavy metals by spectrophotometer several methods are used. In case of Chromium (VI) detection, Diphenyl carbazide Method.

4.14. Preparation of calibration curve of Cr (VI)

(a) Stock solution (1000 PPM) diluted to 10 PPM (1 ml Cr (VI) in 100 ml DW).

(b) Finally diluted this solution 0.1 PPM to 1 PPM (100µl to 1000µl in 10 ml water).

(c) 10 ml diluted solution + 25 µl H3PO4+ 200 µl H2SO4+ 200 µl DPC (Final pH 2.4)

4.15. Estimation of Free radicals' generation under metal stress

The estimation of superoxide and peroxide produced during the experiment were studied by using below explained protocols.

4.16. Superoxide estimation

The estimation of Superoxide radical in Cr (VI), untreated and treated *spirulina* cells was done according to the process of (Elstner and Heupel, 1976). The homogenization of cells took place in phosphate buffer (2 mL) and centrifuged (8000×g). Thereafter, phosphate buffer (0.9 mL) along with hydroxylamine (0.1 mL) was added to the supernatant (1 mL). OD was taken after 20 min of incubation at 530 nm with 1 mL of each N-(1-naphthyl) ethylenediamine dihydrochloride (NEDD) and sulphanilamide. For the superoxide content calculation, a standard curve of sodium nitrite was used (Elstner and Heupel 1976).

4.17. Peroxide Estimation

The untreated and the treated cells were extracted in 5% trichloroacetic acid (TCA) (3.5 mL) for peroxide estimation. At 480 nm the total peroxide in the supernatant was noted. The H_2O_2 standard curve was referred or knowing the amount of total peroxide**.** The level of peroxide in the Cr – treated and untreated cells of *Spirulina* was determined (Sagisaka 1976).

4.18. Superoxide Dismutase Activity

After four days of exposure to Cr metal, cyanobacterial cells were centrifuged and washed twice with a pH 7.8 100mM EDTA-phosphate buffer. Cellular pellets were crushed in an ice-cold mortar with 100 mM EDTA-phosphate buffer, pH 7.8, and the resultant homogenate was centrifuged at 8000 rpm for 20 minutes. The pellets were discarded, and the supernatant was employed as an enzyme source. 0.1 mL of crude extract was then combined with 1.3 µM riboflavin, 13 mM L-Methionine, and 0.05 M Na_2CO_3 (pH 10.2) 63µM-p-nitroblue tetrazolium, yielding a total reaction volume of 3mL. The reaction was carried out at 25ºC in a comparable environment with fluorescent light illumination. The quantity of enzyme was

determined by comparing the rise in absorbance at 560nm in the presence and absence of extract. The identical reaction was conducted in the dark for the blank. The quantity of enzyme required to block the process by 50% was defined as one unit SOD activity. (Giannaopolitis and Ries 1977).

4.19. Peroxide Dismutase Activity

Following treatment, cyanobacterial cells were centrifuged and the pellets were homogenised at 5°C in 2 mL of 100 mM phosphate buffer (pH 7.0). After centrifuging the homogenate for 30 minutes at 8000 rpm, the supernatant fraction was utilised as the enzyme extract. 1 mL 25 mM H_2O_2, 1 mL 100 mM pyrogallol in distilled water, and 1.0 mL enzyme extract were combined to make a 3 mL reaction mixture. After fully mixing the reaction mixture, a change in O.D. was seen at 430 nm for 2-3 minutes. (Gahagen et al., 1968)

4.20. Lipid Peroxidation

Add 0.5 ml of 0.1% (w/v) TCA to homogenise 0.1 gm of leaf tissue. Centrifuge the homogenate for 10 minutes at 4ºC (15000 x g). Collect supernatant and combine 0.5% diluted in 20% TCA in 0.5 mL of supernatant. Incubate for 25 minutes in a 95ºC water bath. Cool the reaction by incubating it on ice. If the solution is not clear, centrifuge for another 5 minutes (15000 x g, 4ºC). Take absorbance measurements at 532 and 600 nm. The OD 600 values are subtracted from the MDA-TBA complex readings at 532 nm, and the MDA concentration is computed using the Lambert-Beer law and an extinction coefficient of M= 155 $mM^{-1}cm^{-1}$. The results are given in mols MDA g^{-1}FW (Heath and Packer 1968).

