Morphogenetic Characterization and Phyco-remediation Potential of Selected Algal Flora Collected from Poonch AJK.

By

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QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN

2024

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A Thesis Submitted to the QUAID-I-AZAM University in Partial Fulfilment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

in

PLANT SCIENCES

DEPARTMENT OF PLANT SCIENCES
QUAID-I-AZAM UNIVERSITY
ISLAMABAD, PAKISTAN
2024


بسم الله الرحمن الرحيم

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This is to certify that the research work presented in this thesis entitled "**Morphogenetic Characterization and Phyco-remediation Potential of Selected Algal Flora Collected from Poonch AJK**" was conducted by Ms. Rooma Waqar under the supervision of **Prof. Dr. Abdul Samad Mumtaz**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan in partial fulfillment for the Degree of "**Doctor of Philosophy**" in the field of Plant Sciences.

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Dedication

My beloved grandparents,

My Parents,

My Uncle,

(Sardar Zarar Ahmed)

&

All My Family

This humble work is a sign of my love to you all!

Special Thanks

"What we do for ourselves dies with us, what we do for other remains and are immortal".

(Albert Pike)

I pay my special thanks to my supervisor,

Prof. Dr. Abdul Samad Mumtaz and host supervisor (Prof. Dr. Feng Chen)

for their guidance

help and valuable assistance.

Acknowledgements

All praises belong to **Almighty ALLAH**, the most merciful, beneficent, creator, and source of all knowledge and wisdom, who enabled me to complete this grand task successfully. I also pay my respect to **Hazrat Muhammad** (Peace and Blessing of Allah Be Upon Him), His faithful companions, who are forever a genuine torch of guidance for humanity.

I feel much pleasure in expressing my heartiest gratitude to my ever-affectionate supervisor **Prof. Dr. Abdul Samad Mumtaz** for his dynamic and friendly supervision and whose inspiring attitude made it very easy to undertake this project. It was only through his guidance that my efforts were fruitful.

My sincere thanks to Prof. Prof. **Dr. Hassan Javed Chaudhary**, Chairman Department of Plant Sciences, Quaid-i-Azam University Islamabad, for providing facilities and possible support during the research activities. I take this opportunity to record my sincere thanks to all the respected teachers of BPS for their unceasing encouragement and suggestions.

I would also thank to extend the wholehearted gratitude to my host supervisor **Prof. Dr. Feng. Chen** (University of Maryland University, USA) for his constant support, useful suggestions and providing access to the different laboratories at the institute of Marine and Environmental Technology USA. I am very pleased to recognize the kind assistance and encouragement of **Dr. Tsvetan Bachvaroff** during my stay at Maryland University, USA. I would like to express my sincere gratitude for the invaluable assistance and support provided by **Prof. Dr. Sohail Yousaf** during my time at Quaid-i-Azam University. I would like to thank my friends Asma Rabbani and Asfa Farooqi for their unwavering support and encouragement. Your presence and enthusiasm meant the world to me.

I would like to extend my sincere appreciation to my all my family members, parents, friends and colleagues for support, assistance, well wishes and their admirable company. I would like, to thank all the ex- and present members of the laboratory of Plant and Algal Genetics, (Quaid-i-Azam University). Also, I want to thank to my lab fellows for their guidance, help and support to the laboratory of Institute of Marine and Environmental Technology, University of Maryland, USA.

Finally, I must specially acknowledge the **Higher Education Commission** of Pakistan for the IRSIP (6 months) without which this project would not have been completed in this current shape.

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List of Abbreviations.

BBM	Bold Basal Media
BG_{11}	Blue Green 11
BLAST	Basic Local Alignment Search Tool
°C	Centigrade
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EB	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
g	Gram
L	Liter
LM	Light microscopy
BBM	Bold Basal media
MEGA	Molecular Evolutionary Genetic Analysis Software Program
ML	Maximum likelihood
mm	millimeter
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
PCR	Polymerase chain reaction

pH	Power of hydrogen
Ph.D.	Doctor of Philosophy
rRNA	Ribosomal RNA
µl	Micro liter
HMs	Heavy metals
$CdCl_2$	Cadmium chloride
Pb $(No_3)_2$	Lead nitrate
KEEG	KYOTO Encyclopedia of Gene and Genomics
DEGs	Differentially expressed genes
CO_2	Carbon dioxide
GIP	Genetic Information Processing
PFM	Protein family's metabolism
TFIID	Transcription Factor IID
SnRNAs	Short nuclear RNAs
GST	Glutathione Transferases
FATB	Fatty acyl-ACP thio-esterases
PFK	Phosphofructokinase
ATP	Adenosine triphosphate
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase

Summary

Microalgae, a source of food and nutrition, has been recently explored for their diverse potential applications across various scientific fields. Despite enormous potential, no baseline research has been conducted on freshwater algae locally. In this study, microalgae samples were collected from stream ecosystem in Hajira Poonch, AJK. In the collected samples, algae were isolated and identified morphologically and genetically. Microalgae enrichment was performed *in vitro* through solid streak plating. Axenic cultures developed on BBM and BG_{11} media. Green algae were commonly found accounting for 72% of the total, while 28% blue green types were also observed in the collection (field samples). During culturing, among green algal types, members of the family Scenedesmaceae were found most frequently, other green taxa were represented by either one or two species only. Among blue green types, *Phormidium* and *Oscillatoria* were observed frequently. Seven strains of green *Desmodemus communis, Desmodesmus subspicatus*, *Scenedesmus dimorphus*, *Scenedesmus bijugas*, *Scenedesmus* sp.1, *Scenedemus* sp.2, and *Scenedesmus* sp.3 were identified that belonged to the family Scenedesmaceae. These strains have been reported for the first time from Hajira Poonch, AJK. The isolated strains of Scenedesmaceae were assessed for their tolerance against heavy metals (Cd and Pb). Two strains (namely *Desmodesmus subspicatus* R3 and *Scenedesmus dimorphus* H9) were identified as the most effective in tolerating the elevated concentrations of Cd and Pb. These were assessed further for their Cd and Pb adsorption capacity under different conditions including pH, contact time, initial concentrations of metal ions in dried and living forms of algae. The experimental data were validated with adsorption kinetic (pseudo first order, pseudo second order and intra particle diffusion), and isotherm (Freundlich, Langmuir and Temkin isotherm) models revealing a chemisorption mechanism homogenously spanning over species surface. The RNA-Seq of *Desmodesmus subspicatus* (strain R3) revealed that 42,347,092, 47,483,530 for control and 39,333,360, 33,492,989 for Cd treated libraries respectively. Analysis further showed clear evidence of 615 differentially expressed genes. Among these, 452 genes were found to be up regulated while 163 genes were down regulated in the Cd stressed sample. The KEGG pathway analysis identified 10 key pathways to be involved in mitigating Cd stress. Metabolic pathways have a higher number of genes and biosynthesis of nucleotide sugars has a lower number of genes. Our results demonstrated that Cd retarded the growth rate in *Desmodesmus subspicatus*, which

was followed by a significant reduction in photosynthetic pigment and a decrease in the expression of genes linked with the photosynthetic system and oxidative phosphorylation. This study provides novel insights for understanding the molecular mechanisms of *Desmodesmus subspicatus* to cadmium-induced stress.

CHAPTER 1

1. Introduction

1.1 Algae

Algae are classified as lower plants since they lack leaves, roots, and stems and have a thallus-like body. Although they have much resemblance with plants but modern classification schemes which are mostly based on molecular phylogenetic systematic do not classify them under Kingdom Plantae (Graham and Wilcox 2000). Algae are further classified as green algae, red algae, brown algae, etc. based on their composition of pigment. Their habitats vary from freshwater rivers, lakes, and ponds with unicellular, multicellular, or colonial organisms to open oceans and rocky shorelines with seaweed. (O'Boyle and Silke 2010). The most prevalent cyanobacterial and microalgal species in freshwater include *Chlamydomonas*, *Chlorella*, *Nostoc*, *Anabaena*, and *Phormidium* species, among others. In addition, they may live on land and in harsh conditions like snow or glaciers in the Arctic or Antarctic (Chu, 2012). According to Ng et al. (2011), *Phormidium*, a cyanobacterium, predominates in the Kuala Lumpur air, most airborne algal species cause allergies in human. Similarly, Zhang et al., (2011) observed *Microcoleus vaginatus* coexist with other cyanobacterial and microalgal species as a dominating species in sand dunes.

Microalgae are among the earliest living organisms on earth. These are thallus-forming creatures that are members of the Kingdom Protista. They are microscopic with the size measured in micrometers. Microalgae are categorized according to the kinds of pigments they have, the chemicals that store energy in them, and the components of their cell wall. They are primarily categorized as diatoms, red algae, and green algae (Brennan and Owende, 2010). Although the number of microalgal species is estimated to be above 50000, only 30000 have been studied so far (Richmond, 2004). Chlorophyll is necessary for photosynthesis in microalgae. They share the same photosynthetic apparatus as plants, but because of their unicellular structure, they have a far higher photosynthetic efficiency. Furthermore, wet habitat not only offers more efficient access to CO_2, water, and nutrients, but also increases their photosynthetic efficiency significantly (Randrianarison, and Ashraf 2017). Morphologically these exist as single cells, small chains of few cells, filaments or trichomes and as a mass of colonies. However, unlike macroalgae, they do not create specialized multicellular structures (Spolaore et al., 2006).

1.2 Isolation methods of algal samples

Filtration, differential centrifugation, serial dilution, micro pipetting, serial dilution, and streaking on agar plates are employed to isolate and purify algal samples (Richmond, 2004).

1.2.1 Filtration

Samples that contain water are filtered through two layers of a sieve, net, or strainer of varying sizes. To remove the unwanted particles from samples, big particles, and trash are eliminated in this separation procedure (Clark and Sigler 1963).

1.2.2 Differential centrifugation

Centrifugation separates cells that are heavier from lighter cells such as bacteria and algae. However, brief centrifugation helps giant dinoflagellates and diatoms sink to the bottom and form a loose pellet. If necessary, the procedure can be done multiple times. The speed and duration of centrifugation differ between species (Watanabe et al., 1998). This procedure permits heavier algae to settle at the bottom, but it is not easy to get a monoalgal culture; thus, additional purification processes are required.

1.2.3 Micropipette isolation

This method is used for isolating microalgae. Glass capillary is commonly used for micropipette isolation. A drop water sample is placed in the slide and examined under a light microscope. Under the microscope, the purity of algal species is important, and the algal cells are gently picked and placed in the capillary. This isolated algal species is placed in a sterile medium drop. This procedure can be done numerous times using capillaries (sterilized) to acquire enough algal cells for appropriate development on plate that contain agar (Andersen and Kawachi 2005).

1.2.4 Streaking cells across agar plates

In this method, samples are placed on the medium enriched with 1.5% agar (Heaney and Jaworski 1977). After some days the plates contain algal growth and form colonies. Then the cells are removed off the agar plates by a loop and streaked again on a new agar plate (Richmond, 2004). This method can be done several times until a pure culture of desired algal species is obtained.

1.3 Raising of mass culture

When the desired algal species is pure, then they are moved to a broth medium to develop small-scale cultures. After inoculation, the culture goes through an adaptation phase known as the lag phase, during which the algae attempt to stabilize the culture environment. At this point, the cells are not capable of dividing. A little color change

can be seen when the cultures are allowed to grow continually. The algal culture is light green, representing that the algal cells have progressed to the stage when they have begun to divide. This is known as the exponential stage. Once the culture has reached full maturity, it is vital to subculture to keep the algae healthy. Then the sample can be aseptically shifted into a flask that contains fresh media and cultured under the same conditions (Devi, 2015).

1.4 Culturing of Algae

1.4.1 Basal Bold medium

Freshwater algae are cultured in Bold Basal medium (BBM). This medium can be made by combining the ingredients in distilled water. Ten milliliters of macronutrients, one milliliter of EDTA, boron, trace metal solution, iron, and one liter of distilled water are combined. For optimal culture growth, the pH of the culture is kept at 6.5. Agar (1.5%) in the broth medium is used to make a solid agar-based medium. Before usage, autoclave the medium (Alfiarty, 2018).

1.4.2 BG_{11} medium

Microalgae are cultured in BG_{11} medium. Four stocks of the culturing medium BG11 were prepared according to the recipe. About 10 ml of 3 stocks were taken into an Erlenmeyer flask and 1ml of the 4th stock which was the stock of trace metals was added (see appendices). The carbohydrates and nitrates were prepared by the addition of Na_2CO_3 (0.02g) and $NaNO_3$ (1.5g). The pH of the medium was adjusted to 7.3 and after it was autoclaved at 121˚C and 15 pa. for 45 min.

1.5 Importance of Microalgae

Today with the advancement of microalgal biotechnology various commercial applications for these organisms are known, for example:

- as food or feed, besides their utility in improving the nutritional value of food,
- their role in the aquaculture industry,
- due to their specific chemical composition these can be a source of high value molecules of industrial use (Spolaore et al., 2006).
- The Triacylglycerol of microalgae and cyanobacteria can be converted into biodiesel.
- Microalgae and cyanobacterial pigments are used in cosmetics. These include astaxanthin, carotenoids, chlorophyll and phycobiliproteins. These pigments perform various functions, for example, astaxanthin has antioxidant activities, anti-aging, anti-inflammatory and even immune-boosting properties (Chen et al., 2016).

- Microalgae have a role in bioremediation and therefore can be used to eliminate heavy metals from contaminated water (Waqar et al., 2023a,b).

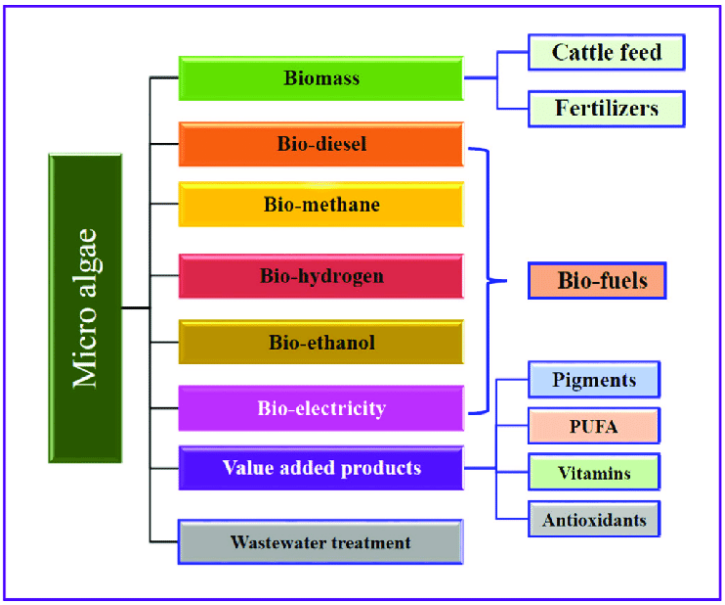


Fig.1.1. Potential of algae in different fields (Enamala et al., 2018).

1.6 Heavy metal pollution

Without water, life is not possible on earth. Water gets polluted by rapid industrialization and urbanization, and it impacts the quality of water and disturbs our ecosystem (Goel, 2006). Today pollution of heavy metals (HMs) is rated as an environmental problem. Different heavy metals like cadmium, mercury, zinc, lead, arsenic, and copper are used by human beings yet these are toxic and badly affect human health and aquatic life (Sharma et al., 2015). Different heavy metals persist in the environment, so the removal of heavy metals may protect public health and the ecosystem (Wang and Chen, 2009). The accumulation of heavy metal in water is caused mainly by increased industrialization, growing human population, and domestic waste run-off. Among such contaminants, heavy metals persistently accumulate in the environment and often exceed the toxic limits. Heavy metals (including lead, cadmium, mercury, and arsenic) impact humans by causing various ailments. Acute toxicity of heavy metals may damage the central nervous system, liver, bones, kidneys, and endocrine glands. It is impossible to avoid exposure to such heavy metals. Although people who are not occupationally exposed to heavy metals, also carry heavy metals in their bodies because of the exposure to contaminated food, air, and beverages. However, it is possible to reduce the toxicity of heavy metals and its risks through choices in lifestyle such as: dietary measures or excretion of ingested heavy metals that reduce the probability of heavy metal uptake (Kumar et al., 2015).

The rise in industrialization along the banks of water bodies is a major cause of heavy metal pollution and adverse effects on human beings. In water, heavy metals translocate into the fish's body and cause toxicity. The fish that live in the heavy metal-polluted water develop a physiological adaptation by accumulating heavy metals in their body. Environmental contamination is the major issue in the ecosystem and scientists have great interest in detecting the various heavy metals and their adverse effects on human beings. Even at low concentrations, heavy metals are toxic, which proves very dangerous to human beings. The concentration of heavy metals in biota has increased through bioaccumulation (Ganagaiya et al., 2001). These pollutants, when released into the environment accumulate into the water bodies and soil (Abida et al., 2009), and often get above the permissible limits.

Table.1.1: WHO Drinking Water Recommendation (Duruibe et al., 2007).

Heavy metal	Max. acceptable conc. (WHO) **(mg/l)**
Magnesium	50
Calcium	50
Zinc	5
Cadmium	0.003
Silver	0.001
Arsenic	0.01
Lead	0.01

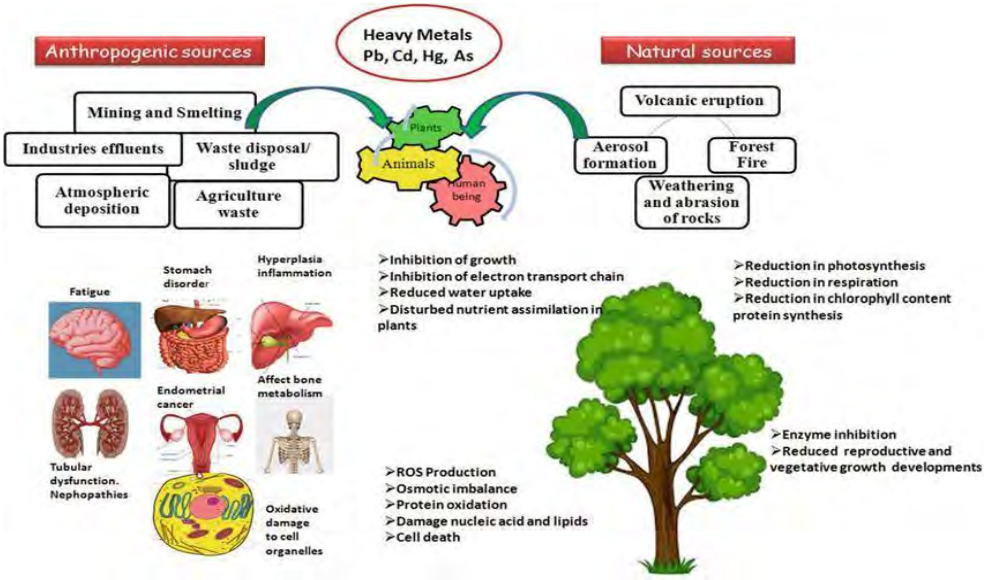


Fig.1.2. Source of heavy metals (natural and anthropogenic) and their effect on the environment (Goutam et al., 2021).

1.7 Removal of heavy metal from water

Different techniques are used to remove heavy metals (HMs) from contaminated water. These are precipitation, filtration, electrodialysis, ion exchange, and bioremediation. In the process of precipitation, the insoluble precipitates of heavy metals are produced, such as hydroxide, sulphides, phosphate, and carbonate. When the heavy metals dissolves in the solution it forms precipitates and eliminated as sludge (Fu et al., 2011). Alternatively, the method of ion exchange is used to treat wastewater (Dizge et al., 2009). In this process, a special ion exchanger is used to eliminate heavy metal ions from the solution. Ion exchangers which are commonly used, are synthetic organic ion exchange resins. Ion exchange resins absorb cations or anions from an electrolyte solution and then release other ions that have the same charge in an equivalent amount. Membrane filtration is a commonly used method to decontaminate heavy metals by using different membranes as filters. These are also known as ultrafiltration, reverse osmosis, and nanofiltration methods used for the removal of heavy metals from water. Ultrafiltration separates metals (heavy metal ions) from the solution based on the pore size and uses a permeable membrane (Vigneswaran et al., 2004). Electrodialysis in which the species having the charge are passed through a membrane (ion exchange) when an electric potential is applied. Membranes are thin sheets made up of plastic, having positive and negative charge characteristics. The solution of ionic species when passes through the cell

compartments, the cations migrate toward the cathode and anions toward the anode, crossing the anion and cation exchange membrane (Chen et al., 2003).

A more recently developed technique called bioremediation is the removal of hazardous waste or heavy metals from the environment using biological agents (Abdallah et al., 2016). Bioremediation is an effective method to deal with contaminated areas. Bioremediation employs different organisms such as fungi, bacteria, algae, and plants to remediate heavy metals from the environment (Kulshreshtha et al., 2014).

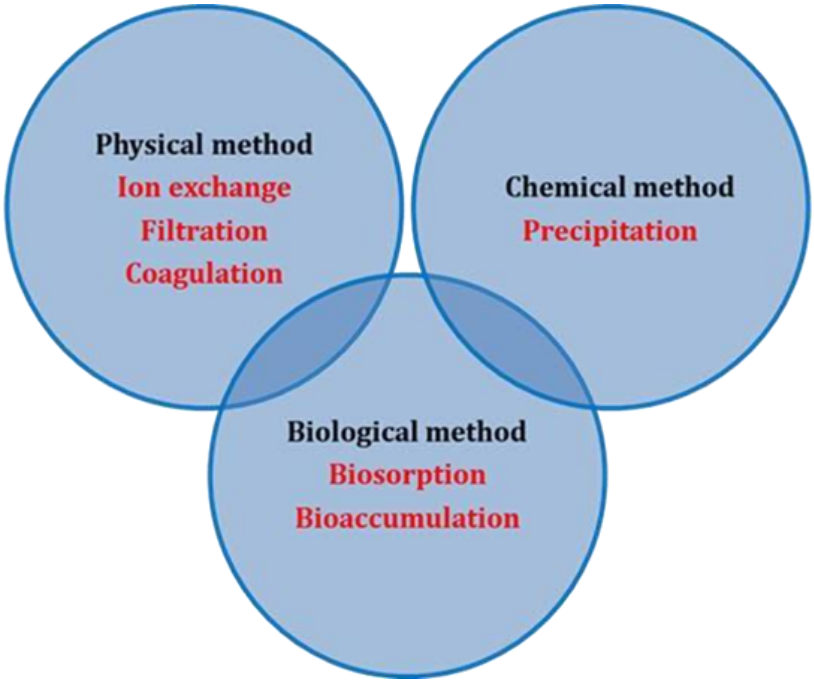


Fig.1.3: Different methods used to remove heavy metal from water.

1.8 Treatment methods for heavy metals remediation

Heavy metal removal methods have limitations, even though they are employed to remove heavy metals. Chemical precipitation is considered a cheap technique for the elimination of heavy metals, but it has some limitations, it is used to treat wastewater in high concentrations, and this method is not effective at low concentrations of heavy metal ions. Chemical precipitation generates sludge in a high volume, and it is very difficult to treat this sludge. The ion exchange method is used to remove heavy metals from polluted water, however, in this method, there is generation of ion-exchange resins, which can result in significant secondary contamination. This is a low-cost technique that is used to treat huge amounts of wastewater with low heavy metal concentrations. This technique is not suitable for large scale. The membrane filtration technique is effective in the removal of heavy metal ions from wastewater, but it is

costly and has a complicated procedure. Electrochemical heavy metal wastewater treatment is regarded to be a quick method that uses few chemicals and generates little sludge. However, electrochemical methods require power in the form of electricity, and their development is limited (Fu and Wang 2011).

Alternatively, bioremediation is a natural process appropriate for wastewater treatment in polluted areas. Bioremediation can prove less expensive as compared to other methods for removing heavy metals from water. Bioremediation completely removes or destroys a variety of pollutants. Many compounds are considered as a hazardous compound and can be converted into harmless products through bioremediation (Kulshreshtha et al., 2014). Although all the above techniques are used for removal of the heavy metals from wastewater, the choice of the most acceptable technique is very important, which thought to be low cost, readily available, and must not cause secon in the ecosystem.

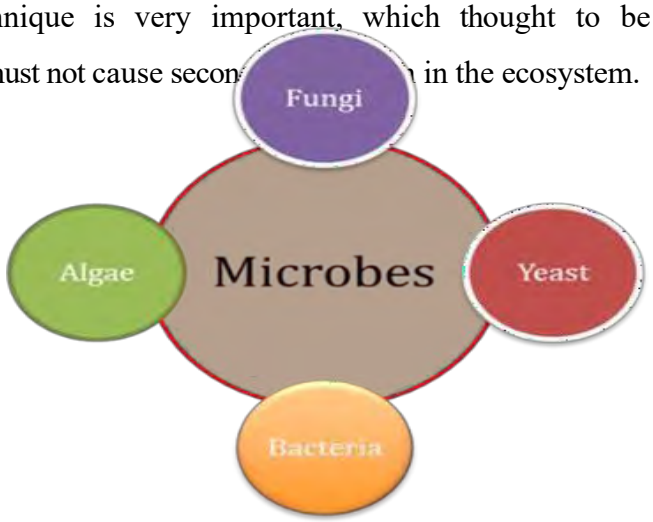


Fig.1.4: Different microbes used for the removal of heavy metals.

1.9 Phycoremediation

Phycoremediation is stated as the process which is used to treat polluted water that contains heavy metals and other contaminants with the help of algal biosorbents (Rawat et al., 2011). Phyco-remediation is a cost-efficient technology as compared to phytoremediation because algae can grow in polluted water due to the availability of different nutrients (Al-Balushi et al., 2012). Algae is an autotrophic organism; it requires more phosphorus and nitrogen for metabolic processes such as protein synthesis. Various algal strains can absorb a considerable quantity of harmful contaminants from wastewater such as pesticides, heavy metals, and other organic pollutants (Oswald, 2003). Algal strains can live in an environment that has high concentrations of heavy metals and other hazardous contaminants; they are the most successful microorganisms for removing heavy metals from wastewater. Algae have a

large surface area and the ability to grow autotrophically and heterotrophically, allowing them to absorb a considerable quantity of contaminants from wastewater. They also can manipulate genetic information (Ahmed et al., 2022).

1.10 Advantages of Phycoremediation

1.10.1 Eco-friendly

Algae species have the potential to be environmentally beneficial. Heavy metals phyco-remediation do not pollute the environment and is not a source of secondary pollutants in the environment. Algae that have been grown in polluted wastewater and harvested from similar conditions can be preserved for future use (Edmundson and Wilkie 2013). Different strains of green algae are used to remove HMs from contaminated water all around the world. Boron and arsenic are absorbed and accumulated by Chlorophyta and Cyanophyta. These algae act as phyco-remediators, removing boron and arsenic from polluted wastewater (Baker, 1981).

1.10.2 Biosorption capacity of algae for heavy metals

Algae are used as a biosorbent because algae are cheap and highly efficient biosorbents. Based on a statistical study of algae in biosorption, it has been determined that algae can absorb 15.3 percent to 84.6 percent which is greater than other microbial biosorbents. Brown sea algae have a high potential for metal absorption, such as Pb, Ni, and Cd because different types of functional groups (amino, carboxyl, hydroxyl, sulfonate, and sulfhydryl) are present on their cell wall which help to eliminate heavy metals from the polluted water (Mustapha and halimoon, 2015).

1.10.3 Metals binding protein

Algae can absorb heavy metals, produce binding proteins, and form complexes when combined with heavy metals. The proteins are involved in the metal binding process and the

proteins are phytochelatins (PC) and Metallothionine (MT). The important role of these proteins is due to the sulfide groups of the cysteine residues, which are present in these proteins. The positive charge of metal (cations) has chemical affinity toward anions which are present in sulfide groups of cysteine, which result in the formation of heavy metal–metallothionein and heavy metal–phytochelatins complexes. Thus, both metallothioneins and phytochelatins can scavenge heavy metals (HMs), minimizing their detrimental effects within the cell (Sharma et al., 2016).

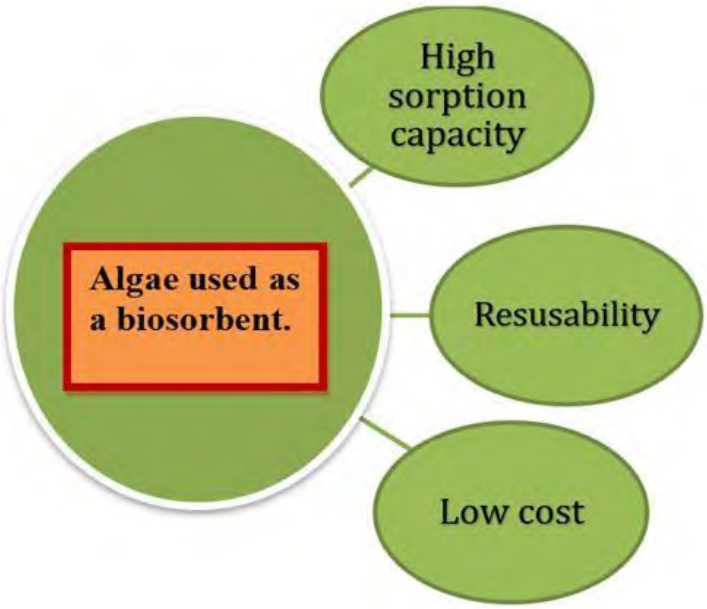


Fig.1.5: Algae used as a biosorbent have several advantages.

1.11 Mechanism of Phycoremediation

Heavy metal can be removed by algae using the following processes:

- Biosorption process
- Bioaccumulation process

1.11.1 Involvement of algal cell wall

Removal of heavy metal by the algal cell is due to its properties in the cell wall. The algal cell wall has a negative charge because of the presence of different functional groups on the cell wall. Different algal species have different cell wall composition and different functional groups, so they have different capacities to remove the heavy metals from the contaminated water. Different functional groups have active sites for binding the positive ions (heavy metal ions). The carboxyl group has a dominant active site and has the highest affinity to bind with metal ions. For example, capsulated and non-capsulated species of cyanobacteria have different capacities to bind with positive ions (heavy metal ions). The cyanobacterial capsule has a high negative charge due to the presence of uronic acids which have a high affinity to bind with positive metal ions as compared to encapsulated cyanobacteria (Silver and Phung 1996).

1.11.2 Biosorption

Biosorption is the process in which the positively charged heavy metal ions are bound to the functional groups that are present on the biosorbent cell wall. Because biosorption is a physical adsorption process, it is not affected by metabolic processes

and can occur in live or dead cells (Bilal et al., 2018).

1.11.3 Mechanism of biosorption

The sorbate binds to the biosorbent during the biosorption process. Natural materials can be utilized as biosorbents, allowing heavy metal ions to be bound either physically (van der Waals forces) or chemically. Biosorbents have a cell wall that contains different functional groups such as carbonyl, carboxyl, amine, sulfonate, thioether, phosphodiester, phenolic, and phosphate which are attached to the heavy metal ions present in polluted water. The important factors that characterize these mechanisms are (Park et al., 2010).

- Oxidation state, ionic radius, and molecular weight of the targeted metal species.
- Biosorbent nature (living or nonliving/amorphous).
- Availability and type of binding sites.
- Different factors such as pH, the concentration of sorbate and sorbent, contact time, and other competing metal ions. The combined effects of the above parameters influence metal speciation means the formation of new metal forms because of biosorption.

The biosorption process includes the mechanisms.

- **Complexation**

The association of two or more species resulting in the formation of a complex is called complexation. Different forms of complexes are formed, and these are mononuclear and polynuclear complexes. Between the metal ion and the ligands, mononuclear complexes are formed, in which the cation (metal ion) is at the central position. Depending on the number of binding groups that are involved, a polynuclear complex is formed that has more than one metal ion in the center, and the metal atom may have a negative, positive, or neutral charge. Because polynuclear comprises several ligands that bind to the monodentate ligand and form complex formation. The metal ions are bound by the ligands with the help of covalent bonds. After the biosorption of antimony (III) by cyanobacteria, FTIR analysis are performed to find the different functional groups that are involved in biosorption process (Wu et al., 2012).

- **Ion exchange**

Ion exchange is the main process in adsorption. During the sorption process, binary metal exchange the ions with the counter-ions which are present on the surface of the

sorbent. A good example of positive charge ion (cation) exchangers are carboxyl groups and imidazole/amino groups are a good example of negative charge ion (anion) exchangers. The process of adsorption of Cr (III), Cu (II), and Cd (II) by blue green algae was studied. The functional groups are responsible for the cation exchange which were present on their surface and identified as carboxyl, phosphate, and hydroxyl groups (Chojnacka et al., 2005).

1.11.4 Bioaccumulation process

It is the process in which the heavy metal ions are translocated into the vacuole across the cell biological membrane where these accumulate in the algal cells. This process occurs only in living cells (Al-Gheethi et al., 2015). In living cells, detoxification processes occur which reduce the toxic effect of heavy metal ions. In the detoxification process, toxic metal ions are changed into non-toxic forms. This method takes place via the precipitation of heavy metal ions such as phosphate, sulfide, or carbonate (Silver and Phung 1996). Nonliving cells act as a storage for heavy metal accumulation via passive transport mechanisms, especially if they have been exposed to pre-treatments that produce pores through which metal ions enter the algal cell and accumulate (Silver and Phung 1996). Heavy metals can be eliminated by algae with polyphosphate bodies. Polyphosphate bodies serve as a storage pool and then detoxify the different metals (Dwivedi, 2012). Algae demonstrated a variety of responses against heavy metal stressors, including the production of metal-binding proteins such as metallothionein, their translocation into vacuoles, and the formation of complexes (Mejare and Bulow 2001). According to previous studies, *Chlorella vulgaris* performed three steps in water contaminated with heavy metals: first metal uptake, second metal toxicity, and finally proline accumulation (Kramer et al., 1996).

1.11.5 Absorbent and adsorbent properties of algae biomass

Algae can absorb and adsorb substances. Heavy metals can be up taken by algae, growing in heavy metal effluent, and then carried into their vacuoles. Different types of heavy metals are removed from wastewater by using *Oedogonium* and *Cladophora* (Kaplan, 2013).

1.11.6 Internal detoxification

Internal heavy metal detoxification has received minimal research in algae as compared to the surface binding and transport. In a polluted environment, algae can activate different physiological processes for resistance in such an environment (Gaur and Rai 2001). Copper is concentrated intracellularly in algae such as the diatoms form

polyphosphate bodies, also known as electron- dense spherical bodies (Soldo et al., 2005). In the detoxification process, the conjugation of heavy metal ions (contaminants) with intracellular molecules, followed by further compartmentation of conjugates, degrades into common cell metabolites, and finally converted to carbon dioxide. The above-mentioned process is maintained by different environmental factors such as salinity pH, temperature salinity, and many others (Ospina-Alvarez et al., 2006).

1.11.7 Metal transformation

Metal transformation is a processes that is involved, in changing the physical or chemical properties of metals. These chemical changes are reduction, methylation, oxidation, and demethylation (Lenis et al., 2007). Methylation has been reported in algae (brown) to detoxify mercury from aqueous medium (Davis et al., 2003). In this process, degradation of metal ions into less toxic form, as in the case of the transformation of tributyltin by green algae. The chemical is butylated into di monobutyltin molecules during this reaction (Toumi et al, 2000). Bacterial transformation is also linked to the conversion of those methyltin derivatives. It has been proposed that arsenosugar breakdown products from algae lead to the production of arsenobetaine. This chemical is found in abundance in the tissues of marine organisms, (Davis et al., 2003). Arsenic may also be taken out by algae and transformed into organo arsenicals, which is part of the detoxification process (Schiewer and Wong 2000).

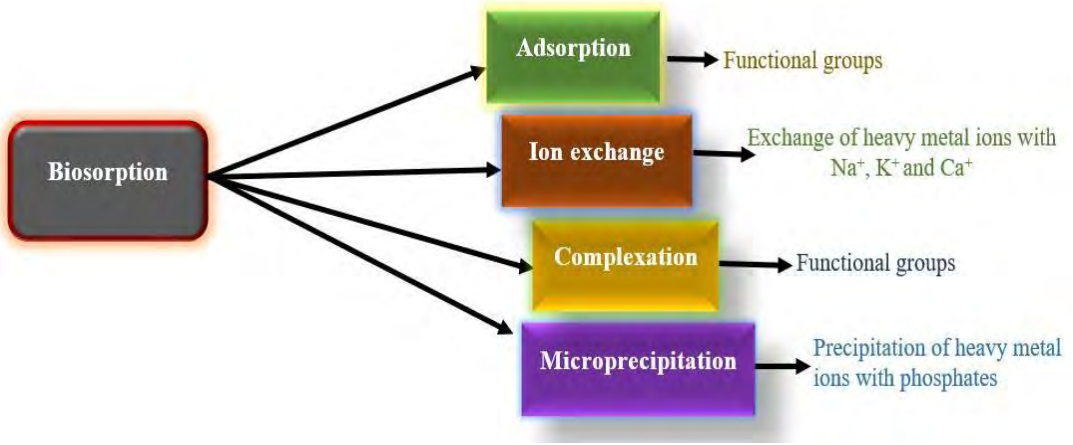


Fig.1.6: Different types of biosorption mechanisms.

1.11.8 Metallothioneins and phytochelatins in heavy metal remediation

Microalgae are gaining interest in a variety of biotechnological applications due to their great genetic, chemical, and functional diversity, as well as their capacity to

grow and produce biomass at quicker rates than other terrestrial plants. Algae produced the metal-binding protein that is essential in phycoremediation. Phycoremediation is the process in which heavy metal ions are removed from polluted sites with the help of algae. Algae can grow faster in different medias and have a wide chemical variety, allowing them to absorb heavy metal ions faster than bigger creatures. *Chlamydomonas, Chlorella,* and *Scenedesmus* are green algae most often utilized to remove heavy metals from polluted water. Heavy metal ions bound to various functional groups that are present on the cell wall of algae (Kumar et al., 2015). Adsorption is combined with a process in which heavy metal ions are transported into the cytosol, resulting in heavy metal compartmentalization into different organelles (Penen et al., 2017). Heavy metal binding proteins have great interest in the elimination of heavy metals from the polluted site. Heavy metals are absorbed, transported, and accumulated within organelles and these processes are regulated by proteins like metallothionein (MT) and phytochelatin (PCs). To enhance the bioaccumulation of heavy metal ions, researchers have recombinantly expressed import– storage systems, that contain active transporters (Diep et al., 2018). Although the aim of genetic modifications is to improve metal absorption in prokaryotes (Deng and Jia, 2011), higher plants (Ueno et al., 2011), and yeasts (Shen et al., 2012), a few research on microalgae have been conducted (Ibuot et al., 2017). In the next decade, heavy metal phycoremediation can be improved by finding different proteins that naturally occur in algae, as well as by using genetic engineering techniques to create mutants that can tolerate and immobilize high concentrations of heavy metals.

1.12 Factors affecting metal sorption in algae

Adsorption of heavy metal ions by algae may be affected by different factors. These factors are pH, live and inactivated biomass, biomass concentration, temperature, and contact time. This part aims to study a few of these factors and their possible effects on the adsorption of metal ions.

A- pH

The major factor that influences the process of adsorption is the pH. The charges which are present on algal surfaces depend upon the pH. By increasing pH (acidic), the algal surface becomes more negative due to the removal of protons from the functional group, so the uptake capacity of metal ions increases (Sheng et al., 2005). By decreasing the acidic pH, the number of binding sites on the algal surface is reduced which decreases the metal uptake. The value of acidic pH lies 0-6. It is

necessary to find an optimum pH that maximizes removal of heavy metal by algae. For the absorption of Cd and Cr (VI) by *Sargassum* sp. and *Padina* sp. the optimum pH was 2 (Sheng et al., 2004). Yu and Kaewsarn (1999) reported that at pH below 2 *Durvillaea potatorum* showed very little sorption of Cu, but the sorption is increased by rising the pH. They reported that when the pH is between 3 and 4 maximum sorption of Cu occurs and the optimum pH of plateau was around pH 5. Ozer et al., (1994) showed that the optimum sorption of Cr (IV) and Pb by green algae is pH 1.0 and 5.0 respectively. The difference in optimum pH of Cr (IV) and Pb is due to the difference in their chemical interaction with cells of algae. When the pH is 5.0, the surface of algal cells has a net negative charge that helps the binding of Pb (II) to surface ligands. The algal cell walls have metal binding groups, and most of these groups are acidic such as carboxyl and their availability are dependent on pH. At acidic pH, these groups have negatively charged and electrostatic interactions between metal ions that have a positive charge, and the anionic algal cell surface is responsible for biosorption of metal. The decrease of sorption property in metal by algae has been observed when there is an extremely acidic pH which means the pH is below 2 (Mehta and Gaur, 2001). The optimum pH for the elimination of heavy metal by the sorption process is different in green and blue-green algae. The optimum pH for the sorption of copper in green algae (*Cladophora prolifera* and *Chlorella vulgaris*) is less than the pH observed in *Microcystis aeruginosa* (Pradhan, et al., 1998 Mehta and Gaur, 2001).

B- Live and inactivated biomass

Most research on the removal of heavy metals by algae has been performed on living cells. Later, this research interest shifted to non-living algae or activated cells (chemical killing or heat killing) for the elimination of heavy metals (Volesky and Holan, 1995; Aksu et al., 1998). Dead cells absorb heavy metals, they do not require nutrients, have high surface area, and are not affected by the environment. Living biomass has the process of biosorption and bioaccumulation (Flouty et al., 2012). Vacuum drying decreased the sorption of uranium by green algae because of the disappearance of effective binding sites for the metal ions which are present on the algal cell surface, as water evaporates from the cells under vacuum conditions. Conversely, it has been observed that the potential of metal adsorption in freeze-dried biomass is greater as compared to live biomass. Carrilho et al., (2000) observed that there is no change in the metal sorbing potential of oven- dried and frozen absorbent,

as compared to living absorbent. Adhiya et al. (2002) reported that the living cells and lyophilized cells of green algae (*Chlamydomonas reinhardtii)* showed similar FTIR spectrum. It has been explained that lyophilization does not change the chemical composition of the cell surface as well as the cell wall. In the dead cells adsorption capacity depends on their pre-treatment and then changes occurred in the cell wall structure (Duddridge and Wainwright 1980). The inactivation (heating killing or chemical killing) method of cells influences the metal adsorption capacity. Heat-killed algal sorbents enhance sorption of metals as compared to chemical killing, which decreases the metal sorption capacity of the adsorbent (Tobin et al., 1984).

C- The influence of contact time

The amount of heavy metal absorbed by algae is influenced by the contact time. Biosorption occurs in two stages: (1) for algae biomass, heavy metal ions adsorb to the cell wall passively, and adsorption of metal ions occurs rapidly, and (2) for living algae, biosorption and accumulation occur slowly into the algal cell. (Vogel et al., 2010). The dried biomass of green algae absorbs over 90% of the dissolved uranium to the cell surface during the first 5 minutes. The green algal biosorbent quickly absorbed free ions of Cd, Hg, and Pb reaching equilibrium within a contact time of 60 minutes (Tuzun et al., 2005). This explains that the adsorption of heavy metal ions by microalgae is a passive process and occurs quickly, even when algal cells are dried. In living algae, the contact time has a greater effect on the adsorption capacity. For example, the adsorption of metal ions by green algae was harvested separately after different days and found that the growth of algae decreased over time, a maximum adsorption capacity was observed in older cultures (Lamaia et al., 2005).

1.13 Desorption and metal recovery

The cost-effectiveness of the method depends on the regeneration of biosorbent and metal recovery. To do this, the sorbed metals must be desorbed, and the biosorbent material must be regenerated for another cycle. In the desorption process, metal is obtained in concentrated form and regenerates the biosorbent (like the original state) for reuse and the biosorbent has no physical changes. This can be done by treating the algal biomass with diluted mineral acids like HCl, HNO_3, and H_2SO_4, and metal is removed in less toxic form (De Rome and Gadd 1987). Organic acids (lactic acid and citric, acetic) and complexing agents (Thiosulphate, EDTA) can also be used for the recovery of metals without affecting the algal biomass (Mattuschka and Straube,

1993).

1.14 Transcriptome analysis of genes involved in the sequestration of metals

The toxicity of microalgae is dependent on the intracellular content of metal ions; therefore, it is important to know the transport systems involved in the cells. Microalgae have evolved an inherent multifaceted system that regulates the sequestration of heavy metals. Different proteins are present in the algal system that help to maintain metal homeostasis inside the cells (Kumar et al., 2015). The following part describes the regulatory role of different proteins that help in the sequestration of heavy metals.

1.14.1 Transporter genes involved in the sequestration of heavy metals

Heavy metal ions once enter the algal cell are chelated by thiol containing molecules preventing them from interfering with important metabolic activities in the cytoplasm. Some key membranes help to transport and then carry these conjugates into vacuoles. MRP and ATM/HMT are two important subclasses of ABC transporters that modulate heavy metal sequestration into the different compartments, which improves the tolerance of heavy metal by algae (Hanikenne et al., 2005). The genome of green algae (*C. reinhardtii*) contains seven MRPs, four of which act as glutathione S-conjugate pumps and are present in the vacuolar compartment that transports complexes (heavy metal-glutathione complexes) into the vacuoles (Hanikenne et al., 2005). ATM/HMT are half-size ABC proteins found in the mitochondrial compartment. Cds1 is identified to play a main function in the tolerance of cadmium by regulating the export of Cd from the mitochondria compartment among the three different ATM/HMT transporters (Blaby-Haas and Merchant 2012). Furthermore, ATM/HMT2 proteins are only found in the mitochondrial compartment, whereas ATM/HMT3 transporters are only present in the vacuolar compartment that help complexes of Cd-phytochelatin sequestration in the vacuoles (Hu et al., 2001). Illumina-based sequencing of green algae treated to lead, inorganic, and methyl mercury (MeHg/IHg) explained the upregulation of ABC proteins, indicating a main role in the heavy metal's uptake (Zheng et al., 2020). Upregulation of MRP2 protein among ABC transporters suggests its role in the vacuolization of mercury that helps in detoxification (Beauvais-Fluck et al., 2017).

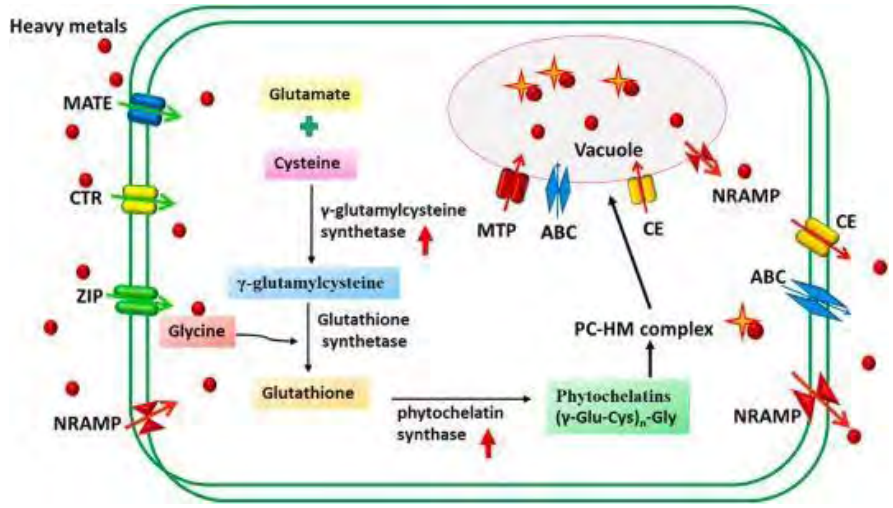


Fig.1.7: Detoxification of heavy metals by algae: transcriptomics analysis (Tripathi et al., 2021).

1.14.2 Heavy metals cause Physiological changes in Microalgae studied by Transcriptome analysis

Once heavy metals enter the algal cell, they are either detoxified or they affect cell physiological health by weakening the process of photosynthesis, slowing down the activity of enzyme, and stopping the cell division (Tripathi and Gaur 2006). The toxicity level in the algal cells is indicated by inhibition of growth. Different heavy metals have been shown to affect membrane of thylakoid and chlorophyll production (Lamaia et al., 2005). Heavy metal ions have also been shown to acidify the cytoplasm and damage the membrane by altering its potential (Conner and Schmid 2003). Different studies have described that heavy metal ions have a negative effect on the growth parameters of microalgae cells (Cheng et al., 2019). However, the experimental data produced through biochemical analysis must be confirmed using modern molecular methods to map even the tiniest cellular disturbance that resulted in microalgae adaptation.