4.21. Non- Enzymatic Antioxidants

4.21.1. Total Phenol content

10 ml culture was treated for 24 hours and then centrifuged at 8000 rpm for 5 minutes. Cells were homogenised at 4^0C in 2mL of 80% methanol. Extract was then centrifuged at 8000 rpm for 2 minutes. To 0.5 mL of supernatant add 2mL of Sodium bicarbonate, 0.3 ml distilled water, 0.2 ml Folin's reagent. Then incubate in water bath till blue colour appears. Absorbance was taken at 750 nm (Singleton and Rossi 1965).

4.21.2. Total proline content

After centrifuging 20 mL of exponential phase culture at 8000 rpm, the pellets were homogenised in 10 mL of 3% sulfosalicylic acid. The extracts were then centrifuged at 8000

rpm for 10 minutes. After that, 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were added to 2 mL of supernatant and incubated at boiling temperature for 1 hour. The standard curve of L-Proline was used for finding the concentration. The resultant combination was extracted with toluene, and proline was spectrophotometrically measured at 520 nm from the organic phase (Bates et al., 1973).

4.22. Protein Isolation and estimation

150 ml culture (Treated and untreated) was centrifuged at 3000 rpm for 10 minutes, pellets were gathered in one tube and 2 ml PBS was added, pellets were washed again with PBS. Pellets were transferred into glass tubes with the help of 400µl PBS. Total volume in glass tubes was maintained to approximately 1ml. further the tubes were kept in -80^{0}C for freezing ($^1/_2$ hr freezing) then thawed by dipping tubes in water at 37^{0}C (this step was repeated 4 to 5 times). The green extract turned purple after freeze thawing. 100µl PMSF ((phenylmethylsulphonyl fluoride) was added from stock solution. Glass powder was added so that the extract is 1-2 mm above glass powder which settles at bottom of tube. Tubes were vortexed for total 10 minutes (in cycle each of 10 sec vortex and 10 sec keeping in ice). The extract was then transferred to Eppendorf tubes. These tubes were then centrifuged at 4^{0}C at 3500 rpm for 5 minutes. Purplish tube supernatant appears on top of the settled glass powder. The supernatant was transferred to another Eppendorf and again centrifuged for 1 minute to collect the residual glass powder. Lastly the protein extract was kept at -20^{0}C. ()

4.23. Pot Experiment (Cyanobacteria as a Biofertilizer)

4.23.1 Experimental organisms and growth conditions

The seeds were surface sterilised for 10 minutes in a 1% sodium hypochlorite solution before being rinsed thoroughly with double distilled water and cultivated on autoclaved petri plates. These petri plates were sometimes watered with double distilled water.

4.23.2. Experimental setup with rice seedlings: The effect of Cr treated *spirulina-based* biofilms on rice crop growth was studied in pots. The studies were conducted in June-July, and the average temperature range of the area during crop development was between 25±5°C.

4.23.3. Immobilization

In Zarrouk's medium, a 1.5% (w/v) sodium alginate solution was made by warming the solution in a water bath. After the solution had cooled to room temperature, 3 mL of 15-day old

cyanobacterial culture was added and properly mixed. Using a syringe canula, the solution was introduced dropwise into 100 mL of 1% $CaCl_2$ solution in laminar air flow. Calcium alginate beads (2 mm diameter size) produced in the $CaCl_2$ solution were hardened in the same solution for 1 hour at 4°C. The beads were collected and thoroughly cleaned with sterile distilled water and Zarrouk's media. These were then moved to flasks containing Zarrouk's media and incubated at 25±5°C with a light intensity of 2400 Lux (Mayashree B Syeim 2004).