1.15 Identify the Significant Research Gaps in the Study of the Molecular Mechanisms and Effectiveness of Algal Morphogenesis and Phycoremediation

Despite the growing interest in algae for their potential applications in the remediation of environment and biotechnology, there are still certain scientific gaps in our knowledge. Not enough research has been done on the morphogenetic potential of algae from Hajira Poonch AJK and their capacity to alter and adapt their form, across a wide range of species and environmental circumstances. More knowledge is required on the genetic, metabolic, and ecological elements that propel algal morphogenesis, especially about how pollutants and environmental stressors affect

these processes. Furthermore, although phycoremediation using algae has shown promise, the effectiveness and processes of pollutant uptake, transformation, and detoxification remain unclear. To close these gaps and determine the genetic underpinnings of these processes, a thorough analysis of the transcriptome profiles of algae is necessary. This research intends to provide new insights into the functional roles of individual genes and their contributions to the environmental adaptation and remediation capacities of algae by combining extensive transcriptome data with morphogenesis and phycoremediation studies. This method improves our theoretical knowledge while incorporating practical implications for increasing the efficiency of algae in biotechnological applications like sustainable resource management and pollution reduction.

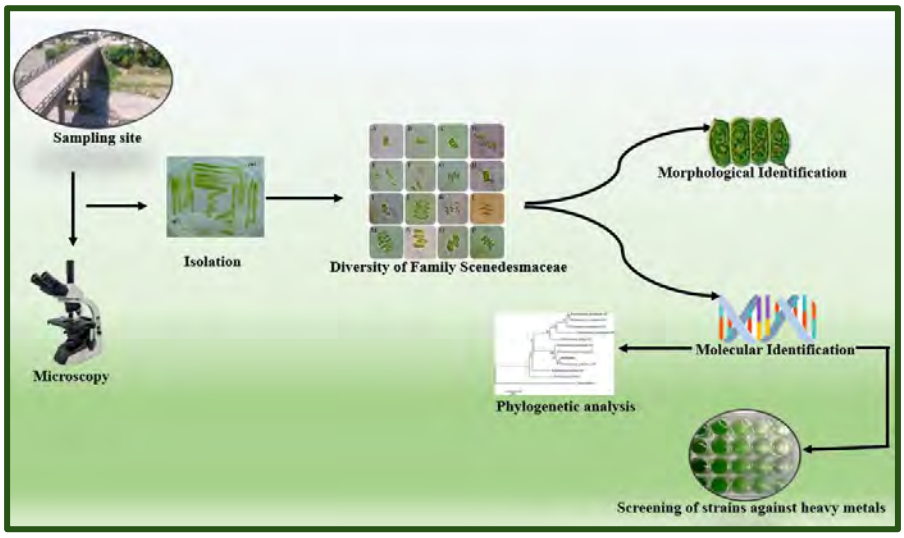
Aims and Objectives

- To collect the algal samples from freshwater streams of Tehsil Hajira Poonch AJK.
- To explore and identify algal flora morphologically and on molecular basis.
- To assess the phyco-remediation potential against Cd and Pb in selected green algae.
- To assess the transcriptome of *Desmodesmus subspicatus* under Cd stress.

CHAPTER 2

Collection, Isolation, and Identification of microalgae from Poonch AJK.

Graphical abstract



2.1 Abstract

Algal samples were collected from Hajira Poonch AJK during the month of August (monsoon season), from sites along stream, contaminated with domestic sewage water, detergents, and agriculture waste including fertilizers. All samples were assessed for the physio-chemical parameters and detection of different heavy metals through atomic absorption. After initial observations, the samples were subjected to isolation, purification, and culturing. Among field samples, the green types were observed frequently contributing a share of 72%, while 28% blue green algae were observed during isolation. Among green algal types, members of the Genus *Spirogyra* were found most frequent in the field samples. Other green taxa were represented by either one or two species only. Axenic cultures obtained in BBM and BG_{11} media, corresponding to distinct species of family Scenedesmaceae were recorded. The diversity of family of Scenedesmaceae were observed during isolation. A regular microscopic observation was conducted besides the molecular analysis based on 18S rDNA, ITS, and ss. The data analysis revealed seven species of family Scenedesmaceae including *Desmodesmus communis*, *Desmodesmus subspicatus*, *Scenedesmus dimorphus*, *Scenedesmus bijugus* and *Scenedesmus* sp.1, *Scenedesmus* sp.2, *Scenedesmus* sp.3.

2.1 Introduction

The biodiversity of freshwater algae was reported by several physiologists. Ashraf et al., (2008) and Dad and Leghari, (2008) described freshwater algae from Hajira and its adjacent area. They collected algal samples during 2007. They identified 74 algal species of algae belonging to 46 genera of 8 phyla. Khalil et al., (2021) determine the role of algae as a bioindicator to assess the water quality. They identified 201 species of algae from different sites of Rawalakot Poonch Azad Kashmir. In this study, Bacillariophyta was shown to be dominant with frequency % 47 followed by Chlorophyta (38%) and Cyanophyta (19%) respectively. Euglenophyta, Charophyta and Dinophyta are minor divisions recorded in this area. Ashraf et al., (2008) also described the algal samples from Hajira (AJK) and its adjacent areas and they also described the seasonal succession. Several studies were made on the seasonal variation of algal species from Bunkhurma, Mirpur, Azad Kashmir. 67 species of freshwater algae were explored from Bunkhurma of Mirpur, Azad Kashmir during different seasons of 2006 (Butt and Leghari, 2007). In District Sudhnoti (AJK) taxonomic study of freshwater algae of Trarkhal was described by the Wazarat and Leghari (2014). The Lichens of Poonch (AJK) was studied by Firdous et al., (2017). They studied Lichen as a bioindicator of air pollution from vehicular emissions. Khalil et al., (2017) study the physico-chemical and biological characteristics of the water in the National Park (Mahasheer) of Azad Jammu & Kashmir (AJK). They collected water samples from different sites in the Mahasheer National Park and for the first time they explored the algal species in that study area.

In the genus *Scenedesmus*, the first species were described by Turnip in 1828. He placed the species of *Scenedesmus* in diatoms, later by Ehrenberg (1834) in Desmidiaceae. Nageli (1849) placed this species in the Chlorococcales family Hydrodictyaceae. Finally, Oltmanns (1904) placed the species in the family Scenedesmaceae. In 1829, Meyen (1829) created the genus name. For this genus Hegewald and Silva in 1988 enumerated taxa or combination. The most important monographs are Smith (1916), Chodat (1926), Kiriakov (1977), Komarek and Fott (1983) and Uherkovich et al., (1995) and for Korea: An (1989) also considered electron microscopy, all other monographs are exclusively based on light microscopy. Genus *Scenedesmus* is a common alga occurring as a pure culture in plankton. Cells occur in combinations of 2, 4 and 8 cells in the colony. The species mostly differ by the

number and type of spines on the cell and texture of the wall. The morphology of a colony depends upon the medium in which cells are growing and can vary by changing the medium (Egan and Trainor, 1989). Members of the genus *Scenedesmus* grow as unicells in the medium which has low phosphorus (Trainor, 1992). The spines are present in some species of *Scenedesmus* help in the flotation of the colonies (Staehelin and Pickett-Heaps 1975). When the medium is deprived of nitrogen, *Scenedesmus* forms zoospores (Trainor, 1963). In dams *Oscillatoria*, *Phacus*, *Euglena* and *Scenedesmus* are indicators of organic pollution (Kumar, 2016). Most of the algae can tolerate various extent of organic pollution in water bodies.

Dominant primary producers are algae in lakes and ponds and are found in water bodies where there is plenty of light for photosynthesis. Important bioindicators of environmental conditions are algae (Stevenson and Smol 2003). For a long time, microalgae have been classified on the basis of morphology. Because of their basic form, most of the green algae have only a few morphological characteristics. However, it has been revealed that the majority of green algal characteristics vary depending on the environment. Then, genetic-based research emerged with somewhat clear connection to morphology (Malavasi et al., 2016). Molecular methods for taxonomic identification of algae are being used as a substitute for morphological identification. DNA bar coding is a method of species identification that classifies algae strains based on their DNA sequence similarity to a sequence database. DNA-based identification is beneficial for revealing cryptic variety at multiple taxonomic levels and identifying species whose morphology is difficult to examine. Most of the molecular research has focused on characterizing biodiversity and systematics. Identifying species using nucleic, mitochondrial, and chloroplast DNA has become common place in recent years. DNA barcoding is a new approach for species identification that uses a short section of DNA (Kowalska et al., 2019). Potential Chlorophyta DNA barcode targets include chloroplast (*rbcL* and *Cp23S*) and nuclear genes (18S rDNA and ITS region) for identification of algae in the environmental samples (Banerji et al., 2018).

According to the evolutionary hypothesis, green algae is proposed to have diverged into two clades: Chlorophyta and Streptophyta. Chlorophyta comprised nearly all forms of green algae, whereas streptophyta included Charophytes and land plants (Leliaert et al., 2012). Chlorophyta members are abundant in marine, freshwater, and

terrestrial environments and exhibit amazing morphological variation. In Chlorophyta four types were acknowledged: Chlorophyceae, the fresh water Trebouxiophyceae, coastal Ulvophyceae and the marine planktonic Parsinophyceae. Pure culture is required for proper morphological description, experimental analysis, and molecular identification. Algae isolation is well established using traditional procedures, dating back to Beijerinck's practice (1890). Some species are relatively easier to isolate, while some are extremely difficult to isolate in the form of pure culture. The serial dilution and agar streaking methods are both extensively employed for species isolation. After which the culturing conditions are critical and require extra care.

Hence for this study the objectives are:

- Collection, isolation, and purification of algal samples from Poonch AJK.
- Microscopic assessment and cultivation of selected algal species.
- Morphological and molecular identification of strains/species of family Scenedesmaceae.
- Screening of selected strains for Cd and Pb (heavy metals) tolerance.

2.2 Materials and Methods

2.2.1 Description of the area

The algal samples were collected from Hajira Poonch (AJK). Hajira is a small town located at latitude 33° 46′ 18.12″ N, longitude 73° 53′ 45.96″ E and an altitude of 3168 feet. Hajira is 17 miles from the city of Rawalakot and 88 miles from the capital of Pakistan (Islamabad). District Poonch has a total area 855 Sq. Kms, and population is 0.411 million. Hajira's total population is approximately 0.1 million. Hajira town is located on both sides of the stream and is surrounded by mountains. Hajira is very close to the line of control and Indian occupied Kashmir. Hajira (Azad Kashmir) is rich in flora with several flowering plant species, high alpine trees, and thick vegetation. Overall, District Poonch has cold winter where temperatures usually drop sub-zero and most of the areas receive snow fall. During the summer few places in the district including Hajira are hot (temperature rising to 30°C).

2.2.2 Sampling sites

This stream is characterized by its proximity to multiple sources of contamination, which has a major effect on its ecological dynamics. The surrounding environment is marked by elevated levels of contamination, including various domestic and agricultural pollutants, which have contributed to water quality of stream. The stream is surrounded by an area enriched with organic matter, a consequence of both natural

processes and anthropogenic activities. The accumulation of organic matter promotes an environment that is rich in nutrients and suitable to a variety of algae populations. Sampling site (stream) is contaminated due to the locals washing their clothes in it by using various detergents.

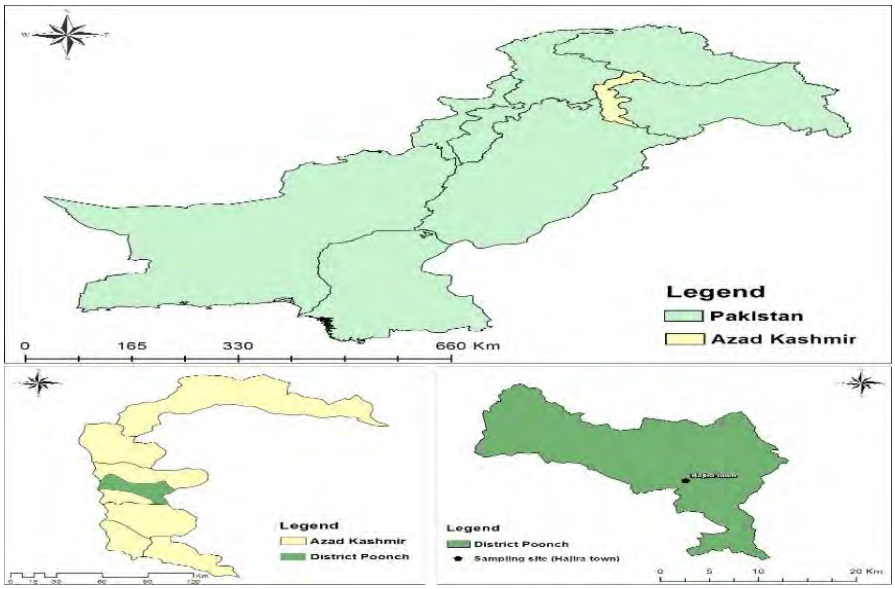


Fig.2.1. Map of collection site.

Fig.2.2. Collection sites of Hajira Poonch AJK

2.2.3 Collection of samples

Falcon tubes were used for the collection of algal samples. Samples were collected during the monsoon. Bottles were labeled according to the sample number, dates, sampling sites. Water was added into the bottles before collection. Water and algal

samples were put into the falcon tubes with the help of forceps and spatula.

2.2.4 Transportation and storage of samples

The samples were transported into the Plant and Algal Genetic laboratory of Quaid-a-Azam university Islamabad. Algal samples were stored in the growth room at 25˚C and were preserved by the addition of 1–2 mL of 4% formalin. Formalin was used to preserve the algal samples to prevent microbial degradation, stabilize cellular structures, and maintain the samples in good condition for further examination (Dell et al., 1999).

2.2.5 Physico-chemical analysis of water

Water samples were analyzed for the determination of physio-chemical parameters at the Chemical Testing Lab, (Water Testing Section) of Qarshi Research International (Pvt) Ltd. Detection of heavy metals were performed at the department of Environmental Sciences Quaid-i-Azam university Islamabad.

2.2.6 Microscopic observations

The samples were examined under a light microscope. A monocular light microscope (Leitz Wetzler, made in Germany) was used to observe morphological characters. The observations were made with a 10x eyepiece and 4x, 10x, 40x, and 100x (with emersion oil) objectives. An ocular micrometer was used to measure the size of individual cells, colonies, and filaments. The specimens were identified using authentic literature and algae base data bank.

2.2.7 Slide preparation

Slides were prepared by mounting a small portion of specimen in water and a cover slip was placed on it. The specimen was observed under a microscope (Jaffer et al., 2019).

2.2.8 Isolation and purification

The plate streaking method was used for isolation and purification of microbes. This method was used to isolate the different species of microalgae, which show distinct morphology on agar plates. For this purpose, solid BG_{11} and BBM media were prepared by adding agar on the broth media and poured the media in autoclaved petri plates. The water sample was sprinkled on the plates. The solid media was prepared by solidifying BBM and BG_{11} media with 1.5% agar. 20ml of this media was poured in each petri plate and allowed to solidify under UV illumination in the laminar flow hood. 500ul of homogenized sample was poured in each plate and was spread with a sterile inoculating loop. The petri plates were sealed with parafilm and were stored at a

temperature of 25ºC under constant illumination of light intensity of 1500 lux from white LED lights for 15 days. After 15 days the agar plates were observed under a light microscope and different colonies were picked and reinoculated into further agar plates using sterile inoculation loops while maintaining a sterile environment (Phang and Chu 1999).

2.2.9 Media used for *in vitro* culturing

Two types of culture media were used to get maximum growth of isolated species of microalgae.

• BG11 Media

Three stocks of the culturing medium BG_{11} were prepared according to the recipe. About 10 ml of these 3 stocks were taken into an Erlenmeyer flask and 1ml of the 4th stock which was the stock of trace metals was added (see appendices). The total volume of the medium was made up to 1000 ml by adding distilled water. The carbohydrates and nitrates were provided by the addition of 0.02g of Na_2CO_3 and 1.5g of $NaNO_3$. The pH of the medium was adjusted to 7.3 to 7.5 and after it was autoclaved at 121˚C and 15 pa for 45 min.

• BBM Media

For BBM medium preparation, five stock solutions were prepared. About 10 ml of stock 1 and 1ml of the remaining 4 stocks were taken in an Erlenmeyer flask. The medium was made up to 1000ml by adding distilled water. The pH was adjusted to 6.8 and the medium was autoclaved at 121˚C and 15 pa for 45 min. BBM medium support maximum strains of green microalgae. The recipe of different media composition is mentioned in appendix section of this thesis.

2.2.10 Biomass production of selected green algae

After confirmation of contamination free growth, the samples were shifted from test tubes to 500ml conical flasks for batch culturing. The inoculated flasks were kept in a growth room at constant temperature, maintained at 25˚ ± 2˚C under continuous white light conditions.

2.2.11 Identification of collected material

The collected specimens of algal species were identified up to the species level with help of standard literature (Beherepatil and Deore, 2013; Ramos et al., 2015; Phinyo et al., 2017; Patil et al., 2018). For the purpose of identification light microscopy was used.

2.2.12 Molecular Identification (DNA Extraction)

DNA extraction was carried out using the CTAB procedure (Murray & Thomson, 1980), with minor modifications. The microalgal culture (5 mL) was centrifuged in a 15 mL falcon. The supernatant was removed, and the pellet was ground in a sterile mortar and pestle with 700ul of warmed CTAB. The liquid was then put into 1.5ml sterile eppendorfs, together with 8ul of sodium dodecyl sulphate (SDS) and 4ul of mercaptoethanol. The suspension was incubated at 65˚C in a water bath for 2 hours. After incubation, 700ul of 24 :1 chloroform isoamylalcohol was added, and the mixture was centrifuged at 12000rpm for 10 minutes at 20˚C. The clear supernatant was transferred to a second set of Eppendorf, and an equal amount of isopropanol was added along with 50ul of ammonium acetate to aid precipitation. The mixture was then stored at 20˚C overnight. The following day, centrifugation was conducted at 12000rpm for 10 minutes at 20˚C. The supernatant was discarded, and the pellet was washed twice with ice-cold ethanol: once with 70% and again with 100%. After washing, the pellet was dried inside an autoclave and suspended in 50ul of PCR water. The quality of the isolated DNA was assessed using a 1% agarose gel. The gel was operated at 95V for approximately 40 minutes before being subjected to UV light using a gel documentation system (BIORAD).

2.2.12.1 Amplification of ITS and ss primers for the identification of green algal strains

The amplification of the ITS and ss gene was carried out using universal primer ITS1, ITS4, ss3 and ss5 for selected species of microalgae. The primers were amplified to 600-750 bp fragment size (White et al., 1990). The primers sequences were as in **Table 2.1 and 2.2.**

Table 2.1: ITS primers for PCR amplification of green algal strains

Forward Primer ITS-1	Reverse Primer ITS-4
5’-TCCGTAGGTGAACCTGCGC-3’	5’-TCCTCCGCTTATTGATATGC-3’

Table 2.2: ss primers for PCR amplification of green algal strains

Forward Primer ss5	Reverse Primer ss3
5’GGTGATCCTGCCAGTAGTCATAT	5’GATCCTTCCGCAGGTTCACCTACGG

GCTTG-3’	AAACC-3’

2.2.12.2 Reaction mixture for PCR

Reaction mixture of 20 µL was prepared for ITS and ss gene amplification. The PCR profile **(Table 2.4)** was set up in PCR thermocycler (BIO-RAD).

Table 2.3: Reaction mixture for PCR

Sr. No	Components	Concentrations
1	Distilled water	11.39 µL
2	Taq polymerase	0.33 µL
3	PCR buffer	2.5 µL
4	dNTPs	0.86 µL
5	Forward primer	0.86 µL
6	Reverse primer	0.86 µL
7	$MgSO_4$	2.2 µL
8	DMSO	0.6 µL
9	DNA	2 µL

Table 2.4: PCR profile.

Steps	Temperature/Time
Temperature initial denaturation	95°C for 5min
Denaturation Temperature	95°C for 30 seconds
Annealing temperature for primer	56°C for 40 seconds
Strand extension temperature	72°C for 1min 30 seconds
Final extension temperature	72°C for 10 min
Total number of cycles	38 cycles
Hold on temperature	4°C for 5 min

2.2.12.3 Phylogenetic analysis

The sequences were examined in Bio Edit software (Hall, 1999) for mistakes and compared to known GenBank sequences using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast). Analysis Sequence contigs were constructed using CLC Main Workbench version 8.1 (QIAGEN Aarhus A/S), and phylogenetic analysis was performed as reported in Inam et al., (2022).

2.2.13 Screening of strains against different concentration of heavy metals

Different algal strains were loaded on 96-well plate with different concentration of Cd and Pb. 96-well plate was placed under the white light at 25˚C. The spectrophotometer or plate reader was used to measure the optical density (OD_{750nm}) of the algal cultures at regular intervals (for 7 days).

2.3 Results

The features of the stream are shown in **Table 2.5**

Table 2.5: Features of stream of Hajira Poonch AJK

Parameters	Stream
Location	Hajira Poonch AJK
Latitude	33.7670° N
Longitude	73.8948° E
Elevation	966m
Vegetation	*Mentha, Grasses, Parthenium, Cannabis sativa*

This stream contains different vegetation and **table 2.6** depicts the physiochemical parameters during the moon soon season. **Table 2.7** shows the concentration of different heavy metals in the water samples and the concentration of Cd and Pb was higher as compared to other metals. Stream contains sewage water, waste (fertilizers from fields) and detergent (local washes their clothes). Different phytoplankton were identified and out of which mostly belonged to the class Chlorophyceae. Among Chlorophyceae, members of the family Scenedesmaceae were found in majority during culturing.

Table 2.6: Physio-chemical Parameters of water samples.

Sr. No	Chemical analysis (Parameters)	Moon soon
1	Appearance	Not turbid
2	Color	Clear
3	Odor	Agreeable
4	Sample Temperature (ºC)	31
5	pH	7.31
6	Electrical Conductivity (µS/cm)	802
7	Turbidity (NTU)	13.64
8	Alkalinity (ppm)	259

9	TDS (ppm)	230
10	Chloride (ppm)	40.21
11	Sulphates (ppm)	3.19
12	Nitrates (ppm)	15
13	Calcium (ppm)	14.47
14	Magnesium (ppm)	6.17
15	Sodium (ppm)	33.45
16	Potassium (ppm)	14.14
17	BOD (mg/L)	8.13

Table 2.7: Heavy metals concentration in the stream of Poonch AJK.

Heavy metals	**Concentration (mg/L)**
Cu	0.01
Cd	0.02
Pb	0.07
Zn	0.002
Ni	0.01
Cr	0.01

2.3.1 Composition, morphology, and identification of microalgae in field samples

2.3.1.1 Chlorophycea (Green Algae)

Based on the morphological observations, following of Chlorophyceae found in study area:

2.3.1.1.1 *Scenedesmus dimorphus*: Cells arranged in fours and without spines. Cells were found in colonial form with four cells. The outer side of the terminal cell is slightly convex and arranged side by side.

2.3.1.1.2 *Scenedesmus* sp.: Cells avoid cylindrical, cells narrow and without spines. Pyrenoid is present in each cell.

2.3.1.1.3 *Spirogyra*: *Spirogyra* is a multicellular green algae, long, filamentous. Cells are arranged end-to-end to form long chains. These chains can be several centimeters in length. Thallus attached or free-floating, intercalary growth, Cells are more than 100 μ in length.

2.3.1.1.4 *Rhizoclonium*: *Rhizoclonium* typically forms a filamentous structure known as a thallus. The thallus consists of a chain of cylindrical cells arranged end to end.

The filaments of *Rhizoclonium* may exhibit branching, forming a network of interconnected filaments.

2.3.1.1.5 *Odogonium*: *Oedogonium* has a filamentous structure, meaning that it consists of long chains of cells attached end-to-end. The filaments can be unbranched or occasionally branched.

2.3.1.1.6 *Ulothrix*: Filaments unbranched, vegetative cells cylindrical or barrel shaped, chromatophore band shaped and occupy whole of cell circumference with more than one pyrenoids with spore.

2.3.1.1.7 *Chlorella*: *Chlorella* has cup shaped chloroplast and has pyrenoids visible which is light green in color, cells are 9-11 μ in diameter and 14-26 μ in length.

2.3.1.1.8 *Ankistrodesmus*: Cells are in the form of colonies of loosely bound unicells. Each cell has a pointed end. Fiber shaped cells spiral or bend.

2.3.1.1.9 *Closterium*: Cells are concave and have rounded apices. Visible pyrenoid is present cells are 29-32 μ wide and more than 100 μ in length.

2.3.1.1.10 *Cosmarium*: Rounded semi cells from the front and has visible pyrenoid. Cell divided into two semi cells produced by constriction in the middle.

2.3.1.1.11 *Microspora*: Rounded cell and had folded chloroplast covering the cell wall, whole filaments are more than 100 μ in length.

2.3.1.1.12 *Chlorococcum*: Reticulate chloroplast, flagellated zoospore formation and in the form of irregular clamp. Cells are 23 μ in width and 25 μ in length.

2.3.1.1.13 *Selenastrum*: Cells are crescent shaped and curved. Cells are arranged with their convex sides facing one another.

2.3.1.2 Cyanophyceae:

The members of Chlorophyceae found in study area are as follows:

2.3.1.2.1 *Phormidium*: Cells are 7 μ wide, but in long more than 100 μ, tapered slightly from end.

2.3.1.2.2 *Oscillatoria*: Trichome rounded at tips, apices are not attenuated, rounded end, cells are 3-4 μ in width and more than 100 μ in length. Filaments are straight, elongate and not constricted at the joints.

2.3.1.2.3 *Nostoc*: Filamentous and unbranched. Filaments are 6µ in diameter and more than 100µ in length. Heterocyst and akinetes are present.

2.3.1.2.4 *Synechococcus*: Cylindrical to rod-shaped. Solitary or irregularly grouped cells, not forming colonies.

2.3.1.2.5 *Chroococcus*: Spherical or oval shaped cell. Cells are 10 μ in diameter and formed colony of 4 cells.

2.3.1.3 Bacillariophyceae

2.3.1.3.1 Diatoms

Diatoms are elongated and single-celled organisms. They are rounded and radially symmetrical. Different types of diatoms were observed in field samples.

2.3.2 The composition of species in field (fresh) samples

Initial observations revealed a total of 19 species, besides a few undetermined types (diatoms). Among these, the green types contributed a share of 72% while 28% were blue green types. Among microalgal types, members of the genus *Spirogyra* were found to be the most abundant. Other taxa were represented by either one or two species only.

2.3.3. Culturing of samples

Green algae responded to BBM medium and blue green algae responded to BG_{11}. Some species of green and blue green algae showed growth in both mediums. The colonies of different species of algae were observed under Stereo microscope and shifted to respective broth media. For comparisons, the no. of species isolated from BBM and BG_{11} are shown in **Table 2.8.**

2.3.4 Observations of species

2.3.4.1 Observation of 2CL sample

Field samples: *Spirogyra*, *Chlorella* were observed.

***in vitro* culturing:** The BBM showed growth of green algae. Among the green algae. *Chlorella*, *Chlorococcum*, *Scenedesmus hystrix* were observed in BBM and BG_{11} medium.

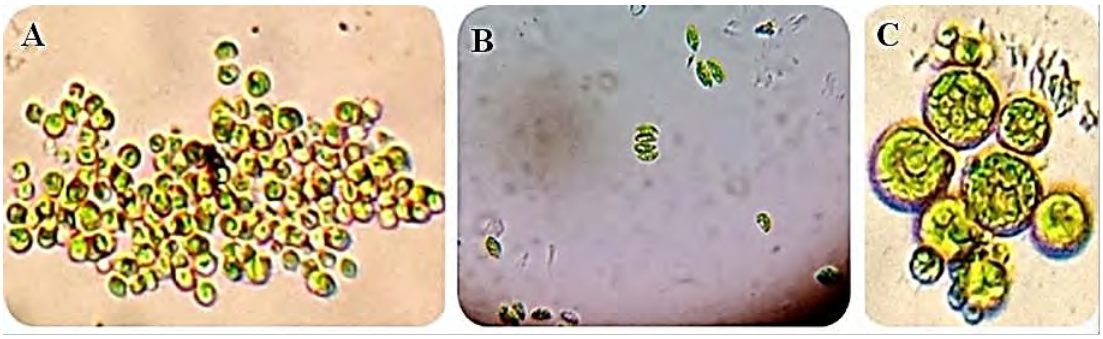


Fig.2.3: (A) Growth of *Chlorella* in BBM media. (B) Unicells and four celled coenobium of *Scenedesmus hysterics* in BBM media. (C) Culture of *Chlorococcum* in BBM medium.

2.3.4.2 Observation of R1 sample

Field samples: *Phormidium* was observed along with *Synechococcus*.

***in vitro* culturing:** The BBM showed no growth of algae. While BG_{11} medium showed the growth of *Scenedesmus acutiformis, Phormium* and *Synechococcus*.

Fig. 2.4: (A) *Scenedesmus acutiformis* (B) *Phormium* (C) *Synechococcus* in BG_{11} media.

2.3.4.3 Observation of R2 sample

Field samples: *Spirogyra and Ulothrix* were observed in field samples.

***in vitro* culturing:** BBM medium showed the mix culture of green (*Scenedesmus ecornis*) and blue green algae (*Phormidium*), while BG_{11} medium produced the *Phormium* sp.

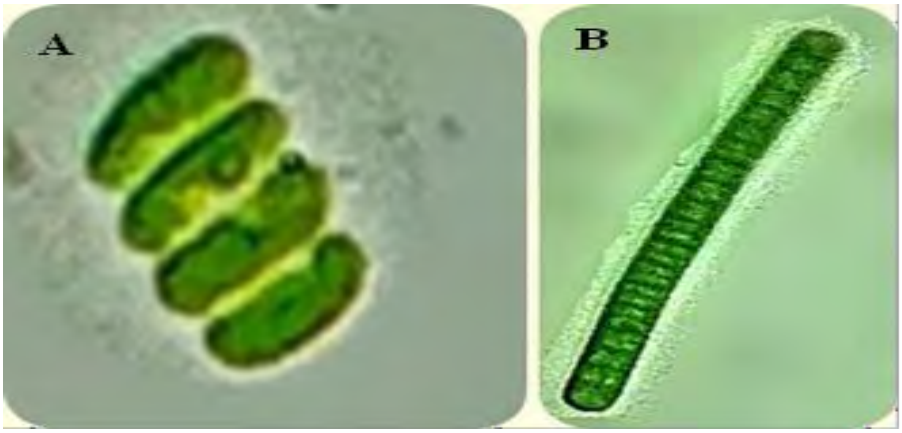


Fig.2.5: (A) *Scenedesmus ecornis*) in BBM (B) *Phormium* in BG_{11}.

2.3.4.4 Observation of R3 sample

Field samples: *Oedogonium* and *Rhizoclonium* were observed in field samples.

***in vitro* culturing:** BBM medium showed *Desmodesmus subspicatus* and BG_{11}

medium produced the growth of *Nostoc*.

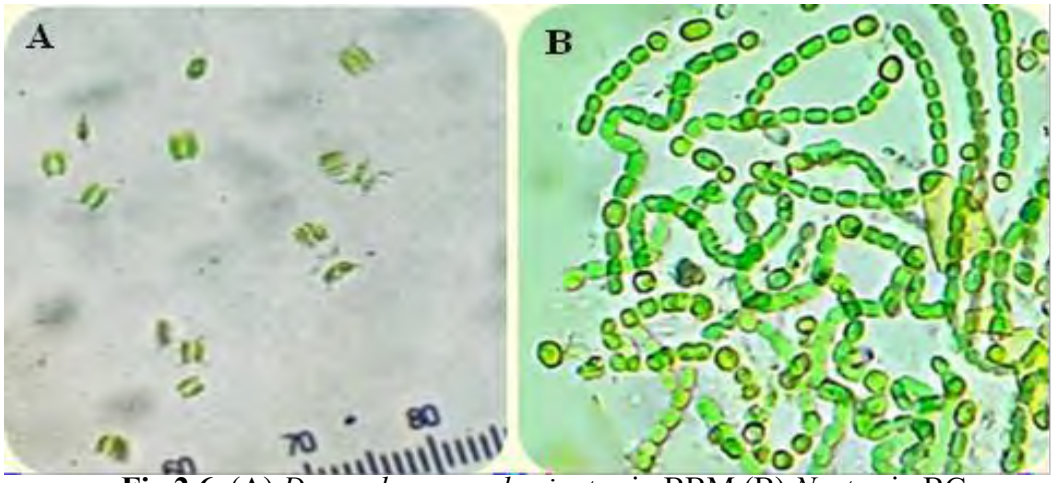


Fig.2.6. (A) *Desmodesmus subspicatus* in BBM (B) *Nostoc* in BG_{11}.

2.3.4.5 Observation of H1 sample

Field samples: *Ankistrodesmus* and *Spirogyra* were observed in field samples.

***in vitro* culturing:** *Scenedesmus acunae, Chlorococcum* and *anabaena*, was observed in BBM medium and BG_{11} medium showed the growth of only *Anabaena* sp.

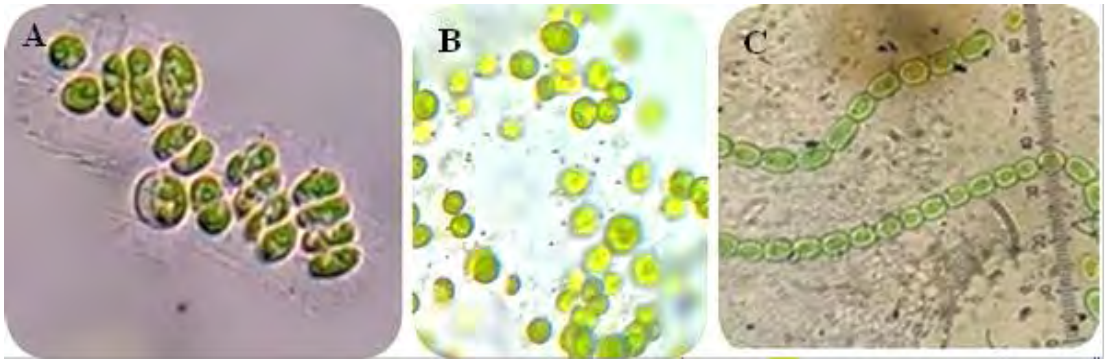


Fig.2.7. (A) *Scenedesmus acunae* (B) *Chlorococcum* and (C) *Anabaena* in BG11.

2.3.4.6 Observation of H2 sample

Field samples: *Cladophora*, *Synechococcus* and *Chroococcus* were observed in field samples.

***in vitro* culturing:** *Scenedesmus* sp. had growth in BBM medium while *Chroococcus* growth was observed in BG_{11}. *Synechococcus* showed growth in both media (BBM and BG_{11}).

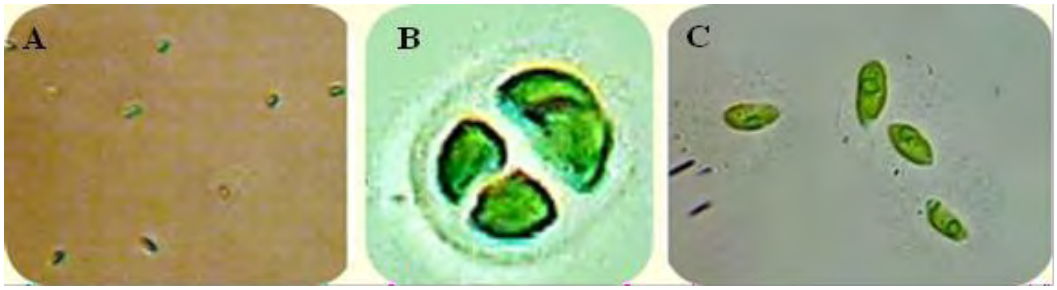


Fig.2.8. (A) *Synechococcus* (B) *Chroococcus* in BG_{11} (C) *Scenedesmus* sp. in BBM medium.

2.3.4.7 Observation of H3 sample

Field samples: *Microspora* and *Scenedesmus* sp. was observed in field samples.

***in vitro* culturing:** *Scenedesmus ellipticus* and *Nostoc* were observed in BG_{11} medium. No growth was observed in BBM medium.

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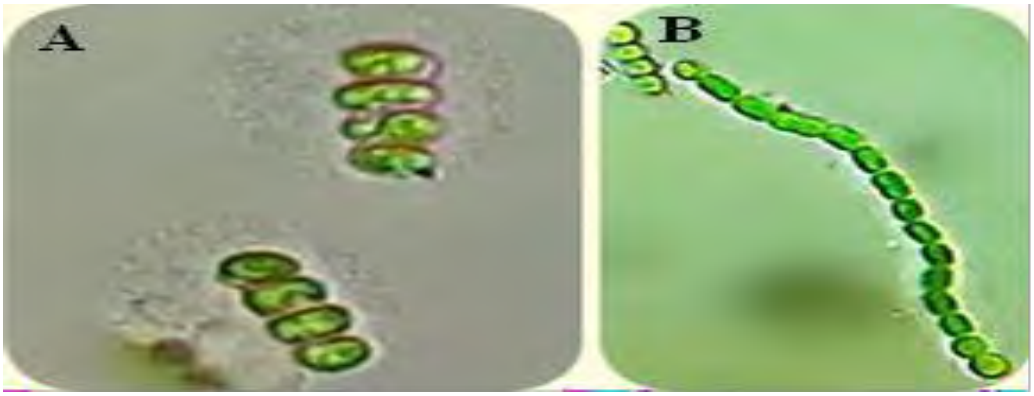


Fig.2.9. (A) *Scenedesmus ellipticus* (B) *Nostoc* in BG_{11}.

2.3.4.8 Observation of H4 sample

Field samples: *Scenedesmus* sp., *Closterium* and *spirogyra* were observed in field samples.

***in vitro* culturing:** The BBM showed the growth of *Closterium* and *Scenedesmus dimorphus.* There was no growth in BG_{11} medium.

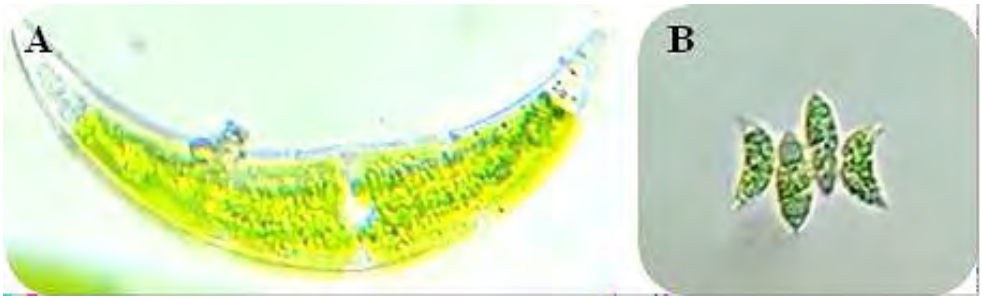


Fig.2.10. (A) *Closterium* **(B)** *Scenedesmus dimorphus* in BBM medium

2.3.4.9 Observation of H5 sample

Field samples: *Oscillatoria* and *Cosmarium* were observed in field samples.

***in vitro* culturing:** *Chlorococccum*, *Scenedesmus rubescence* and *Chlorella* was observed in BBM and BG_{11} medium.

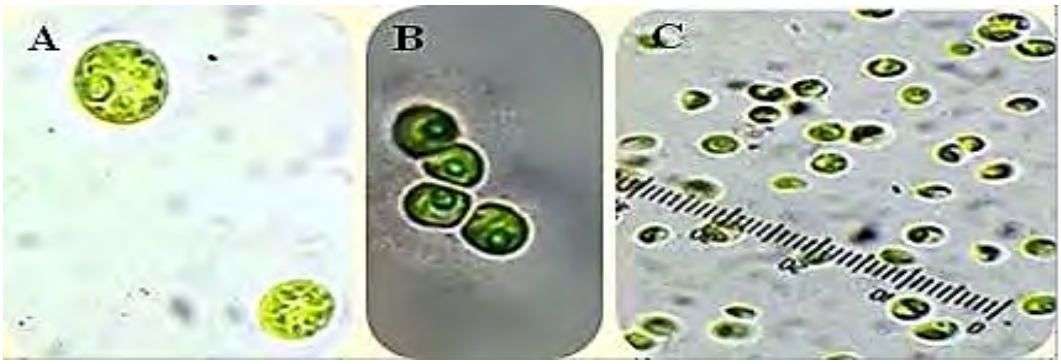


Fig.2.11. (A) *Chlorococccum* (B) *Scenedesmus rubescence* (C) *Chlorella* in BBM.

2.3.4.10 Observation of H6 sample

Field samples: *Chlorococcum* and *Selenastrum* were observed in field samples.

***in vitro* culturing:** *Chlorococcum* and *Scenedesmus javanensis* showed the growth in BBM medium. *Oscillatoria* appeared in BG_{11} medium.

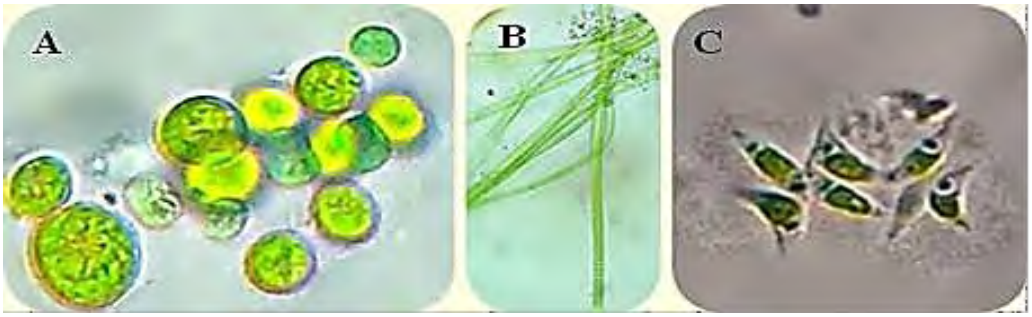


Fig.2.12. (A) Growth of *Chlorococcum* in BBM medium (B) *Oscillatoria* in BG_{11} (C) *Scenedesmus javanensis* in BBM medium.

2.3.4.11 Observation of H7 sample

Field samples: *Oscillatoria* and *Nostoc* were observed in field samples.

***in vitro* culturing:** *Oscillatoria* and *Cosmarium* observed in both BBM and BG_{11} medium while *Scenedesmus incrassatulus* observed in BG_{11} medium.

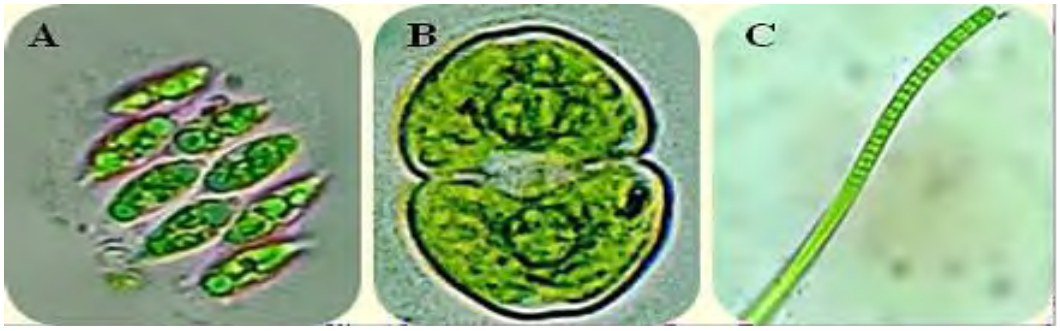


Fig.2.13. (A) *Scenedesmus incrassatulus* in BG_{11} medium (B) *Cosmarium* and (C) *Oscillatoria* in BG_{11}.

2.3.4.12 Observation of H8 sample

Field samples: *Oscillatoria* and *Chlorella* were observed in field samples.

***in vitro* culturing:** BG_{11} medium produced *Oscillatoria*, while *Scenedesmus* sp. had growth observed in BBM.

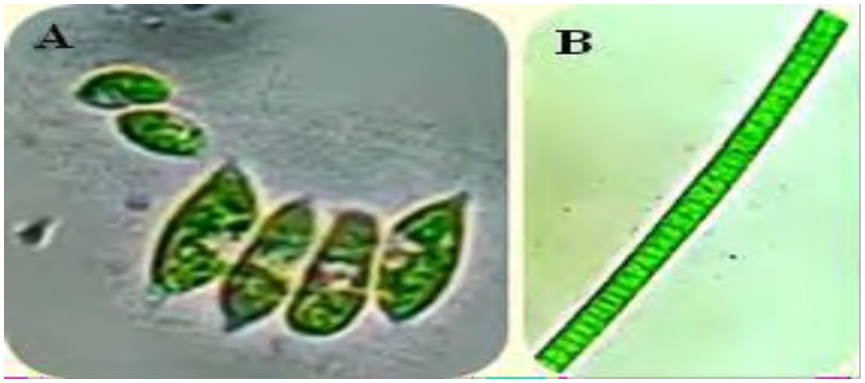


Fig.2.14. (A) *Oscillatoria* in BG_{11} medium **(B)** *Scenedesmus* sp. in BBM medium.

2.3.4.13 Observation of H9 sample

Field samples: *Rhizoclonium, Ankistrodesmus* and *Oscillatoria* were observed in field samples.

***in vitro* culturing:** *Scenedesmus dimorphus*, *Ankistrodermus* and *Oscillatoria* medium showed growth in both BBM and BG_{11} medium.

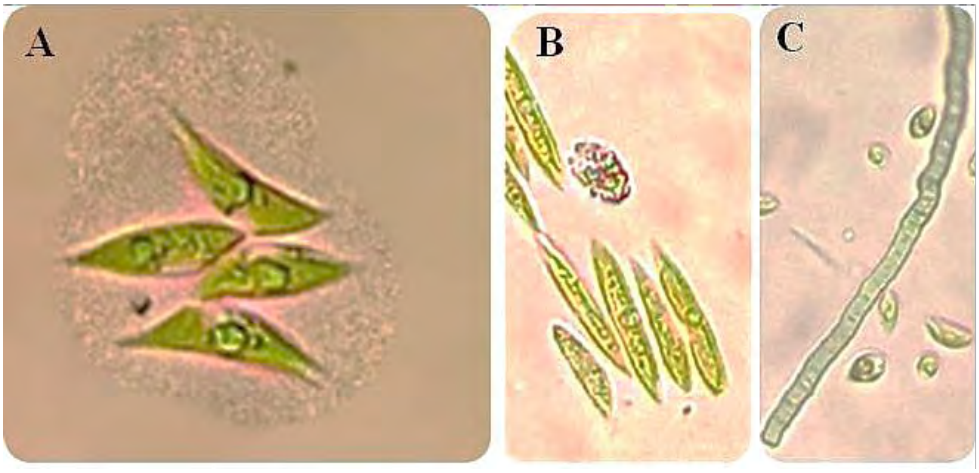


Fig.2.15: (A) *Scenedesmus dimorphus* **(B)** *Ankistrodermus* **(C)** *Oscillatoria* in BBM.

2.3.4.14 Observation of H10 sample

Field samples: *Chlorella* was observed in field samples.

In vitro culturing: *Chlorella* showed growth in BBM medium. *Phormidium* was observed in BBM medium and *Desmodesmus communis* appeared in both BBM and BG_{11} medium.

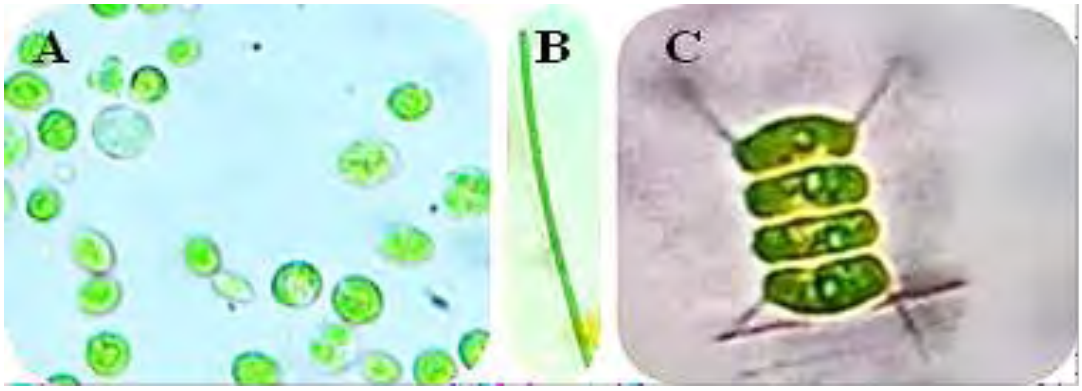


Fig.2.16: (A) *Chlorella* in BBM **(B)** *Phormidium* **(C)** *Desmodesmus communis* in BG_{11}.

2.3.4.15 Observation of sample 15

Field samples: *Oscillatoria* and *Spirogyra* were observed in field samples.

In vitro culturing: *Scenedesmus bijugus* and *Chlorococcum* medium showed growth in both BBM and BG_{11} medium.

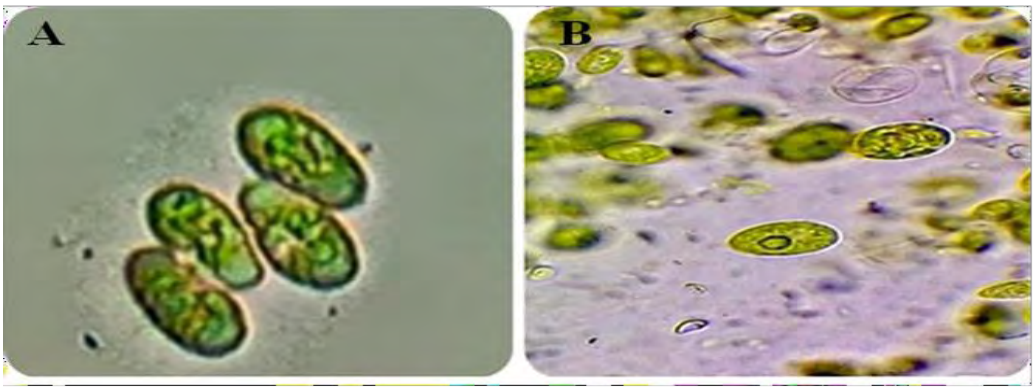


Fig.2.17: (A) *Scenedesmus bijugus* **(B)** *Chlorococcum* in BBM.

2.3.4.16 Observation of sample 4

Field samples: *Spirogyra* were observed in field samples.

***in vitro* culturing:** *Scenedesmus sp.* and *Chlorella* showed growth in both BBM medium.

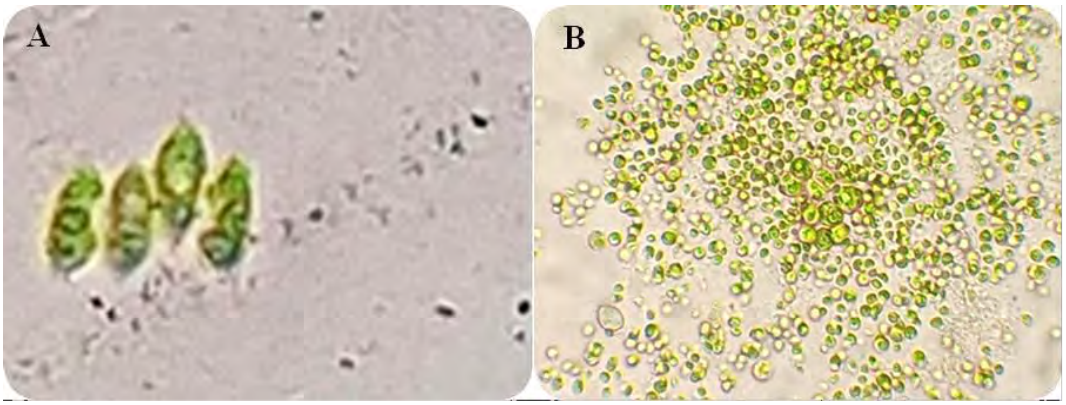


Fig.2.18: (A) *Scenedesmus* sp. **B)** *Chlorella* in BBM.

Table 2.8: Culturing response and observed algal species of mother samples on BBM and BG_{11} media.

Sample Code	BBM	Observed species	BG_{11}	Observed species
2CL	✓	*Chlorococcum, Chlorella Scenedesmus hystrix*	✓	*Scenedesmus hystrix*
R1	✓	*Scenedesmus acutiformis Phormidium*	✓	*Phormidium Synechococcus*
R2	✓	*Scenedesmus ecornis Phormidium*	✓	*Phormidium*

R3	✓	*Desmodesmus subspicatus*	✓	*Nostoc* *Desmodesmus subspicatus*
H1	✓	*Scenedesmus acunae,* *Chlorococcum* *Anabaena*	✓	*Anabaena*
H2	✓	*Synechococcus* *Scenedesmus* sp.	✓	*Chroococcus* *Synechococcus*
H3			✓	*Scenedesmus ellipticus,* *Nostoc*
H4	✓	*Closterium* *Scenedesmus dimorphus*		
H5	✓	*Chlorococccum*, *Scenedesmus rubescence* *Chlorella*	✓	*Scenedesmus rubescence* *Chlorella* *Chlorococcum*
H6	✓	*Chlorococcum* *Scenedesmus javanensis*	✓	*Oscillatoria*
H7	✓	*Oscillatoria Cosmarium*	✓	*Scenedesmus incrassatulus* *Oscillatoria, Cosmarium*
H8	✓	*Scenedesmus* sp.	✓	*Oscillatoria*
H9	✓	*Scenedesmus dimorphus* *Oscillatoria, Ankistrodermus*	✓	*Ankistrodermus* *Oscillatoria* *Scenedesmus dimorphus*
H10	✓	*Desmodesmus communis* *Chlorella*	✓	*Phormidium* *Desmodesmus communis*
15	✓	*Scenedesmus bijugus* *Chlorococcum*	✓	*Chlorococcum* *Scenedesmus bijugus*
4	✓	*Scenedesmus sp.* *Chlorella*		

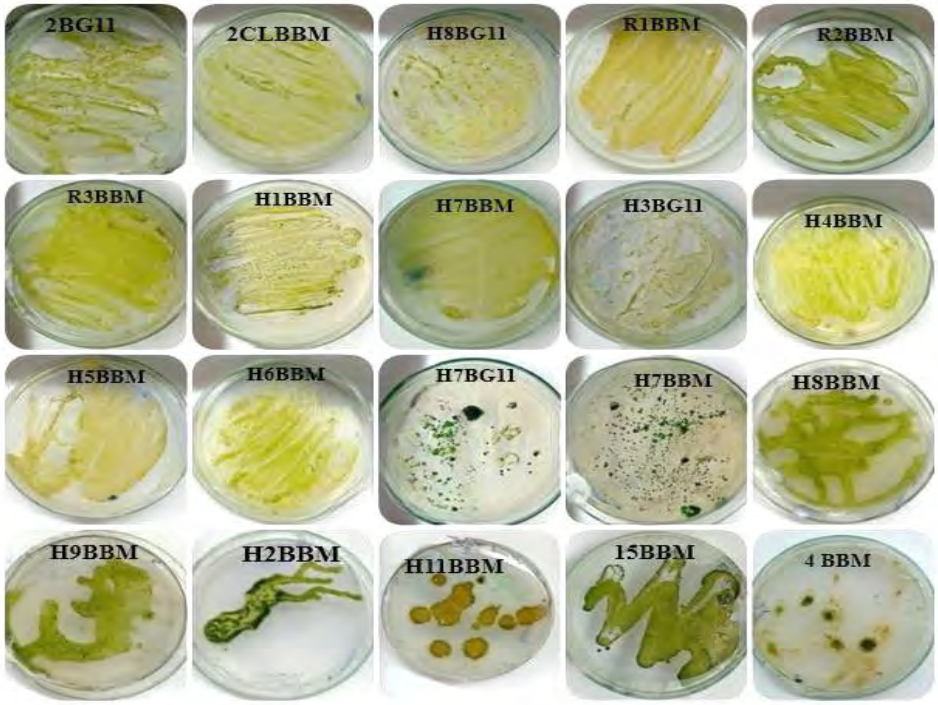


Fig.2.19. The *in vitro* growth of algae in petri plates with agar-based media

2.3.5 Diversity of Family Scenedesmaceae

Also, an assessment of the diversity of Family Scenedesmaceae from the stream of Hajira Poonch AJK indicated a higher extent of 16 species of this family during monsoon. Systematic position of Family Scenedesmaceae according to Guiry and Guiry (2013) is as follows:

- Empire Eukaryota
- Kingdom Plantae
- Phylum Chlorophyta
- Class Chlorophyceae
- Order Sphaeropleales
- Family Scenedesmaceae

Colonies of 2,4 or 8 cells. Cells are arranged side by side, zigzag, linearly. Cell bodies are elliptical, crescent in shape or spindle in shape. Some species have spines. Cell wall is usually smooth.

2.3.5.1 *Scenedesmus hystrix*

Characters: Colony of 2, 4 or 8 cells. Cells are arranged in a linear manner. Cells are oblong, cylindrical with obtuse ends. The length of cell is 19.2µm and width is 5.8µm **(Fig 2.20A)**.

2.3.5.2 *Scenedesmus acutiformis*

Characters: 4 celled colony. Cells are arranged in a linear manner. The shape of cells is fusiform. The Cell wall is smooth. Cells have acute poles. Teeth or spines are not present. The length of the cell is 17.4 µm long and width is 12.5 µm **(Fig 2.20B)**.

2.3.5.3 *Scenedesmus ecornis*

Characters: Colony of 4 cells. Cells are arranged in a linear manner. Cells are elliptical, straight and rounded. Only one pyrenoid is present. Spines or teeth are not present. There is no thickening in the cell wall. Cells are 7.5-17 µm long and 4 -6.1µm wide **(Fig 2.20C)**.

2.3.5.4 *Scenedesmus acunae*

Characters: 4 celled cenobia. The shape of cells is ellipsoid to oblong cells and have rounded poles. Outer cells have convex shape and inner are straight. Length of 4 celled cenobia is 8.5- 15 µm and 4-6 µm wide **(Fig 2.20 D)**.

2.3.5.5 *Scenedesmus obliquus*

Characters: Colony of 2,4 or 8 cells. Cells are arranged in a linear series. The shape of cells is fusiform. Cells have acute ends. Cell wall smooth. Spines or teeth are not present. The 4 cell colonies are 7.5 µm long and 3.4 µm wide **(Fig 2.20 E)**.

2.3.5.6 *Scenedesmus ellipticus*

Characters: 4 or 8 celled colony. Cells are elliptical to oblong. Cells are arranged in a linear manner. Cells have rounded poles. Teeth and spines are not present. Length of cell is 12.5-17.5 and width is 5-6.5 **(Fig2.20 F).**

2.3.5.7 *Scenedesmus dimorphus*

Characters: Colonies of 2,4 and 8 celled Cells are arranged in a single or alternating manner. Cells are lunate with acute and sharp apices. Spines or teeth are not present. The colonies of 2 Cells are 5.6 µm long and 1.5µm wide (**Fig 2.20G)**.

2.3.5.8 *Desmodesmus communis*

Characters: 2 or 4 celled colonies and sometimes 8 celled. Colony of 2 celled is 9.4 µm long; cells are arranged linearly; cells are oblong to ellipsoid. In the 4 celled colony, terminal cells are broader and more or less straight curved spines. Spines on the poles of the terminal cell. Inner cells are without spines. The colonies of 4 cells are 8.1µm broad, 16.8 µm long and spines are 6.1µm long **(Fig 2.20H)**.

2.3.5.9 *Scenedesmus rubescens*

Characters: Colony of 4 cells. Cells are in circular shape. Colonies are arranged linearly or in alternate manner. Pyrenoid is present at the side of the cell. Spines or teeth are not present. The length of the colony is 6.1 µm and width is 2.3 µm **(Fig 2.20I)**.

2.3.5.10 *Desmodesmus subspicatus*

Characters: Colony of 4 cells. Polar and lateral spines are present. Polar spines are slightly curved and lateral spines are straight. Cell dimension is 10 µm long and 4 µm wide (4 celled) **(Fig 2.20J)**.

2.3.5.11 *Scenedesmus javanensis*

Characters: Colony of 4 or 8 cells. Cells are fusiform. Cells arranged alternately contact with their apices. Cells are 25-29.5 µm and 4-7.5 µm wide. Cells are without spines or teeth **(Fig 2.20K)**.

2.3.5.12 *Scenedesmus dimorphus*

Characters: Colony of 4 cells. Cells have pointed tips. Laterally connected cells but without alignment of the edges. 9.3–12.4 µm long and 4.1–5.7 µm width **(Fig 2.20L)**.

2.3.5.13 *Scenedesmus incrassatulus*

Characters: Colony of 4 or 8 cells. Cells are attached side by side in one or two rows. Cells are spindle shaped. Spines or teeth are not present. The outer cells slightly curved inward and the inner side are slightly concave. Cells have pointed tips. Colony of 8 Cells 20.5 µm long, 15.5 µm wide **(Fig 2.20M)**.

2.3.5.14 *Scenedesmus* sp.

Characters: Colony of 4 cells. Cells are cylindrical or oblong ellipsoid with pointed apices. Teeth are present at the end of cells. The colony of 4 cells is 14.3 µm long and 5.1 µm wide **(Fig2.20N)**.

2.3.5.15 *Scenedesmus bijugus*

Characters: Colony of 4 or 8 cells. Cells are pointed ends. Colonies are arranged in an alternate manner. Spines or teeth are not present. The length of the colony of 4 cells is 17.8-27.5 µm, and 15.2-25.1 µm wide **(Fig2.20O)**.

2.3.5.16 *Scenedesmus prismaticus*

Characters: Colony of 4 cells. Cells are arranged in a linear manner. Cells are prismatic with pyramidal end faces. Cells have sharp pointed ends. Cells are 12.2 µm long and 5.5 µm broad **(Fig 2.20 P)**.

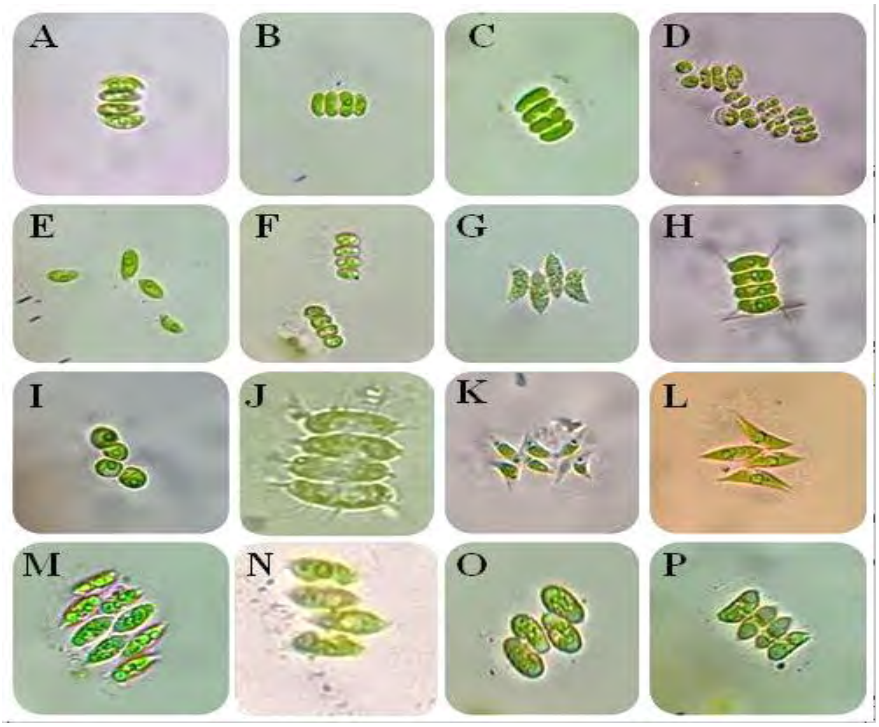


Fig.2.20. Diversity of family Scenedesmaceae (A). *Scenedesmus hystrix* (B). *Scenedesmus acutiformis* (C). *Scenedesmmus ecornis* (D). *Scenedesmus acunae* (E). *Scenedesmus obliquus acunae (*F). *Scenedesmus ellipticus* (G). *Scenedesmus dimorphus* (H). *Desmodesmus communis* (I). *Scenedesmus rubescens* (J). *Desmodesmus subspicatus* (K). *Scenedesmus javanensis* (L). *Scenedesmus dimorphus* (M). *Scenedesmus incrassatulus* (N). *Scenedesmus* sp. (O). *Scenedesmus bijugus* (P) *Scenedesmus prismaticus.*

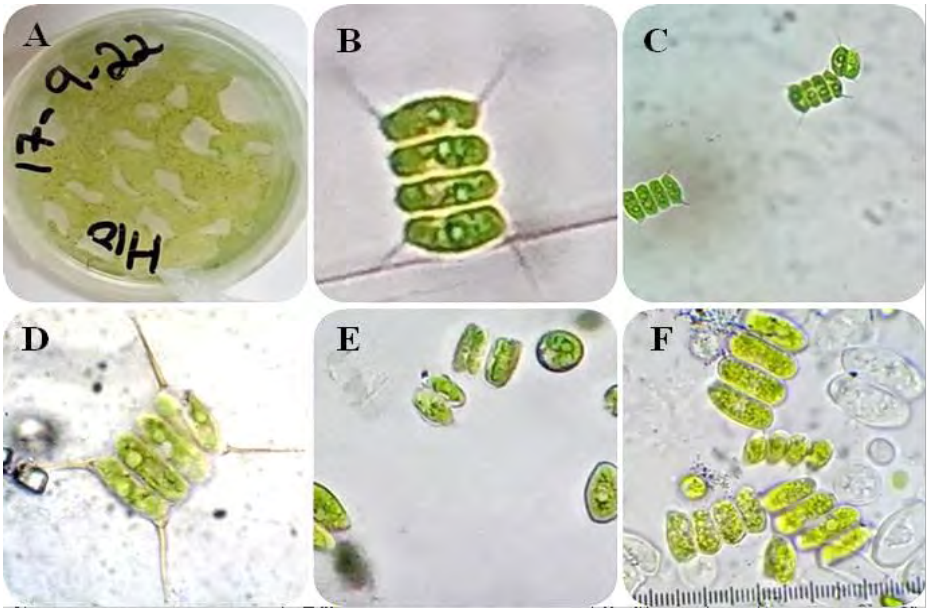
2.3.6 Morphological and Molecular characterization of selected species of Family Scenedesmaceae

2.3.6.1 *Desmodesmus communis* (Scenedesmaceace) (strain H10)

Morphological description: Colonies of 2-4 cells, linear; cells are elongated with widely or cone-like rounded ends, with 4 spines (fig 3.21B), each at the apices of terminal cells. Spines are 7.3µm long. cell size: 17µm in length and 7 µm diameter (4 celled). 10µm in length and 5µm in diameter (2 celled).

Diagnostic characters: The cell walls are ornamented with spines **(Fig 2.21B and C)**, and sometimes warts and ribs (although these are harder to see). *Desmodesmus* may also occur as a unicell or in pairs **(Fig 2.21C and E)** under some conditions.

Site details and habitat: This species was isolated from the running stream. Other taxa found in the sample were: *Chlorella, Phormidium*.



Culturing conditions: *Desmodesmus* sp. responded in BBM and BG_{11} medium.

Fig.2.21: (A) The colonies of H10 on BBM agar medium. (B-D) Terminal spines and pyrenoid in *Desmodesmus communis* (E) *Desmodesmus* in unicells and pair (F) *Desmodesmus communis* under 100x.

Molecular and Phylogenetic analysis

The strain H10 provided an 18SrRNA sequence of 1829 bp for this study. The similarity of our sample to *Desmodesmus communis* was tested using 18rRNA by performing BLASTIN searches at the NCBI Database, and the results showed 97.8% similarity with the query coverage 90%. The ML, NJ and MP methods were used to build the phylogenetic trees. The NJ **(Fig 2.22)** was created using the sequence of a green algal strain downloaded from NCBI, as well as the sequence of the examined strain. Phylogenetic analysis reveals that H10 forms a clade with *Desmodesmus communis*.

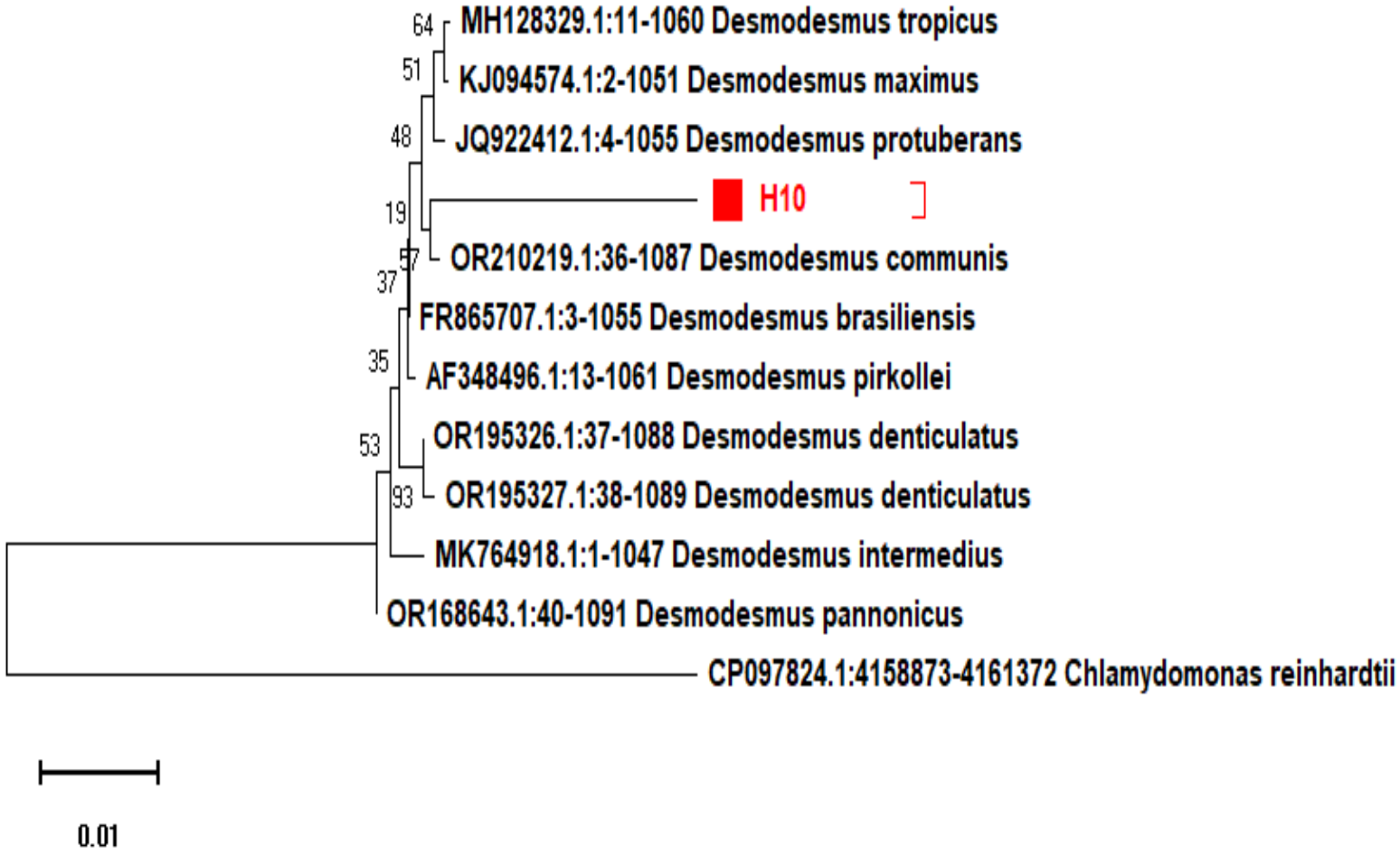


Fig.2.22: Phylogenetic analysis *Desmodesmus communis* (strain H10) based on neighbor joining (NJ) method.

2.3.6.2 *Desmodesmus subspicatus* (Scenedesmaceace) (strain R3)

Morphological description: Cells are oval shaped. The two-celled coenobium are common, but four celled coenobia are also present. Polar and lateral spines are present **(Fig 2.23B and E)**. Polar spines are slightly curved and lateral spines are straight **(Fig 2.23E).** Lateral spines are present in outer cells. Cell dimension is 10 µm long and 4 µm wide (4 celled). Cell dimension is 7 µm long and 3 µm wide (2 celled). Polar spines are 4.3µm long and lateral spines are 3.5 µm long.

Diagnostic characters: The cell walls are ornamented with spines, (polar and lateral spines). *Desmodesmus subspicatus* may also occur in pairs under some conditions **(Fig 2.23B and C).**

Site details and habitat: This species was isolated from the running stream. Other taxa found in the sample was: *Nostoc*.

Culturing conditions: *Desmodesmus* sp. responded in BG_{11} and BBM medium.

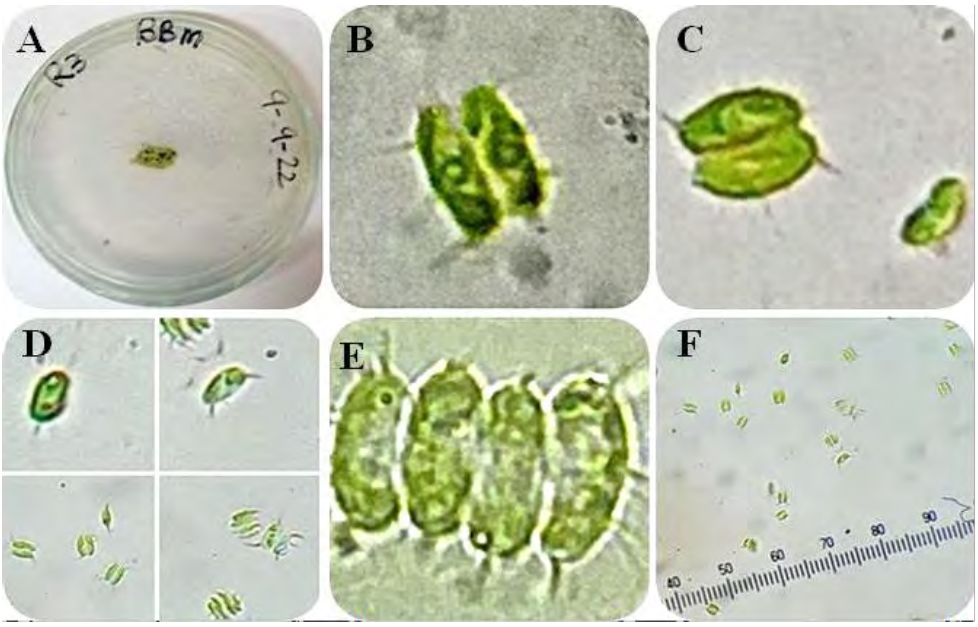


Fig.2.23: (A) The colonies of *Desmodesmus subspicatus* on BBM agar medium. (B-D) Green algae in unicells and pair (E) Terminal and lateral spines in *Desmodesmus subspicatus.* (F) *Desmodesmus* in 2 celled and 4 celled cenobium.

Molecular and Phylogenetic analysis

In this study 18SrRNA sequence of 2119 bp were obtained from the strain H9. The similarity of our sample was checked using 18rRNA by performing BLASTIN searches at the Database NCBI, and the results showed 95.3% similarity with the query coverage 98 % to *Desmodesmus subspicatus*. The phylogenetic trees were constructed

using the ML NJ, and MP method. The NJ **(Fig 2.24)** constructed with the sequence of green algal strain downloaded from NCBI including the sequence of investigated strain. Phylogenetic analysis shows R3 makes a clade with *Desmodesmus subspicatus*.

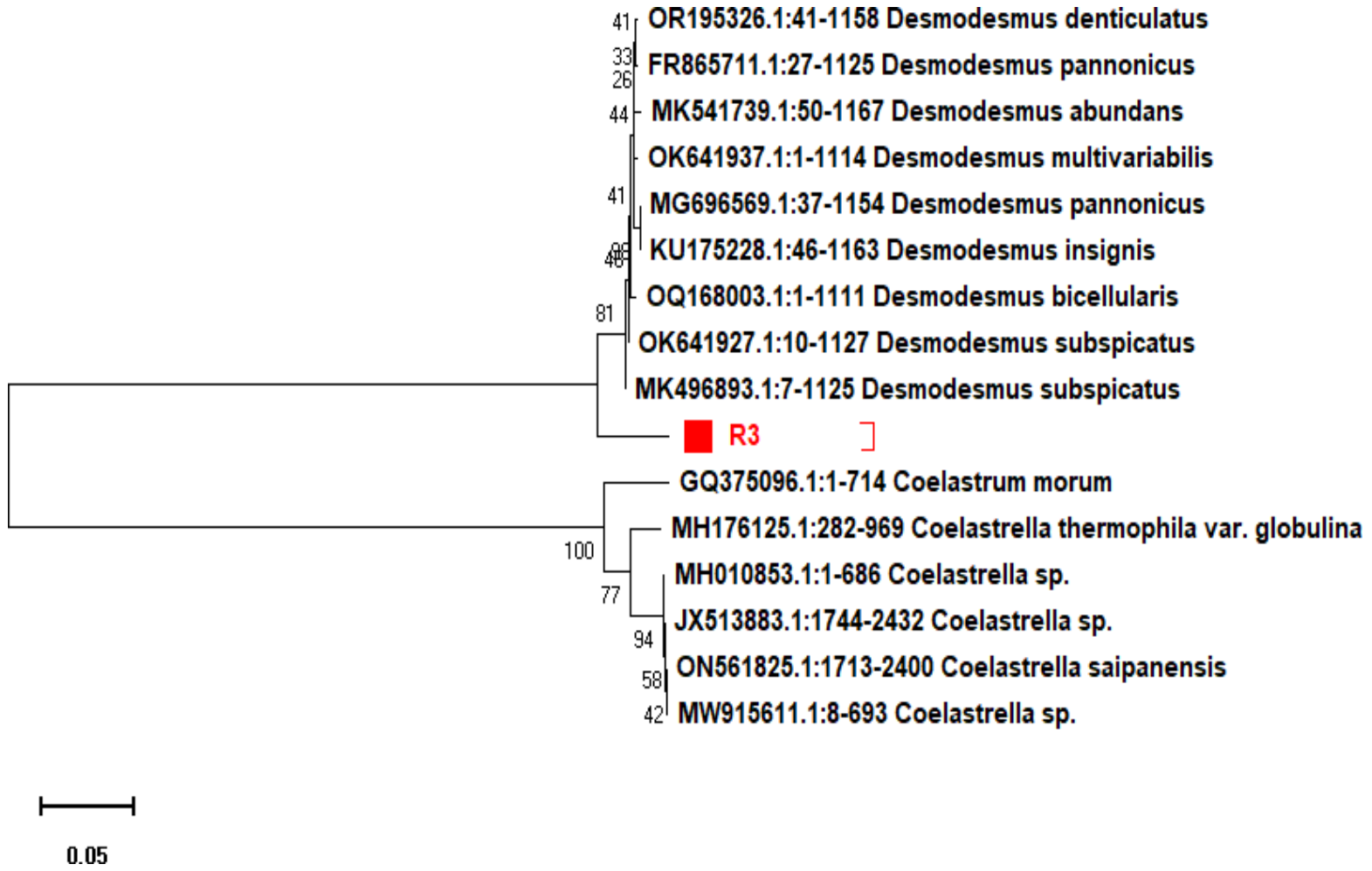


Fig.2.24: Phylogenetic analysis *Desmodesmus subspicatus* (strain R3) based on neighbor joining (NJ) method.

2.3.6.3 *Scenedesmus dimorphus* (Scenedesmaceace) (strain H9)

Morphological description: Four celled coenobium and have pointed tips **(Fig 2.25B-E).** Laterally connected cells but without alignment of the edges. 9.3–12.4 µm long and 4.1–5.7 µm width. Large round-shaped cells resembling sporangia with autospores were also common **(2.25Fig C).**

Diagnostic characters: *Scenedesmus* forms colonies consisting of two or four cells, but also have 8, and are occasionally unicellular.

Site details and habitat: This species was isolated from the running stream. Other taxa found in the sample were: *Ankistrodermus* and *Oscillatoria.*

Culturing conditions: *Scenedesmus* sp. responded in BG_{11} and BBM medium.

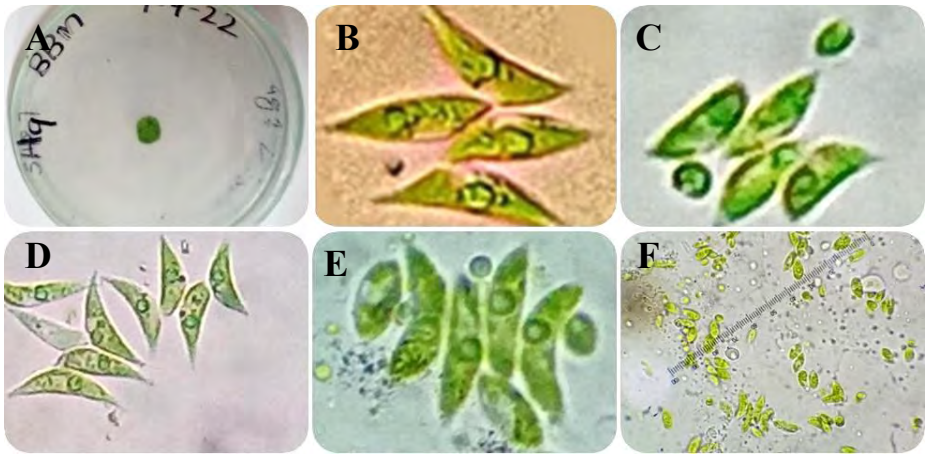


Fig.2.25: (A) The colonies of *Scenedesmus dimorphus* on BBM agar medium (B-D) *Scenedesmus* four celled cenobium (D-F) Different cell formation of *Scenedesmus sp.*

Molecular and Phylogenetic analysis

In this study 18SrRNA sequence of 807 bp were obtained from the strain H9. The similarity of our sample was checked using 18rRNA by performing BLASTIN searches at the Database NCBI, and the results showed 95 % similarity with the query coverage 97.7 % to *Scenedesmus dimorphus*. The phylogenetic trees were constructed using the ML, NJ, and MP method. The NJ tree **(Fig 2.26)** constructed with the sequence of green algal strain downloaded from NCBI including the sequence of investigated strain. Phylogenetic analysis shows H9 makes a clade with *Scenedesmus dimorphus.*

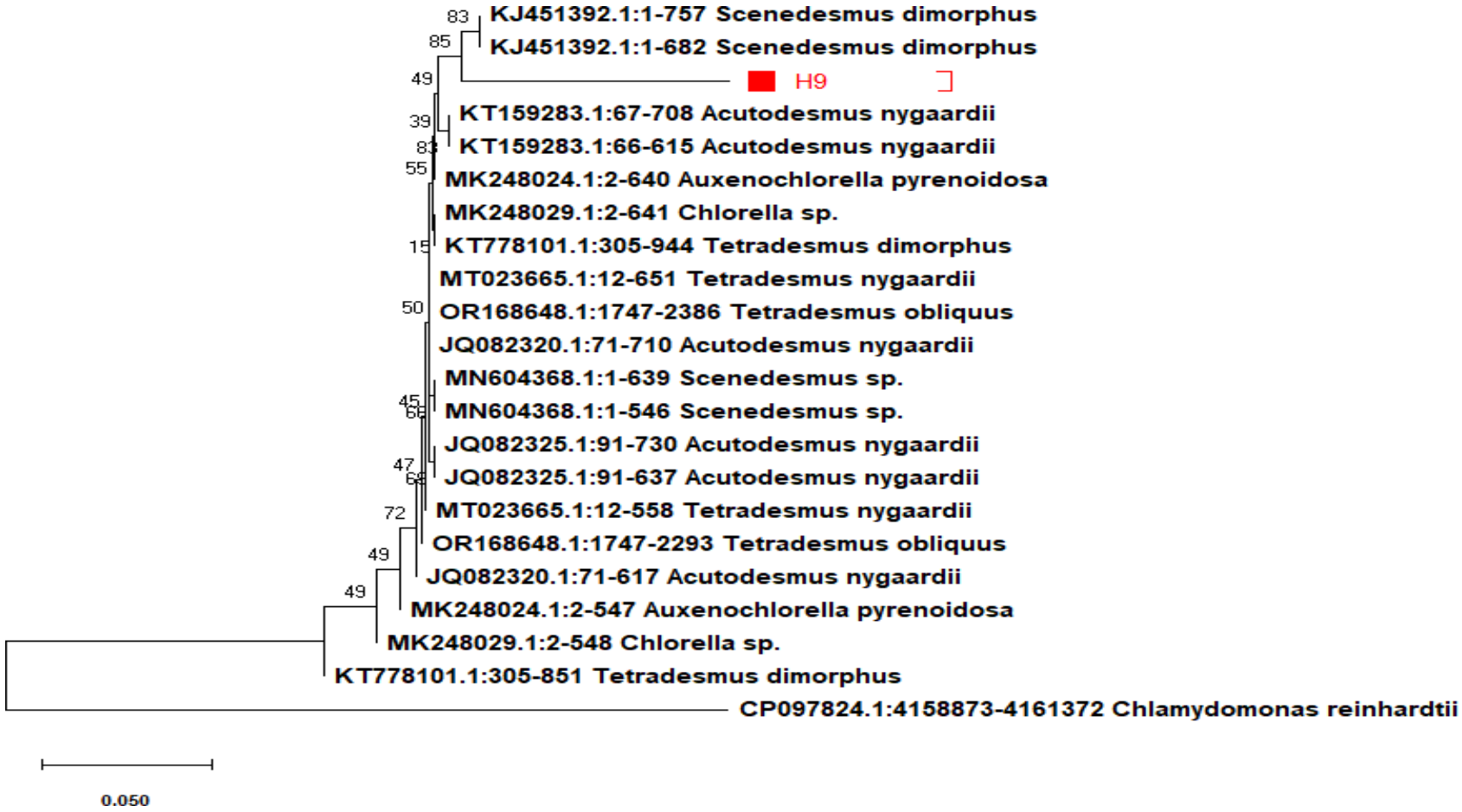


Fig.2.26: Phylogenetic analysis *Scenedesmus dimorphus* (strain H9) based on neighbor joining (NJ) method.

2.3.6.4 *Scenedesmus sp.*1 (Scenedesmaceae) (strain H2)

Colony of 2,4 or 8 cells. Cells are arranged in a linear series. The shape of cells is fusiform. Cells have obtuse ends **(Fig 2.27C-E).** Cell wall smooth. Spines or teeth are not present. The 4 cell colonies are 7.5 µm long and 3.4 µm wide **(2.27Fig E).**

Diagnostic characters: Ovoid cells, mostly in the form of colony of 4 cells arranged side to side. The cell walls are not ornamented with spines or warts.

Site details and habitat: This species was isolated from the running stream. Other taxa found in the sample were: *Synechococcus* and *Chroococcus.*

Culturing conditions: *Scenedesmus* sp. responded in BG_{11} and BBM medium.

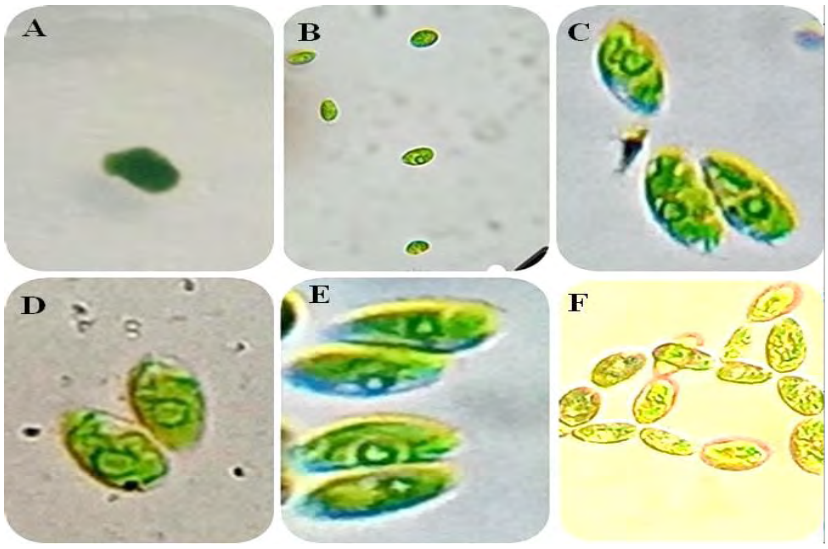


Fig.2.27: (A) The colony of *Scenedesmus* on BBM agar medium. (B-D) *Scenedesmus* in unicell and pair (E) formation of four celled cenobium of *Scenedesmus* sp. (F) Chain like formation of *Scenedesmus* sp.

Molecular and Phylogenetic analysis

In this study 18SrRNA sequence of 1411 bp were obtained from strain H2. The similarity of our sample was checked using 18rRNA by performing BLASTIN searches at the Database NCBI, and the results showed 98.9% similarity with the query coverage 96 % to *Scenedesmus* sp. The phylogenetic trees were constructed using the ML, NJ, and MP method. The NJ tree **(Fig 2.28)** constructed with the sequence of green algal strain downloaded from NCBI including the sequence of investigated strain. Phylogenetic analysis shows H2 makes a clade with *Scenedesmus* sp.

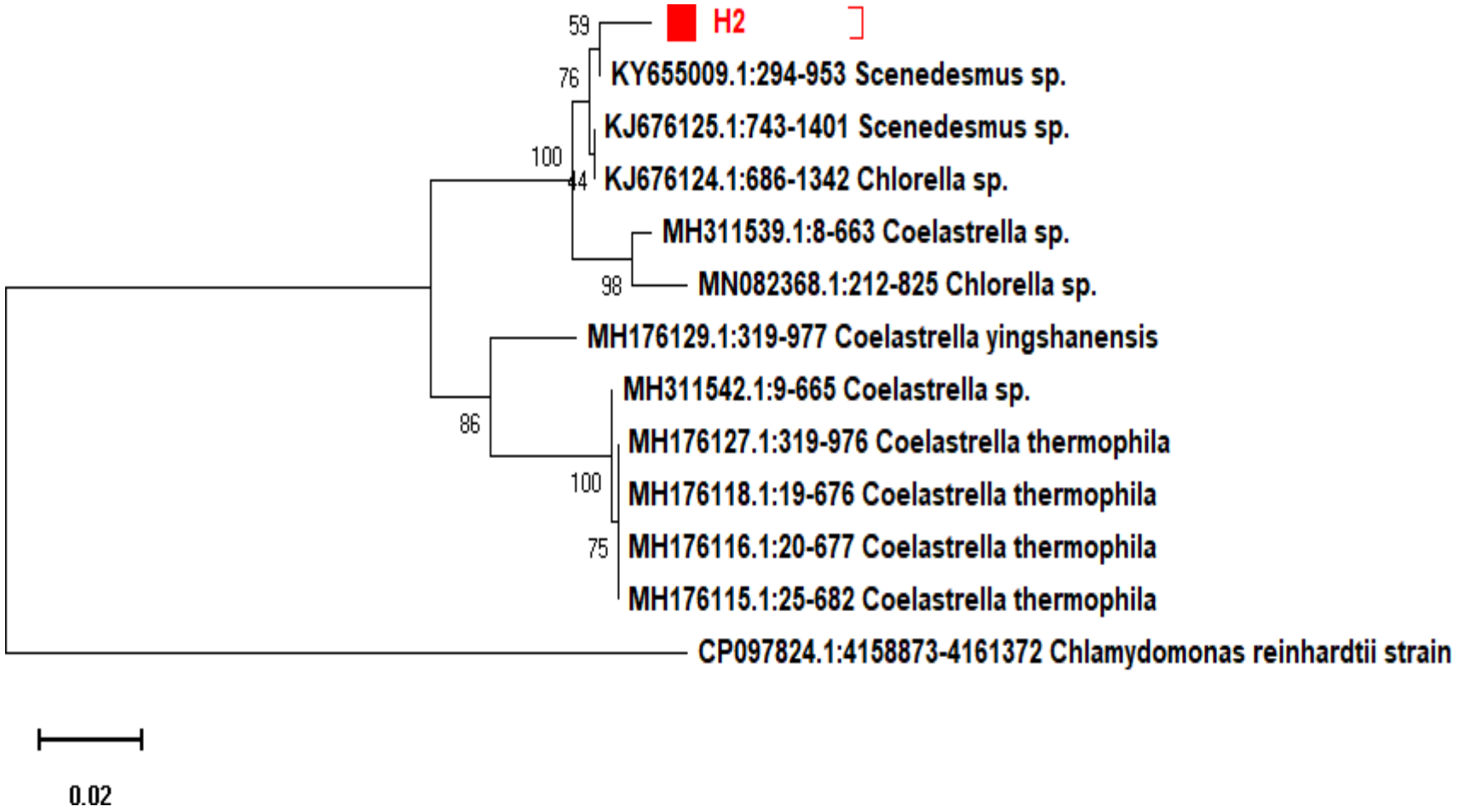


Fig.2.28: Phylogenetic analysis *Scenedesmus* sp.1 (strain H2) based on neighbor joining (NJ) method.

2.3.6.5 *Scenedesmus* sp2. (strain 4) (Scenedesmaceace)

Colony of 2, 4 or 8 cells. Cells are arranged in a linear manner **(Fig 2.29D).** Cells have pointed ends and pyrenoid id present in each cell **(Fig 2.29C).** The length of the cell is 19.2µm and width is 5.8µm.

Diagnostic characters: The cell walls are without spines **(Fig 2.29B-D),** and *Scenedesmus sp*. may also occur as a unicell (initial phase) **(Fig 2.29B)** or in pairs under some conditions.

Site details and habitat: This species was isolated from the running stream. Other taxa found in the sample was: *Chlorella.*

Culturing conditions: *Scenedesmus* sp. responded in BG_{11} and BBM medium.

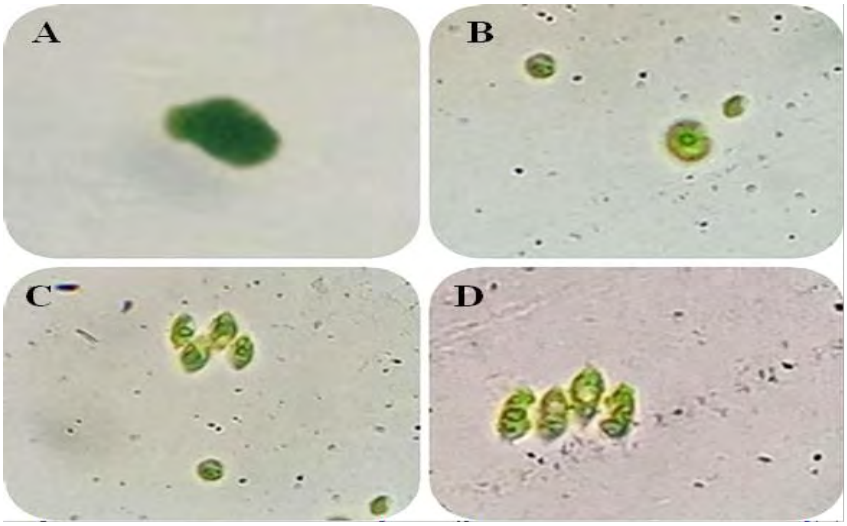


Fig.2.29: (A) The formation colony of *Scenedesmus* sp. on BBM agar medium. (A) *Scenedesmus* sp. in unicell (initial phase) (C-D) Four celled cenobium of *Scenedesmus* sp.

Molecular and Phylogenetic analysis

In this study 18SrRNA sequences of 693 bp were obtained from strain 4. The similarity of our sample was checked using 18rRNA by performing BLASTIN searches at the Database NCBI, and the results showed 99.8 % similarity with the query coverage 96 % to *Scenedesmus* sp. The phylogenetic trees were constructed using the ML, NJ, and MP method. The NJ tree **(Fig 2.30)** constructed with the sequence of green algal strain downloaded from NCBI including the sequence of investigated strain. Phylogenetic analysis shows strain 4 makes a clade with *Scenedesmus* sp.