Figure 1: Immobilized beads of *Limnospora Fusiformis* IUAF001

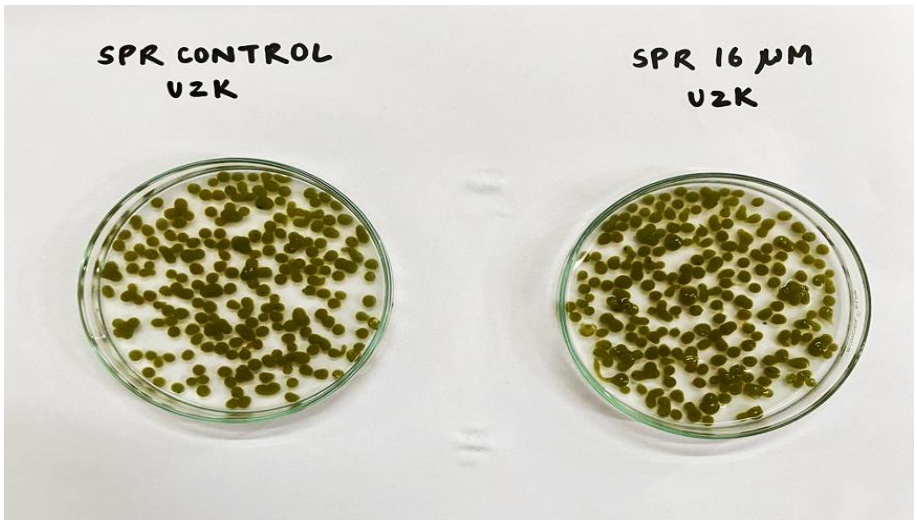


Figure 2: Immobilized beads of *Limnospora Fusiformis* TICT 01

4.24. Estimation of effect on growth of rice crop with immobilized cultures

For the estimation of the rice plant growth under treated immobilized culture beads was done by measuring the root and shoot length of the crop in comparison to the control at the end of the experiment.

4.24.1. Statistical analysis

All experiments were done with mid-log phase cells and repeated thrice to ascertain the duplicability of the outcomes. Values are denoted as ± Standard error mean (n = 3) (SEM). Tests of significance were applied by one-way analysis of variance (ANOVA), followed by Dunnett's test with control to focus on the efficacy of different formulated groups (Agarwal et al., 2018) with the help of Graph Pad Prism 5.

5. Results & Discussion

5.1.Growth Study

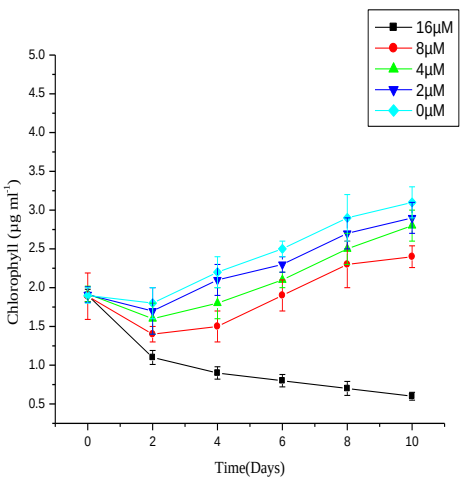


Fig.3. Effect of different Cr concentrations on the growth of *Spirulina platenesis IUAF001*. Values are means ± SE with n=3.

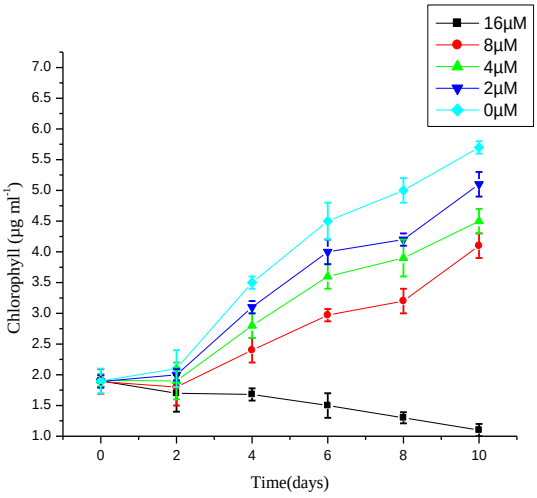


Fig.4. Effect of different Cr concentrations on the growth of *Spirulina platenesis TICT01*. Values are means ± SE with n=3.