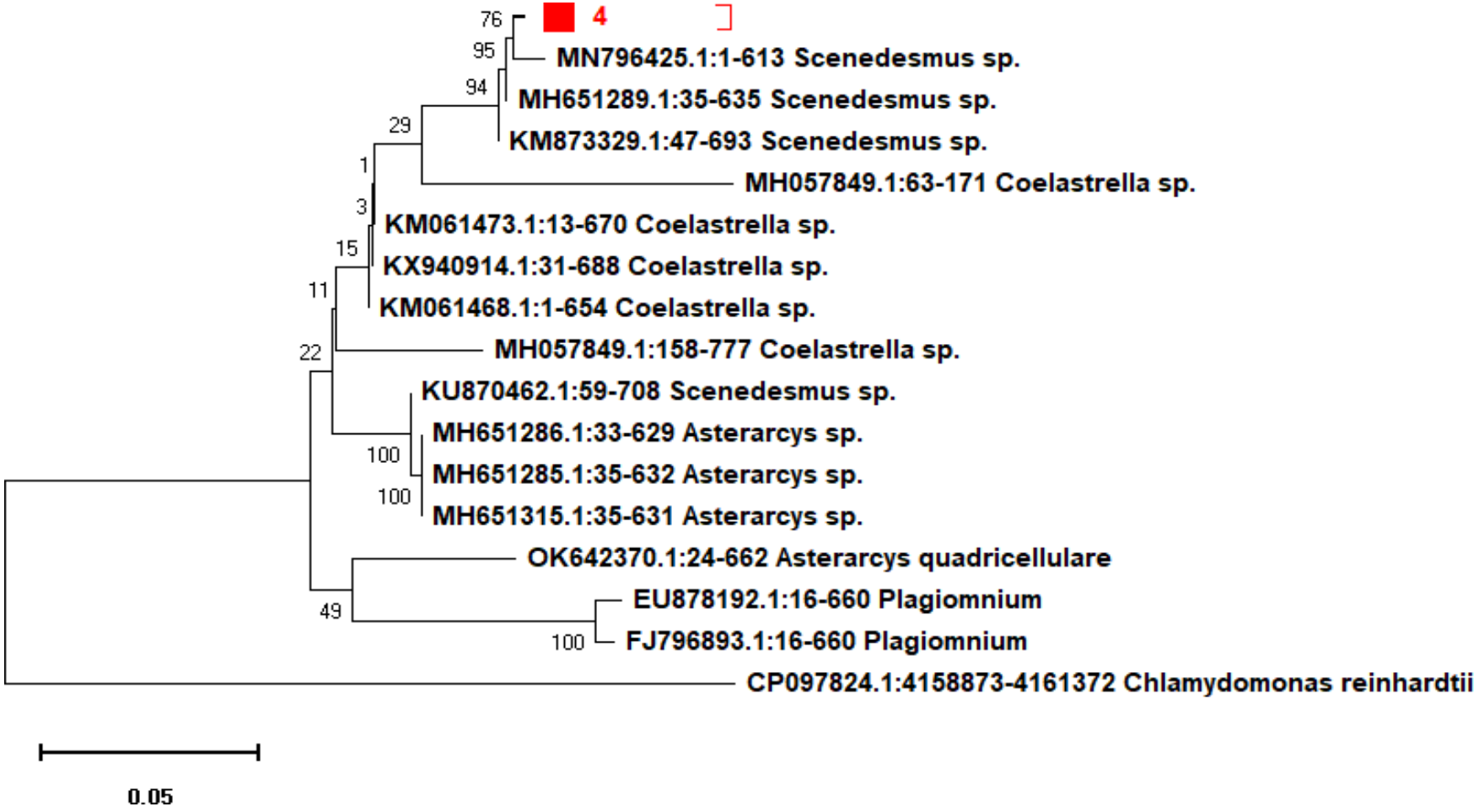


Fig.2.30: Phylogenetic analysis *Scenedesmus* sp. (strain 4) based on neighbor joining (NJ) method.

2.3.6.6 *Scenedesmus* sp3. (Strain 2CL)

Colony of 2 or 4 cells. Cells are arranged in a linear manner. Cells have rounded ends. The length of the cell is 20.2µm and width is 6.4µm.

Diagnostic characters: Spines are not present in *Scenedesmus* sp. **(2.31C-D).**

Site details and habitat: This species was isolated from the running stream. Other taxa found in the sample were: *Chlorella and Chlorococcum.*

Culturing conditions: *Scenedesmus* sp. responded in BG_{11} and BBM medium.

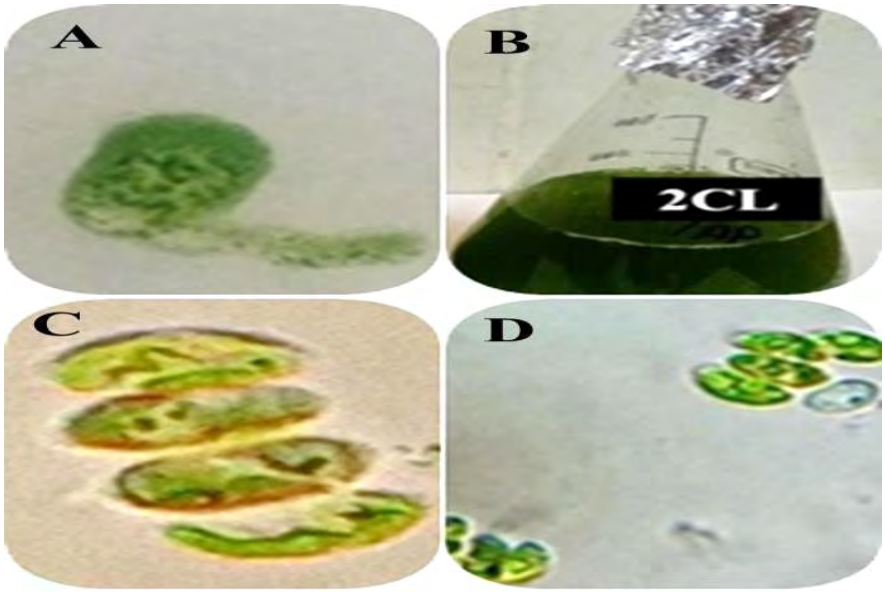


Fig.2.31: (A) The formation colony of *Scenedesmus* on BBM agar medium. (B) Division of one cell into two (C-D) Two and four celled cenobium of *Scenedesmus* sp.

Molecular and Phylogenetic analysis

In this study 18SrRNA sequence of 693 bp were obtained from the strain 2CL. The similarity of our sample was checked using 18rRNA by performing BLASTIN searches at the Database NCBI, and the results showed 99.8 % similarity with the query coverage 95 % to *Scenedesmus* sp. The phylogenetic trees were constructed using the NJ, ML and MP method. The NJ **(Fig 2.32)** constructed with the sequence of green algal strain downloaded from NCBI including the sequence of investigated strain. Phylogenetic analysis shows 2CL makes a clade with *Scenedesmus* sp.

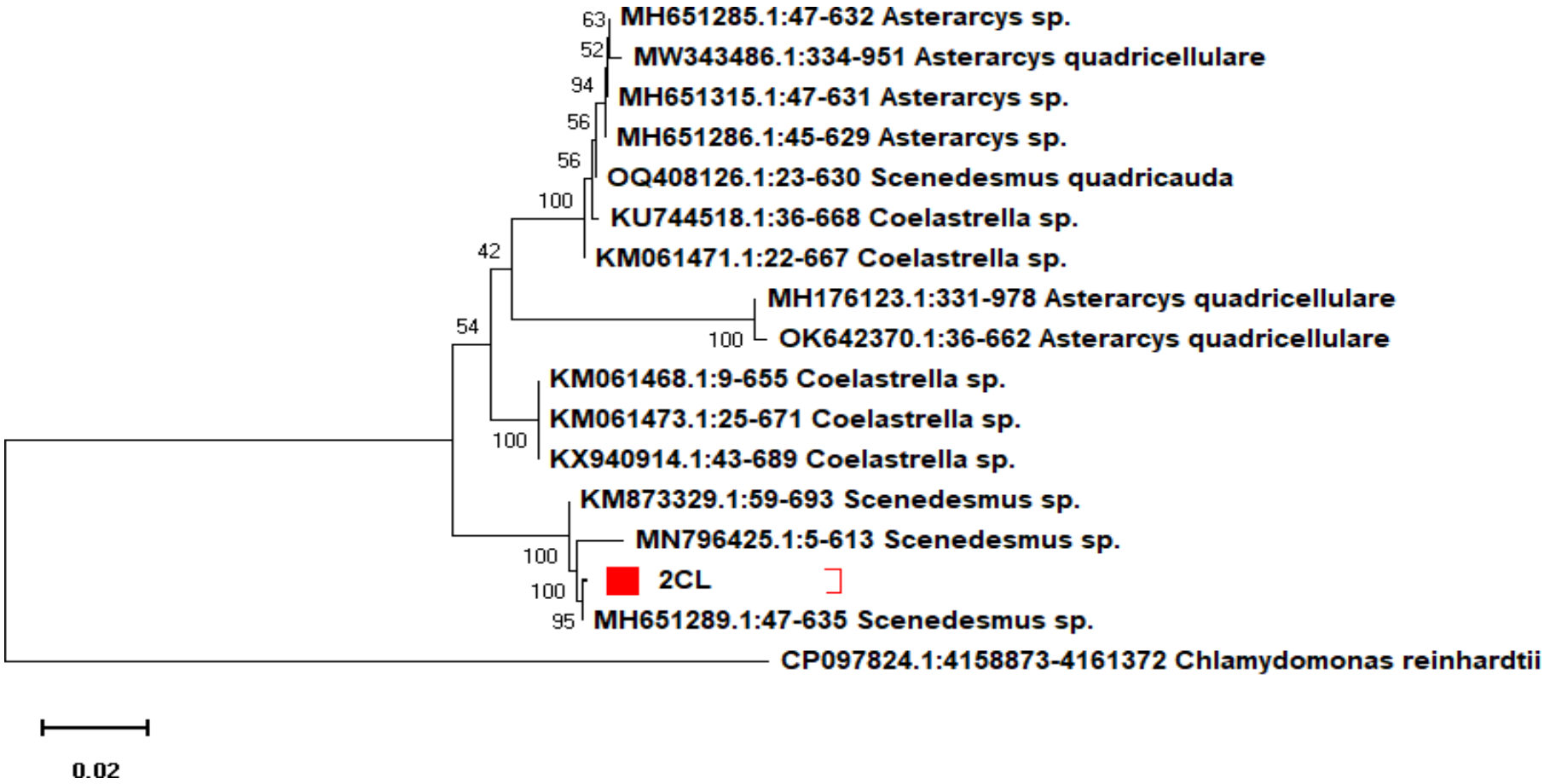


Fig.2.32: Phylogenetic analysis *Scenedesmus* sp. (strain 2CL) based on neighbor joining (NJ) method.

2.3.6.7 *Scenedesmus bijugus* (strain 15)

Morphological description: Colony of 4 or 8 cells. Cells are ellipsoid with rounded ends. Colonies are flat and arranged in an alternate manner. Spines or teeth are not present **(Fig 2.33B).** The length of the colony of 4 cells is 17.8-27.5 µm long, and 15.2-25.1 µm wide.

Diagnostic characters: 4 celled cenobium is present. Spines are absent in cell wall.

Site details and habitat: This species was isolated from the running stream. Other taxa found in the sample were: *Chlorococcum.*

Culturing conditions: *Scenedesmus* sp. responded in BG_{11} and BBM medium.

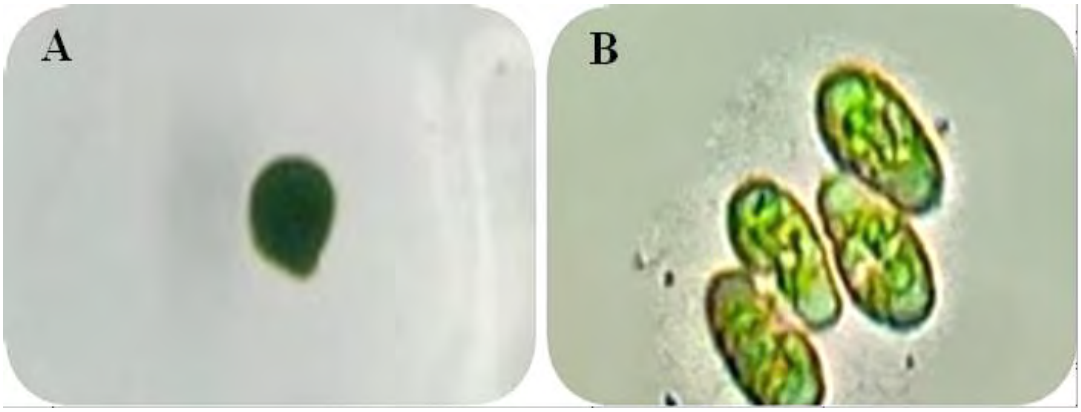


Fig.2.33: (A) The formation colony of *Scenedesmus* on BBM agar medium. (B) Four celled cenobium of *Scenedesmus* sp.

Molecular and Phylogenetic analysis

In this study 18SrRNA sequences of 784 bp were obtained from strain 15. The similarity of our sample was checked using 18rRNA by performing BLASTIN searches at the Database NCBI, and the results showed 99.5 % similarity with the query coverage 88 % to *Scenedesmus bijugas*. The phylogenetic trees were constructed using the ML, NJ, and MP method. The NJ **(Fig 2.34)** constructed with the sequence of green algal strain downloaded from NCBI including the sequence of investigated strain. Phylogenetic analysis shows strain15 makes a clade with *Scenedesmus bijugas*.

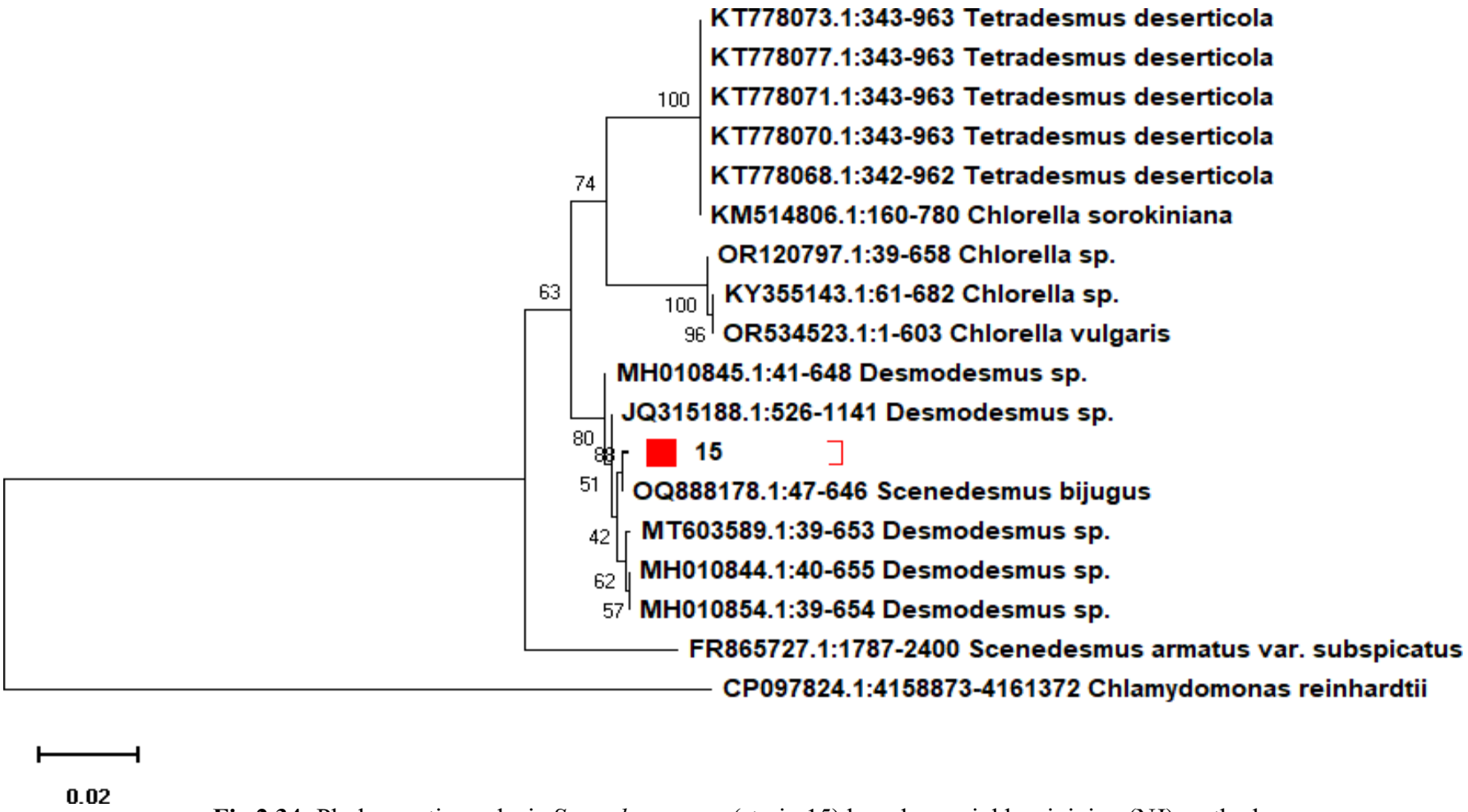


Fig.2.34: Phylogenetic analysis *Scenedesmus* sp. (strain 15) based on neighbor joining (NJ) method.

2.3.7 Screening of strains against different concentration of heavy metals.

Screening of algal strains against heavy metals on a 96-well plate aims to assess the tolerance or sensitivity of different agal strains to various concentrations of heavy metals. **Table 2.9 and 2.10** show the tolerance of green algal strains against the different concentration of Cd and Pb after 7 days.

Table 2.9: Screening of strains against different concentration of Cd.

Concentration	H9	R3	H10	H2	4	2CL	15
5 ppm	0.699	0.601	0.582	0.453	0.433	0.418	0.401
10 ppm	0.578	0.543	0.462	0.421	0.421	0.404	0.399
15 ppm	0.554	0.521	0.432	0.404	0.389	0.387	0.375
20 ppm	0.498	0.426	0.401	0.376	0.355	0.344	0.339
30 ppm	0.425	0.351	0.321	0.353	0.349	0.342	0.332
40 ppm	0.401	0.329	0.315	0.310	0.309	0.278	0.273
50 ppm	0.389	0.301	0.290	0.288	0.288	0.276	0.255

H9: *Scenedesmus dimorphus* R3: *Desmodesmus subspicatus* H10: *Desmodesmus communis* H2*:* *Scenedesmus* sp.1 4: *Scenedesmus* sp. 2 2 CL: *Scenedesmus* sp.3 15: *Scenedesmus bijugus*. (O.D values represent the tolerance of algal strains against different heavy metals).

Table. 2.10: Screening of strains against different concentration of Pb.

Concentration	H9	R3	H10	H2	4	2CL	15
5 ppm	0.732	0.645	0.642	0.490	0.487	0.467	0.445
10 ppm	0.634	0.578	0.532	0.476	0.465	0.445	0.423
15 ppm	0.601	0.555	0.487	0.454	0.443	0.431	0.411
20 ppm	0.587	0.578	0.476	0.421	0.419	0.401	0.396
30 ppm	0.553	0.453	0.433	0.387	0.387	0.386	0.374
40 ppm	0.534	0.429	0.417	0.374	0.365	0.362	0.353
50 ppm	0.517	0.403	0.388	0.353	0.342	0.334	0.319

H9: *Scenedesmus dimorphus* R3: *Desmodesmus subspicatus* H10: *Desmodesmus communis* H2*:* *Scenedesmus* sp.1 4: *Scenedesmus* sp. 2 2 CL: *Scenedesmus* sp.3 15: *Scenedesmus bijugus*. (O.D values represent the tolerance of algal strains against different heavy metals).

2.4 Discussion

The section 2.3 provides a thorough description collection, morphological, and molecular identification of algal samples, as well as their subsequent screening

against different heavy metals. The sampling involved collection of algal specimens from a range of aquatic environments to study a diverse array of species. Initial morphological identification allowed for the identification of algal samples based on morphological characteristics. This was followed by molecular characterization techniques that confirmed species-level taxonomy and identified genetic traits associated with heavy metal resistance. The detailed screening results revealed the capacity of family Scenedesmaceae to tolerate different heavy metals, underscoring their potential role in bioremediation.

Azad Kashmir, with its varied topography and climatic conditions, provides a conducive environment for growth. The freshwater resources, including rivers, lakes, and ponds, support a rich diversity of algae, including green and blue green algae which are abundant colonizers in these habitats. Despite being a terrain with relatively higher rainfall compared to arid regions, freshwater remains a critical resource for survival in Azad Kashmir, especially considering the importance of water availability for the diverse flora and fauna of the region. This is a first-time effort to study diversity of algae from stream of Poonch AJK. The life-supporting features of the stream habitat, including rainfall, temperature, electrical conductivity, and pH, support the microalgae in Poonch AJK. The region is influenced by the monsoon, especially during the summer months, bringing rain and increased humidity. Hajira Poonch AJK generally experiences a temperate climate, with hot summers and cold winters. During visits to the stream of Poonch AJK, the water was clear.

The stream is surrounded by agricultural fields that are being newly introduced, and these fields are irrigated through canals connected to a nearby stream. Unfortunately, the stream has been polluted by the infiltration of sewage water, posing a significant threat to the ecosystem because of incorporation of both nutrients and chemical contaminants. This situation has provided a unique opportunity to investigate and compare the impacts of human activities on the diversity of green and blue-green algae in this area. Agricultural practices, including the utilization of synthetic fertilizers, had a substantial impact. Specifically, the electrical conductivity (EC) in this stream was measured within the range of 802 ds/m, **(Table 2.6)** and it is an exceeding level of EC as reported in previous literature (Dhanam et al., 2016), indicating a concentration of ions, nutrients, and dissolved solid contents. Similar findings were noted by various researchers, that describe the physico-chemical properties of Bijapur (Hulyal and Kaliwal 2011). Additionally, the pH was recorded at

7.31, and the atmospheric temperature was 31°C. Interestingly, the pH value during the monsoon season was not elevated, possibly due to the dilution effect of rainwater (Nandan and Patel 1992). The concentration of chloride serves as a crucial parameter for identifying sewage contamination, with a recorded value of 40.21 mg/l in the stream. Chloride ranks among the major inorganic anions in water (Harsha et al., 2017) and is commonly found in discharges from sewage, and irrigation waste (Manivasakam, 2003). Nitrate levels were observed at 15 mg/l, with higher concentrations noted during the monsoon season, aligning with previous literature findings (Dhanam et al., 2016).

Nitrate contributions to freshwater result from sewage discharge, and runoff from agricultural fields. The elevated concentration during the monsoon season is associated with the influx of nitrogen-rich floodwater, contributing to substantial sewage volume. This period experiences the highest nitrate-nitrogen concentration, known to support the formation of blooms (Anderson et al., 1998). Biological Oxygen Demand (BOD), measuring the oxygen consumed by microorganisms in the aerobic oxidation of organic matter. Jain and Dhanija, (2000) consider BOD an essential parameter in aquatic ecosystems for establishing the status of pollution in water. Higher value of BOD results in the presence of numerous microbes in water bodies accelerating their metabolic activities, with increased concentrations of organic matter. In this study, BOD ranged between 8.13 mg/l **(Table 2.6)** which indicates that water is moderately polluted. Unpolluted water typically has a BOD of less than 1.00 mg/l, moderately polluted water falls between 2.00-9.00 mg/l, and heavily polluted water surpasses 10.00 mg/l (Adakole, 2000).

2.4.1 Diversity of Phytoplankton in the field samples

Phytoplankton is the major producer of aquatic ecosystems, shows great diversity. In the field samples, the plankton studies showed that a total of 18 species of plankton belonging to green and blue green algae. Among the Chlorophyta (green algae), members of the genus *Spirogyra* were found to be the most abundant. Other taxa were represented by either one or two species only. Hegde and Sujata, (1997) and Nirmal Kumar et al. (2005) reported that high water temperature supports the growth of Chlorophyceae. In the current study, thirteen species of Chlorophyceae were recorded in field samples such as *Spirogyra, Chlorella. Chlorococcum, Scenedesmus, Scenedesmus dimorphus, Rhizoclonium, Odogonium, Ulothrix, Ankistrodesmus, Closterium, Cosmarium, Chlorococcum* and *Selenastrum*. The members of

Chlorophyta are free living and planktonic, mostly confined lakes, rivers, streams and are attached to submerged plants (Huisman et al., 2005). Chlorophyta is the second dominant group among the plankton. The presence of Chlorophyta might be due to high dissolved contents in the water. High turbidity has an adverse effect on the diversity of phytoplankton by absorbing solar energy in the surface layer on water and thus impairing photosynthesis which causes a sharp fall in phytoplankton density. High rain fall dilution in the water bodies, also playing an important role in the growth of phytoplankton. This finding was also made by Trivedy and Goel, (1989). The seasonally resistant most common genera were *spirogyra*, *Oedognium*, *Ulothrix* and *Zygnema*, which were found to survive in all the four seasons (Ali, 2006).

Among these, Cyanophyta comprised of 5 species were recorded and the stream of Poonch AJK showed a predominance of filamentous types such as: *Phormidium*, *Oscillatoria*, *Nostoc*. Zarina et al. (2010) reported the distribution, and the growth of blue-green algae was most abundant in the aquatic and terrestrial environment. Stagnant water with 7.5 pH, high temperature (up to 48 °C) with rainfall and sunlight, are the most suitable conditions for their growth. They were mostly found in summer, but some of also occurred in winter. Higher temperature triggers the maximum growth of filamentous and non-filamentous blue green algae.

2.4.2 *In vitro* culturing

The examination of naturally occurring and cultured samples holds significant importance in taxonomic studies. However, cultivating natural samples presents certain drawbacks, emphasizing the need to cultivate these samples in simplified and standardized culture conditions within shorter and more simplified cycles. It has been noted that wild strains, cultured under 'natural' conditions, often play a more crucial role in this type of investigation compared to the transfer into monospecific culture under standardized conditions. The latter can lead to modifications, or restrictions in morphology and life cycles (Komarek, 2013). The taxonomic implications of these different stages during life cycles and vegetation cycles still require proper elucidation, and the impact of wild morphological forms on taxonomic affiliation remains uncertain. Various types of green algae and blue green algae exhibited distinct reactions to *in vitro* culture. Blue green algae were cultured using different methods and standard culture media. The first documentation of axenic cultures for microalgae dates back to 1890, provided by the Dutch microbiologist Beijernck. Over time, culturing procedures have seen improvements through advancements in various

biological fields. Various media and solid agar plating techniques were employed for cultivating algal species (Lee and Shen 2003). Notably, green algae exhibited a greater response to BBM, while BG_{11} media prompted response from blue-green algae. Some species of both green and blue-green algae responded to both BBM and BG_{11} medium (**Table 2.8**).

2.4.3 Exploring species diversity within the Family Scenedesmaceae: Implications as pollution indicators in aquatic environment

In the present study, diversity of family Scenedesmacae was observed during *in vitro* culturing in the stream of Poonch AJK **(Table 2.8)**. *In vitro* culturing typically involves providing specific growth conditions optimized for the target organisms. The conditions favor the growth of certain Scenedesmaceae species while inhibiting others. As a result, the cultured samples have the diversity of family Scenedesmaceae present in the natural environment. In the present study 16 species of family Scenedesmaceae are reported from Poonch AJK. The members of the family Scenedesmaceae indicate that ponds and streams have a high level of nutrient content because species of this family prefer these conditions for their survival and growth. These species are thought to be indicators of highly contaminated water with organic waste. In some studies, similar results have been explained about the diversity of *Scenedesmus* in highly contaminated organic water (Kumar et al., 2016). The stream of Poonch contains high phosphate and nitrates concentrations, thus increasing the growth of phytoplankton **(Table 2.6)**.

The diversity of phytoplankton and their association as a biological indicator in water quality assessment has been discussed by Clesseri et al., (1998). *Euglena, Scenedesmus, Microcystis* and *Oscillatoria* were found in organically polluted waters described previously by Nandan and Aher (2005). Singh and Balasingh, (2011) discussed that different species of algae like *Scenedesmus, Oscillatoria, Chroococcus, Navicula* and many others were reported as a pollution indicator. Kshirsagar (2013) studied the algal species sensitive to water pollution in river Mula. The results showed the dominance of *Chlorella vulgaris, Oscillatoria limosa, Scenedesmus quadricauda* and *Melosira granulate* throughout the year. These genera are considered as an indicator of organic pollution in fresh water. Blum (1957) observed that in the river Ganges there is organic pollution due to entry of sewage and concentration of nitrates was increased due to organic matter. Furthermore, in the study of palmer in 1969 higher concentration of nitrogen content was recorded, when there is maximum rainfall,

since nitrogenous substances were entered into the stream by water runoff and genus *Scenedesmus* seemed to be abundant at the season of rain fall. Palmer reported 60 most pollution tolerant genera of algae. Among these, genus *Scenedesmus* is one of most important genus as a pollution indicator. 12 species of genus *Scenedesmus* are considered pollution tolerant. These are: *Scenedesmus quadricauda, Scenedesmus obliquus, Scenedesmus dimorphus, Scenedesmus acuminatus Scenedesmus opoliensis, Scenedesmus abundans, Scenedesmus acutus, Scenedesmus armatus, Scenedesmus bijuga, Scenedesmus longus, Scenedesmus bijugatus* and *Scenedesmus falcatus.*

2.4.4 Morphological and Molecular identification of selected species of Family Scenedesmaceae

Isolation of new strains from the environment also includes identification, which allows for a better understanding of their physiology. For a long time, microalgae were identified on the basis of their morphology. However, due to morphological plasticity in microalgae, the use of molecular identification techniques is required. Morphological observation can be ambiguous and less accurate for species-level identification than more advanced methods such as taxonomic phylogenetic analysis. Many strains showed similar morphologies, making it impossible to separate them using taxonomic and morphological analysis (Lee et al., 2018). Therefore, relying simply on microscopic observation would make it difficult to taxonomically position of various microalgae. As a result, it was preferred to go with molecular authentication and compare the results to morphological evaluations. The nuclear conserved region containing the molecular marker ITS and 18S, a well-known ribosomal sequence used in DNA barcoding and eukaryotic phylogenetic analysis that was successfully applied to microalgae, was used to genetically identify seven algal strains (Heege et al., 2015; Ferro et al., 2018). Recently, numerous new species and genera have been recognized in the Scenedesmaceae family, exhibiting a high degree of diversity among its members worldwide (Markert et al., 2012; Hoshina, 2014). When examining the phylogenetic relationships within the Scenedesmaceae, the ITS1 and ITS2 sequences are important alternative markers. The markers ss3 and ss5 are also important for the identification of green algae (Khaw et al., 2020). Kessler et al. (1997) divided *Scenedesmus* into two subgenera, *Scenedesmus* for non-spiny forms and *Desmodesmus* for spiny forms, based on 16S rRNA gene sequence, DNA base composition, and DNA/DNA hybridization studies. Later, An et al. (1999) verified a clear difference between them by analyzing the ITS-2 rDNA sequence and divided

into two different taxa.

Desmodesmus (family **Scenedesmaceae**) is one of the most common genera in fresh and brackish waters around the world (Lurling, 2003). Because of their great degree of polymorphism, these organisms can withstand a wide range of environmental circumstances, as indicated by their widespread distribution (Egan and Trainor 1991). *Desmodesmus*-like strains have spines, but *Scenedesmus* spp. have a smooth, no ornamented cell wall. *Desmodesmus* was differentiated from Scenedesmus using ITS2 sequences (An et al. 1999). However, the classification of *Desmodesmus* species remains one of the oldest issues in green microalgal systematics (Shubert et al., 2014). Many *Desmodesmus* strains and species demonstrate significant morphological plasticity in response to nutritional availability, environmental signals, and culture conditions (Shubert et al., 2014). The morphological study of strain H10 revealed that they belong to the genus *Desmodesmus*. Within the 18S rRNA and ITS phylogenies, every sequence that grouped into robust clades with strain H10 is identified as *Desmodesmus communis*. This strain has been isolated from Europe to Asia, according to Kaplan-Levy et al. (2016). These sequences were aligned with 3410 and showed up to 99% identity. The *rbcL*-based phylogeny supports the earlier findings, as 3410 clustered with a *Scenedesmus quadricauda* sequence, which most likely belonged to a non-revised *D. communis* (Kaplan-Levy et al., 2016). The strain R3 encountered in this investigation were morphologically heterogeneous, with the following characteristics: four-celled cenobium, lateral spines, and terminal spines. The morphological data of strain R3 revealed a relationship to species of the genus *Desmodesmus*. Strain R3, with strong node support, was identified as a *Desmodesmus subspicatus*. This strain was found in the diverse freshwaters throughout the world and identified on the base ITS and 18S rRNA (Hegewald, 2000; Bhatt and Tamta 2013). The molecular categorization and morphological taxonomy of strain R3 was consistent (Hegewald et al., 2001).

Scenedesmus Mayen is a chlorococcalian freshwater green algae. It is either single-celled or colonial, having two (typically four or eight) 16 to 32-celled coenobia. In colonial form, cells are organized linearly in one row (4 celled) or alternately in 2 to 3 rows (8 or more celled), connecting only to lateral walls or in the subpolar region. The shape of cell is from spherical to ellipsoidal, elongate, or fusiform, measuring 3-78 µm long and 2-10 µm broad. The apices can be capitate, obtuse, acute, or tapering, and the cell wall is ribbed, granulated, or dented, without spines. The nucleus is

single, as is the chloroplast, which is parietal and contains a single pyrenoid. Asexual reproduction occurs through non-motile autospores; sexual reproduction is uncommon, having only been documented in *S. obliquus* (Komarek and Fott, 1983; Skaloud, 2008; Sakthivel, 2016). Guiry and Guiry (2014) recorded around 447 *Scenedesmus* taxa globally, with 94 recorded in Korea. The majority of them were recognized using typical key features such as cell form and size, cell arrangement in the coenobia, and the location of spines on the coenobia. However, the morphological variety of its coenobia, cells, and cell wall sculptures has resulted in the description of numerous taxa, and the current system is quite complex (Comas and Komárek 1984). Following the 1960s, some researchers documented morphological variations in *Scenedesmus* (Comas and Komárek 1984; Hegewald and Silva 1988). The numerous structures of *Scenedesmus* cell walls that are not visible under light microscopy and are studied using an electron microscope. According to Hegewald and Silva (1988), Komarek and Ludvik (1971) and Hindak (1990) employed these electronic microscopical criteria for taxonomy.

In the current investigation, four strains with different morphological characteristics were discovered. Among these, the morphological characters of strain H9 were similar to *Scenedesmus dimorphus* which featured a four-celled cenobium with a pyrenoid at the center (Patil et al. 2018). Phylogenetically, it was similar to the *Scenedesmus* group. Another strain, H2, had pointed ends and chain-like formation. These characteristics were consistent with previous descriptions of the genus *Scenedesmus* (Beherepatil and Deore 2013). Strain 2CL differed in a few characters, such as the outer cells being curved in comparison to the inner cells and having rounded ends. Strain 4 was organized alternately and had sharp tips, but strain 15 had rounded tips and scattered cytoplasm (Beherepatil and Deore 2013). The phylogenetic studies **(Fig 2.28, 2.30, 2.32, 2.34)** found that five four strains are clearly and closely related to the Genus *Scenedesmus*. Based on appearance and molecular analysis, the four strains obtained were identified as *Scenedesmus* sp. (Gour et al., 2016; Akgul et al., 2017).

Conclusion

- A survey of stream located in Hajira Poonch AJK revealed a rich source of microalgae.
- The stream is surrounded by an area which is rich in organic matter. Heay metals

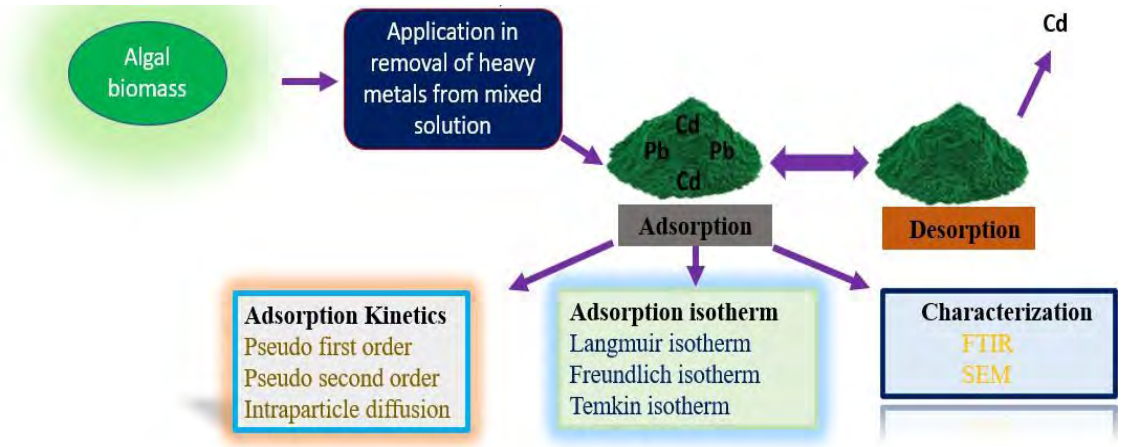
(Cd and Pb) was detected in higher concentration as compared to other metals.

- Members of the family Scenedesmaceae are frequently observed during *in vitro* culturing. 16 species of family Scenedesmaceae were observed during isolation along with other green and blue green algae. The diversity of species from family Scenedesmaceae in a stream indicates the indicator of pollution in the water body.
- Based on morphological and molecular characterization *Desmodesmus communis*, *Desmodesmus subspicatus*, *Scenedesmus dimorphus*, *Scenedesmus bijugus, Scenedesmus* sp.1, *Scenedesmus* sp.2, *Scenedesmus* sp.3 are reported first time from Poonch AJK.
- Among these strains *Desmodesmus subspicatus* and *Scenedesmus dimorphus* were best resistant against Cd and Pb.

CHAPTER 3

Biosorption Potential of dried biomass of *Desmodesmus subspicatus* and *Scenedesmus dimorphus* from Contaminated Water

Graphical Abstract



3. Abstract

Industrialization, urbanization, and natural processes have potentially accelerated the pace and level of heavy metals in the aquatic environment. Recently, modern strategies for heavy metal treatment in wastewater have received the specific attention of the scientific community. The present study aimed to assess the dried biomass of two strains as a low-cost adsorbent to remove cadmium (Cd) and lead (Pb) from aqueous solutions. It involved the optimization of pH, contact time and initial concentration of metal ions. Data collation revealed that an optimum contact time for both metals was 60 min, with an adsorption capacity of 63 (Cd) and 66 mg/g (Pb) for *Desmodesmus supspicatus.* The biosorption process was quick and the equillibrium time for the above-mentioned metals was recorded as 90 and 60 minutes, and percentage removal of 85 and 83 mg/g for S*cenedesmus dimorphus* respectively. Different models were applied to the equilibrium data. The pseudo 2nd order described the best adsorption of Cd and Pb for both strains. The equilibrium data were computed with various isotherms. Langmuir isotherms better suit for the adsorption of the above-mentioned metals for *Desmodesmus* and *Scenedesmus* sp. Hence, the maximum adsorption capacity of *Desmodesmus* and *Scenedesmus* sp. for Cd (64.1 and 128 mg/g) and Pb (62.5 and 102 mg/g) respectively. The mechanism of biosorption was validated

through a comparative FT-IR and Scanning Electron Microscopy of raw and metal-loaded algal biomass based on cell morphological changes. To study the reusability of adsorbent, adsorption- desorption of Cd and Pb ions was repeated three times using HCl. These results did not noticeably change in adsorption capacity during the three cycles for both strains. Using HCl (0.1 M), desorption of both metals was achieved more than 70% in three cycles for both strains. This work presented a long-term bioremediation approach for heavy metal pollutants in wastewater. This research could be seen as an interdisciplinary approach to large-scale heavy metal remediation. In addition, growing microalgae in wastewater produces animal feed and biodiesel. When compared to other conventional methods for environmental remediation and the manufacture of valuable products, the use of microalgae is a more efficient and cost-effective method.

3.1 Introduction

Toxic (heavy) metals constitute the most hazardous moieties found persistently in the environment (Gong et al., 2022). Heavy metals are the contaminants that impact humans and animals (Sharma et al., 2021; Waheed et al., 2021). Heavy metals (HMs) are present in the natural ecosystem due to increased industrial and anthropogenic activities as shown in **Fig 3.1.** Heavy metals that are toxic to the surroundings include cadmium and lead (Nateras-Ramírez et al., 2021). Despite this, Cd has been excessively used in pesticides, fertilizers, plastics, mining, welding, and refining (Luo et al., 2021). Similarly, lead is used in batteries, cables, steel, paint, and plastic industries (Al-Homaidan et al., 2016; Njati and Maguta 2019). Discharge of waste from these production units causes heavy metal contamination in the aquatic system. Prolonged exposure to Cd can cause hepatic injury, hypertension, lung damage, renal dysfunction, and teratogenic consequences in humans (Singh et al., 2006; Cheng et al., 2017). Likewise, the effects of lead exposure may damage the brain, kidneys, and liver, in addition to developing ancillary issues, such as loss of appetite, gastrointestinal, anemia, and mental defects in children (Wang et al., 2013; Farooqi et al., 2020; Farooqi et al., 2022;). Due to being non-degradable and accumulative, heavy metals persist in the food chain with biological consequences, as already mentioned (Jalilian et al., 2020).

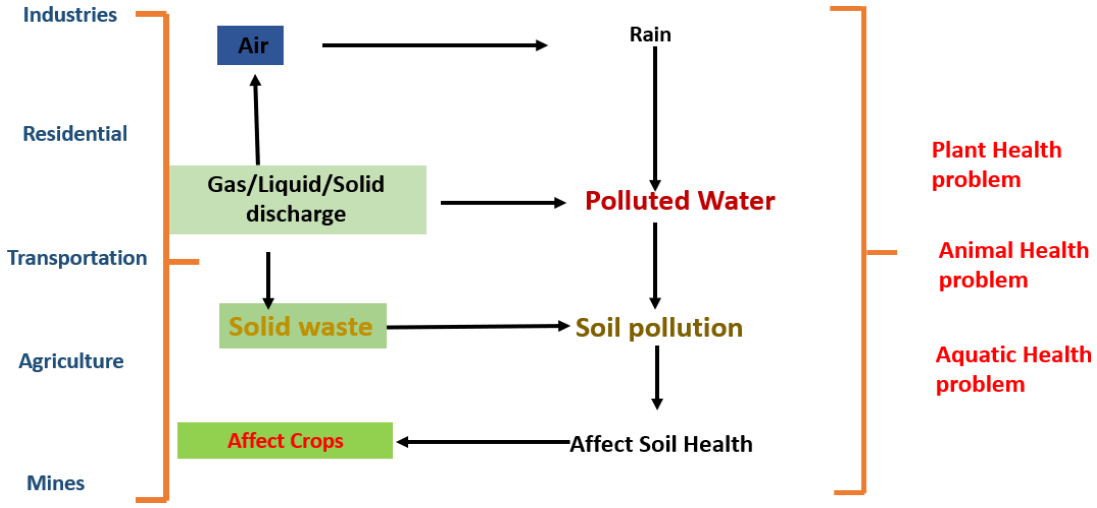


Fig.3.1. Sources and effects of heavy metals.

To avoid adverse impacts of heavy metals, it is imperative to purify wastewater before it is discharged into the surroundings (Ahluwalia and Goyal 2007; Barakat, 2011). Heavy metals can be eliminated from wastewater through a variety of processes. These may include ion exchange, coupled processes of oxidation/reduction, lime precipitation, membrane technology, electrochemical treatment, filtration, and reverse osmosis (Barakat, 2011; Awwad et al., 2020). There are some limitations of these methods, such as not being more efficient at low metal concentrations and producing toxic sludge (Chong et al., 2000; Ahmadzadeh et al., 2021). Therefore, new techniques are needed that eliminate heavy metals from the ecosystem, ideally in an economical and environmentally friendly way (Benabdallah et al., 2017). Biosorption, as an alternative, provides the advantage of safe and efficient means of heavy metal removal from aquatic bodies, including wastewater, either in catchment areas or in ponds (Hussain et al., 2021). Consequently, this research is focused on findings, effective and economically viable biosorbents to deal with these persistent heavy metals for biosafe ecosystem.

The process of biosorption is complex and is affected by the type of microbes, either in the living or amorphous form (Madrid and Cámara, 1997). Since 2009, algae and cyanobacteria have been recognized as a preferred means to eliminate metal pollutants (Chojnacka et al., 2005; Priyadharshini et al., 2021). Algae, bacteria, fungi, and yeast all exhibit the uptake capacity of metal ions either by bioaccumulation or biosorption (Qin et al., 2020; Sharma et al., 2021). The application of amorphous biomass as an adsorbent has been promising in removing or recovering pollutants from aquatic environments (Taniguchi et al., 2000). Besides accumulating pollutants,

algae are a broad category of organisms in aquatic environments that contain chlorophyll and prepare their food, thus boosting the water quality (Davis et al., 2003; Farooqi, et al., 2021; Kausar et al., 2021). Algae as a biosorbent are efficient even at low levels of metal ion concentration (Mehta and Gaur, 2005), thus offsetting disadvantages associated with commercial resins at lower metal concentrations (Eccles, 1999). Different processes, such as coordination, electrostatic attraction, complexation, and microprecipitation, participate in the binding mechanism of heavy metal in the process of adsorption. At the same time, ion exchange has significantly influenced the binding of adsorbates to biosorbents (Bilal et al., 2018). Therefore, algal biomass is a reliable system for eliminating heavy metals offering dual capacity for biosorption and bioaccumulation. Biosorption is a passive phenomenon that essentially involves the chemical interaction of adsorbate and adsorbent (Manzoor et al., 2019), while bioaccumulation is an active process that occurs when there is intracellular incorporation of metal ions (Rangsayatorn et al., 2002; Mustafa et al., 2021). There have been numerous prior studies that have used microalgal species such as *Stigonema* sp., *Aphanothece halophytica*, *Spirulina*, *Chlorella Vulgaris*, *Chlamydomonas reinhardtii*, *Neochloris oleoabundans*, *Chlorococcum* sp., *Chroococcus paris*, *Scenedesmus* sp., and *Phormidium* sp., as biosorbents (Chu and Phang, 2019; Gu and Lan, 2021). These species have maximum adsorption capacities (q_{max}) exceeding 50 mg g^{-1} against Cd, Pb, Zn, Cu and Hg. These findings show that microalgae are an effective alternative for removing these contaminants when compared to other biosorption materials (Wang et al., 2018; Mustapha et al., 2019).

In this study, the dried biomass of local strains of *Desmodesmus subspicatus* and *Scenedesmus dimorphus* were used for the first time as a biosorbent for the removal of cadmium and lead from the aqueous medium. The present study aimed to assess the individual capacity of each of the dried biomass as sorbent of cadmium and lead. Batch experiments were carried out to find out the optimum conditions i.e. optimum pH of the medium, contact time, the initial concentration of cadmium and lead ions, and adsorbent dose, to assess the efficiency of adsorption of metals from aqueous solution to decipher the adsorption mechanism involved in the process. Furthermore, the desorption of loaded (metal) biosorbents were also investigated.

3.2 Materials and Methods

3.2.1 Preparation of adsorbents

The biomass of *Desmodesmus* and *Scenedesmus* sp. was harvested at the exponential phase of growth and centrifuged at 5000 rpm (10 min) to obtain a pellet. The pellet was washed with sterilized deionized water, dried at 40 °C, ground, sieved (100 µm), and stored (4 °C) for future use (Abdel-Aty et al., 2013). A schematic presentation of production and processing of biomass is shown in **Figure 3.2.**

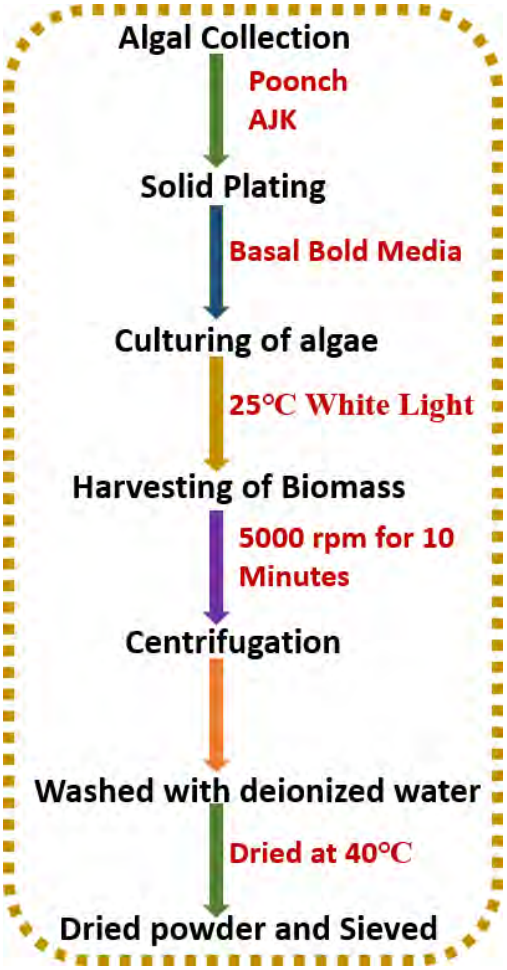


Figure 3.2. Schematical presentation of production and processing of biomass.

3.2.2 Reagents Preparation

The stock solutions of Cd and Pb and serial dilutions were prepared by dissolving $CdCl_2$ and Pb $(NO_3)_2$ (1000 mg/L) in deionized water. The pH (827 Metrohm benchtop pH meter, USA) of both solutions was adjusted between 2.0 and 8.0 (Mirghaffari et al., 2015).

3.2.3 Biosorption Experiments

Batch experiments were performed to examine the impact of pH, contact time, initial concentration of cadmium and lead ions, and sorbent dosage in the aqueous solutions. The optimum pH was studied using 100 mg/L Cd and Pb concentration at a contact time of 60 min. The impact of contact time was examined by sample collection at specified intervals of time (5, 10, 15, 20, 30, 60, 90, and 120 min) at the 100 mg/L of metal concentration, pH 6 for cadmium, 5 for lead and biomass dosage 1 g/L. After optimizing the pH of the medium and contact time, tests were performed with different initial concentrations of metal ions, keeping the time interval (60 min) constant. 1 g/L of dried algal biomass was mixed with a metal ion solution with a concentration ranging between 20 and 120 mg/L at optimized pH for cadmium and lead in a batch experiment at 23 °C. Another experiment was performed at optimum conditions to know the influence of biosorbent dosage, 0.5–2 g/L. Biosorption tests were performed in 10 mg/100 mL of metal solutions within a specific pH range and then mixed with algal biomass and agitated at 150 rpm with two replicates. Metal solution mixed with biosorbent was filtered, and the final concentration level of metals in the medium was assessed by atomic absorption spectrophotometry (Al-Rub et al., 2006) (Agilent Technologies, MC187906, Petaling Jaya, Selangor, Malaysia). The following formulas **(Equation 1)** were used to compute the amount of sorbate adsorbed on the biosorbent, which described the efficiency of metal adsorption (as mg/g and removal percentage) (Mirghaffari et al., 2015; Kumar et al., 2018).

$$q_e = V\frac{C_i - C_e}{M} \qquad (1)$$

Here, in **Equation (1)**, q_e quantifies the adsorbed ions on the biosorbent, where C_i is an initial and C_e is the final level of metal concentration, V denotes the solution volume, whereas M represents the biomass of sorbent (g).

3.2.4 Analysis of Adsorption Kinetics

To decipher the biosorption mechanism, the sorption data were assessed against pseudo 1st order (Lagergren, 1898) and pseudo 2nd order (Ho and McKay, 1999). Finally, the intraparticle diffusion model (Weber and Morris, 1963) was applied to determine the rate constants and order of reactions for the processes. The adsorption kinetics were tabulated as shown in **Table 3.1**.

Table 3.1: The linearized form of kinetics model and isotherms.

Kinetic Model	Linearized Equation	Graphical Dependence
Pseudo 1st order	$\text{Log}\,(q_e - q_t) = \log q_e - \frac{k_1 t}{2.303}$	log ($q_e - q_t$) vs. time
Pseudo 2nd order	$\frac{t}{q_t} = \frac{1}{k_2 qe^2} + \frac{1}{q_e} t$	t/q_t vs. time
Intra-Particle diffusion	$q_t = k_i t^{0.5} + I$	q_t vs. time $^{0.5}$

Where k_1 and K_2 represent the pseudo first and second order rate constants (mg g^{-1}). The plot between log (q_e-q_t) and time revealed a straight line, and the slope's value, -$\frac{k_1}{2.303}$ is used to derive the k_1 value. k_i denotes rate constant of the Intra-particle diffusion as mg g^{-1} min^{-1}. The graph between (q_t) against time ($t^{0.5}$) must be linear and is deemed a rate-limiting step when the line crosses through the origin.

3.2.5 Adsorption Isotherms

The sorption isotherm models provide vital knowledge on the sorption process. Here, sorption data are evaluated using Langmuir, Freundlich, and Temkin isotherms. The Langmuir isotherm suggests adsorbate monolayer adsorption on adsorbent surfaces (Çolak et al., 2011). The Langmuir adsorption isotherm (Chinedu et al., 2012) is specified by **Equation 2.**

$$\frac{1}{q_e} = \frac{1}{q_{max}} + \frac{1}{b\, q_{max} C_e} \qquad (2)$$

q_e is the quantity of biosorbent adsorbed at equilibrium (mg g^{-1}) onto the biomass of biosorbent), q_{max} is a constant that relates to the maximum amount of biosorbent that can be adsorbed by the biomass of algae forming a monolayer (mg g^{-1}), b is the Langmuir constant (l/mg) for binding adsorbate on the sorbent sites, and C_e is equilibrium biosorbent concentration in medium following the biosorption process (mg l^{-1}).

Freundlich isotherm is used to assess the biosorption processes that take place on heterogeneous surfaces. The surface heterogeneity, active sites, and their energies are defined using this isotherm model (Ozturk, 2007). The Freundlich adsorption isotherm (Chinedu et al., 2012) can be presented as:

$$In\, q_e = InK_F + \left(\frac{1}{n}\right) In\, C_e \qquad (3)$$

Where q_e is the quantity of sorbate adsorbed on the sorbent, K_F denotes the Freundlich isotherm constant that measures the capacity of biosorption (a linear plot of ln q_e vs. ln C_e), Concentration of sorbate (mg l^{-1}) at equilibrium is C_e, and n is a

constant that links the effectiveness and energy of adsorption. The biosorption capability of the biosorbent that is binding to the sorbate raises the value of K_F. The value of 1/n is an important ratio for determining the suitability of adsorption.

Temkin isotherm states that increasing surface coverage reduces the adsorption heat for all molecules in the layer. This model is only applicable for moderate concentration ranges. The equation of this isotherm (Areco and Dos 2010) is as follows:

$$q_e = B\ (K_t C_e) \qquad (4)$$

The interaction between adsorbate molecules with sorbent sites may impact the adsorption properties. Temkin adsorption is define as the concentration of sorbate molecules adsorbed on the sorbent rises, the adsorption heat of all molecules in the adsorption layer decreases linearly, suggests that the adsorbate and adsorbent may interact mutually (Areco and Dos 2010).

3.2.5 Desorption of Cd and Pb Ions from Metal Loaded Adsorbent

To effectively desorb the metal ions and restore the adsorbent for reuse, the reusability of the adsorbent was assessed by using the following techniques. To conduct the consecutive experiment of adsorption and desorption, the repetition of cycles (3 cycles) was done using the mentioned above preparations. Desorption studies were conducted by mixing loaded sorbent (metal) with desorption medium (0.1 M HNO_3, 0.1 M HCl, 0.1 M H_2SO_4) and were agitated at 150 rpm for 60 min. The amount of metal ions was determined by AAS (Tuzun et al., 2005). The percentage of desorption efficiency was calculated by using **Equation (5)** (Ezeonuegbu et al., 2021).

$$\text{Desorption efficiency } \% = \frac{\text{Amount of metal desorbed}}{\text{Amount of metal adsorbed}} \times 100 \qquad (5)$$

3.26 Characterization

The treated and untreated biomass of *Scenedesmus* sp. were examined with a PerkinElmer Spectrum 65 FTIR within the wave number 4000–500 cm^{-1} under ambient conditions. The evaluation involved nine samples (R3 control, R3 Cd, R3 Pb, H9 control, H9 Cd, H9 Pb) of R3 and H9 species. This method was used to find the functional groups relevant to the biosorption of metal ions by the biosorbent. Scanning electron microscopy (JSM5910, JEOL Japan) was employed to examine the morphology before and after exposure to Cd and Pb.

3.3 Results and Discussion

3.3.1 Effect of pH on Metal Adsorption

pH is considered a main factor that influences the process adsorption of metal ions in mixed solutions (Gaur and Dhankhar, 2009). The pH of mixture impacts the binding sites that exist on the algal surface and the chemistry of the adsorbate present in the medium, as depicted in **Fig. 3.3** (Arief et al., 2008). Increased adsorption of Cd and Pb was seen at pH 6.0 and 5.0 for *Desmodesmus* sp. respectively. The uptake capacity of the *Desmodesmus* sp. increased rapidly from 2 to 6 for Cd and 2 to 5 for Pb. **Fig. 3.3A** clearly shows an increase in the biosorption process (62 and 65 mg/g adsorption capacity) as the pH was increased and leveled off at pH 6.0 and 5.0 for Cd and Pb in the case of *Desmodesmus* sp. (Tuzun et al., 2005). Biosorption capacity of Cd and Pb was low at initial (2-4) pH for *Scenedesmus* sp. but significantly increased when the pH of the solution increased to 7 (in the case of Cd) and 6 (in the case of Pb) with the adsorption capacity of 80 and 82 mg/g **(Fig 3.3B)**. On further increase in pH, the biosorption capacity remained unchanged (Dirbaz and Roosta, 2018). However, the optimum pH value for maximum metals uptake for both strains differed. As a result, all further experiments were performed at respective optimum pH for both strains. The observation mentioned above could be described by the fact that at lower pH the hydrogen ions and metal ions compete to form ligands at the surface of the sorbent (because of the charge repulsion) (Arief et al., 2008). Normally, an increase in pH value increases the sorption of metal ions because the proton concentration decreases and the algal surface gets negatively charged, maximizing the biosorption of metal ions (Abdel-Aty et al., 2013). The maximum adsorption efficiency of metals occurred at different pH due to the different characteristics of the metals, e.g., electronegativity and the availability of metal ions that were better adsorbed on the binding sites of the sorbent (Chen et al., 2008).

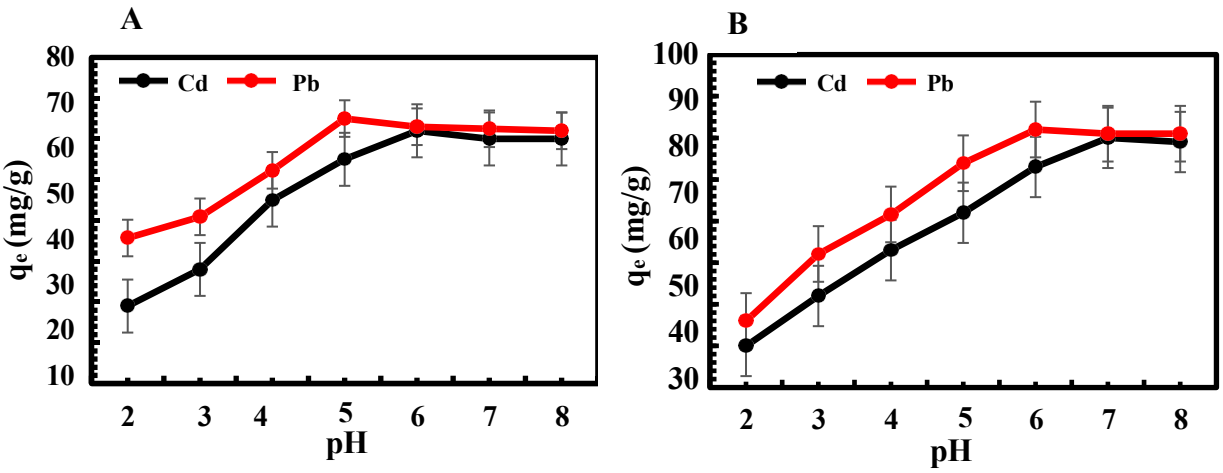


Figure 3.3. Influence of solution pH on biosorption of metal ions on the dried biomass of (A) *Desmodesmus* sp. and (B) *Scenedesmus* sp. Each point is the mean of three data and vertical bars denote standard error within triplicate.

3.3.2 Effect of contact time on biosorption of metals ions

The effect of contact time greatly influences the adsorption process (Abdel-Aty et al., 2013). **Fig. 3.4** depicts the effect of contact time on the adsorption of Cd and Pb onto the dried biomass of algal strains. Results revealed that the biosorption of metals attains equilibrium approximately after 60 minutes of contact with the adsorption efficiency of 58 (Cd) and 60 mg/g (Pb) for *Desmodesmus* sp. Similar outcomes have been noted for the green algal biomass of *Chlorella vulgaris* (Kumar et al., 2018). 60 and 90 minutes for the Cd and Pb ions were the optimum contact time in the biosorption of *Scenedesmus* sp. Furthermore, in the first 20 minutes, the reaction rate was fast, gradually decreasing and finally leveling off with no significant changes beyond 60 and 90 minutes (Abdel-Aty et al., 2013). Previously, 60 minutes has also been reported as an optimum contact time for the adsorption of cadmium (Abdel-Aty et al., 2013) and lead (Mirghaffari et al., 2015) by the dried biomass of blue-green and green algae.

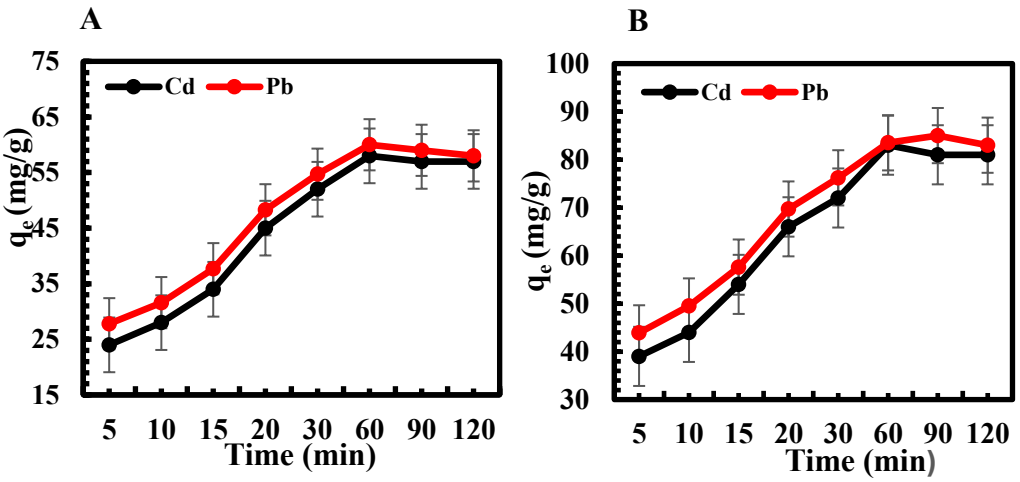


Fig. 3.4 Effect of contact time on the adsorption of metal ions by (A) *Desmodesmus* sp. and (B) *Scenedesmus* sp. Each point is the mean of three data and vertical bars denote standard error within triplicate.

3.3.3 Effect of initial concentration of Cd and Pb on Metal Adsorption

The impact of initial concentration of metal ions on the biosorption capacities of Cd and Pb in the presence of dried biomass of *Desmodesmus* sp. is depicted in **Fig. 3.5A**. It was noted that the biosorption of Cd and Pb ions increased with an increase in the initial concentration of metal ions and then it attains an equilibrium. Consequently, no significant changes were observed because of the saturation of binding sites with the metal ions on the surface of the biosorbent (Sun et al., 2012). The maximum adsorption capacity of metal ions (61 and 64 mg/g for Cd and Pb) on the dried biomass of *Desmodesmus* sp. was obtained approximately at an initial metal ions concentration of 100 mg/L (Katircioglu et al., 2008). The effective adsorption of cadmium and lead by dry biomass of *Scenedesmus* sp. increases with increasing concentration of Cd and Pb with the adsorption capacity of 78 and 83.2mg g^{-1} and attains equilibrium as shown in Figure **(3.5B)** (Kumar et al., 2018). An increase in initial Cd ion concentration decreases the mass transfer resistance between the sorbate and sorbent. This, consequently, assisted the approaching adsorbate to active sites, thus improving the capacity to adsorb. At that point, the saturation of binding sites is achieved in the stipulated time with no further adsorption.

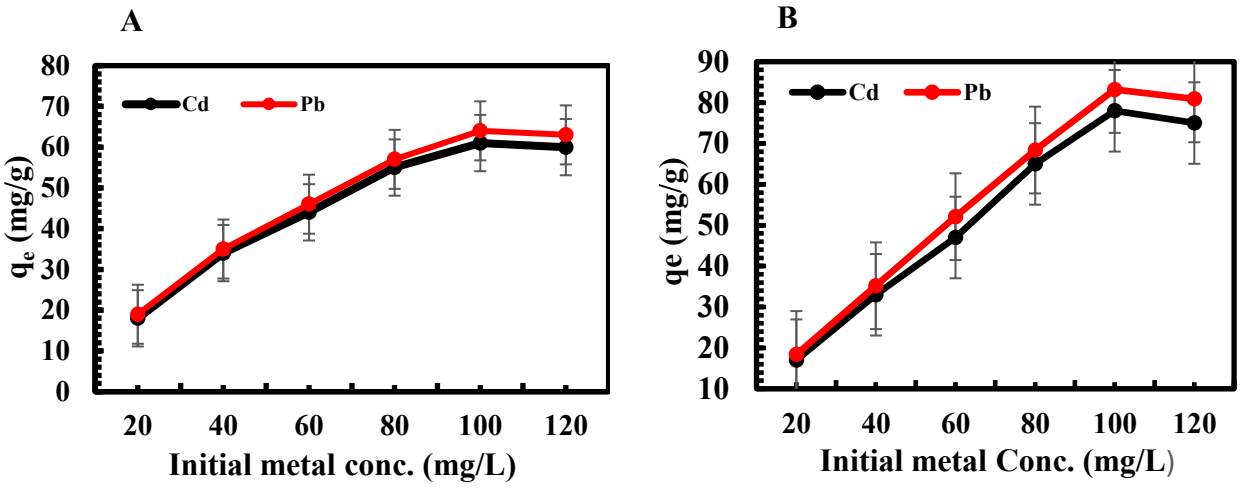


Figure 3.5. Effect of initial metal conc. on adsorption of metal ions on the dried biomass of **(A)** *Desmodesmus* sp. and **(B)** *Scenedesmus* sp. Each point is the mean of three data and vertical bars denote standard error within triplicate.

3.3.4 Adsorption kinetics

The determination coefficients (R^2) for pseudo 1st order and intra particle diffusion model were found significantly less for Cd and Pb in the biosorption of two algal strains as shown in **Table 3.2** (Areco and Dos 2010). The experimental and calculated values of qe **(Table 3.2)** did not closely match with each other (Mirghaffari et al., 2015). It is also depicted from the R^2 values, pointing to the fact that the biosorption of these metals onto *Desmodesmus* sp. and *Scenedesmus* sp. did not follow pseudo 1st order and intra particle diffusion model (Oliveira, et al., 2008). The R^2 (determination coefficient) values of pseudo 2nd order was close to unity (0.9952 (Cd); 0.9956 (Pb) in case of *Desmodesmus* sp. and 0.997 (Cd); 0.998 (Pb) for *Scenedesmus* sp. (Mirghaffari et al., 2015). The calculated values of q_e for *Desmodesmus* sp. are 62.5 (Cd) and 62.8 mg/g (Pb) showing agreement with q_e exp (experimental values) in pseudo 2nd order kinetics. A similar trend was found for *Scenedesmus* sp. i.e. 83 and 85 mg/g for Cd and Pb **(Table 3.2).**

Table 3.2: A comparison among rate constants of different kinetics models.

Strains	Metal	Pseudo 1st order			Pseudo 2nd order			Intraparticle diffusion		
		K_1	q_e	R^2	K_2	q_e	R^2	B	R^2	$q_{e\ exp}$
***Desmodesmus* sp.**	**Cd**	-0.0345	27.51	0.831	27297	62.5	0.995	3.9	0.783	58
	Pb	-0.0294	22.8	0.713	36090	62.8	0.995	3.6	0.755	60
***Scenedesmus* sp.**	**Cd**	-0.0308	3.39	0.742	91980	86.9	0.997	4.6	0.798	83
	Pb	-0.0317	3.19	0.789	11525	88.4	0.998	4.9	0.797	85

The collected data revealed that the adsorption of Cd and Pb on the dried biomass of *Desmodesmus* sp. follows the pseudo 2nd order of kinetics (Bulgariu and Bulgariu, 2012; Kaleem et al., 2023). It could be presumed from the results mentioned above that the metal-strains biosorption mechanism was likely controlled by the chemosorption process involving covalent bonds or ion exchange between the sorbent and sorbate until all active sites were engaged. Similar findings were described by (Gupta and Rastogi 2008) in the material deployed to eliminate pollutants. The intraparticle diffusion model helped to define the mechanism of adsorption. The value of R^2 coefficient values for Cd and Pb in both strains were not close to unity (Kumar et al., 2018), and the trend line was not observed to pass through the origin, which clearly indicated that this model is not supported by the data (Freundlich, 1907; Mane et., 2007). The kinetics models are presented in **Fig 3.6.**

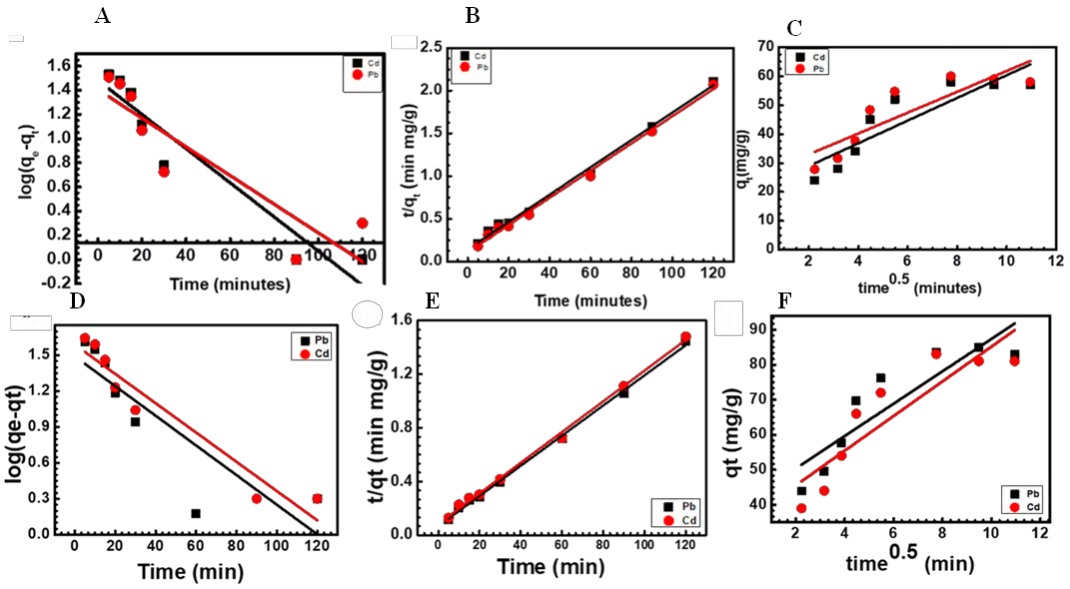


Figure 3.6. (A and D) Pseudo 1st order, (B and E) pseudo 2nd order and (C and F) intra particle kinetics models for Cd and Pb adsorption on the dried biomass of *Desmodesmus subspicatus* and *Scenedesmus dimorphus*.

3.3.5 Adsorption isotherms

The parameters of the adsorption isotherm are displayed in **Table 3.3** explain the biosorption of metal ions is followed by the Langmuir sorption isotherm due to higher R^2 values of cadmium and lead when compared to other isotherms (Khan et al., 2016). The value of q_{max} calculated by Langmuir isotherm was observed at 64.1 (Cd), 62.5 mg/g (Pb) and 128 (Cd) and 102 (Pb) for *Desmodesmus subspicatus* and *Scenedesmus dimorphus*. respectively, as shown in **Table 3.3** (Mirghaffari et al., 2015). The Langmuir model indicates the homogeneous surface of the biosorbent and the monolayer adsorption of positive ions on the surface of microalgae. The 1 / n values **(Table 3.3)** obtained were 0.361; 0.623 (Cd) and 0.286; 0.487 (Pb) for both the algal strains respectively, which ranged between 0 and 1, indicating Cd and Pb adsorption is favorable (Lokeshwar and Joshi, 2009). Therefore, this investigation demonstrates that the Langmuir model is well fitted to the observed data as manifested through various isotherms (Waqar et al., 2023). The plots of different isotherms are shown in **Fig. 3.7**.

Table 3.3. Adsorption isotherm parameters for the biosorption of metal ions using biomass of *Desmodesmus* and *Scenedesmus* sp.

Strains	Metal	Langmuir Constants			Freundlich Constants			Temkin Constants	
		1/b	q_{max}	R^2	K_F	1/n	R^2	K_{id}	R^2
***Desmodesmus* sp**	**Cd**	5.153	64.1	0.994	572.1	0.361	0.947	13.20	0.974
	Pb	2.275	62.5	0.986	1250.5	0.286	0.925	11.00	0.962
***Scenedesmus* sp**	**Cd**	9.826	128	0.990	186.6	0.623	0.917	26.06	0.911
	Pb	7.708	102	0.990	774.9	0.487	0.902	22.09	0.936

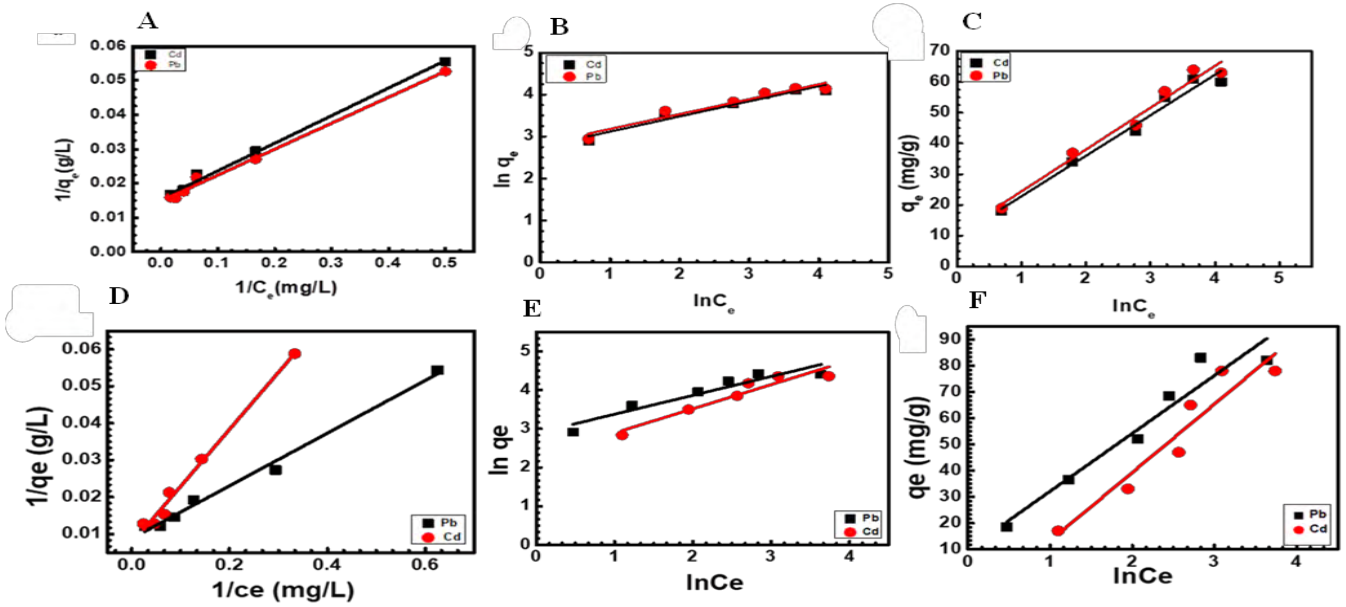


Figure 3.7. (A and D) Langmuir, (B and E) Freundlich, and (C and F) Temkin model for Cd and Pb sorption on the dried biomass of *Desmodesmus* and *Scenedesmus* sp.

In addition, **Table 3.4** compares the biosorption capacity of *Desmodesmus subspicatus* and *Scenedesmus dimorphus* for Cd and Pb with that of other biosorbents. *Desmodesmus* and *Scenedesmus* sp. have a better biosorption capacity for these metal ions than other adsorbents as shown in **Table 3.4.** Therefore, it is noteworthy that both strains have the high potential for removing metal ions from an aqueous solution.

Table 3.4: A comparison of adsorption capacity between different sorbents and *Desmodesmus subspicatus* and *Scenedesmus dimorphus.*

Biosorbent	**Biosorption capacity (mg/g)**		
	Cd	**Pb**	**References**
U. lactuca	29.2	34.7	(Sarı and Tuzen, 2008)
Rhizopus arrhizus	27.0	56.0	(Fourest and Roux, 1992)
Chlorella minutissima	11.1	9.74	(Roy et al., 1993)
Caulerpa lentillifera	4.7	28.7	(Pavasant et al., 2006)
Mucor rouxii	8.5	35.7	(Yan and Viraraghavan, 2003)
Desmodesmus sp.	64.1	62.5	Current study
Scenedesmus sp.	128	102	Current study

3.3.6 Desorption Studies

To demonstrate the reusability of the sorbent, the desorption of Cd and Pb ions (adsorbed) from the sorbent was examined using different desorption solutions. Results show that HNO_3 and HCl are effective agents for desorption as shown in **Fig. 3.8.** But HNO_3 damages the adsorbent at high concentration (Deng et al., 2008). The desorption of both metals by *Desmodesmus subspicatus* and *Scenedesmus dimorphus* was more than 75% (Katircioglu et al., 2008; Mirghaffari et al., 2015). Under acidic conditions, the metal desorption caused protonation of the biosorbent surface, allowing the desorption of ions (positively charged) from the biosorbent. Some recently performed experiments reported HCl as a desorbing agent (Katircioglu et al., 2008; Kumar et al., 2018). In addition, an effective prospective sorbent to eliminate heavy metal ions must have good adsorption and recovery ability of metal ions (Mondal et al., 2017).

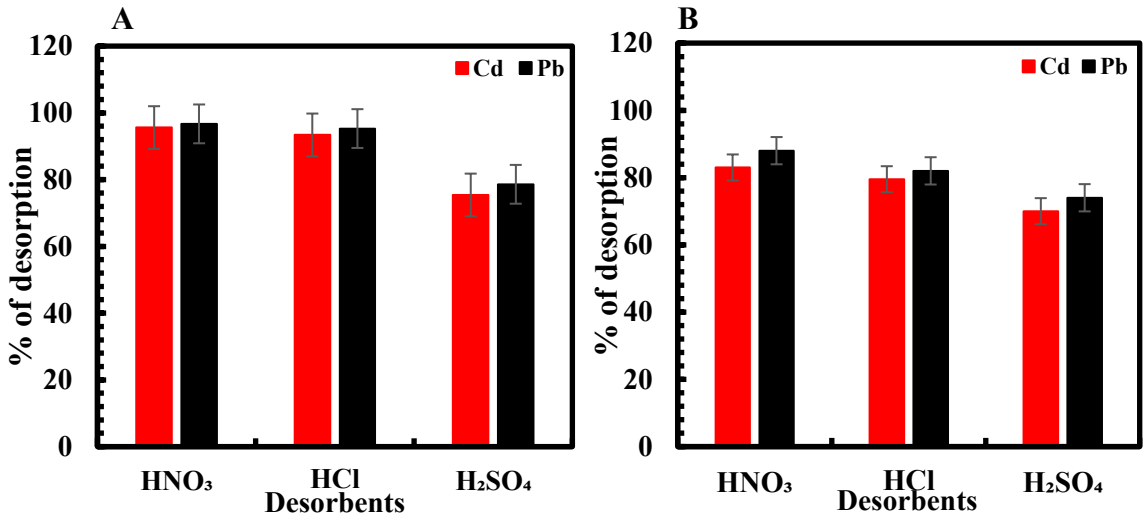


Fig. 3.8. Percentage of desorption of (A) *Desmodesmus subspicatus* and (B) *Scenedesmus dimorphus*. by using different desorbents.

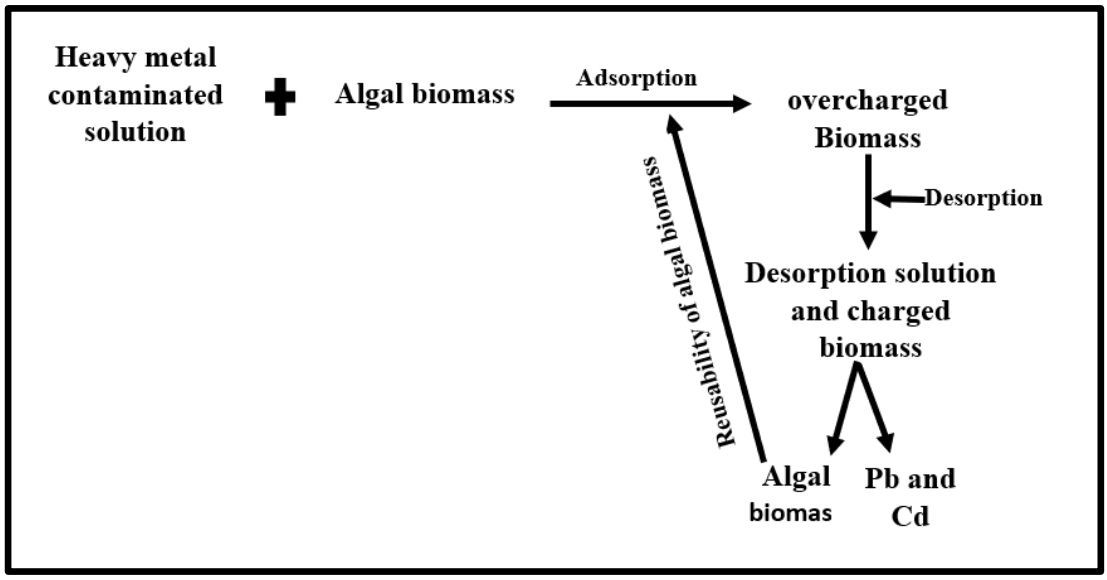


Fig. 3.9. Strategy for desorption and regeneration and reusability of algal sorbent.

3.3.7 Adsorption mechanism

A comparative analysis of FTIR spectra in the case of pre-adsorption and post-adsorption samples revealed a possible mode of adsorption mechanism for two strains (R3 and H9). **Fig. 3.10 (A)** depicts the functional groups that are involved in the process of adsorption of *Desmodesmus* sp. The control **Fig. 3.10 (A)** represents the peak (black) at 3306 $cm^{-1,}$ demonstrating the presence of N-H or O-H functional groups (Pan et al., 2007). The C-H stretching vibrations are in the 2932-2842 cm^{-1} (Mungasavalli et al., 2007). The C=O stretching vibration band appears at 1754 cm^{-1} for carbonyl functional groups (Rao et al., 2007). The absorption band of C=O of amide linkage appeared in the range of 1646 and 1539 cm^{-1} (Mungasavalli et al., 2007). The absorption band located near 1243 cm^{-1} corresponds to C-N functionality (Rao et al., 2007) and the band at 1012 cm^{-1} indicates the existence of C-O stretching band (Baral et al., 2007). The FTIR spectrum shows metal ions bound to the functional groups of sorbents, and many changes were noted (red and blue peaks) from the original spectrum (black peak, **(Fig. 3.10A).** The N-H /O-H band is shifted from 3306 cm^{-1} to 3189 cm^{-1}(Cd) and 3277cm^{-1}(Pb) which depicts the interaction of metal ions with amide and hydroxyl groups (Sun et al., 2019). The band's disappearance at 1754 cm^{-1} indicates metal binding to the carbonyl group in both metals. The complex band of amide C-N is shifted from 1243 to 1290 cm^{-1} in Cd. Similarly, the C-O stretching band shifts from 1012 cm^{-1} to 1042 cm^{-1} and 1059 cm^{-1} for Cd and Pb, respectively.

The FTIR spectra of raw *Scenedesmus* sp. **Figure 3.10B** revealed an absorbance peak (black) at 3184 cm^{-1}, which corresponded to N-H stretching vibration. Alcoholic O-H stretching vibration was attributed to the peak at 2935 cm^{-1} and N-H binding vibration to the peak at 1625 cm^{-1}. The FTIR spectra demonstrated that the surface of *Scenedesmus* sp. was covered with hydroxyl and amines (Ferreira et al., 2011). The functional groups associated with metal sorption were determined by comparing the spectra of raw (black peak) and metal loaded *Scenedesmus* sp. (red and blue peaks). In the metal-loaded spectrum (red and blue peaks) displayed in **Figure 3.10B,** the relative intensity of the N-H bands decreased, indicating that Pb and Cd interacted with amine. Furthermore, the O-H absorption band at 2935 $cm^{-1}$was discovered to shift at 2944 (Pb) and 2918 cm^{-1} (Cd). Similarly, the absorption band shifted from 1625 cm^{-1} to 1629 (Pb) and 1627 cm^{-1} (Cd). This result can be due to Pb and Cd's preferred interaction with amine groups (Sheng et al., 2004). As a result of these findings, the hydroxyl and amine groups on the surface of *Scenedesmus* sp. were found to be involved in the biosorption process. The results support a sufficient efficiency of metal surface binding capability. The spectrums explain the existence of main functional groups like carbonyl, hydroxyl, amide, and amines primarily involved in metal adsorption. Previous research using microalgae has validated these findings (Sun et al., 2019).

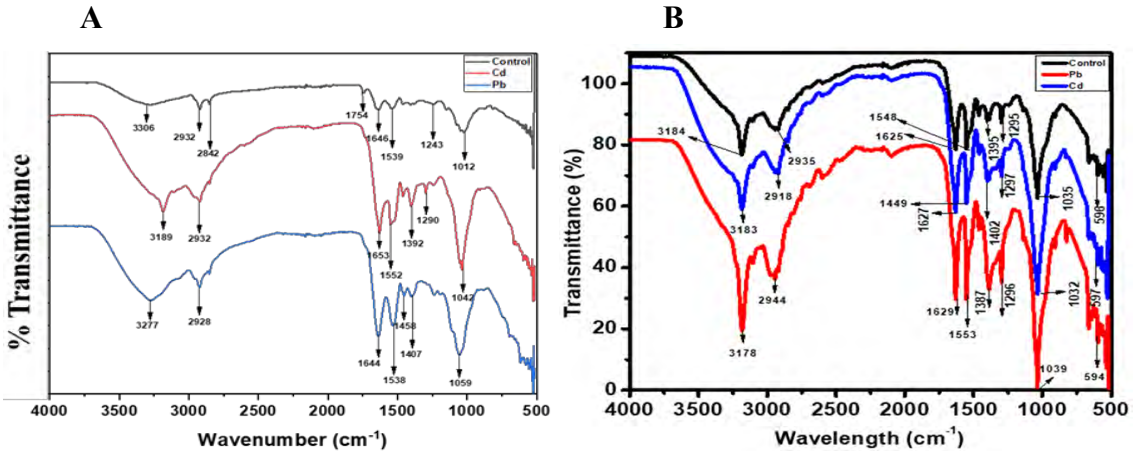


Fig.3.10. FTIR spectra of unloaded and loaded curves with Cd and Pb of (A) *Desmodesmus subspicatus* and (B) *Scenedesmus dimorphus*.

Scanning electron microscopy (SEM) of the raw biomass of *Desmodesmus subspicatus* and *Scenedesmus* sp. **(Fig. 3.11A-F)** revealed the porous nature of cell surface with a transparent layer on the outer surface of the cell wall (El-Sheekh et al.,

2019). The porous surface of the cell wall increases the surface area of the sorbent and improves the biosorption of metal ions (Kumar et al., 2018). The mono- graphs of *Desmodesmus subspicatus* **(Fig. 3.11A, B, C)** demonstrate that the algae-based biosorbents reduce the surface porosity when brought in contact with metal ions, forming a flat structure (Mirghaffari et al., 2015). In the monograph of *Scenedesmus* sp. **(Fig. 3.11D, E, F)** the cells were smooth and had specific dimensions before exposure **(Fig. 3.11D);** however, after the adsorption of metal ions, they were damaged and enlarged **(Fig. 3.11E and F)**. This could be because of precipitating of Pb and Cd ions on the cell surface and becoming attached to the functional groups (Ghoneim et al., 2014).

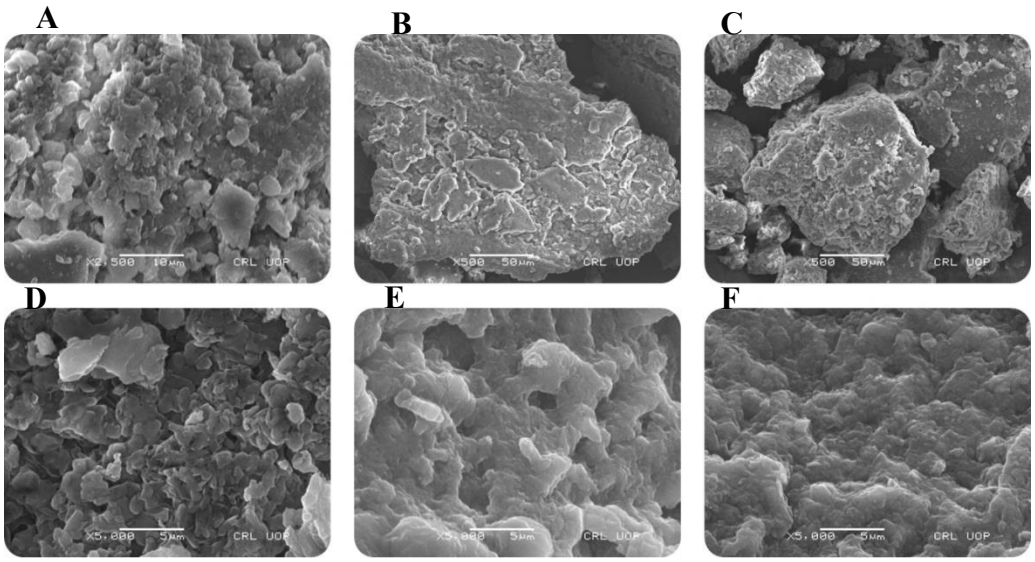


Fig. 3.11. SEM micrograph of *Desmodesmus subspicatus* and *Scenedesmus* sp. **(A and D)** virgin biomass **(B and E)** after Cd biosorption, and **(C and F)** after Pb biosorption.

Results of FTIR further revealed the morphological changes that have occurred due to the interlinking of Cd and Pb and the functional groups present on the algal cell wall (Arief et al., 2008). Possible mechanisms for the biosorption of metal ions from the aqueous system are shown in **Fig. 3.12.**

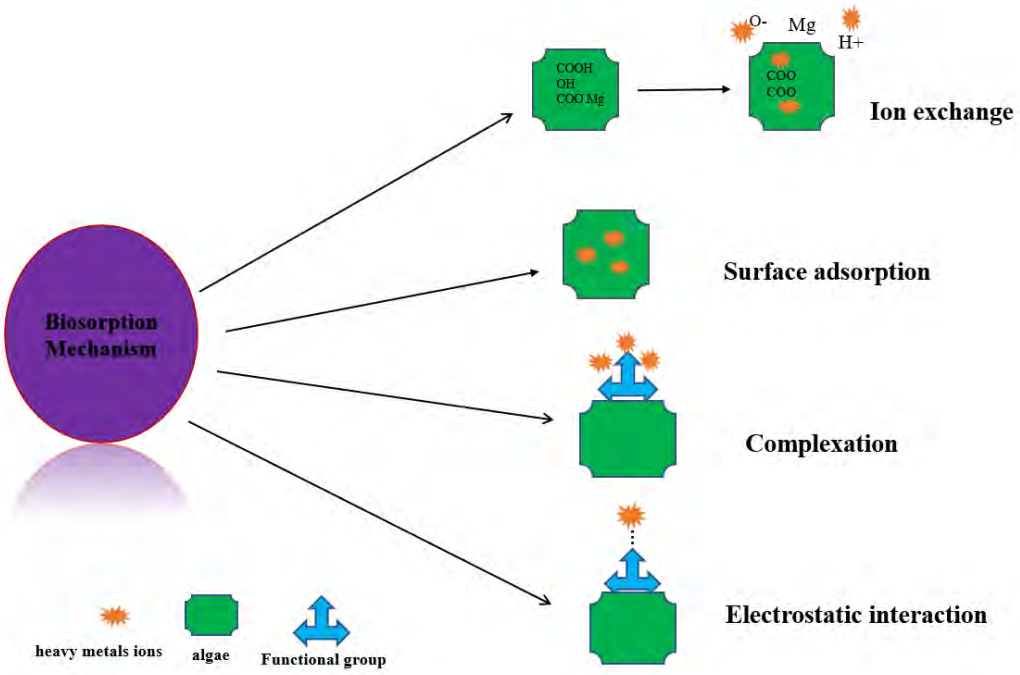


Figure 3.12. Different mechanisms in the biosorption process. Metal ions adsorb to the surface of algae in surface adsorption. Ion exchange occurs between adsorbent (algae) and adsorbate when the light metal ions, such as magnesium, already attached to a functional group, are released, followed by heavy metal ion adsorption onto algae Different functional group on algal cell wall plays a main role in binding of metal ions in complexation and electrical attraction (through van der Waals interaction).

Conclusion

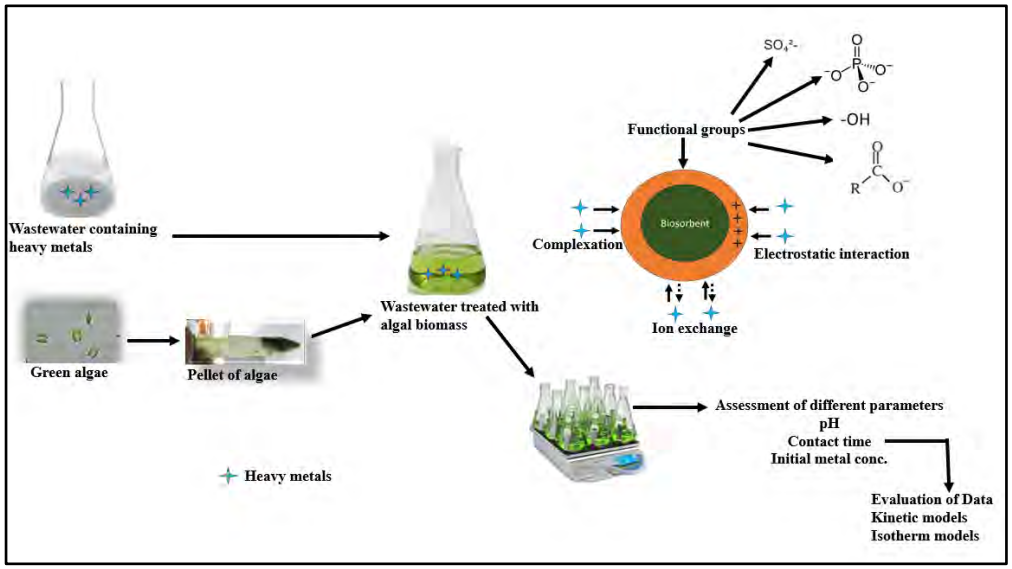
- Experimental evidence suggests that *Desmodesmus subspicatus* and *Scenedesmus dimorphus* can be used as an efficient sorbent for the removal of metal ions.
- Biosorption capacity of Cd (62 mg/g); Pb (65 mg/g) for *Desmodesmus subspicatus* and 80 (Cd mg/g); 82 (Pb mg/g) for *Scenedesmus* sp. at optimized conditions. The biosorption process correlated with the pseudo 2nd order kinetiscs model and adsorption equilibrium data showed the best fit with the Langmuir model. The value of the q_{max} of dried biomass of *Desmodesmus* sp. for Cd and Pb was 64.1 and 62.5 mg/g it was 128 mg/g (Cd) and 102 mg/g (Pb) for *Scenedesmus* sp.
- Desorption of metal ions from the biosorbents was more than 75 % by using hydrochloric acid. FTIR data showed the role of carbonyl, hydroxyl, amine, and amide groups present on the surface of the dried biomass of *Desmodesmus* and *Scenedesmus* sp. in the adsorption process.

- SEM data showed morphological changes in the treated and untreated dried biomasses of two algal strains which revealed the mechanism of efficient adsorption for the elimination of metal ions from wastewater.