5.2. Calibration Curve of Chromium (VI)

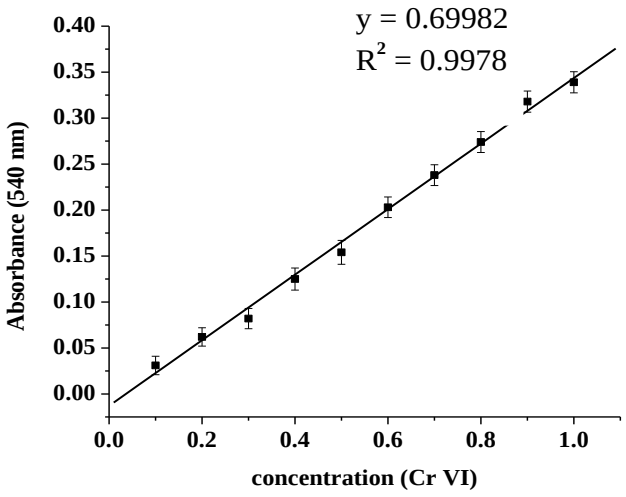
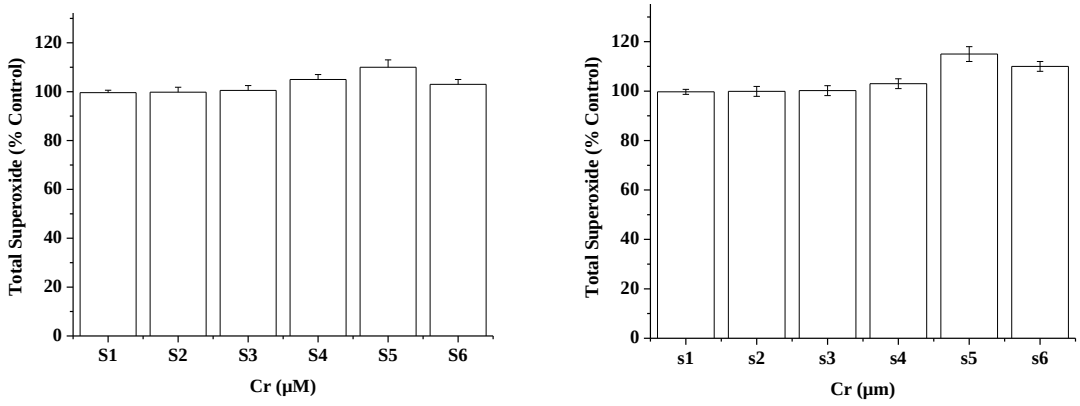


Figure 5: Calibration Curve for Cr (VI)

5.3. Superoxide estimation

Total superoxide was measured in two *Spirulina* strains (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TICT 01 after 4 days of exposure to Cr (0-32 µM) and the results obtained are presented in Figure1 (a) and (b). The dose-dependent increment was found in the free radicals i.e., Superoxide (1-35 %) in the *Spirulina*. Low Cr concentration was unable to promote the total superoxide content even after four days of exposure whereas the high doses (8µM and 16µM) of Cr stimulated ROS production significantly. Similar studies were performed by (N.L. Loseva et al., 2003) in which the quantity of superoxide generated by chlorella cells increased in a concentration-dependent manner as soon as they were exposed to the mycoplasma and its supernatant at zero time. Whereas in our studies the superoxide concentration increased and then decreased on increasing Cr concentration.