CHAPTER 4

Bioremediation capacity of living *Desmodesmus subspicatus* and *Scenedesmus dimorphus*: for the adsorption of Cd and Pb from wastewater. Kinetics and Isothermal analysis

Graphical abstract



4. Abstract

Green algae are promising adsorbents used in the removal of heavy metals from aqueous solutions. In the present work, two algal strains were studied in terms of the physiological reactions to high cadmium (Cd) and lead (Pb) concentrations. In addition, the Cd and Pb binding capability and mechanisms behind metal biosorption by two algal strains are discussed. Chemisorption with a high initial sorption rate is used in the ion binding process. In the present study, *Desmodesmus subspicatus* and *Scenedesmus dimorphus*. have significant cadmium adsorption capacity and are suitable for industrial-scale applications. The adsorption behavior and mechanism of Cd and Pb ions were examined. Batch biosorption tests revealed that pH 7.0 and 6.0, 90-minute contact time, and initial ion concentration of 100 and 120 mg L^{-1} were the best adsorption conditions for Cd and Pb in the case of *Desmodesmus* sp. For *Scenedesmus* sp. the best adsorption capacity was at pH 6, contact time of 120 minutes and 120mg L^{-1} was the initial metal concentration of Cd and Pb ions. The adsorption process for both strains corresponded closely to pseudo-second-order kinetics, which were mostly based on chemisorption for both strains. Langmuir isotherms accurately represented the biosorption process and maximum biosorption capacities of 83.3 (Cd) and 71.4 mg/g (Pb) for *Desmodesmus*

subspicatus; 212 (Cd) and 232 mg/g (Pb) for *Scenedesmus* sp. respectively. FTIR was used to establish that cell wall components comprising amine groups played a crucial role in the adsorption of Cd and Pb ions. Hence, *Desmodesmus* sp. (R3) and *Scenedesmus* sp. (H9) have significant Cd and Pb adsorption capacity and are suitable candidate for industrial scale applications.

4.1 Introduction

Heavy metal contamination is a severe hazard to the ecosystem. Because of their widespread usage in agriculture, industry, and the home, Cd and Pb are two of the most serious water contaminants. Pb is used to make metal items (such as pipes), batteries, paint, and pigments (Ebrahimi et al., 2020). On the other hand, Cd is used to make paint, batteries, copper alloy, zinc refining and fertilizer (Ali et al., 2019) These metals are mostly found in industrial effluents (untreated), where they affect soil and aquatic environments. Cd and Pb enter the food chain from the environment and bioaccumulate in living organisms. (Banik et al., 2018; Chandrashekharaiah et al., 2021). Heavy metals cause cancer and are highly poisonous to cells; they disrupt cellular enzymes and function and produce a variety of health problems in humans. Metal ion cleanup by any means is desperately needed in the current study due to substantial health risks and detrimental effects on the ecosystem. Heavy metal removal from contaminated water streams and many physicochemical processes, such as filtration, solvent extraction, precipitation, reduction, and chemical oxidation, are used. Traditional technologies are hindered by high operating and maintenance expenses, high energy needs, unpredictability in removal, and enormous amounts of secondary sludge produced. Microbial remediation, which uses bacteria, fungi, algae, and other microorganisms, is an alternative strategy that is renewable, sustainable, and environmentally beneficial (Chandra et al., 2020).

Removal of metal ions by microorganisms can occur via both metabolically dependent and metabolically independent processes. Metals can be detoxified and accumulate inside metabolically active living cells (Chandra et al., 2020). In recent years, one such unique and green strategy has been the use of biosorption and bioaccumulation mechanisms of microbial agents for heavy metal removal (Banik et al., 2018). The removal of heavy metals, on the other hand, has been completely employed in recent years. Algae can remove heavy metals from contaminated water via biosorption and bioaccumulation. Biosorption is defined as a passive process in which cations bind to the surface (functional group) of a biosorbent (Davis et al., 2003). Metal ions are

collected inside the cell with the help of active transporters by living adsorbents in a process known as bioaccumulation (Mrvcic et al., 2012). The process of bioaccumulation is complex and irreversible. As a technique for neutralizing and collecting additional metals, bioaccumulating strains generate substances rich in thiol groups. Metal-tolerant strains are good for the adsorption and bioaccumulation of metal ions (Deng and Wilson 2001). Algae are becoming more popular because of their functional groups on their cell wall, larger surface area per volume, better metal removal capacity, and adsorbed metal regeneration, recovery, and recycling. As a result, the algal bioremediation strategy is regarded as both cost-effective and environmentally friendly (Pahlavanzadeh et al., 2010).

Furthermore, different functional groups (carboxyl, amine, hydroxyl, acetyl, phosphate) found in microbe cell walls interact with cations and remove metal ions via biosorption (Fashola et al., 2016). Live bacteria are better than dead microbes for heavy metal bioremediation because they can remove metals through adsorption and bioaccumulation (Filote et al., 2021). Thus, bioremediation by living microbes is closely tied to microorganism development. Our prior research indicated that organisms need to tolerate a reasonable level of harmful heavy metals for efficient bioremediation. Higher Pb and Cd concentrations were reported to impact photosynthesis and growth in green algae (Purushanahalli et al., 2021). In bacteria, algae, and fungi, higher concentrations of Cd and Pb were found in nucleic acids, transcription factors, and proteins (Fashola et al., 2016). During the process of bioremediation, organisms should be exposed to optimal pH, nutritional conditions, and temperature for their growth (Asira, 2013).

Many researchers have examined heavy metal bioremediation employing various dried biomasses of algae as adsorbents (Zabochnicka-Swiątek and Rygala 2017). In contrast, bio removal by living algal cultures is dependent on culture conditions, culture growth, and metal concentration toxicity. *Desmodesmus* and *Scenedesmus* sp. are fresh water green algae with enormous potential for the treatment of pollution. To accurately estimate the bioremediation capacity of a metal, it is necessary to define the algal cultures while simultaneously evaluating the bio-removal and biomass production in synthetic solution. Cd is a highly hazardous metal at extremely low concentrations when compared to other metals. Before the biosorption study of Cd and Pb ions in synthetic water, it is vital to understand the reaction of algae to varying metal ion concentrations, growth parameters, and bio removal capacities. The

objectives of the present study were to assess the tolerance of Cd and Pb stress on *Desmodesmus* and *Scenedesmus* sp. to record an impact on the development and photosynthetic efficiency of these strains and to understand the possible mechanisms adopted by these strains to mitigate any possible toxicity incurred.

4.2 Materials and Methods

4.2.1 Cultivation of algal strains

The algal strains were isolated from the stream of Hajira Poonch AJK. The samples were cultured by the method described in previous literature (Piotrowska-Niczyporuk et al., 2012). The cells of two algal strains (*Desmodesmus subspicatus* and *Scenedesmus dimorphus*) were cultivated in conical flasks with modified BBM medium. To avoid contamination, all instruments and media were sterilized at 12°C for 45 minutes prior to the experiment. To avoid agglomeration, all flasks were shaken manually twice daily during the cultivation period. Furthermore, according to a prior study, the position of the flasks was varied at random to reduce any possible effect of light intensity (Shen et al., 2021).

4.2.1.1 Culture medium

BBM was used for culturing *Desmodesmus* and *Scenedesmus* sp. The BBM recipe is presented in the appendices section. Before use, the media was autoclaved at 121°C for 45 minutes at 15 psi.

4.2.1.2 Cadmium and lead solution preparation

In order to make the Cd and Pb solutions, 1000 ppm mother liquor was made by combining cadmium chloride and lead acetate with sterile water. The sterilization of Cd and lead solutions was accomplished by filtering via 0.22 mm pore size filter (Mirghaffari et al., 2015).

4.2.1.3 Effect of Cd and Pb on the growth of algae

Three biological replicates of the test cultures (*Desmodesmus* and *Scenedesmus* sp.) were grown in 250 mL flasks. Each flask received an adequate volume of Cd and Pb solution from the stock to obtain the final target concentrations of cadmium chloride (5, 10, 15, 20, 30, 40and 50 ppm) (Qader and Shekha 2023) and lead acetate (15, 30, 60, 90, 120, and 180 ppm) (Abd El-Hameed et al., 2018). As a control (0 ppm), algae cultivated in conditions free of heavy metals were employed. Respective green cultures were put into each flask to maintain at 0.05 OD and incubated for 12 days under conventional culture conditions. Daily monitoring of OD @ 750 nm in cultures was used to determine algal growth. Chlorophyll and carotenoid were measured by

using the equation described in previous literature (Shen et al., 2021).

4.2.1.4 Light microscopy

To investigate the effects of Cd and Pb, the morphology of treated cells (15 ppm, 50 ppm Cd and 90, 180 ppm Pb), and those in control sample were investigated under a light microscope (Leitz Wetzler, Germany). The observations were made with a 10x eye piece and 40x and 100x (with emersion oil) objectives.

4.2.1.5 Determination of photosynthetic content

For the assay of photosynthetic pigments, 1 ml of cell suspension was centrifuged at 15000 × g for 7 minutes, the supernatant was discarded, and precooled (at -20°C) 1 ml methanol was added to the pellet (Zavrel et al., 2015). The mixture of pellet and methanol was then incubated in the dark for 60 minutes or longer at 4°C until the pellet turned white. The absorbance of the supernatant was measured at 470, 665, and 720 nm (Wellburn, 1994). The quantities of 'chlorophyll a' and carotenoids were determined using the formulas described in previous literature (Ritchie, 2006).

4.2.1.6 Determination of dry weight

The measurement of dry algal biomass was carried out in the same manner as described previously (Sorokin, 1973), with slight modifications. The flasks containing the algae culture were agitated, and representative aliquots of algae suspension were obtained. The algal cells were centrifuged (4000 rpm for 10 minutes) to separate from the culture medium. The cell deposit is then resuspended in distilled water and centrifuged again. Cells were transferred into a glass dish after being cleaned once in distilled water. Then, the cells were dried in an oven at 65-70°C. The weight of the biomass was measured after the dish had cooled to room temperature. The dry weight of cells was calculated for each replicate by subtracting the weight of dried biomass from the weight of the dish and is represented by per unit volume.

4.2.2 Assessment of Cd and Pb biosorption

4.2.2.1 Preparation of biosorbent

All the studies were carried out with a 15-day-old algal culture. The culture media was withdrawn from the algal cultures after they were centrifuged at 10,000 × g for 5 minutes. This procedure was performed two to three times, and a cell pellet weighing 1 g was obtained. Finally, the pellet was rinsed with sterile deionized water to remove the impurities (Liyanage et al., 2020).

4.2.2.2 Batch analysis to optimize the experimental parameters in Cd and Pb biosorption Optimization of experimental parameters

Different parameters, such as solution pH, contact time and initial concentration of metal ions, influence Cd and Pb sorption in aqueous media. The influence of pH on Cd and Pb adsorption was examined using different pH regimes (2.0 to 8.0). Incubation for various durations of time (5, 10, 15, 20, 30, 60, 90, and 120 minutes) was used to determine the impact of contact time, while other parameters were kept constant in this experiment. The effect of the initial metal conc. on the biosorption of Cd and Pb was investigated using different metal ion concentrations. (20, 40, 60, 80, 100, 120 mg/L) under constant conditions (pH and contact time). The biomass was constant throughout the experiment. All subsequent sorption studies were conducted under the following conditions: pH 7 for Cd and 6 for Pb, contact time of 90 minutes, and initial cadmium concentrations of 100 and 120 mg/L for Cd and Pb, respectively. For *Scenedesmus* sp. experiment was carried out at pH 6, contact time of 120 minutes and initial metal concentration of 120mg L^{-1}. All tests were carried out in triplicate in an orbital shaker at 150 rpm in Erlenmeyer flasks (250 ml) that contained 100 ml of solution. Following incubation, test solutions were filtered, and the resulting filtrates were evaluated for residual Cd and Pb using an atomic absorption spectrometer (Agilent Technologies, MC187906, Malaysia) (Mirghaffari et al., 2015; Kumar et al., 2018).

4.2.2.3 Adsorption kinetics modeling

Biosorption kinetics discuss the biosorption rates and characteristics that influence the maintenance of equilibrium over time (Imran et al., 2021). In this study, pseudo 1st order (Lagergren, 1898), pseudo 2nd order (Ho and McKay 1999), and intraparticle diffusion models (Weber and Morris 1963) were used to predict biosorption kinetics, and the equations **(1, 2 and 3)** are shown below:

$$Log\ (q_e - q_t) = \log q_e - \frac{k_1 t}{2.303} \quad (1)$$

$$\frac{t}{q_t} = \frac{1}{k_2 qe^2} + \frac{1}{q_e} t \quad (2)$$

$$q_t = k_i t^{0.5} + I \quad (3)$$

In the above Equations **(1, 2, 3)** equation, k_1, k_2 and k_i are the rate constants.

4.2.2.4 Adsorption isotherms modeling

Adsorption isotherms were used to study the distribution of molecules among distinct

phases at equilibrium. They discuss surface characteristics, probable adsorption mechanisms, and adsorbent affinities. The Freundlich Langmuir and Temkin models are commonly used. The Langmuir model, which considers the uniform distribution of energy over all sites, is used to calculate monolayer adsorption (Imran et al., 2021; Langmuir, 1918). **Equation 4** specifies the Langmuir adsorption isotherm (Chinedu et al., 2012).

$$\frac{1}{q_e} = \frac{1}{q_{max}} + \frac{1}{b\, q_{max} C_e} \qquad (4)$$

where q_{max} is the maximum adsorption amount (mg g^{-1}) of cations absorbed at equilibrium, q_e is the adsorbent's equilibrium adsorption, and C_e is the residual heavy metal at equilibrium.

The Freundlich model is used to explain the heterogeneous adsorption on adsorbent surfaces. The **equation (5)** of the Freundlich model is described as follows (Chinedu et al., 2012):

$$In\, q_e = InK_F + \left(\frac{1}{n}\right) In\, C_e \qquad (5)$$

where q_e is the amount of sorbate adsorbed on the sorbent and n is the empirical factor of adsorption intensity/heterogeneity. The slope and intercept of the curve were used to calculate the values of the Freundlich parameters using the linear version of the Freundlich equation. According to the Temkin isotherm, the adsorption heat is reduced for all molecules in the layer as the surface coverage increases. This model can only be used for moderate concentration ranges. This isotherm Equation (6) (Areco and Dos 2010) is as follows:

$$q_e = B\, ln(K_t C_e) \qquad (6)$$

4.2.2.5 FTIR

Changes (before and after positive ion attachment) in functional groups on the sorbent surface were determined using FTIR analysis (Murtaza et al., 2019). FTIR was utilized to analyze the infrared spectra at 4000-400cm^{-1}.

4.2.2.6 Statistical analysis of data

Three independent experiments (biological replicates) were carried out for toxicity at different metal conc., pigment content, and dry biomass determination for Cd and Pb removal. The data in the respective figures for each analysis are expressed as the mean ± standard deviation of triplicate measurements. Statistical analyses were conducted using Origin Pro 8.5, and Excel software.

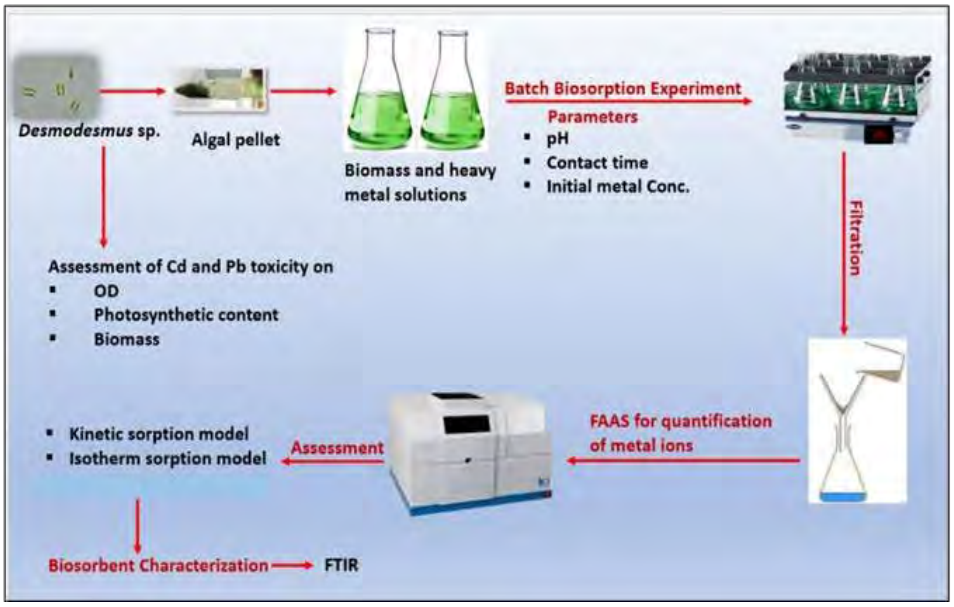


Fig. 4.1. A schematic diagram showing the experimental design to assess the toxicity of metal ions, growth parameters and bio removal of Cd and Pb by using algal strains.

4.3 Results and Discussion

4.3.1 Cultivation of algae and effect of Cd and Pb stress on their growth

Cd and Pb stress resulted in substantial variations in growth parameters (OD) of *Desmodesmus subspicatus* and *Scenedesmus dimorphus* as compared to the control **(Fig.4.2 A, B, C and D)** (Chandrashekharaiah et al., 2021). Cd and Pb had no growth inhibitory impact, especially at low doses, as shown in **Fig. 4.2 A, B, C and D**. When subjected to 15 ppm Cd (0.064) and 90 ppm Pb (0.078), growth production was comparable to that of the control (0.159) in *Desmodesmus* sp. at 72h and 96h (Shen et al., 2021). There were substantial differences (significant decrease) in growth parameter from 15 ppm Cd concentration after 48 hours and 90 ppm Pb concentration after 72 hours of exposure in *Desmodesmus* culture. When exposed to 50 ppm Cd and 180 ppm Pb, the optical density (OD_{750}) was 0.014 and 0.02 (after 12 days) for cadmium and lead, in *Desmodesmus* sp. respectively, showing that Cd and Pb had an

inhibitory influence on the growth of green microalgae **(Fig 4.2A and B)**. Cd and Pb at higher concentration from 20 to 50 mg/L (for Cd) and 90 to 180 mg/L (for Pb) the growth of *Scenedesmus* sp. was decreased as compared to control, but at higher concentration of Cd and Pb, at 50 mg/L (0.024; **Fig. 4.2C)** and Pb at 120 mg/L (0.031; **Fig. 4.2D**), it causes a significant decrease on the cell concentration of *Scenedesmus* sp. on 12 days. When the Cd and Pb concentration reached concentration of 20 (Cd) and 60 (Pb) mg/L the growth is almost constant after the 4^{th} days in *Scenedesmus* sp. **(Fig. 4.2C and D**). However, a further increase in concentration of metal ions resulted in a sharp decrease in growth of *Scenedesmus* sp. Cd and Pb caused significant effects and dose-dependent on the growth *Desmodemsus* and *Scenedesmus* sp. During the 12-day culture period, the cells developed well in the control group. However, the growth curve lagged, and the growth rate gradually decreased as the metal content increased (Shen et al., 2021). Abd El-Hameed et al., (2018) found that increased Pb concentrations reduce the optical density of blue green algae. Cell density was reduced in diatoms treated with increasing concentrations of Cd and other metals (Sbihi et., 2012). (Wang et al., 2021) presented a similar study in which the optical density of green algae decreased as cadmium exposure increased.

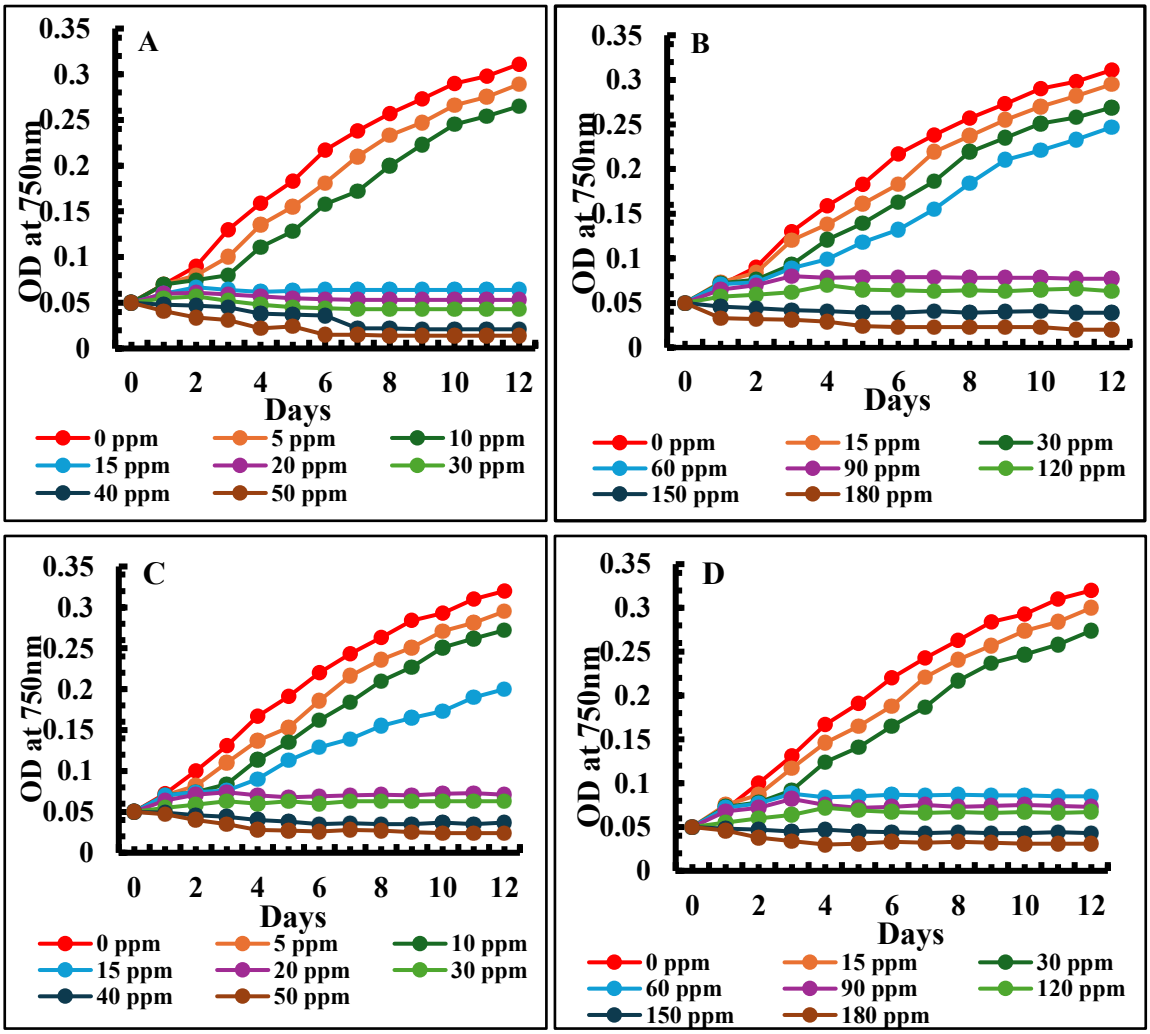


Fig.4.2. Effect of different concentrations of (A) Cd (B) Pb and (C) Cd and (D) Pb on the growth of *Desmodesmus* and *Scenedesmus* sp.

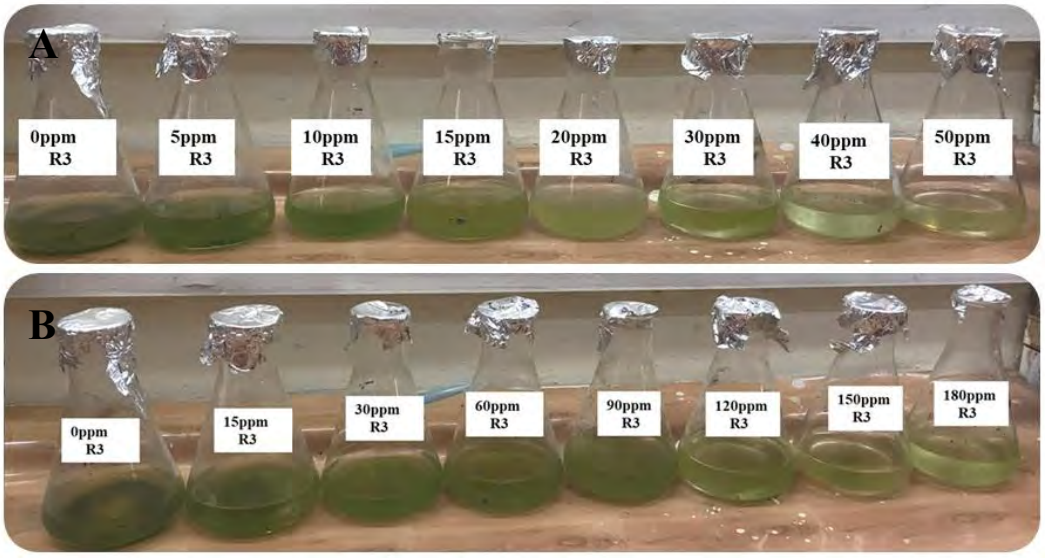


Fig.4.3 Effect of different concentrations of (A) Cd and (B) Pb on the growth of *Desmodesmus* sp. R3 after exposure for 12 days.

4.3.2 Cell morphological changes observed through light microscopy

Light microscopy data revealed the morphological changes on the cell surface caused by the stress of Cd and Pb, as shown in **Fig. 4.4 and 4.5**. Some of the prominent changes included: disappearance of spines (*Desmodesmus* sp.) in samples treated for 96 hours (**Fig. 4.4C-F**) compared to the control **(Fig. 4.4A-B)**; Four- celled coenobium was lost in the treated group of *Scenedesmus* sp. **(Fig. 4.5C-F)** as compared to the control group **(Fig. 4.5A-B)**. Previously there were few studies that suggest inducing phenotypic plasticity in members of the genus *Scenedesmus* exposed to heavy metal ions. Monahan, (1976) briefly explained the loss of coenobium in *Scenedesmus sp*. observed in its population (unicellular) in the presence of toxic concentrations of heavy metals. In *Scenedesmus* sp. cell color changed to yellow **(Fig. 4.5C-F).** Previous research on the morphological alterations caused by the divalent heavy metals studied indicated a sequence of change from four-cell coenobia to two-cell and exhibiting yellow color. Except for the unicellular forms exposed to Cd, a state that could not recovered due to irreversible damage to cells, the final populations were able to regain their normal morphology (Pena- Castro et al., 2004).

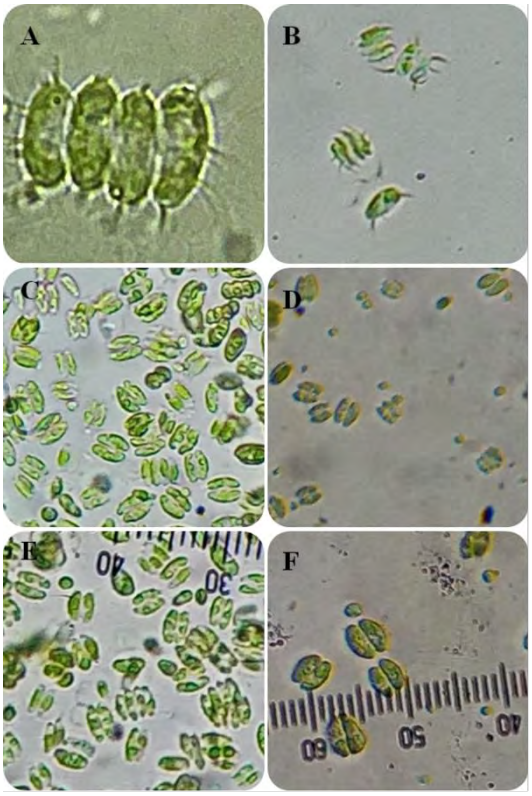


Fig.4.4. Light microscopy images of *Desmodesmus* sp. (A-B) Control (C-D) treated with 15 and 50 ppm Cd (E-F) and treated with 90 and 180 ppm Pb after 96 hours.

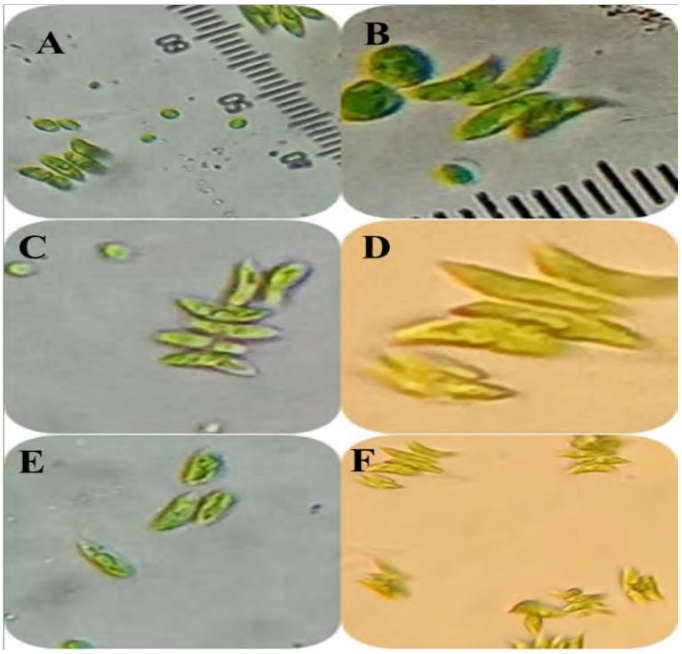


Fig.4.5. Light microscopy images of *Scenedesmus* sp. (A-B) Control (C-D) treated with 20 and 50 ppm Cd (E-F) and treated with 60 and 180 ppm Pb after 96 hours.

4.3.3 Pigment content estimation

Exposure to high metal concentrations resulted in significant reduction in 'chlorophyll a' and carotenoid content in *Desmodesmus* sp. as shown in **Fig. 4.6A and C**. In general, *Desmodesmus* was more susceptible to Cd and Pb at high dose as compared to *Senedesmus* sp. *Desmodesmus* showed the high pigment loss/damage when exposed to high Cd concentration i.e. 50 mg/L (0.2 and 0.019 µg/ml for total 'chlorophyll a' and carotenoid respectively). *Desmodesmus* showed the lowest pigment level when exposed to 180 mg/L of Pb (0.3 and 0.027 µg/ml for 'chlorophyll a' and carotenoid respectively).

In *Scenedesmus* sp. the decrease in photosynthetic content was observed at a concentration of 20 ppm for Cd and 60 ppm for Pb upon an exposure of 72 hours. Total chlorophyll content of *Scenedesmus* sp. exposed @ 15 and 60 ppm of Cd and Pb, decreased significantly from those in controls **(Fig 4.6Band D)**. The lowest 'chlorophyll a' (0.3 µg/ml for Cd and 0.36 µg/ml for Cd and Pb) and carotenoid contents (0.023 µg/ml for Cd and 0.027 for Pb) were found in algae exposed to highest concentration i.e. 50 ppm of Cd and 180 ppm of Pb which indicated that heavy metals may have induced the deactivation of some proteins, leading to a reduction in photosynthetic electron transfer (Samadani et al., 2018). The loss in 'chlorophyll a' content as metal ion concentration increased here was attributed to

metal oxidation and inhibitory impact on pigments, confirming earlier findings (Kupper et al., 2003; Rai et al., 2013). In green algae, chlorophyll content loss has been observed when exposed to chromium and copper (Chen et al., 2012; Rai et al., 2013). Since 'chlorophyll a' is an indication of algal growth, Cd has been shown to influence "Chlorophyll a" production in *P. lanceolatum* (Sbihi et al., 2012). As Cu or Cd concentrations increased, the 'chlorophyll a' content of *Chlorococcum* sp. decreased (Qiu et al., 2006). El-Hameed, (2018) highlighted how pigment content decreased when exposed to varying concentrations of lead.

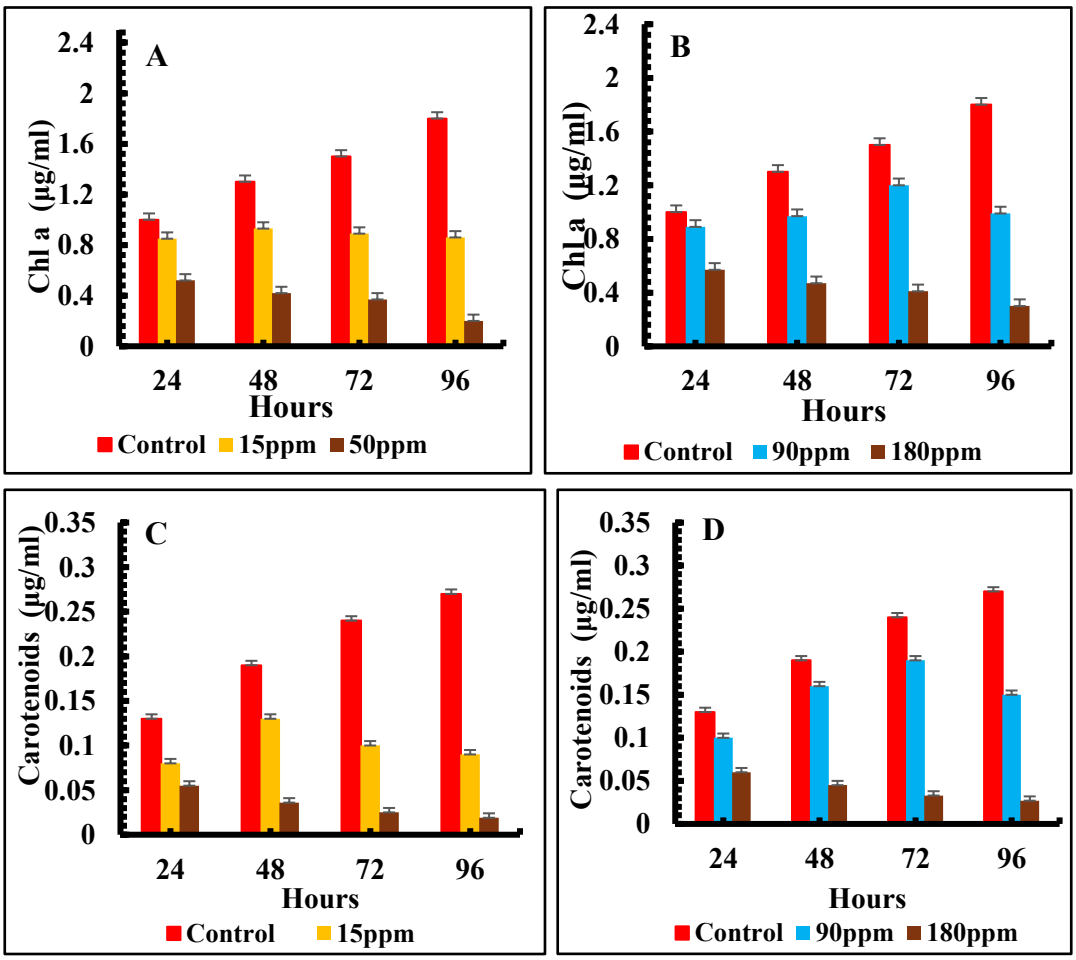


Fig. 4.6. Changes of chlorophyll a (A and B) carotenoid (C-D) in *Desmodesmus* and *Scenedesmus* sp. under Cd and lead stress during cultivation time. All the values mean ± SD. (n = 3).

4.3.4 Biomass

There was a difference in the biomass values during the incubation period in *Desmodesmus* sp. and *Scenedesmus* sp. depending upon the metal ion concentration as shown in **Fig. 4.7A, B, C, and D**. In the case of *Desmodesmus* sp. the highest biomass value was recorded in the control/untreated group at 96 hours of incubation, @ 84

mg/L for Cd and Pb. It was shown that at 72 and 96 hours of exposure @ 15 ppm in Cd and 90 ppm in Pb, the biomass begins to decrease and reaches the declining/lag phase @ 96 hours of incubation at maximum metal concentration. The lowest value (21 and 24 mg/L) was recorded after 96 hours of treatment (50 and 180 ppm) for Cd and Pb, respectively **(Fig4.7A and B)**. In *Scenedesmus* sp. the highest value of biomass was recorded in control treatment after 96 hours of incubation (89 mg/L). The lowest value was recorded in the treatments @ 50 and 180ppm after 96hrs, it was (25 and 27mg/L) for Cd and Pb. At low concentration of Cd and Pb the biomass continues to increase. It was found to decrease @ 20 ppm of cadmium and @ 60 ppm of Pb at an exposure of 96 hours **(Fig4.7C and D)**. Similar studies have been described previously (Wang et al., 2021), where a reduction in biomass was observed with an increase in the Cd concentration.

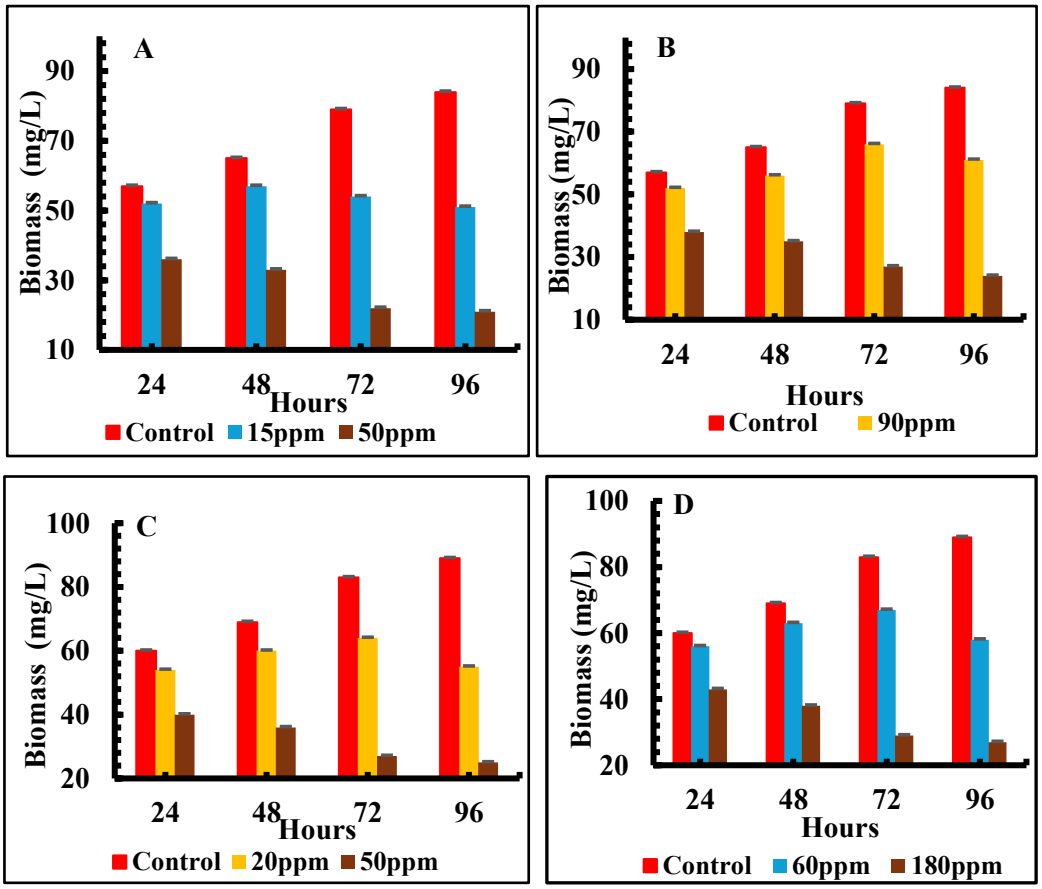


Fig.4.7. Effect of different concentrations of (A and C) Cd and (B and D) Pb on the biomass of *Desmodesmus* and *Scenedesmus* sp. All the values are the mean ± SD. (n = 3).

4.3.5 Optimum parameters for enhanced biosorption capacity

4.3.5.1 Impact of pH on biosorption

Since protons and metallic ions compete for the active sites, the pH value is an important determinant in metal ion adsorption by the microalgae. Usually, pH affects the surface characteristics of microalgae as well as the chemistry of metal ions in aqueous solution (Monteiro et al., 2012). **Figure 4.8A** depicts the cadmium and lead adsorption capability of *Desmodesmus* sp. at various pH values (ranging from 2 to 8). The adsorption capacity of *Desmodesmus* sp. for cadmium and lead improved as the pH was increased from 2.0 to 7.0 for Cd and 2.0 to 6.0 for lead and it remained stable as the pH increased. The adsorption capacity of *Desmodesmus* sp. was 61 and 79 mg/g at pH 7.0 and 6.0, respectively. (Rzymski et al., 2014). In the adsorption of *Scenedesmus* sp. the pH was increased from 2 to 6 for both metals (90 mg/g for Cd and 95 mg/g for Pb) then adsorption capacity did not change **(Fig 4.8B)** (Cheraghpour et al., 2020). However, because of the high concentration of hydronium ions, the functional groups of algae were protonated at low pH. However, as the pH increases, deprotonation of negatively charged groups resulted in electrostatic attraction of cations to the cell surface (Monteiro et al., 2012). Rzymski et al., (2014) reported maximum cadmium removal by blue green algae at a neutral pH value. A pattern consistent with this data can also be found in a previous study by Rangsayatorn, (2002), who reporting that pH 7.0 is the best in the case of blue green algae Cd for reduction.

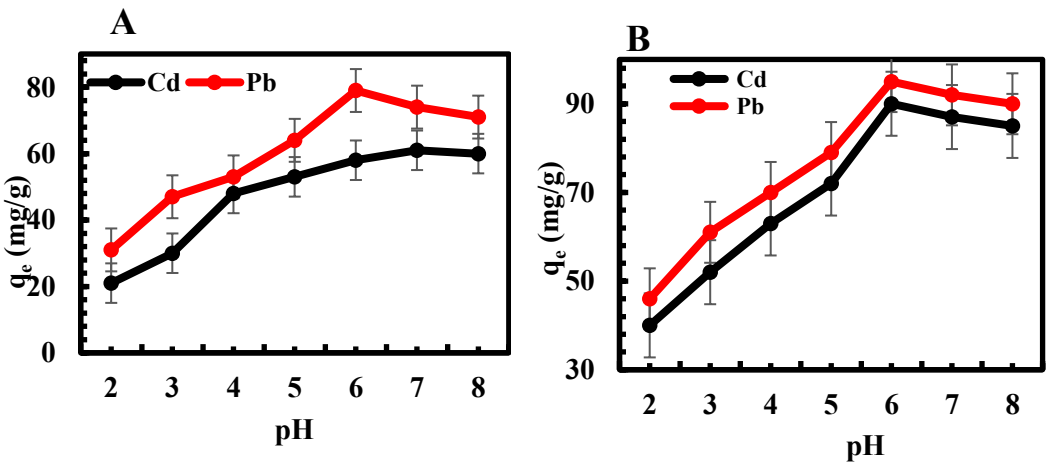


Fig.4.8. Effect of pH on the adsorption capacity of Cd and Pb by *Desmodesmus* and *Scenedesmus* sp. (Data are presented as the means ± SEs (n=3).

4.3.5.2 Effect of contact time on the biosorption

Contact time has been identified as an important element influencing metal ion adsorption capacity. In *Desmodesmus* sp. within 90 minutes, both metals had

adsorption capacities of 62 and 75 mg/g, as shown in **Fig. 4.9A**. Furthermore, the biosorption of Cd and Pb by *Desmodesmus* sp. reached 57 and 66 mg/g in 60 minutes, respectively, with the maximum adsorption reaching within 90 minutes. Meanwhile, when the contact duration was increased from 90 to 120 minutes, there was no significant increase in Cd and Pb adsorption. Hence, the optimal contact time was 90 minutes in the case of *Desmodesmus* sp. In the case of *Scenedesmus* sp. the biosorption of Cd and Pb gradually increased with increase in contact time, reaching a maximum of 100 and 105 mg/g at 120 min for both metals and remained constant thereafter **(Fig 4.9B)**. Hence, the adsorption equilibrium time chosen for further study was 90 and120 mins. for *Desmodesmus* and *Scenedesmus* sp. (Limcharoensuk et al., 2015) respectively. Cheng et al., (2017) described a similar trend of gradual increase in adsorption in the live material attaining an equilibrium @ 105 minutes. Many previous studies found that *S. obliquus* (Chen et al., 2012) *Spirulina platensis* (Rangsayatorn et al., (2002), and several brown algae (Montazer-Rahmati et al., 2011) achieved maximum removal of heavy metals within 2 hours. However, due to the toxic nature of Cd and Pb, the initial metal ion concentration should not be high enough to hamper the growth of algal cells. *Desmodesmus* sp., for example, suffered lethality at high concentrations of cadmium and lead. As a result, short-term biosorption of heavy metals by algae has an advantage in the treatment of aqueous environments with high initial heavy metal concentrations (Zhang et al., 2016).

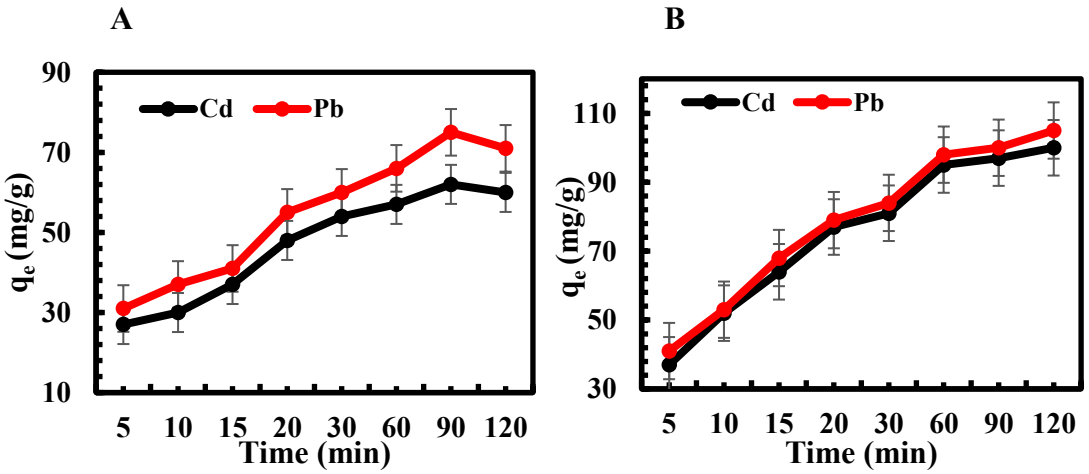


Fig. 4.9. Effect of contact time on the adsorption capacity of metal ions by *Desmodesmus* and *Scenedesmus* sp. (Data are presented as the means ± SEs (n=3).

4.3.5.2 Effect of the initial metal ions concentration on biosorption

Figure 4.10 depicts the adsorption capacity of algal strains and the initial Cd and Pb

concentration (from 20 to 120 mg L^{-1}). With increasing initial metal concentration, in *Desmodesmus* sp. the metal adsorption capacity of both metals grew significantly **(Fig. 4.10A)** (Cheng et al., 2017). At 100 and 120 mg/L, the maximum biosorption capacity can be as high as 65 and 70 mg/g for Cd and Pb **(Fig. 4.10A)** in *Desmodesmus* sp.; while in *Scenedesmus* sp. the maximum adsorption capacity was attained at 120 mg/g (97 and 101 mg/g) **(Fig. 4.10B)**. The removalarefficiency of microalgae is heavily dependent on the heavy metal content. The number of binding sites remained constant with a constant biomass content, whereas the number of Cd ions increased with increasing Cd concentration (Bhat et al., 2008). The efficiency can grow further with increasing metal ion concentration in the adsorption process, but only when the biomass is low. This adsorption event is driven by a greater driving force that overcomes all cadmium mass transfer resistance between the aqueous and solid phases. As a result, a higher initial metal concentration leads to more metal ion adsorption (Edris et al., 2014). Ghimire et al (2008) discovered that when the metal ion concentration increased, adsorption also increased until equilibrium is attained at a high concentration of metal ions. In the adsorption of green algae, the metal ion adsorption increased as the initial concentration of cations in the medium increased (Bayramoglu and Arica 2011). As a result, the initial Cd concentration had a considerable effect on the biosorption capacity. (Chen et al., 2009) and (Edris et al., 2014) discovered similar results.

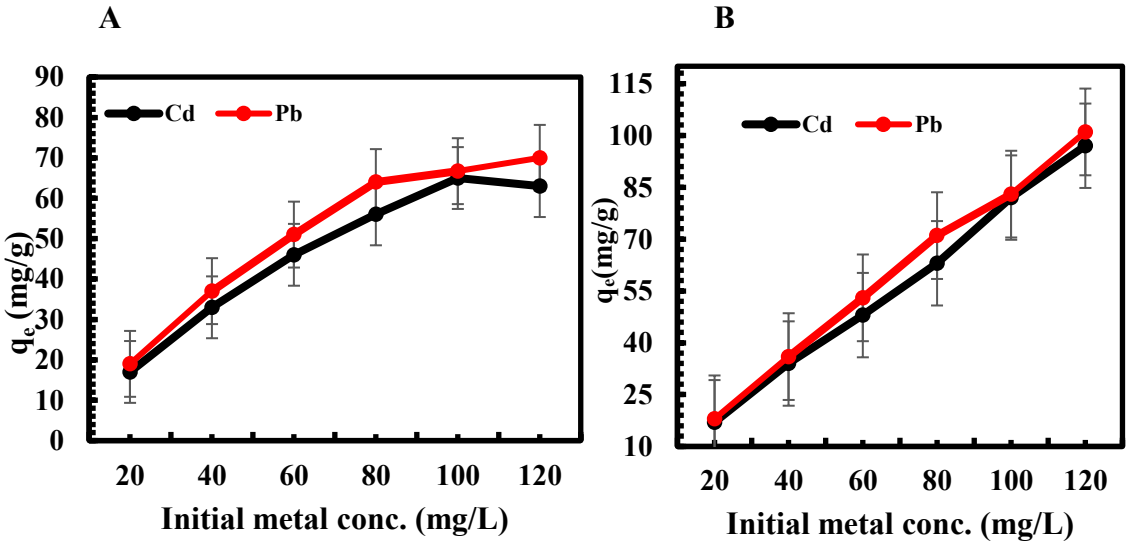


Fig. 4.10. Effect of contact time on the adsorption capacity of metal ions by *Desmodesmus* and *Scenedesmus* sp. (Data are presented as the means ± SEs (n=3).

4.3.5.3 Biosorption kinetics

Different kinetics models were employed to explain the experimental data of the adsorption kinetics of metal ion adsorption on *Desmodesmus* and *Scenedesmus* sp. The parameters and kinetic curves of several models are shown in **Fig. 4.11; 41.12** and **Table 4.1**, respectively. The adsorption results fit well with the pseudo second order model, with R^2= 0.996 and 0.994 for Cd and Pb, in *Desmodesmus* sp.; R^2= 0.996 and 0.994 for Cd and Pb, in *Scenedesmus* sp. respectively, which was higher than the pseudo first order and intraparticle diffusion models. The pseudo- second-order model produced equilibrium adsorption values (q_e) of 65.7 (Cd) and 78.7 mg/g (Pb), which were similar to the experimental values (62 for Cd and 75 for Pb). In *Scenedesmus* sp. qe values were 108.4 (Cd) and 112.6 (Pb) which depicted a nice agreement with experimental values 100 (Cd) and 105 (Pb). Similar findings were reported for the biosorption of Pb in *Bacillus* strains, with second-order kinetics outperforming first-order kinetics (Colak et al., 2011). As a result, the adsorption kinetics followed the pseudo second order, indicating that the mechanism of adsorption between metal ions and the sorbent was mostly chemical adsorption. The results show that the rate of Cd and Pb biosorption in two algal strains was controlled by chemical interactions between functional groups and positive ions (Bulgariu and Bulgariu 2012; Kaleem et al., 2023).

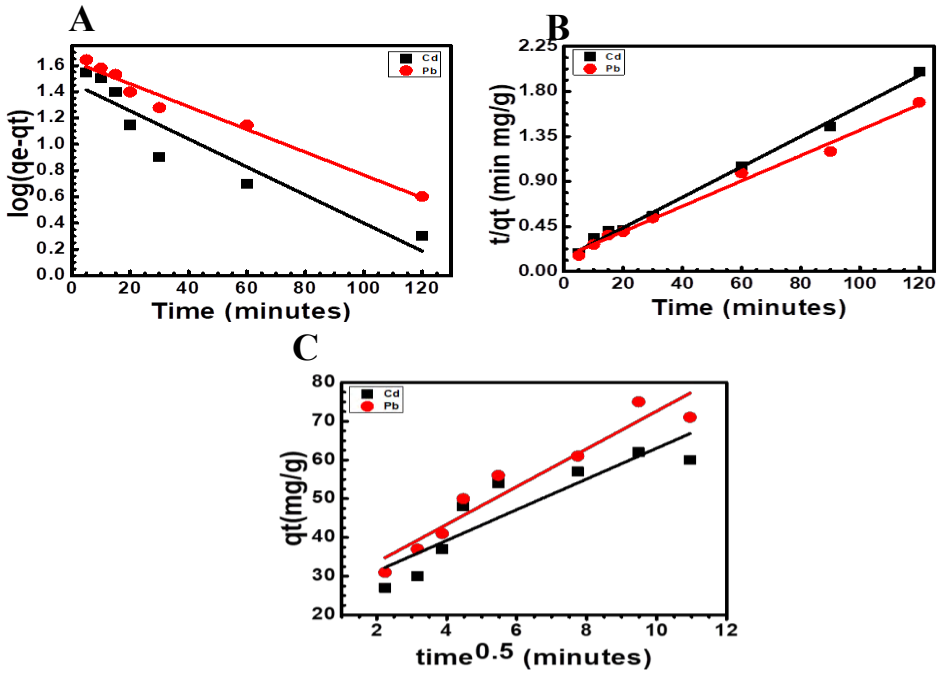


Fig.4.11. (A) Pseudo-first order (B) pseudo-second order (C) intraparticle diffusion model for Cd adsorption by *Desmodesmus* sp.

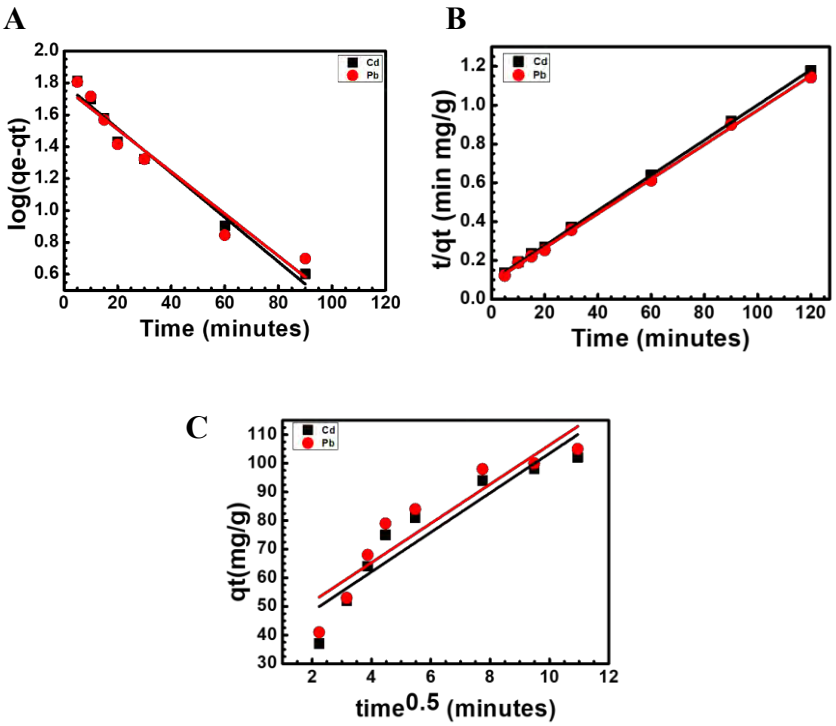


Fig.4.12. A) Pseudo-first order (B) pseudo-second order (C) intraparticle diffusion model for Cd adsorption by *Scenedesmus* sp.

Table 4.1: A comparison among different rate constants of adsorption kinetics.

Strains	Metal	Pseudo 1st order			Pseudo 2nd order			Intraparticle		
		K_1	qe	R^2	K_2	q_e	R^2	k_{id}	R^2	qe exp
***Desmodesmus* sp**	**Cd**	-0.029	3.31	0.856	32109	65.7	0.996	3.963	0.813	62
	Pb	-0.027	4.62	0.773	48551	78.7	0.994	4.817	0.924	75
***Scenedesmus* sp**	**Cd**	-0.0348	5.85	0.978	13186	108.6	0.999	6.678	0.845	100
	Pb	-0.0332	6.31	0.972	146119	112.4	0.999	6.841	0.863	105

4.3.5.5. Biosorption isotherms

Biosorption linearized models were used to illustrate the relationship between Cd and Pb adsorption (q_e) and the metal residual concentration in solution (C_e) (**Fig. 4.15; 4.16; Table 4.2).** The R^2 values show that the *Desmodesmus* sp. data for both Cd and Pb obtained in this experiment did not suitably fit the two isotherms, particularly the Freundlich and Temkin models. The values of the Langmuir coefficient constants found greater for Cd and Pb. This revealed that *Desmodesmus* sp. has a higher affinity for Cd and Pb biosorption due to a stronger binding approach. The Langmuir isotherm's applicability to the biosorption of Cd and Pb by *Desmodesmus* sp. (Langmuir: $R^2 = 0.996$ for Cd; $R^2 = 0.997$ for Pb) and *Scenedesmus* sp. (Langmuir: $R^2 = 0.988$ for Cd; $R^2 = 0.993$ for Pb) demonstrated that the monolayer biosorption distribution of active sites on the surface of *Desmodesmus* sp. existed under the experimental conditions (Doshi et al., 2007). As indicated in Table 4.2, the q_m values produced by green algae using the Langmuir model were (83.3 mg g^{-1} *Desmodesmus*; 212 mg g^{-1} *Scenedesmus* sp.) for Cd and (71.4 mg g^{-1} *Desmodesmus* sp.; 232 mg g^{-1} *Scenedesmus* sp.) for Pb. In this previous study, the mechanisms involved in Cd removal by *S. obliquus* were discussed based on Langmuir isotherm parameters (Zhang et al., 2016; Kaleem et al., 2023).

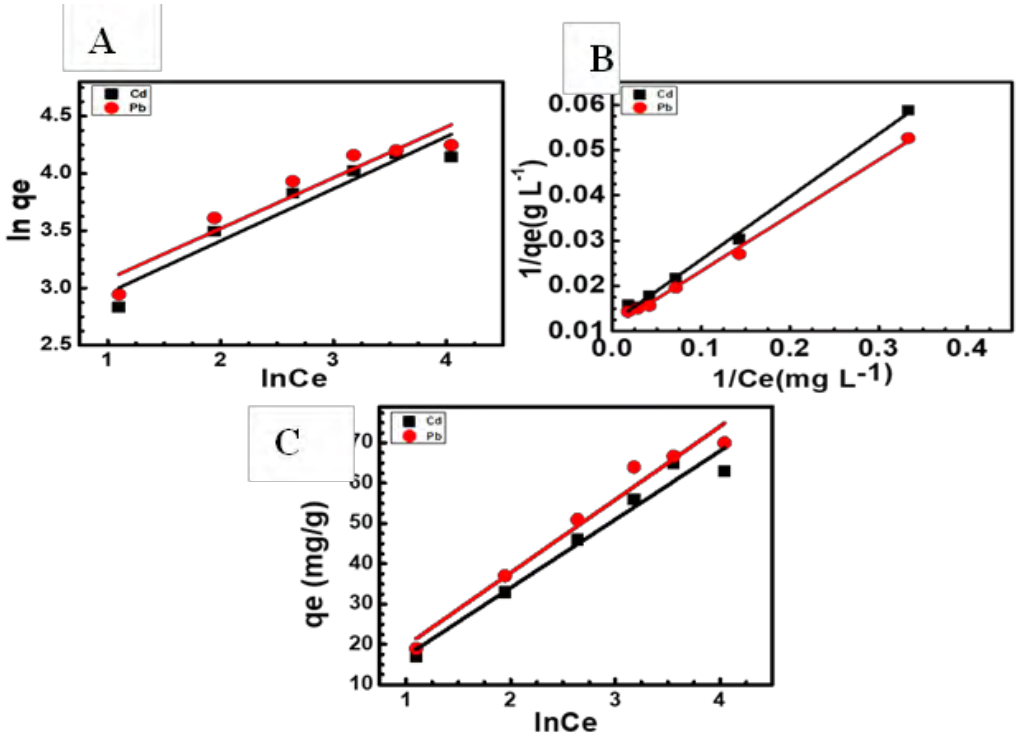


Fig.4.13. (A) Freundlich (B) Langmuir (C) Temkin isotherm for Cd and Pb adsorption by *Desmodesmus* sp.

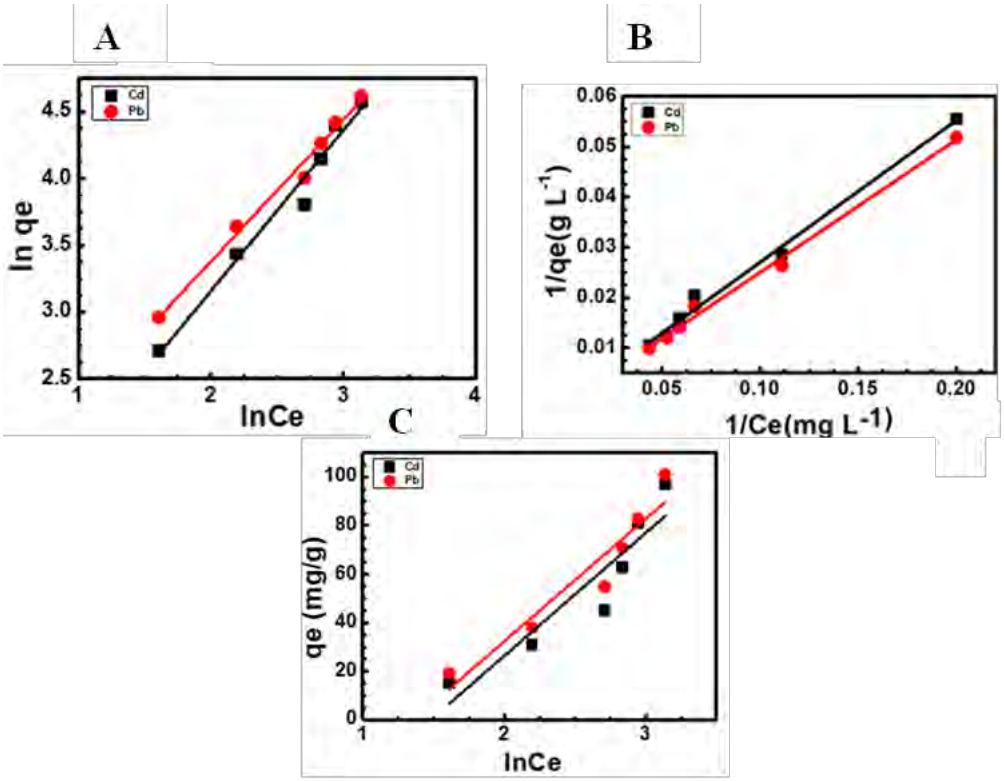


Fig.4.14. (A) Freundlich (B) Langmuir (C) Temkin isotherm for Cd and Pb adsorption by *Scenedesmus* sp.

Table 4.2: Parameters of the model for the adsorption of metals by two algal strains.

Strains	Metal	Langmuir Isotherm			Freundlich Isotherm			Temkin Isotherm	
		1/b	q_{max}	R^2	K_F	1/n	R^2	B	R^2
***Desmodesmus* sp.**	**Cd**	11.5	83.3	0.996	318	2.199	0.920	16.96	0.964
	Pb	2.77	71.4	0.997	1316	3.080	0.919	13.28	0.973
***Scenedesmus* sp.**	**Cd**	34	212	0.988	92.9	0.811	0.974	36.08	0.8841
	Pb	23	232	0.993	322	0.274	0.965	35.03	0.9653

4.3.5.6 Comparison with Other Biosorbents

The comparison of algal strains with different biosorbents used for Cd and Pb biosorption is shown in **Table 4.3**. It is clear that *Desmodesmus* and *Scenedesmus* sp. have a high uptake of Cd and Pb in comparison to other biosorbents. Therefore, it is evident to state that biosorption by *Desmodesmus* and *Scenedesmus* sp. is an effective method in removing Cd and Pb from industrial wastewater.

Table 4.3: A comparison of the adsorption capacities of several sorbents and green algal strains.

Biosorbent	Biosorption capacity (mg/g)		
	Cd	Pb	References
Aspergillus fumigatus	6.28	21.5	(Khamesy et al., 2016)
Spirogyra insignis	22.9	51.5	(Romera et al., 2007)
Chlorella minutissima	11.1	9.74	(Roy et al., 1993)
Mucor rouxii	8.5	35.7	(Yan and Viraraghavan 2003)
Desmodesmus sp.	83.3	71.4	Current study
Scenedesmus sp.	212	232	Current study

4.3.5.7 FTIR

The adsorption mechanism and interaction of metal ions with algal strains (biomasses) were investigated. FT-IR was used to analyze control and treated biomass with the aim to identify the functional groups involved in metal adsorption. Cd and Pb ions attached to the functional groups present on the surface of algal biomass are shown in **Fig. 4.15.** The FT-IR spectrum of *Desmodesmus* sp. **(Fig. 4.15A)** showed an absorbance peak at 2844 cm^{-1} (black), which indicates C-H stretching vibrations (Sharma and Bhattacharyya 2005). The amide linkage of C=O appeared in the range of 1640 and 1537cm^{-1}. The absorption band located near 1239cm^{-1} corresponds to the functionality of C-N, and the band at 1059 cm^{-1} indicates the presence of a C-O stretching band (Waqar et al., 2023b). Many changes (blue and red peak in Fig. 3.16) are noted when Cd and Pb ions are bound to the functional groups when compared with the control (black peak).

The absorption band shifted from 1640 cm^{-1} to 1638 cm^{-1} (Cd) and 1637 cm^{-1} (Pb) (Sheng et al., 2008). This result indicates the interaction of Cd and Pb with amine groups. The C-N band shifted from 1239 cm^{-1} to 1393 cm^{-1} in Pb. The relative intensity of the C-O stretching band changed from 1059 cm^{-1} to 1043 cm^{-1} and 1145 $cm^{-1,}$ indicating the interaction of Cd and Pb. In this study, the carbonyl and amine groups on the surface of *Desmodesmus* sp. were found to be involved in the adsorption process. These findings have been explained in the previous literature (Waqar, et al., 2023a).

The FTIR spectrum **(Fig. 4.15B)** of *Scenedesmus* sp. showed an absorbance peak (black) at 3182 cm^{-1}, which corresponded to N-H stretching vibration. 2912 showed the peak of O-H. N- H binding vibration revealed to the peak at 1626 cm^{-1}. S=O stretching vibration was depicted by the peak at 1035cm^{-1}. Sulfoxide and amines groups were present at the surface of *Scenedesmus* sp. (Shokri et al., 2015). Blue and red peak in fig **(4.15B)** showed that metal interacts with the functional groups on the surface of *Scenedesmus* sp. The N-H stretching peak 2912 cm^{-1} shifted to 2915 cm^{-1} in the biosorption of Cd. N-H binding vibration shifted to 1625 cm^{-1} (Cd) and 1632 cm^{-1} (Pb). The sulfoxide peak shifted from 1035 cm^{-1} to 1030 cm^{-1} (Cd) and 1032 cm^{-1} (Pb). Similar results have been described previously too (Sun et al., 2019).

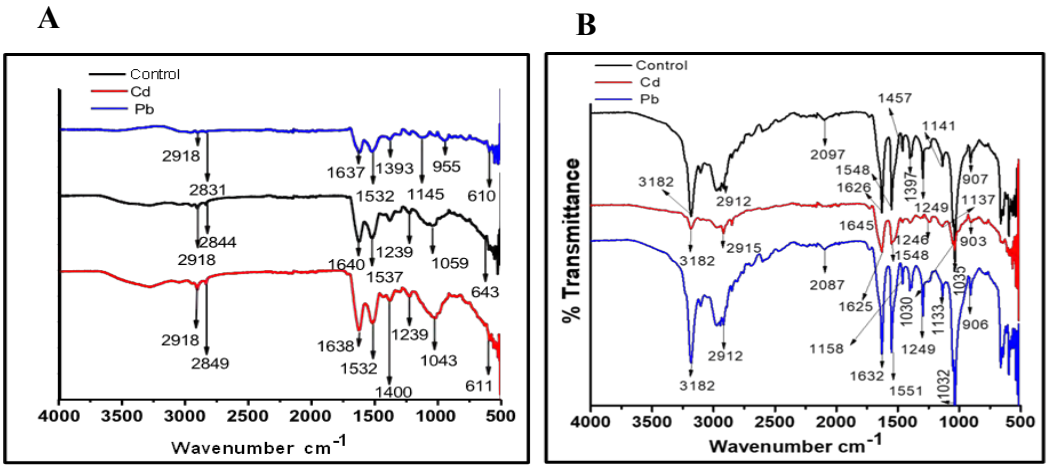


Fig.4 .15. FTIR analysis of control and treated samples of (A) *Desmodesmus* and (B) *Scenedesmus* sp.

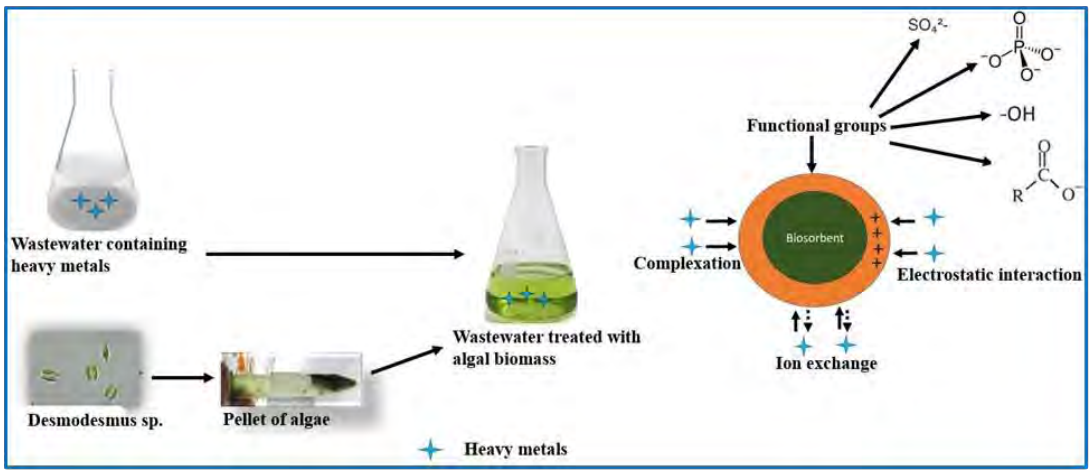


Fig.4.16. Different mechanisms of biosorption of heavy metal ions by algae.

Conclusion

- Two green algal strains are unique and can efficiently bind cations (Cd and Pb) from water. The high concentration of Cd and Pb ions in the medium had an

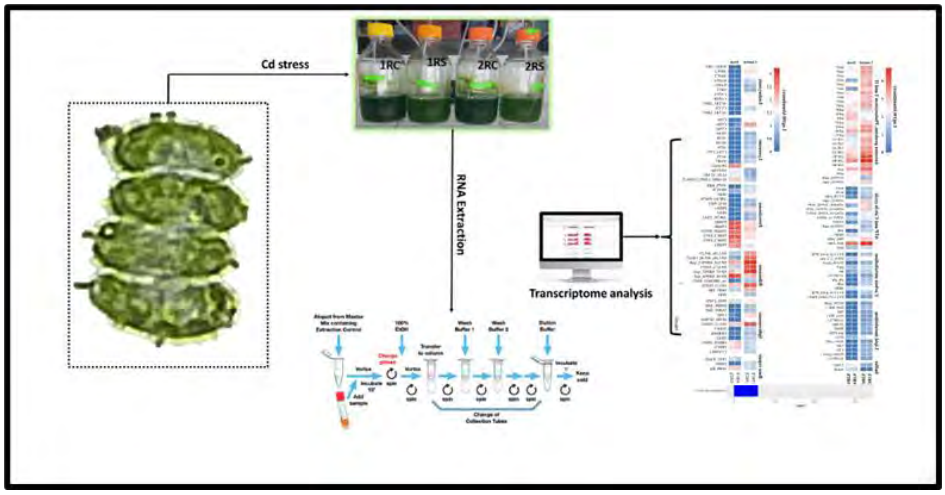
impact on *Desmodesmus* and *Scenedesmus* sp.

- Low concentrations of metal ions had a negligible inhibitory effect on the green algal cultures. However, the loss of 'chlorophyll a' and decline in biomass was observed at higher concentrations of metals.
- Many parameters, including pH, contact time, and initial metal ion concentration, influence the process of sorption. The biosorption of Cd and Pb followed pseudo-second-order kinetics, mainly based on chemical adsorption.
- *Desmodesmus* and *Scenedesmus* sp. are the appealing candidates for industrial-scale remediation of metal-polluted water.

CHAPTER 5

Transcriptome analysis of *Desmodesmus subspicatus* exposed to cadmium stress.

Graphical abstract



5. Abstract

Global ecosystems face a significant threat from heavy metal pollution, which is a widespread hazard. In recent decades, the use of microalgae's adaptive properties to remediate harmful heavy metals has been recognized globally. Transcriptomics investigations have revealed mechanistic insights into the dynamics of cellular events under heavy metal stress, to better understand microalgal strategic responses. Multiple studies have established the pathways of Cd toxicity in *Desmodesmus subspicatus* at the physiological level, but nothing is known about their transcriptome basis. In the current investigation, *Desmodesmus subspicatus* was treated with 15ppm cadmium chloride for 96 hrs. The results show that the optical density and Fv/Fm of *Desmodesmus subspicatus* started to decrease after 48 hrs of Cd exposure. These physiological results supported the impact of cadmium on *Desmodesmus subspicatus*. RNA-Seq revealed that 42,347,092, 47,483,530 for control and 39,333,360, 33,492,989 for Cd treated libraries respectively. 615 gene present significantly different between control and treatment experiment. 452 gene is significantly down regulated and 163 is significantly upregulated at 72hrs of Cd stress. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed 10 main pathways to be involved. Among these 10 pathways, metabolic pathways have a higher number of genes and biosynthesis of nucleotide sugars has a lower number of genes. Our findings demonstrated that Cd retarded the growth rate in *Desmodesmus subspicatus*, which was followed by a significant reduction in photosynthetic pigment and a decrease in the expression of genes linked with the photosynthetic system and oxidative

phosphorylation. The photosystem was affected due to the down regulation of genes which suppressed chlorophyll biosynthesis. This analysis reports the first transcriptome of *Desmodesmus subspicatus*, offering a thorough understanding of the molecular pathways used by microalgae at the transcriptome level to detoxify the effects of Cd.

5.1. Introduction

The technological breakthroughs over the past several decades have raised crucial concern about environmental safety (Qian et al., 2020). For instance, the heavy metal deposition in aquatic and terrestrial ecosystems is facilitated by the unchecked release of wastewater from cosmetics, paints, dyes, mining, and battery-making industries, besides the agricultural waste (chemical fertilizers) (Karabourniotis et al., 2020). Ecology has been harmed by heavy metal biomagnification to the upper echelons of the food chain, endangering human life (Ouyang et al., 2018). A wide range of species depend on heavy metals, including cobalt, nickel, manganese, copper, iron zinc, and chromium, to regulate vital biological processes. However, substituting toxic heavy metals like cadmium, mercury, arsenic, and lead for these vital metals and micronutrients results the manipulations in genetic makeup through DNA mutations, neurological dysfunction and immune system, and a variety of tumors (Tamas et al., 2014). These dangerous heavy metals (HMs) cause protein aggregation and related diseases by binding to free thiols and different functional groups of proteins or by displacing essential ions in metalloproteins (Jaishankar et al., 2014). Therefore, before being directly discharged into natural water bodies, wastewater containing heavy metals must be treated.

The main species that are immediately exposed to the hazardous chemical makeup of wastewaters are the microbial consortiums found in aquatic water bodies. The microbial community is made up of microorganisms that can survive and/or eliminate harmful substances like heavy metals from their environment. These microorganisms belong to several classes and include bacteria, fungus, algae, and archaea (Kumar et al., 2015). It has been noted that the algal systems are the most resilient and effective at withstanding this kind of abiotic stress out of all of them. The two groups of algae that make up the algae system are macroalgae and microalgae. For effective heavy metal removal efficiencies, both have been extensively researched. Seaweed, or macroalgae, has been widely reported to be an effective bio-sorbent for eliminating heavy metals; nevertheless, because to its slower growth rate, sessile nature, and offshore cultivation, researchers face a few technical difficulties (Charrier et al., 2017). Microalgae are photosynthetic organisms that are highly favored for heavy metal cleanup due to their simpler structure and short

production period. Further, presence of different functional groups present on the cell surface of algae and large surface area increases its chance of removing different heavy metals (Leong et al., 2020).

Algae are a remarkable class of phytoplankton that can effectively remove hazardous heavy metals from polluted areas and convert them into less harmful forms through biotransformation. For instance, dimethylarsinate, monomethylarsonate, and arsenosugars, are less dangerous forms of inorganic arsenic that can be produced by microalgae (Hasegawa et al., 2019). According to Singh and Kumar (2020), biological redox processes also change the oxidation states of HMs to facilitate further detoxification. In response to heavy metal toxicity, microalgae either control the expression of specific genes that are involved in the absorption, sequestration, or detoxification of certain proteins or transporters (Brulle et al., 2010). In addition, microalgae have evolved an inherent set of defense mechanisms that include both high- and low-molecular-weight antioxidants to withstand the oxidative stress brought on by heavy metal invasion (Lu et al., 2019). Owing to the present need, physiological research, and omics-based different technologies transcriptomics, metabolomics, and genomics are being used more in tandem to obtain mechanistic insight into how microalgae respond to external abiotic stress (Zheng et al., 2020).

Studies using comparative transcriptomics are becoming more and more prevalent across all the existing omics approaches (Salama et al., 2019). Transcriptomics provides an advantage over other omics techniques, such as the development of sequencing technologies that make it feasible to create and build microarrays and provide a snapshot of all the genes present in the entire genome of an organism that is not a model but is nevertheless ecologically significant (Brulle et al., 2010). Furthermore, methods such as Illumina-based de-novo RNA sequencing, which does not necessitate prior knowledge of the test organism's gene sequence, provide a reliable way to profile an organism's genetic expression and provide interactive networking under specific toxicant exposure conditions (Ozsolak and Milos 2011). A common aquatic model organism used to study the toxicity of heavy metals is the green algae *Chlamydomonas reinhardtii* and *Chlorella sorokiniana* (Wang et al. 2018). According to Simon et al. (2008), Cd may bind to organic molecules including glutamate, and cysteine, then inactivate protein to cause a variety of negative impacts on the algae. It has been shown to suppress the growth of algae, cause ultrastructural changes in cells, such as cytoplasmic electron-dense granules, interfere with

the chlorophyll synthesis and chloroplasts (Tonon et al., 2018), and increase the activity of enzymes such as catalase, and superoxide dismutase, that are signs of oxidative stress, (Wang et al. 2018). Instead, Cd can interfere with membrane transporters to disrupt food absorption or calcium signalling pathways, as well as remove calcium or zinc from the active sites of enzymes (Chen et al. 2018).

The tools provided by transcriptomics technologies enable us to uncover the molecular mechanisms behind the toxicity of Cd and to gain a thorough description of the cellular response following exposure to contaminants (Beauvais-Fluck et al. 2016). Few research has investigated the transcriptome changes caused by Cd exposure in green algae (Puente-Sanchez et al., 2018). According to Jammers et al. (2013), *Chlamydomonas reinhardtii* exposed to Cd for over 48 hours may exhibit the upregulation and down regulation of genes. These genes were linked to DNA replication, protein metabolism, energy metabolism and oxidative stress. According to Regier et al. (2013), 500 lg/L of Cd can cause *Elodea nuttallii* to down-regulate 158 genes and up-regulate 54 genes. The genes that are most strongly up- regulated fall into the section of metabolism of energy reserves, which include "ion transport," signal transduction and transport ATPases. The categories of cellular import, homeostasis, and detoxification showed the highest enrichment for downregulated genes (Regier et al., 2013). These results suggested that different algae species might react differently to Cd exposure. Thus, examining the transcriptomics response to Cd exposure in additional algae species, such *Chlorella*, might expand our knowledge of the molecular mechanisms behind Cd toxicity. The above-mentioned Cd exposure values also resemble moderately or substantially Cd-polluted water, hence, to determine the toxicity of Cd, the environmentally appropriate concentration must be used. By revealing the molecular mechanisms behind the toxicity of contaminants, transcriptomics technologies may be able to assist in locating their interaction sites. No research has conducted on the transcriptomics response of *Desmodesmus subspicatus* exposure to Cd stress. The aims of this study are to construct a transcriptome database of *Desmodesmus subspicatus* and to investigate the significantly expressed genes under Cd-toxic condition.

5.2. Materials and Methods

5.2.1 Reagents Preparation

The stock solutions of Cd were prepared by dissolving $CdCl_2$ (1000 mg/L) in deionized water. The 15ppm concentration of Cd was prepared by diluting the stock solution.

5.2.2 Algal culture and CO_2 manipulation

Desmodesmus subspicatus (strain R3) strain culture was maintained in the laboratory at the Institute of Marine and Environmental Technology (IMET), University of Maryland Centre for Environmental Science. The strain R3 culture was grown in BG_{11} medium with 10 % CO_2, and the medium was prepared and autoclaved by using water (deionized), which was 0.22-µm filtered (Rippka et al., 1979). The experimental setup contained two groups: control (without Cd) and stressed (15 ppm Cd). The experiment was run in duplicate. After incubation of R3 strain to the exponential phase, algal cells were inoculated into four bottles at the OD_{750} value of 1.113. Tested cultures were grown in glass bottles (1L) with 800ml medium (BG_{11}) and placed at 25°C with an intensity of light (600µE/m2/s) that are controlled by fluorescent light (54-watt white). Cell density (optical density at 750nm), (Fv/Fm), and pH were monitored at 4, 24, 48, 72, and 96 hours then once each day afterwards.

5.2.3 RNA extraction

For RNA extraction, a 50ml culture was harvested and then centrifuged at 4000 rpm for 10 minutes at 4°C. After the process of centrifugation, the supernatant was discarded, and the pellet was immediately stored at −80°C for RNA extraction. The total RNA extraction was extracted by using an Omega EZNA Plant RNA extraction kit (Norcross, GA 30071 USA) following the manufacturer's protocol. The concentration of RNA was quantified using a Nanodrop (ND-2000 spectrophotometer; Thermo Scientific).

5.2.4 Library construction and RNA sequencing

The mentioned procedure yielded total RNA, so it is important to extract the mRNA from it. To achieve this, poly(A) mRNA was isolated from the total RNA samples utilizing oligo (dT) magnetic beads, followed by fragmentation using a 5× fragmentation buffer. Subsequently, the first strand of cDNA was synthesized through a reaction involving N6 primers, dNTPs, random short mRNA fragments, and RNase inhibitors. The second cDNA strand was then synthesized using GEX buffer, dNTPs, DNA polymerase, and RNaseH. The resulting double-stranded cDNAs were purified with a PCR Purification Kit, followed by the addition of a single nucleotide A (adenine). Adapters were ligated to the cDNA using a PCR Purification Kit, and the obtained products were purified through gel electrophoresis (1% agarose) before being amplified through PCR. Lastly, the R3 cDNA library was sequenced on the BGISEQ-500 platform with 100 nucleotide paired-end reads at the Institute for Genome Sciences of the University of Maryland USA.

5.2.5 Transcriptome bioinformation analysis

The raw reads were subjected to quality control using Trimmomatic 0.36 (LEADING:10, TRAILING:10, SLID-INGWINDOW:4:20, MINLEN:70) (Bloger et al., 2014). Fastqc was used to evolute the read quality (Chen et al., 2017). Trimmomatic 0.36 was used to remove low-quality reads (LEADING:10, TRAILING:10, SLID-INGWINDOW:4:20, MINLEN:70). The clean raw data was assembled with Trinity soft (Bloger et al., 2014). The ORF (Open Reading Frames) was predicated with the TransDecoder **(**Haas et al., 2013**).** The CD-HIT (Fu et al., 2012) was used to set up a redundant transcriptome gene set. To improve the accuracy of gene prediction, the transcriptome gene set was mapped to Uniprot and Pfam database with Blastp (Finn et al., 2010) to obtain a final gene set. The gene set was annotated using the KEGG and eggnog databases (Cepas et al., 2019; Kanehisa et al., 2004). Salmon v 0.13.1 was applied to calculate the TPM abundance of each gene (partro et al., 2017; Uritskiy et al., 2018). The detailed information is shown in **Fig 5.1.**

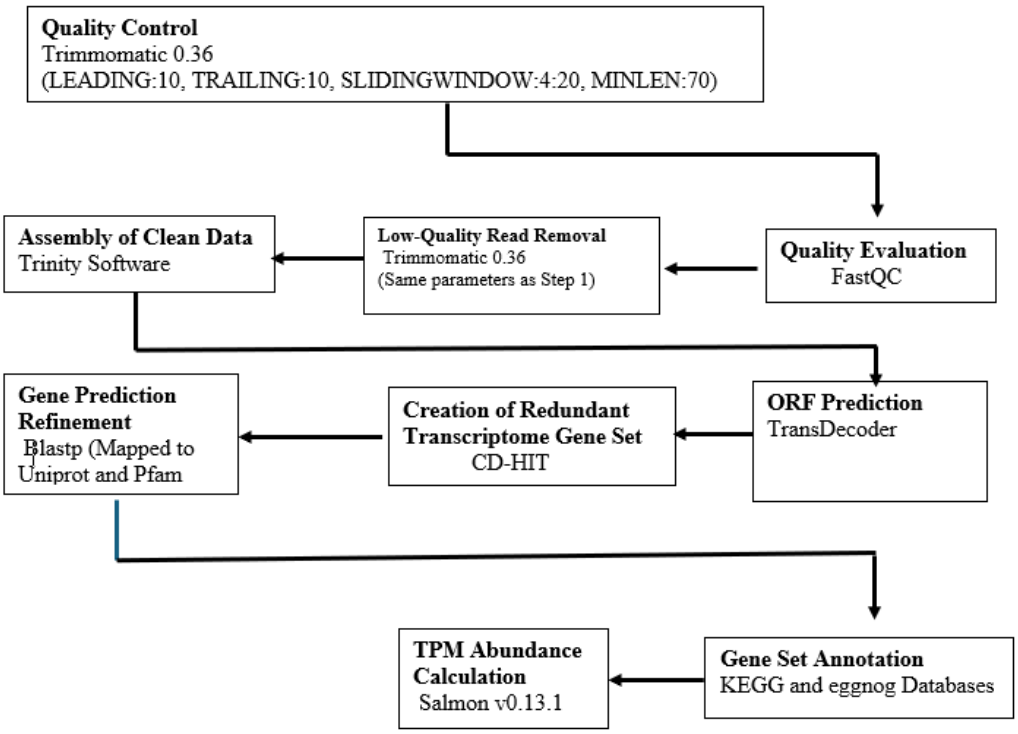


Fig. 5.1. Illustration of the flowchart of bioinformatics analysis.

5.2.6 Statistics and analysis

All the calculations and plots were carried out in an R environment (version 4.3.3). All figures were generated using the ggplot2 (version 3.5.0) package. The edgeR package (Wickham et al., 2016) was used to analyze the significantly different genes between the

control and treat cultures. Heatmaps were produced using the pheatmap package (Gu, 2022).

5.3. Results

5.3.1 Physiological responses to Cd exposure

The control and stressed samples had different growth trends, and the growth rate of the control group exhibited a slight increase compared to the treated group **(Fig. 5.2).** On the first two days, the optical density (OD) of the control and treated groups increased as shown in **Fig. 5.2A**. From day 03 (72hrs), the OD value of the treated group decreased. The OD values of the control and stressed samples were 2.09 and 1.17, respectively, on the 4th day of cultivation. These data suggest that with 10% CO_2, the control culture grows faster compared to the culture treated with Cd. Interestingly, the maximum quantum efficiency (Fv/Fm) in the control increased with time and showed no photoinhibition, but the Fv/Fm of the stressed group remained low (~0.54) for 48h and started to decrease at 72 h **(Fig. 5.2B).** At the end of the experiment (96 h), the Fv/Fm in the Cd-stressed sample reached 0.417 while the Fv/Fm in the control was 0.777 **(Fig. 5.2B)**. The pH values in the control group were higher as compared to the treated group because rapid growth of algal culture in the control adsorbed CO_2 quickly **(Fig. 5.2C)**.

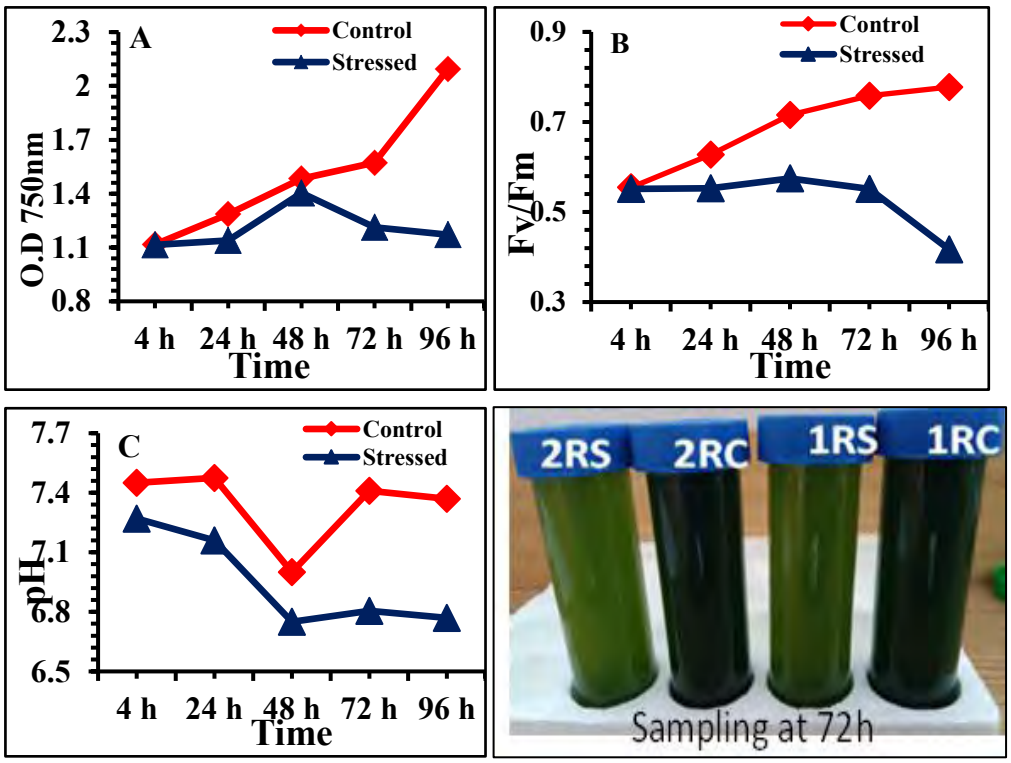


Fig. 5.2. Physiological responses of R3 under 15 ppm Cd stress and without stress conditions. (A) Growth curves; (B) Fv/Fm (C) pH trends.

5.3.2 The sequence data summary

After removing the low-quality reads, 42,347,092 and 47,483,530; 39,333,360 and 33,492,989 clean reads were obtained for the control and Cd-treated libraries, respectively. The assembled contigs are 162, 790 and ORF is 165,139 **(Table 5.1)**.

Table 5.1: Summary of sequencing data for control and treated samples.

	1RC	**2RC**	**1RS**	**2RS**
Total sequence	43,461,574	48,570,956	40,408,927	34,487,268
Clean data (reads)	42,347,092	47,483,530	39,333,360	33,492,989
Assembled contigs		162,790		
ORF number		165,139		

5.3.3 Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed to assess the overall gene expression patterns among the four samples: two control samples (1RC and 2RC) and two stressed samples (1RS and 2RS). The first principal component (PC1) accounted for the majority of the variance in the data, effectively separating the control samples from the stressed samples. The control samples (1RC and 2RC) clustered together along PC1 and the stressed samples (1RS and 2RS) also formed a distinct cluster along PC1. This suggests that the primary difference in gene expression between the control and stressed conditions is effectively captured by PC1. These results demonstrate that the PCA successfully differentiated the stressed and control conditions, highlighting the distinct transcriptional responses to stress as shown in **Fig 5.3.**

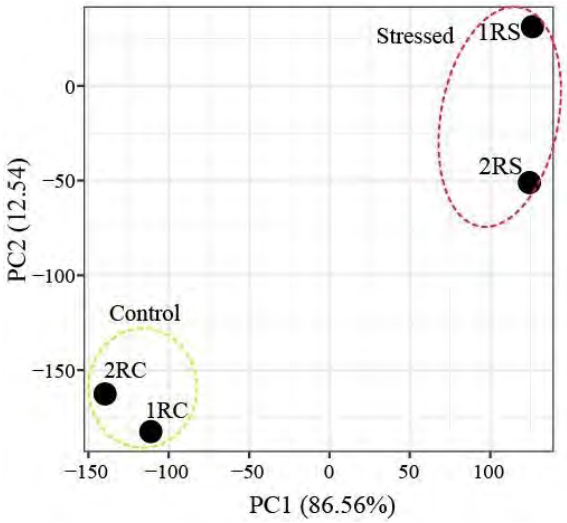


Fig. 5.3. Principal Component Analysis (PCA) of gene expression data from control and stressed samples.

5.3.4 Differentially expressed genes in *Desmodesmus subspicatus*

The predicated transcriptome gene set was annotated using the KEGG database and obtained 4099 different gene families (KEGG Orthology). Difference analysis found that there are 615 genes significantly different between control and treat cultures. Detailed, the expression of 452 and 163 unigenes was upregulated and downregulated, respectively, at 72 h compared to the control **(Fig 5.4)**.

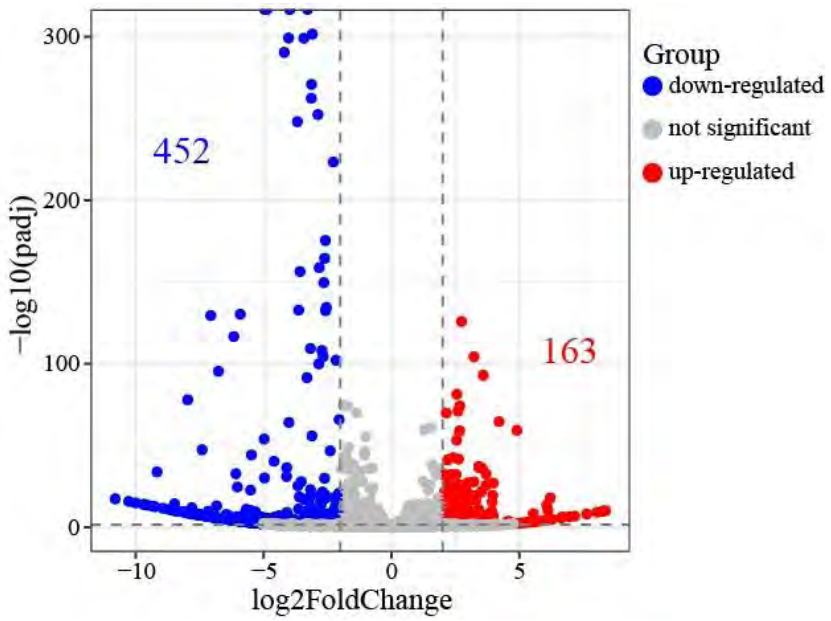


Fig. 5.4. A volcano plot was constructed to visualize the differentially expressed genes (DEGs) in *Desmodesmus subspicatus* when comparing control and stressed samples.