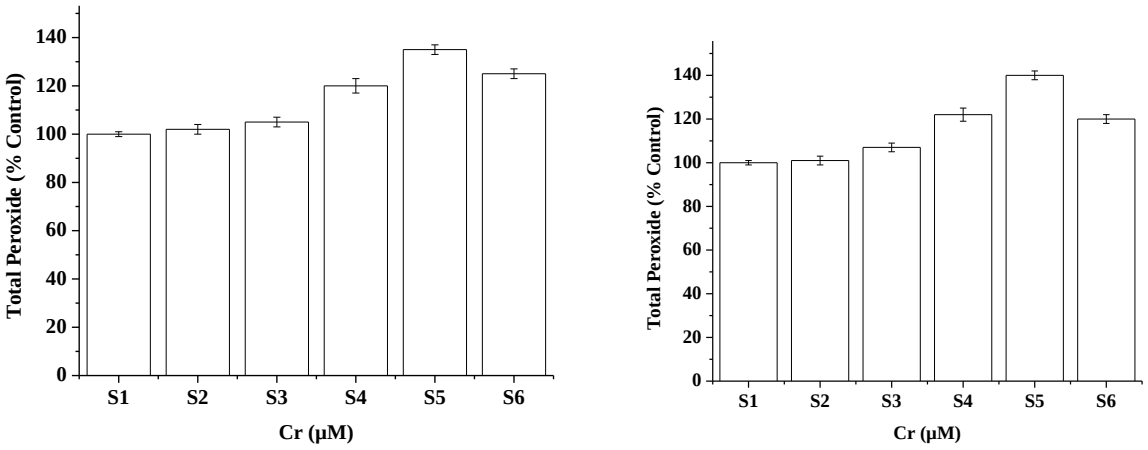


(a) (b)

Figure 6: The Effect of different Cr concentration (S1, S2, S3, S4, S5 and S6 represents the metal concentration 0, 2, 4, 8,16 and 32 µM respectively) on total superoxide of *spirulina* strains. (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TCIT-01.

5.4. Peroxide estimation

Total peroxide was measured in two Spirulina Strains (a*) Limnospora Fusiformis* IUAF 001 and (b) *Limnospora Fusiformis* TICT 01 after 4 days of exposure to Cr (0-32 µM) and the results obtained are presented in Figure1 (a) and (b). The dose-dependent increment was found in the free radical i.e., peroxide (3-37%) in the *Spirulina*. Low Cr concentration was unable to promote the total peroxide content even after four days of exposure whereas the high doses (8µM and 16µM) of Cr stimulated ROS production significantly.

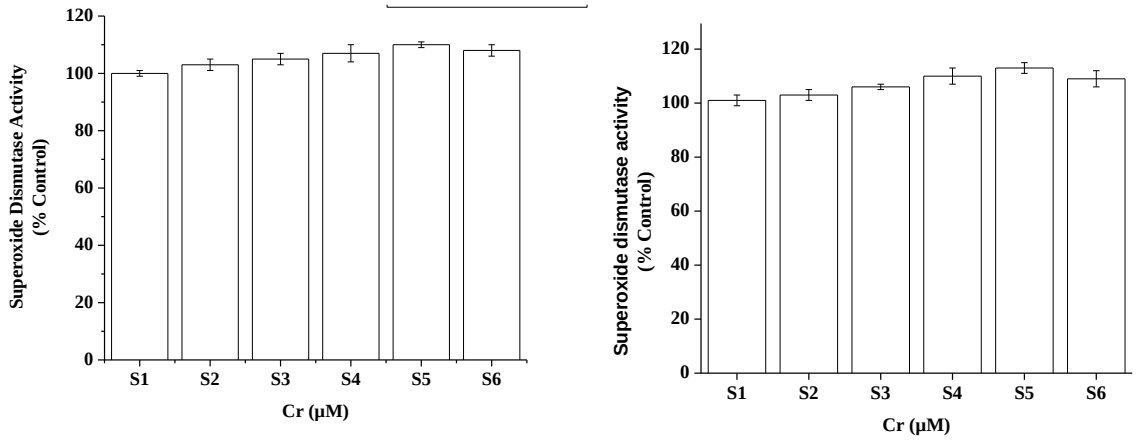


(a) (b)

Figure 7: The Effect of different Cr concentration (S1, S2, S3, S4, S5 and S6 represents the metal concentration 0, 2, 4, 8,16 and 32 µM respectively) on total peroxide of *spirulina* strains. (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TCIT-01.

5.5. SOD (Superoxide Dismutase)

In (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TICT 01 the activity of SOD at Cr treatment is represented in Figure 1 (a), (b). The SOD activity in untreated Spirulina were 6.9 ± 0.25 Unit (mg protein)-^{1}. Low concentrations of Cr (2 µM and 4 µM) did not show a substantial increment in the SOD activity whereas 16µM concentration stimulated the antioxidant activity by 27%. In similar studies were performed by (U.N. Rai et al., 2003) in which the maximum SOD was recorded at 1 µg/ml^{-1} Cr concentration, however, at the concentration above 5 µ/ml^{-1} the SOD content decreased substantially whereas in our studies the maximum SOD was recorded at 16µM and decreased substantially at 32µM.

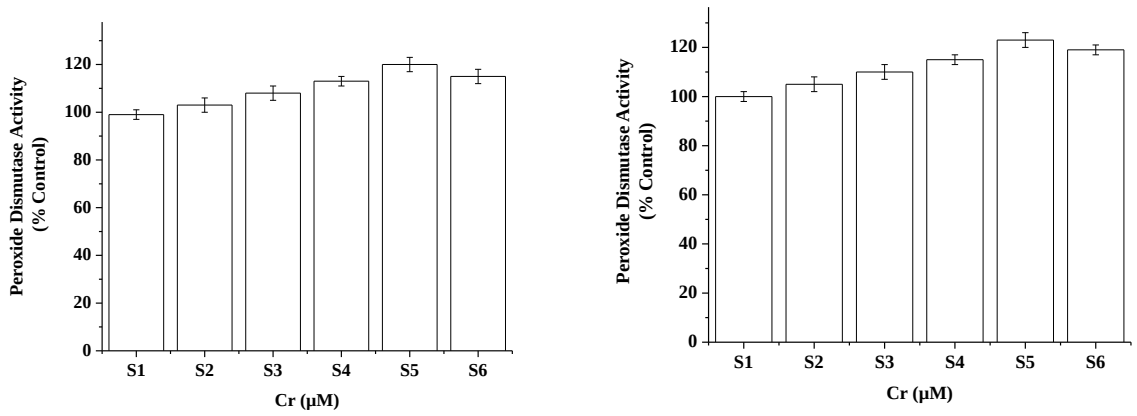


(a) (b)

Figure 8: The Effect of different Cr concentration (S1, S2, S3, S4, S5 and S6 represents the metal concentration 0, 2, 4, 8,16 and 32 µM respectively) on total superoxide dismutase activity of *spirulina* strains. (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TCIT-01.

5.6. POD (Peroxide Dismutase)

In *Spirulina* the activity of POD under the treatment of Cr have been represented in Figure 4 (a) and (b). The POD activity in untreated *Spirulina* was 1.5 ± 0.02 change in OD430 (mg protein)-1 min-1. Low concentrations of Cr (2 µM and 4 µM) did not show a substantial increment in the POD activity whereas high concentration (16 µM) stimulated the antioxidant activity by 18%.

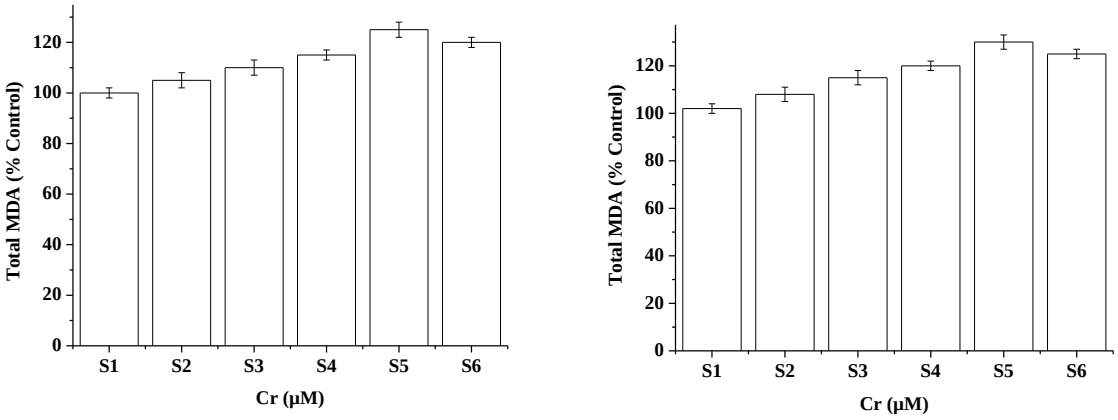


(a) **(b)**

Figure 9: The Effect of different Cr concentration (S1, S2, S3, S4, S5 and S6 represents the metal concentration 0, 2, 4, 8,16 and 32 µM respectively) on total peroxide dismutase activity of *spirulina* strains. (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TCIT-01.

5.7. Lipid Peroxidation

The data pertinent to membrane damage was measured with respect to the concentration of lipid peroxidation (LP) presented in Figure 5. (a) and (b) was analysed in terms of total MDA concentration in the cells and dose-dependent increment in MDA content was observed. The supplementation of Cr metal at a concentration of 0, 2, 4, 8,16 and 32 µM showed an enhancement in MDA content by 5%, 10%, 15%, and 25%, respectively in *Spirulina*. Similar studies were performed by (U.N. Rai et al., 2003) in which an enhanced MDA level was detected with the increasing concentration of Cr. 15 $\mu g/ml^{-1}$ was found to be the highest.

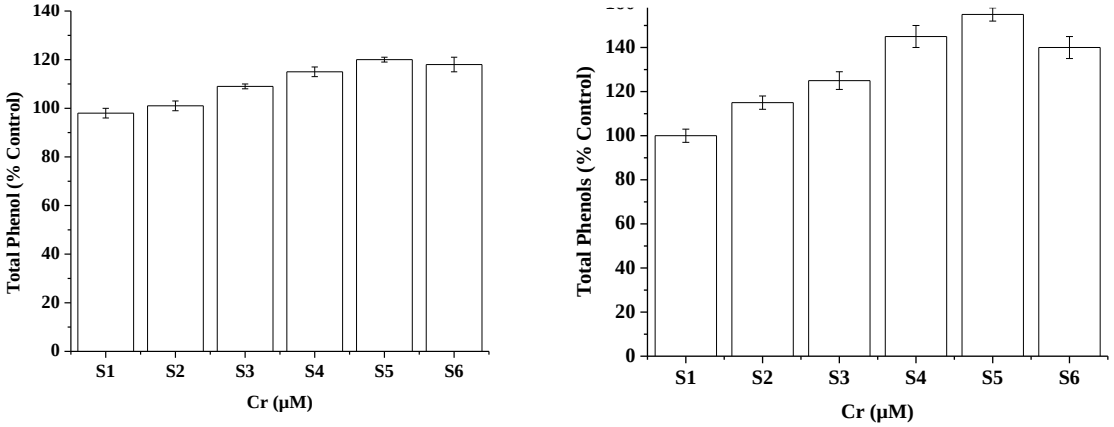


(a) (b)

Figure 10: The Effect of different Cr concentration (S1, S2, S3, S4, S5 and S6 represents the metal concentration 0, 2, 4, 8,16 and 32 µM respectively) on total MDA content of *spirulina* strains. (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TCIT-01.

5.8. Phenol

The data in Figure 6 (a) and (b) represent an alteration in phenol content in treated Spirulina at different Cr concentrations (0, 2, 4, 8,16 and 32 µM). After 4 days of Cr treatment, a dose-dependent increment in free phenol was observed but the enhancement was insignificant at low doses of Cr (2 and 4 µM). An enhancement in total phenol content by 45 and 60 % at 8 µM and 16 µM, respectively was observed.

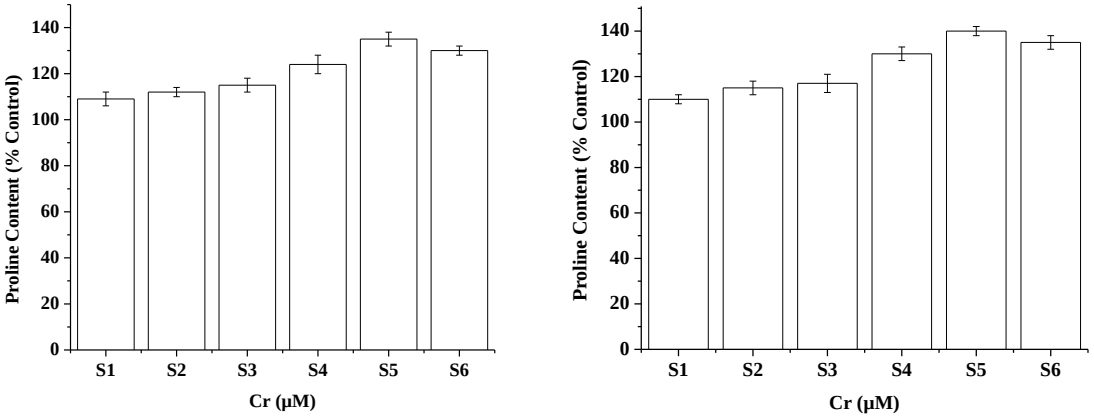


(a) **(b)**

Figure 11: The Effect of different Cr concentration (S1, S2, S3, S4, S5 and S6 represents the metal concentration 0, 2, 4, 8,16 and 32 µM respectively) on total phenol content of *spirulina* strains. (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TCIT-01.

5.9. Proline

The data in Figure 7 (a) and (b) represent an alteration in proline content in treated Spirulina at different Cr concentrations (0, 2, 4, 8,16 and 32 µM). After 4 days of Cr treatment, a dose-dependent increment in free proline was observed but the enhancement was insignificant at low doses of Cr (2 and 4 µM). A significant increment of 34 and 41 % in proline content was observed. In similar studies the proline content increased under Hexavalent chromium stress conditions in comparison to control. It was maximum in presence of 5ppm chromium in Lyngbya then started decreasing gradually (Adinath et al., 2001) whereas in our studies it was maximum at 16µM and decreased at 32µM concentration.



(a) **(b)**

Figure 12: The Effect of different Cr concentration (S1, S2, S3, S4, S5 and S6 represents the metal concentration 0, 2, 4, 8,16 and 32 µM respectively) on total Proline content of *spirulina* strains. (a) *Limnospora Fusiformis* IUAF001 and (b) *Limnospora Fusiformis* TCIT-01.

5.10. Protein Estimation

Table 4: Quantitative estimation of strain IUAF 001

S. No.	Nm	µg/ ml	µg/µl
1	0.739	71.93064	0.071931
2	1.685	167.2167	0.167217
3	1.432	141.7333	0.141733
4	1.291	127.3441	0.127121
5	1.064	104.6664	0.104666
6	0.585	56.41895	0.056419

Table 5: Quantitative estimation of strain TICT 01

S. No.	Nm	µg/ml	µg/µl
1	1.523	150.8993	0.150899
2	0.849	83.01042	0.08301
3	0.552	53.09501	0.053095
4	0.652	62.01252	0.062327
5	0.888	86.9387	0.086939
6	0.686	66.5922	0.066592

5.11. Pot experiment

The rice crops were used as an experimental crop for estimating the effect of the immobilised culture as biofertilizers. The treatment of the crop with immobilised beads was done for a duration of 45 days. A significant increase in the root and shoot length was observed for both the strains.

In strain 1 *Limnospora Fusiformis IUAF001* an increase of 3.1 cm was seen in root and 9 cm in shoot in comparison to the control.

Figure 13: Increment in root and shoot length of strain TICT 01in control and treated condition

In strain 2 *Limnospora fusiformis* TICT 01. An increase of 2 cm was seen in root and 20.6 cm in shoot in comparison to the control.

Figure 14: Increment in root and shoot length of strain TICT 01in control and treated condition

6.Conclusion

Spirulina exhibited moderate toxicity towards the test metal, chromium and tolerated it to a significant level thereby making it a suitable model organism for toxicity testing and investigating the underlying mechanisms to avert the metal stress-mediated biochemical changes. The intrinsic abilities of *Spirulina* to adsorb Cr from the soil will help in its reduced bioavailability to increase the crop productivity. Nevertheless, these original data do warrant further research to utilize *Spirulina* as a bio-fertilizer which can help farmers to use acidic fallow land for agricultural use enabling more sustainable and efficient production in the field of agriculture.

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