5.3.5 KEGG pathway enrichment analysis

Upon combining all KEGG pathways significantly enriched by DEGs, 10 key pathways were identified **(Fig 5.5).** The results revealed that KEGG pathways associated with different metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environment, biosynthesis of cofactors, biosynthesis of amino acid, Carbon metabolism, 2-Oxocarboxylic acid metabolism, fatty acid metabolism, nucleotide metabolism and biosynthesis of nucleotide sugar. The KEGG pathway analysis showed that metabolic pathways have the highest gene number **(Fig 5.5)**. In the metabolic pathways different pathways are involved as shown in supplementary file **(Fig 5.6)**. These pathways are carbohydrate metabolism, nucleotide metabolism, lipid metabolism, secondary metabolite biosynthesis, energy metabolism, amino acid metabolism, glycan biosynthesis and metabolism, Xenobiotic degradation and metabolism, vitamin and cofactor metabolism Among these pathways, the highest number of genes related to photosynthesis and oxidative phosphorylation were identified as shown in **Fig 5.6.**

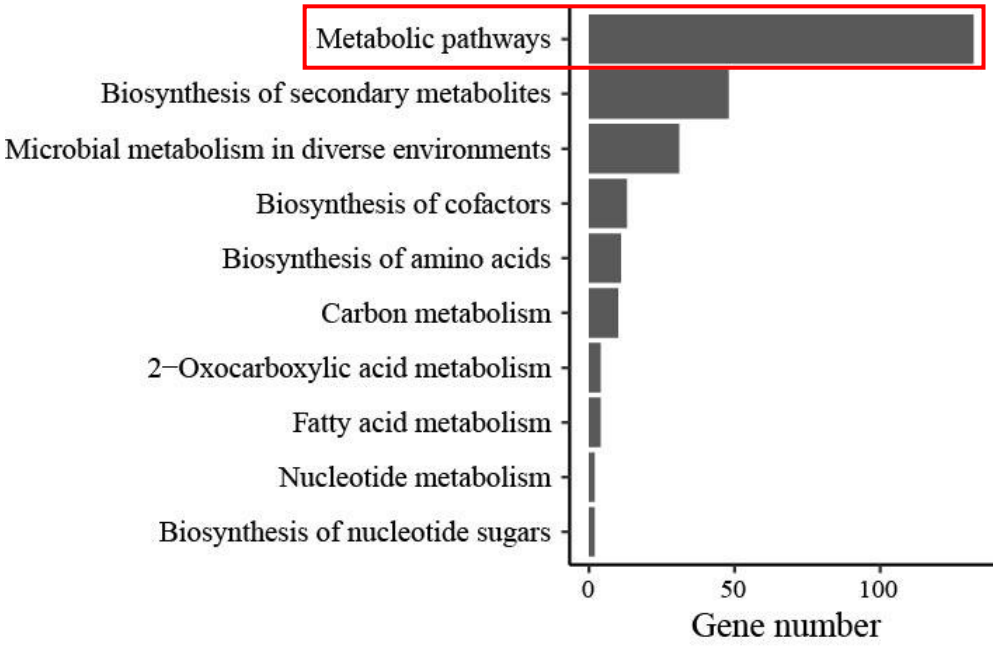


Fig. 5.5. Significant KEGG pathway enrichment of DEGs at 72h.

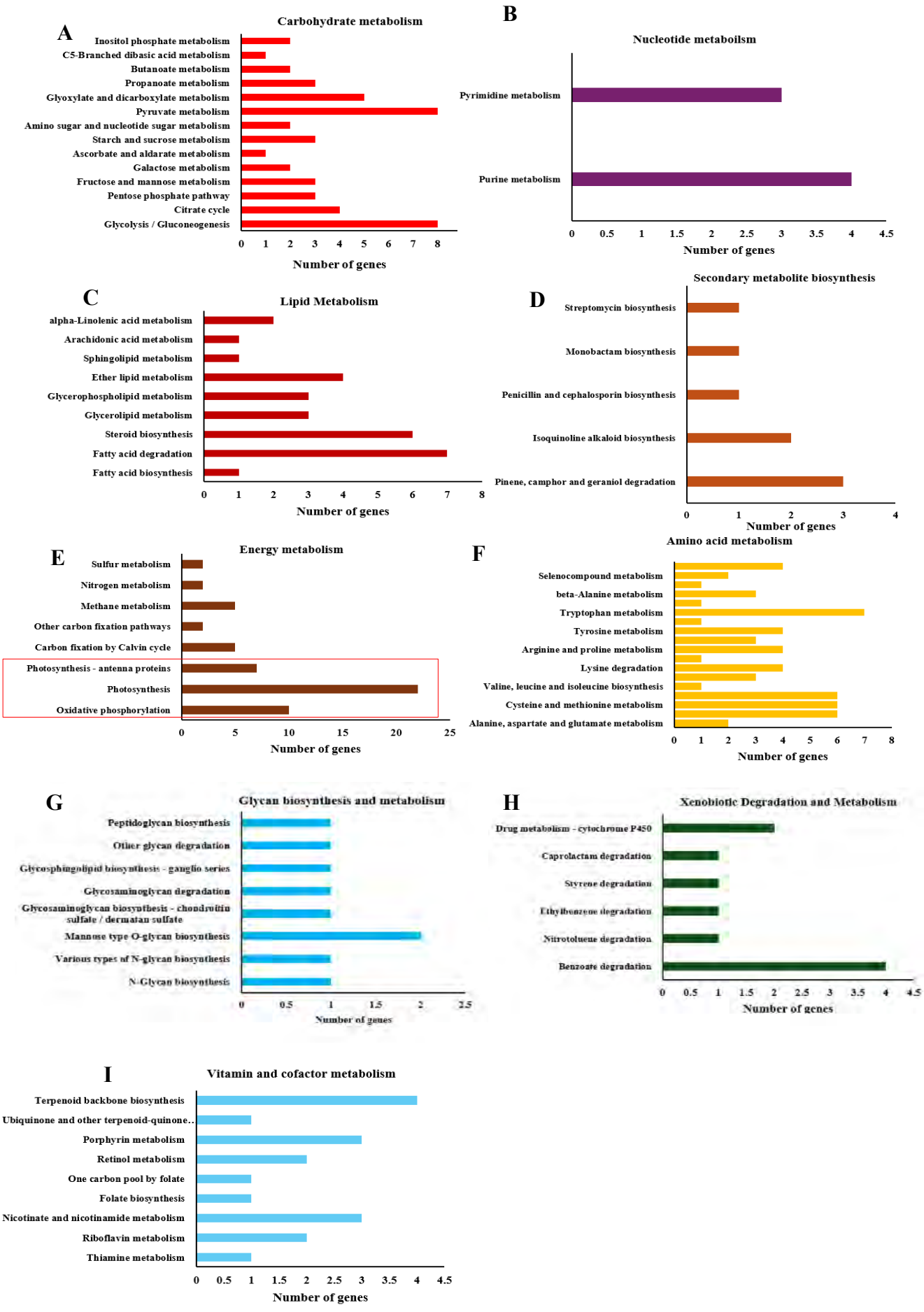

A
Carbohydrate metabolism
Inositol phosphate metabolism
C5-Branched dibasic acid metabolism
Butanoate metabolism
Propanoate metabolism
Glyoxylate and dicarboxylate metabolism
Pyruvate metabolism
Amino sugar and nucleotide sugar metabolism
Starch and sucrose metabolism
Ascorbate and aldarate metabolism
Galactose metabolism
Fructose and mannose metabolism
Pentose phosphate pathway
Citrate cycle
Glycolysis / Gluconeogenesis
0 1 2 3 4 5 6 7 8
Number of genes
B
Nucleotide metaboilsm
Pyrimidine metabolism
Purine metabolism
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5
Number of genes
C
Lipid Metabolism
alpha-Linolenic acid metabolism
Arachidonic acid metabolism
Sphingolipid metabolism
Ether lipid metabolism
Glycerophospholipid metabolism
Glycerolipid metabolism
Steroid biosynthesis
Fatty acid degradation
Fatty acid biosynthesis
0 1 2 3 4 5 6 7 8
Number of genes
D
Secondary metabolite biosynthesis
Streptomycin biosynthesis
Monobactam biosynthesis
Penicillin and cephalosporin biosynthesis
Isoquinoline alkaloid biosynthesis
Pinene, camphor and geraniol degradation
0 1 2 3 4
Number of genes
E
Energy metabolism
Sulfur metabolism
Nitrogen metabolism
Methane metabolism
Other carbon fixation pathways
Carbon fixation by Calvin cycle
Photosynthesis - antenna proteins
Photosynthesis
Oxidative phosphorylation
0 5 10 15 20 25
Number of genes
F
Amino acid metabolism
Selenocompound metabolism
beta-Alanine metabolism
Tryptophan metabolism
Tyrosine metabolism
Arginine and proline metabolism
Lysine degradation
Valine, leucine and isoleucine biosynthesis
Cysteine and methionine metabolism
Alanine, aspartate and glutamate metabolism
0 1 2 3 4 5 6 7 8
Number of genes
G
Glycan biosynthesis and metabolism
Peptidoglycan biosynthesis
Other glycan degradation
Glycosphingolipid biosynthesis - ganglio series
Glycosaminoglycan degradation
Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate
Mannose type O-glycan biosynthesis
Various types of N-glycan biosynthesis
N-Glycan biosynthesis
0 0.5 1 1.5 2 2.5
Number of genes
H
Xenobiotic Degradation and Metabolism
Drug metabolism - cytochrome P450
Caprolactam degradation
Styrene degradation
Ethylbenzene degradation
Nitrotoluene degradation
Benzoate degradation
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5
Number of genes
I
Vitamin and cofactor metabolism
Terpenoid backbone biosynthesis
Ubiquinone and other terpenoid-quinone..
Porphyrin metabolism
Retinol metabolism
One carbon pool by folate
Folate biosynthesis
Nicotinate and nicotinamide metabolism
Riboflavin metabolism
Thiamine metabolism
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5
Number of genes

Fig. 5.6. KEGG analysis (Metabolic pathways) results of *Desmodesmus subspicatus* exposure to Cd stress. KEGG-based classification of the (A) Carbohydrate metabolism (B) Nucleotide metabolism (C) Lipid metabolism (D) Secondary metabolite biosynthesis (E) Energy metabolism (F) Amino acid metabolism (G) Glycan biosynthesis and metabolism (H) Xenobiotic degradation and metabolism (I) vitamin and cofactor metabolism.

5.3.6 Specific differentially expressed genes in response to Cd

5.3.6.1 Carbohydrate and Energy Metabolism

In *Desmodesmus subspicatus* metabolic pathways were downregulated under cadmium stress. The expression of genes PckA, PCK, eno, PGAM, gpmA, glpX, ahr, ALDH7A1, pfp, involved in the glycolytic pathway is significantly downregulated in stressed samples **(Fig 5.7).** This indicates a suppression of the primary pathway responsible for glucose breakdown and energy production in the form of ATP. Phosphofructokinase, or PFK, is an enzyme involved in glycolysis, the metabolic route that transforms glucose to pyruvate. Glycolysis is an essential step for cellular energy production. Phosphofructokinase plays a critical role in glycolysis control because it catalyses one of the essential processes. Cadmium stress also leads to the downregulation of genes (mdh, MDH1, PRK, PrkB, rbcS, cbbS, glpX) associated with the Calvin cycle in stressed samples, the critical process of carbon fixation in photosynthetic organisms **(Fig 5.7).** This downregulation indicates a decreased capacity for carbon assimilation and subsequent sugar synthesis, lowering total photosynthetic efficiency. The tricarboxylic acid (TCA) cycle, or citrate cycle, experiences a similar downregulation of mdh, MDH1, pckA, PCK, fumA and fumB genes under cadmium stress **(fig 5.7).** This cycle produces energy by oxidizing acetyl-CoA obtained from carbohydrate, lipids, and proteins. The reduction of TCA cycle genes suggests a possible disturbance in cellular respiration and energy metabolism. These data demonstrate that cadmium exposure has a severe metabolic impact on algae, altering critical pathways involved in energy production and carbon assimilation**.**

5.3.6.2 The genes related to Photosynthesis and oxidative phosphorylation

5.3.6.2.1 PSII and PSI activity and photosynthetic electron transport

Photosynthesis was the most significantly enriched pathway in Cd exposure groups. In the present study, 28 genes related to photosynthesis **(Fig 5.7)** and 10 genes related to oxidative phosphorylation **(Fig 5.7)** were identified. Among these 28 genes, 10 down regulated genes (in stressed samples) were involved in photosystem I (PS I) and 7 genes were involved in photosystem II (PS II). Among these 7 genes of PS II only 2 genes were

upregulated, and the rest of genes were downregulated in the Cd stressed samples. Other genes related to light harvesting complex, plastocyanin, cytochrome and ATPase subunit. Among 10 genes related to oxidative phosphorylation, the ATPF0A and atpB series genes (H+ transporting ATPase subunit was upregulated while rest of genes nuoA, nuoK, CYTB, PetB, ATPF1G, atpG, ATPeF0C, ATP5G, ATP9, ATPeF1A, ATP5E, ATP1, ATPeF1E, ATP5E, ATP15, ATPeV1A, ATP6A and COX17 were downregulated **(Fig 5.7).**

Downregulation of photosynthetic genes in algae is a preventive and adaptive response to Cd stress that aims to reduce oxidative damage, conserve energy, protect cellular machinery, and divert resources towards survival and detoxification processes. This deliberate adjustment in gene expression helps algae cope with the detrimental effects of cadmium and boosts their chances of survival under stressful settings. The activation of two genes, ATPF0A and atpB, may be part of a larger adaptive response process in which algae alter their metabolic pathways to cope with heavy metal stress. By boosting oxidative phosphorylation, algae may effectively manage larger metabolic demands while sustaining critical biological functions under stressful situations. The downregulation of genes nuoA, nuoK, CYTB, PetB, ATPF1G, atpG, ATPeF0C, ATP5G, and ATP9 during Cd stress is most likely due to a combination of energy conservation strategies, mitochondrial damage protection, oxidative stress reduction, metabolic shifts, and intricate regulatory responses to maintain cellular homeostasis under heavy metal toxicity **(Fig 5.7)**.

5.3.6.3 Protein, nucleotide, and lipid metabolism

Transcripts involved in the metabolism of nucleotide were significantly downregulated in Cd exposure group **(Fig 5.7)**. For the Cd exposure group, malate dehydrogenase (mdh, MDH1) and S-adenosylmethionine decarboxylase, (speD, AMD1; K01611;) cystathionine beta-synthase (CBS; K01697) and cystathionine gamma-lyase (CTH; K01758) were down-regulated **(Fig 5.7)**. These genes participated in the metabolism of cysteine and methionine. For Cd exposure group, monoamine oxidase, cystathionine beta-synthase, cystathionine gamma-lyase, 2,3-bisphosphoglyerate-dependent phosphoglycerate mutase, aldehyde dehydrogenase family 7 member A1 were down-regulated that involved in the pathways of glycine, serine and threonine metabolism **(Fig 5.7)**.

Genes involved in the degradation of fatty acids such as glutaryl-CoA dehydrogenase, (598), acetyl-CoA acyltransferase (598), 3hydroxylacyl-CoA dehydrogenase/ enoyl-CoA hydratase/ 3 hydroxybutyrl-CoA epimerase (598), Delta 3-Delta2-enoyl-COAisomerase

(598) were significantly down-regulated by Cd exposure. The down regulation in the genes PEMT, psd, PISD, BTA1 is involved in metabolism of glycerophospholipid **(Fig 5.7)**. The gene prenylcysteine alpha-carboxyl methylesterase was upregulated involved in the terpenoid backbone synthesis, while hydroxymethylglutaryl-CoA reductase, farnesyl diphosphate synthase and protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha were downregulated. Ribonuclease III, U4/U6 small nuclear ribonucleoprotein, rRNA 2'-O-methyltransferase fibrillarin was down regulated, while ribonuclease P/MRP protein subunit POP1 gene was upregulated involved in biogenesis of protein.

5.3.6.4. Stress-causative and stress-responsive mechanisms

Several genes implicated in stress-responsive regulation, such as Base excision repair, Proteasome, Lysosome, Endocytosis, two component system, Ubiquitin mediated proteolysis was significantly dys-regulated in Cd exposure groups. For the Cd exposure group, genes involved in the base excision repair such as flap endonuclease -1 FEN1; K04799, RAD2, K04070) and poly [ADP-ribose] polymerase 1 (PARP1, K24070) were significantly down-regulated. The mutM, fpg (formamidopyrmidine DNA glycosylase) was up regulated in Cd stresses group **(Fig 5.7)**.

Genes associated with ubiquitin-mediated proteolysis such as culin 3 (CUL3, id K03869) and ubiquitin-protein ligase (UBE3C, idK10589) were up-regulated, while the rest of genes were down regulated. Gene involved in proteasome assembly such as proteasome subunit beta-5 (PSMA4, idK02737), proteasome subunit beta-1(PSMB6, idK02738), 26Sproteasome regulatory subunit N10 (PSMD4, RPN10; idK03029), 26Sproteasome regulatory subunit T3 (PSMC4, RPT3; K03063), proteasome regulatory subunit T3, (PSMC3, RPT5; K03063), proteosome activator subunit T5 (PSMC3, RPT5; id k03065) was up-regulated **(Fig 5.7)**. Their upregulation demonstrated increased ubiquitin-mediated proteolysis after Cd- exposure.

Gene involved in two component system such as ubiquinol-cytochrome c reductase cytochrome b subunit (id K00142) and uridylyl transferase (K00990) was down-regulated.

The lysosome genes DNASE2; deoxyribonuclease II, GNPTAB; UDP-N-acetylglucosamine-lysosomal-enzyme, GLB1, ELNR1; beta-galactosidase, SCARB2, LIMP2, CD36L2; lysosome membrane protein 2 were up regulated while rest of genes were downregulated **(Fig 5.7)**.

5.3.6.5 Sulphur metabolism

ETHE1; sulfur dioxygenase and SQOR; eukaryotic sulfide quinone oxidoreductase were upregulated in Cd stress samples as compared to control. Cadmium stress can cause oxidative stress and affect the sulphur metabolism in algae **(Fig 5.7)**. Up-regulation of ETHE1 and SQOR indicates an increased need to detoxify sulfide, which can accumulate under stressful situations. This adaptive reaction allows the algae to survive and grow while reducing the negative consequences of cadmium exposure.

5.3.6.6 Genetic information processing

For the Cd exposure group, gene implicated in pre-mRNA-splicing factor ATP-dependent RNA helicase DHX38/PRP16 (PRP16, id K12815), U4/U6 small nuclear ribonucleoprotein PRP31 (PRPF31, id K12844), beta-catenin-like protein 1 (CTNNBL1, id K12864), were up-regulated, while remaining genes exhibited downregulation **(Fig 5.7)**.

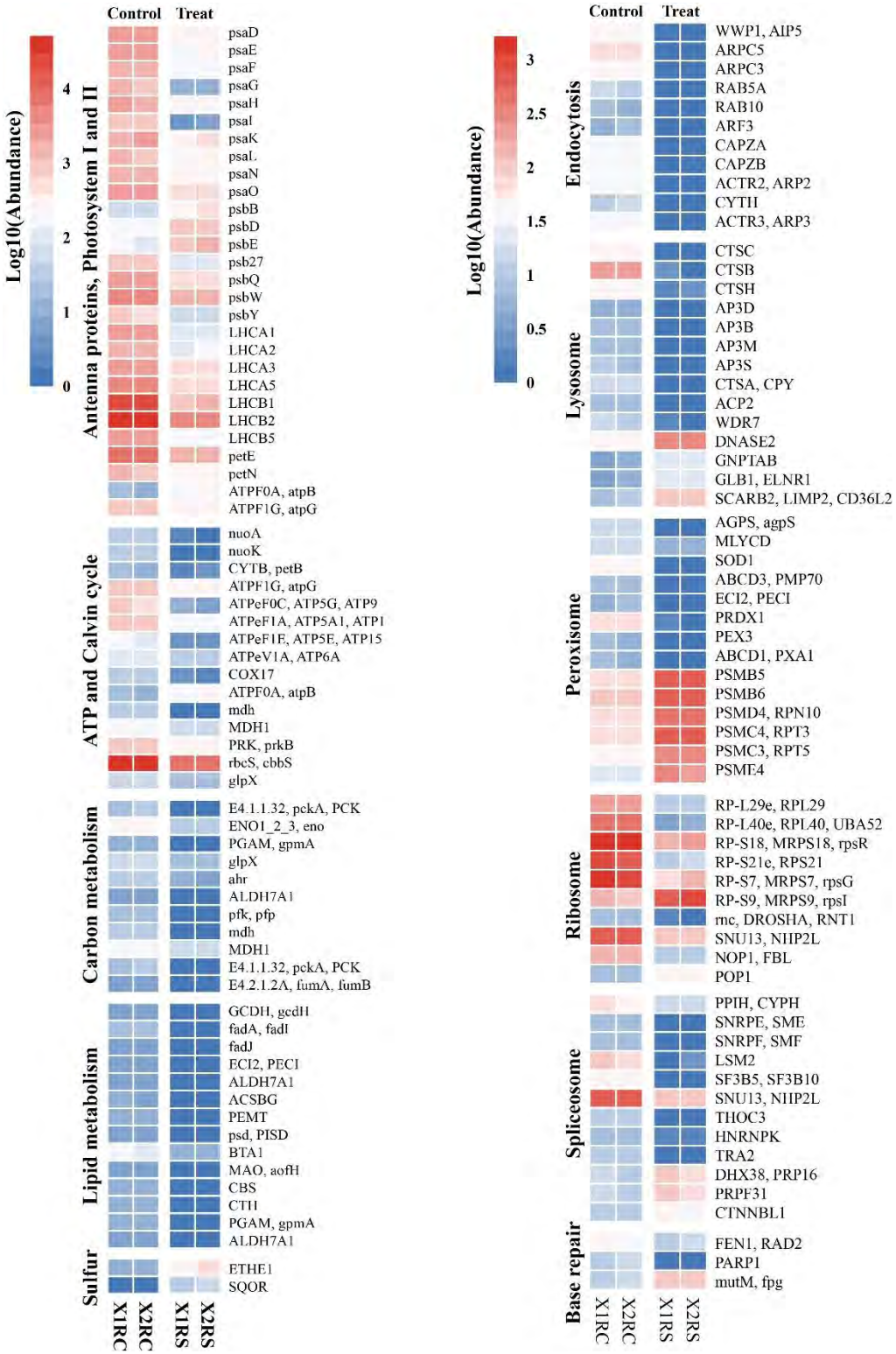


Fig. 5.7. Heat map diagram of relative abundance of genes in control and stressed samples. 1RC and 2RC=control 1RS and 2RS=stressed.

5.4. Discussion

There are few studies on the effects of heavy metals on the members of the family Scenedesmaceae and most of them focus on physiological parameters such as growth,

photosynthetic content, and antioxidant enzymes (Bascik-Remisiewicz et al., 2009; Chandrashekharaiah et al., 2021). The present study discussed how Cd affected the physiological parameters (growth and photosynthesis) of *Desmodesmus subspicatus*, as well as the transcriptome. Cd is a hazardous heavy metal that plays a main role in numerous physiological processes, including photosynthesis and electron transport chains. However, the impact of Cd can occur when organisms consume high levels of Cd (Dias et al., 2013). In the current investigation, the Cd concentration of 15ppm has an impact on *Desmodesmus subspicatus*, with growth and Fv/Fm in *Desmodesmus* sp. drastically falling after 48 hours **Fig 5.2B.** The results show that the 15 ppm Cd concentration in BG_{11} medium with 10% CO_2 exceeded the requirements of development in *Desmodesmus* sp. that cause the oxidative stress in this species. Similar findings were reported by Cheng et al. (2016). In green algae, photosynthesis is one of the most essential functions, and it is commonly targeted for environmental stressors (heavy metals). The Fv/Fm ratio reflects the effectiveness of the photosynthesis by expressing the highest quantum efficiency of PSII in the electron transport chain (Baker, 2008). In this study the Fv/Fm value declined in the Cd treatment as exposure time increased (at 72hrs), showing that Cd was the caused suppression in photosynthesis. Chen et al. (2015) found similar results with sodium metal. To explore the additional regulatory mechanisms associated with heavy metal (Cd) toxicity stress in *Desmodesmus subspicatus*, RNA-seq technology was employed as a powerful tool for globally discovering genes and molecular mechanisms responding to the stress condition (Wang et al., 2017).

5.4.1 Global patterns of transcription in response to Cd stress

However, advances in high-throughput sequencing technology have identified numerous Cd-tolerant genes in different species (Benton et al., 2011; Yang et al., 2015). This progress has considerably enhanced our knowledge of the molecular mechanisms underlying algal response to Cd stress. This sequenced transcriptome of *D. subspicatus* yielded 42,347,092, 47,483,530 for control and 39,333,360, 33,492,989 for Cd treated libraries clean reads, respectively **Table 5.1**. The results indicated that the majority of DEGs were implicated in crucial biological processes, including molecular, metabolic, and cellular functions, aligning with findings reported by Wang et al. (2016). In this investigation, a total of 615 genes significantly different between control and treat cultures. Detailed, the expression of 452 and 163 unigenes was upregulated and

downregulated, respectively, at 72 h compared to the control **(Fig. 5.4)**. The primary transcript groupings were associated with replication, recombination, and splicing. Hanawalt (2007) emphasized the necessity of genome replication and maintenance for a cell's survival. Despite the active nucleotide sequencing and protein metabolism in *Desmodesmus subspicatus*, many DEGs identified in our study may still require functional validation. The KEGG pathway database is a systematic investigation of inner metabolic pathways in the cell and activities of gene products (Wang et al., 2016). The KEGG pathway database is the most extensively utilized database for identifying enriched pathways. The KEGG pathway database is a valuable collection of human-generated maps that show the current understanding of molecular interaction for genetic, metabolic, and environmental information processes and so on. Furthermore, KEGG can be viewed as a virtual biological system that contains many sorts of information required for the restoration of a living species (Kanehisa and Goto 2000).

5.4.2 Cd negatively affected energy metabolism

During the growth of microalgae, photosynthesis is essential to produce starch (carbohydrate), protein, and lipid. The results of the KEGG enrichment analysis showed that genes that were differentially expressed (DEGs) were partially enriched in pathways relevant to photosynthetic organisms, including "Photosynthesis," "Carbon fixation in photosynthetic organisms," and "Photosynthesis antenna proteins." Chlorophyll-encoding genes (P680 and P700) in the reaction centres of photosystems I (PSI) and II (PSII) showed similar expression patterns in both short- and long-term responses to cadmium (Cd) treatment. Initially, Cd treatment reduced the expression of genes that produce the photosystem I P680 reaction centre proteins D1 and D2. After 48 hours, their expression grew dramatically, but then decreased with continued treatment. PSII is made up of a reaction centre complex and a light-harvesting complex (LHC II). It utilizes light energy to split water molecules on one side of the thylakoid membrane's interior cavity and reduce plastoquinone (Girolomoni et al., 2019). It establishes a proton gradient on both sides of the thylakoid membrane. A pigment study of *Chlamydomonas reinhardtii* found that reactive oxygen species (ROS) degraded the PSI light-harvesting complex (LHC) in high salt conditions, as well as the PSII protein involved in oxygen release (Subramanyam et al., 2010;). Differentially expressed photosynthetic genes (DEGs) are critical for giving cadmium resistance. The detrimental effects of Cd on green algae primarily affect the

photosynthetic system. Cadmium can reduce the expression of genes involved in chlorophyll production.

Chlorophyll is essential for absorbing light energy during photosynthesis, and any disturbance in its production can greatly reduce the efficiency of the process. Photosynthetic species are extremely sensitive to metal compounds, which can affect a variety of physiological systems in higher plants, including water intake, respiration, mineral nutrient absorption, and photosynthesis (Burzynski and Zurek, 2007). In this investigation, 28 transcripts associated with the photosynthetic system were discovered. Among these 28 genes, 10 were downregulated (in stressed samples) and were related with photosystem I (PS I), while 7 were linked to photosystem II. Only two of these 7 PS II genes were elevated, whereas the rest were downregulated in the Cd-stressed cells. The findings suggest that photosynthesis-related genes could be a crucial focus for future Cd stress studies. In our findings, the PsbB, psbD, and psbE genes were higher elevated in Cd-stressed samples than in controls **(Fig 5.7)**. *Desmodesmus* sp. survival in cadmium-contaminated environment may be dependent on the regulation of these genes, which may contribute to Cd tolerance. Baron et al. (1995) discussed how Cu directly inhibits photosynthesis by preventing electron transport, notably inside PSII. Geiken et al. (1998) discovered that Cd affects the oxygen-evolving complex in leguminous plants, resulting in PSII disassembly, whereas Herbette et al. (2006) discovered that Cd inhibits PSII protein expression in Arabidopsis. According to Zhou et al. (2006), Cd's inhibitory site in M. *aeruginosa* is most likely found at the ferredoxin/NADP+-oxidoreductase enzyme, which is located at the end of electron transport chain.

Previous investigation found that Cu (at doses of 0.5 and 1.5 μM) and Cd (at values of 1.0 and 2.0 μM) reduced the abundance of the psbA gene in *Chlorella vulgaris*. The psbA gene encodes the D1 protein, which is an important component of photosystem II (PSII). A decrease in psbA mRNA transcripts may result in poorer PSII activity and electron transfer rates in *C. vulgaris*, as evidenced by a fall in chlorophyll content. Differentially expressed genes (DEGs) involved with photosynthesis are critical for cadmium tolerance, as photosynthetic pigments are the principal targets of Cd toxicity (Yu et al., 2018). A prior investigation on Cd toxicity in green algae found DEGs in cells exposed to 2.5 ppm Cd for 6 and 96 hours. After 6 hours of incubation, it was found that 11 ribosomal genes and three photosystem-regulating genes (psbA, PetF, and psbP) had been upregulated (Zhu et al., 2019).

Several studies have shown that cadmium (Cd) can reduce photosynthetic activities in green algae such as, *Chlamydomonas reinhardtii* and *Microcystis aeruginosa* (Aksmann et al., 2014; Rihab et al., 2017; Samadani et al., 2018). Nonylphenol has also been demonstrated to reduce photosynthetic activity in green algae (Gao and Tam, 2011). However, there is little knowledge on the molecular processes by which Cd influences the photosynthetic system, and the findings of this study could help fill that gap.

Further research has shown that copper (Cu) inhibits the physiological proliferation of algal cells (Beauvais-Flück et al., 2019). Few research has used to investigate cellular toxicity pathways by Cu in microalgae on the molecular level. For example, when *C. reinhardtii* was exposed to 106 μM Cu for 2 hours, the transcriptome changed, with 624 genes upregulated and 773 genes downregulated. The impacted genes were predominantly involved with pathways related to gene regulation, redox homeostasis, growth, and photosynthesis, with significant inhibition of genes related to photosystem I (PSI).

5.4.3 Oxidative phosphorylation

Cells use enzymes in the metabolic process known as oxidative phosphorylation to oxidise molecules. The oxidation process produces energy that promotes the synthesis of ATP from inorganic phosphate and ADP (Powell and Somero, 1986). Most aerobic species contain this pathway because it takes place in the mitochondria and is more energy-efficient than other fermentation processes such as anaerobic glycolysis (Guo et al., 2016). Reactive oxygen species are produced by oxidative phosphorylation, even though it is a crucial component of metabolism, these ROS can generate more radicals, which can damage cells (Powell and Somero, 1986). In current study, ten differentially expressed genes involved in oxidative phosphorylation may be useful targets for studying oxidative stress. Among them, ATPF0A and atpB genes, which are related with H^+ carrying ATPase subunits, were increased (**Fig. 5.7)**. In contrast, the remaining genes were downregulated, possibly causing considerable cellular harm. According to Lu et al. (2014), downregulation of photosynthesis and ATP synthase is an important indication of photoinhibition. The F-type H^+-ATPase on chloroplast thylakoid membranes is a multi-subunit complex that generates ATP via light-induced electron transport and proton gradients (Mellwig and Böttcher, 2003). This enzyme uses proton electrochemical gradients to synthesise ATP from ADP and inorganic phosphate (P_i). An increase in chlorophyll accumulation improves light quantum fixation, ATP production, photosynthetic phosphorylation which is consistent with the observed overexpression of ATPF (ATP synthase) in chloroplasts and the results of ATP accumulation assays in

protein expression profiles. Furthermore, earlier research has demonstrated that increased expression of several ATPF genes increases photophosphorylation (Sa et al., 2020).

5.4.4 Regulation of Carbon metabolism pathways: Glycolysis, Pentose Phosphate Pathway, TCA Cycle, and Fatty acid synthesis

The carbon metabolism pathway consists mostly of glycolysis, the pentose phosphate pathway, the TCA cycle, and fatty acid synthesis. **Figure 5.7** shows changes in gene expression associated with these processes. All the genes involved in these processes were downregulated. The genes associated in mannose and fructose metabolism, starch and sucrose metabolism, pyruvate metabolism glycolysis/gluconeogenesis, dicarboxylate metabolism glyoxylate and, and the citrate cycle (TCA cycle). In the previous literature, two genes involved in energy production were altered by Cd exposure these are pyruvate metabolism dysregulation (ACS) and glycolysis downregulation (TPI). This shows that Cd exposure may influence ATP production, however the overall effects are unknown. Downregulation of ADPGlc causes the buildup of alpha-D-glucose-1P, a precursor for sucrose and starch synthesis (Cereijo et al., 2018).

This downregulation is related with an increase in energy reserves, such as sucrose and starch metabolism, corresponding with observations in *Elodea nuttallii* subjected to 500 µg/L of Cd for 24 hours. Jamers et al. (2013) discovered that in green algae exposed to 114.8 µg/L Cd for 24 hours, two genes were activated and four were downregulated in the energy metabolism process. However, this study made no distinction between energy production and the energy reserves from carbohydrate pathways. When there is comparison to the Cd exposure group, 4-nNP had a similar effect on the energy reserves route by downregulating ADPGlc. Three more genes were linked to energy production dysregulation: pyruvate metabolism upregulation (PFLD), glycolysis downregulation (TPI), and TCA cycle dysregulation (IDH3). Suppressing the TCA cycle has a considerable impact on ATP generation (Zhang and Bryant, 2014). According to previous study, Cd and Cu can reduce CO_2 absorption by altering the enzymes activity involved in the photosynthetic carbon-reduction cycle. Sheoran et al. (1990) discovered that Cd inhibits 3-phosphoglyceric acid kinase (PGK), an enzyme involved in the photosynthetic carbon reduction cycle, in leaves of leguminous plant.

Burzynski and Zurek (2007) found that Cd and Cu decreased protein synthesis in cucumber cotyledons in addition to inhibiting PGK and glyceraldehyde-3-phosphate

dehydrogenase, another enzyme in this cycle. According to Stiborová et al. (1986), the effects of both metals on PGK in maize leaves were comparable. For plants to grow and develop, rubisco, the carboxylase that causes the rate-limiting step in carbon fixation, is necessary. Cu and Cd suppress the transcription of the rbcL gene, which encodes the major subunit of Rubisco, according to this study, which measured the abundance of the gene using real-time PCR. Therefore, it is postulated that Cu and Cd may hinder CO_2 assimilation by inhibiting gene expression on mRNA in addition to lowering enzyme activity that encode enzymes particularly rbcL.

5.4.5 Sulphur metabolism

Differently expressed genes (DEGs) relevant to the antioxidant system, including those involved in "antioxidant activity" and "cellular oxidant detoxification," were linked to Cd exposure. Plant antioxidant mechanisms are predicted to play a critical role in reducing Cd stress. Superoxide dismutase (SOD) transforms oxygen radicals (O^{2-}) into hydrogen peroxide (H_2O_2), while other enzymes including L-ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD), work cooperatively to eliminate hazardous H_2O_2 (Zhang and Reynolds, 2019). Additionally, sulfur-containing antioxidants such as thioredoxin (Trx) may aid in Cd detoxification (Smiri et al., 2011; Zhang et al., 2023a). Cyanobacteria require sulphur compounds for metabolism, growth, and resistance to oxidative stress, as well as for secondary metabolism. The biosynthetic pathways of sulphur compounds in cyanobacteria use enzyme systems comparable to those in bacteria, green algae, and plants, indicating a wide range of operations within a single phylum (Karvansara et al., 2024). In this work, transcriptome analysis revealed that Cd exposure caused coordinated regulation of gene expression for involved genes in sulphate and nitrogen metabolism. The expression of genes involved in sulphur metabolism increased **(Fig. 5.7)**. This increase provided the raw materials required to synthesise critical components such as cysteine, which is essential for metal-chelating proteins, resistance-related metalloproteins, and ABC transporters. This reaction is an early, short-term strategy for dealing with Cd stress (Tian et al., 2022). Cadmium (Cd) can improve nitrogen and sulphur absorption by enhancing the expression of their transporters in reaction to Cd stress. This regulation ensures that there is an adequate supply of nitrogen and sulphur to meet increased glutathione synthesis and energy demands, boosting metal tolerance. It has been observed that numerous genes involved in sulphur metabolism are rapidly altered after Cd exposure. For example, genes that code for sulphate transporters, such as Sultr1;1 and Sultr2;1, are quickly upregulated in the sulphur assimilation pathway (Herbette et al., 2006).

Furthermore, after Cd treatment, genes like MYO-129790 and MyO-129780, which are concentrated in the sulphur transport pathway, largely encode enzymes involved in biosynthesis of cysteine.

Cysteine is an essential part of metal-bound metallothionein, acting as a precursor for glutathione and metal chelate synthesis (Hendrix et al., 2017). Its expression increases dramatically in response to Cd exposure in the short term, emphasising its importance in chelation. However, cysteine expression declines over time, most likely due to the cells activating alternate defence mechanisms, such as those involving the photosynthetic system. Similarly, Cd treatment reduced the expression of genes encoding ammonium and nitrate transporters in *E. nuttallii*, demonstrating that metals have a broad impact on nutrient absorption (Regier et al., 2013; Beauvais-Flück et al., 2018). Our findings demonstrated that genes involved in nitrogen metabolism, such as those encoding nitrate transporters, were downregulated with Cd exposure. Previous study indicates a link between sulphur and nitrogen metabolism. Furthermore, ATP-binding proteins involved in nitrogen transport and ABC transporters associated to sulphur metabolism exhibited similar modulation in both short- and long-term Cd treatments, demonstrating their connection (Tian et al., 2022).

5.4.6 Transcriptional factor affected by Cd stress

Tanscriptional factors (TFs) are proteins that play an important role in controlling the process of transcription, translation, and spliceosome. They bind to DNA sequences and regulate the the process of transcription, affecting how genes are turned on or off in response to environmental conditions (Xian et al., 2020). In our study, DHX38, PRP16, PRPF31 and CTNNBL1 genes were found to be up regulated during Cd stress (**Fig. 5.7)**, which indicates that above-mentioned TFs are involved against Cd response by *Desmodesmus* sp. The findings of this study show that the greatest number of genes were observed in spliceosome processing. Most genes that are involved in genetic information processing, specifically for transcription, translation, splicing, and DNA repair (Yamada et al., 2018).

PRP31, ZOP1 and STA1 are essential for both stress and developmental responses. Under cold stress, PRP31 necessary not only for pre-mRNA splicing but also for regulating the expression of genes responsive to cold. Our findings show that the splicing machinery serves multiple functions, including, gene control, pre-mRNA splicing and stress response and transcriptional gene silencing, (Du et al., 2015). RNA splicing is critical for mRNA

maturation in humans. The spliceosome is made up of functional proteins, such as the RNA-protein complex that includes precursor mRNA, five small nuclear ribonucleoprotein particles (snRNPs) (, U4/U6, U1, U5 and U2), and several non-snRNP protein components. (Yuan et al. 2005). PRPF 31, a splicing factor, interacts with U4/U6 di-snRNP and U5 snRNP (Schaffert et al., 2004). PRPF31 knockdown with siRNA lowers the expression of retina-specific mRNA transcripts. Other genes associated with splicing factors include RNA processing genes (PRPF19, PRPF8, PRPF3, and PRPF4), phototransduction genes (GNAT1/2, RHO, and RP1), photoreceptor cell structure genes (FSCN2, SEMA4 and ROM1,), and transcription factors (CRX) (Azizzadeh Pormehr et al., 2020). Non-snRNP splicing factors, including CWC27 and DHX38, are linked to autosomal recessive retinitis pigmentosa (ARP) (Amjad et al., 2014). In immortalised retinal pigment epithelial cells, knocking down CWC27, an uncharacterized splicing factor, or CWC22, a binding protein for CWC27, causes increased expression of inflammation-related genes and decreased expression of mitochondrial enzyme genes involved in oxidative phosphorylation. This imbalance causes increased immunological responses and oxidative damage (Busetto et al., 2020).

5.5. Proposed toxic molecular mechanism for *Desmodesmus subspicatus* exposure to Cd

Cd exposure would initially influence the photosystem because it reduces chlorophyll biosynthesis by downregulating related genes. This inhibition can induce reactive oxygen species (ROS) by inhibiting photosystems I and II, oxidative phosphorylation, and disrupting cellular redox equilibrium. The higher ROS levels cause DNA damage and protein breakdown. Concurrently, RNA transcription becomes dysregulated, resulting in a cascade of gene expression alterations. These changes have an impact on a variety of metabolic pathways, including secondary metabolite biosynthesis, microbial metabolism in a variety of environments, cofactor biosynthesis, amino acid biosynthesis, carbon metabolism, 2-oxocarboxylic acid metabolism, fatty acid metabolism, nucleotide metabolism, and sugar biosynthesis. Notably, in the Cd exposure group, genes associated to photosynthesis and oxidative phosphorylation significantly downregulated **(Fig 5.7)**.

Second, Cd exposure causes ROS-induced activation of sulphur metabolism genes **(Fig 5.7)**. A detailed investigation into the adverse effects of heavy metals and organic contaminants on algae is required. Overall, the transcriptome profiles provide useful information about the mechanisms of toxicity, detoxification, adaptation, and repair in

Desmodesmus subspicatus exposed to Cd at environmentally relevant doses. The toxicological and resilience pathways for cadmium in *Desmodesmus subspicatus* can be identified by analysing the effects on cell signalling networks. Furthermore, transcriptomics has the potential to uncover biomarkers of heavy metals or organic contaminants for application in field investigations and ecological risk assessments as early warning signs in ecosystems.

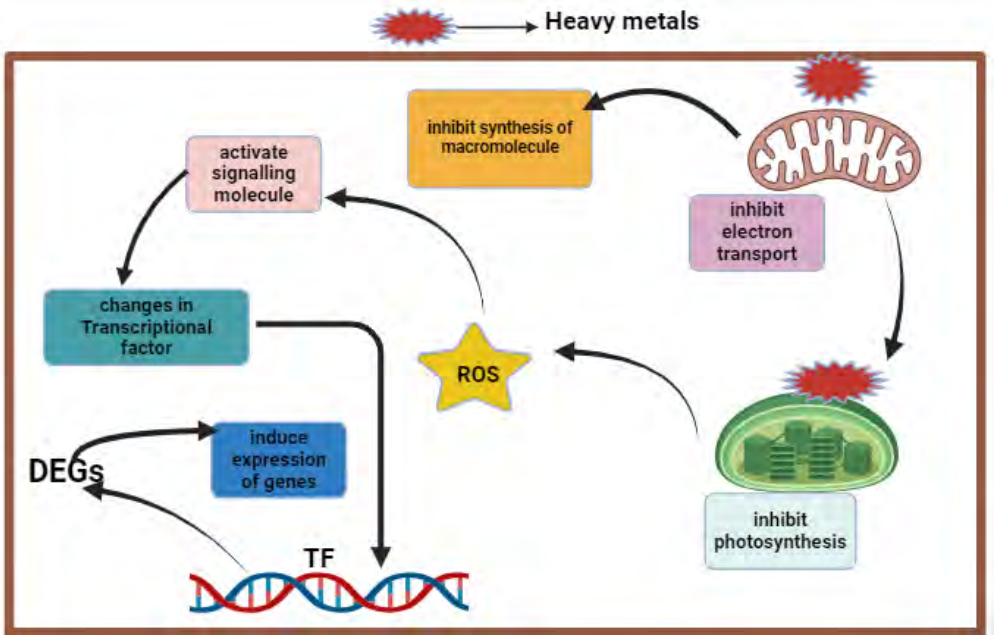


Fig. 5.8. Schematic representation of induction of expression of genes in response to heavy metals.

Conclusion

- This study is an essential reference for future investigations on heavy metal tolerance in green algae, as well as a valuable source of transcriptome data from a non-model species surviving in harsh environments.
- The current study revealed the identification of multiple genes that are involved in Cd tolerance in *Desmodesmus subspicatus*.
- The results of transcriptome analysis, indicate the exogenous Cd affected multiple genes involved in metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environment, biosynthesis of cofactor, biosynthesis of amino acid, carbon metabolism, 2-oxyocarboxylic acid metabolism, fatty acid metabolism, nucleotide metabolism, biosynthesis of nucleotide sugar.

- 615 gene present significantly different between control and treatment experiment. 452 gene is significantly down regulated and 163 is significantly up regulated at 72hrs during Cd stress.
- KEGG pathway analysis showed 10 main pathways to be involved. Among these 10 pathways, metabolic pathways have a higher number of genes.
- Exogenous Cd down-regulates many photosynthetic and oxidative phosphorylation genes, according to transcriptome research.
- The findings show that Cd exposure slows the growth of *Desmodesmus subspicatus*, dramatically reduces photosynthetic pigment concentration, and suppresses the expression of photosynthetic system genes.
- This chapter serves as an important reference for future heavy metal tolerance studies in green algae, as well as rich transcriptome data from a non-model species that has evolved to harsh environments.

CHAPTER 6

CONCLUSIONS

Pakistan has unique geographical and environmental characteristics that encourage widespread biodiversity. The plant flora of Poonch, AJK has been well studied. However, algae have gained a little attention in the Hajira Poonch AJK. The current study focuses on the collection, isolation, and characterization of native algae species.

1. AJK has diversity of algae. However, this area is not well explored hence there is a chance to explore the algal species.

2. During the isolation different species of algae has been identified. These strains belonged to different species of algae which include 72% of green and 28% of blue green algae. Different species of diatoms has been recorded during the study.

3. Based on *in vitro* culturing, 16 species of family Scenedesmaceae has been recorded along with other green and blue green algae. The species of family scenedesmaceae include: *Scenedesmus hystrix, Scenedesmus acutiformis, Scenedesmus ecornis, Scenedesmus acunae, Scenedesmus obliquus acunae, Scenedesmus ellipticus, Scenedesmus dimorphus, Desmodesmus communis. Scenedesmus rubescens, Desmodesmus subspicatus, Scenedesmus javanensis. Scenedesmus dimorphus, Scenedesmus incrassatulus, Scenedesmus* sp.*, Scenedesmus bijugus, Scenedesmus prismaticus* recorded first time from Hajira Poonch AJK.

4. Seven species of family Scenedesmaceae (*Desmodesmus communis* (strain H10), *Desmodesmus subspicatus* (strain R3), *Scenedesmus dimorphus* (strain H9), *Scenedesmus* sp. 1 (strain H2) *Scenedesmus* sp.2 (strain 2CL), *Scenedemus* sp.3 (strain 4) and *Scenedesmus bijugus* (strain 15) characterize morphologically and on molecular basis.

5. Screening of different strains of algae has been studied on 96-well plate against different heavy metals (Cd and Pb). Among these *Scenedesmus dimorphus*, *Desmodesmus subspicatus* showed a better response against Cd and Pb as compared to other strains.

6. The biosorption of Cd and Pb was studied by *Desmodesmus subspicatus* and *Scenedesmus dimorphus* in the form of dried and living biomass.

7. The value of the q_{max} of dried biomass of *Desmodesmus* sp. for Cd and Pb was 64.1 and 62.5 mg/g; for *Scenedesmus* sp. it was 128 mg/g (Cd) and 102 mg/g (Pb).

8. In case of living adsorbent the higher concentrations of Cd and Pb ions had an impact to *Desmodesmus subspicatus* and *Scenedesmus dimorphus*. The loss of chlorophyll and decline of biomass were observed at higher concentrations of metals.

9. The value of the q_{max} of living biomass of *Desmodesmus* sp. for Cd and Pb was 83.3 and 71.4 mg/g; for *Scenedesmus* sp. it was 212 mg/g (Cd) and 232 mg/g (Pb) in case of living biomass.

10. The biosorption of both metals followed pseudo-second-order kinetics, mainly based on chemical adsorption. The experimental data were well explained by the Langmuir isotherm.

11. FTIR data showed the role of carbonyl, hydroxyl, amine, and amide groups and sulfoxide, present on the surface of *Desmodesmus* and *Scenedesmus* sp. in the adsorption process.

12. In the transcriptome analysis of *Desmodesmus* sp. comparison of control and stress result in identification of 615 significant differentially expressed genes.

13. The number of down regulated genes was higher than up regulated genes in stressed sample.

14. The KEGG pathway analysis results in 10 main pathways of KEGG databases.

15. Metabolic pathways have a higher number of genes and biosynthesis of nucleotide sugars has lower number of genes.

16. Cd retarded the growth rate in *Desmodesmus subspicatus*, which was followed by a significant reduction in photosynthetic pigment and a decrease in the expression of genes linked with the photosynthetic system and oxidative phosphorylation.

17. The upregulation of ETHE1 and SQOR suggests a heightened requirement for detoxifying sulfide, which may accumulate under stressful conditions.

FUTURE RECOMMENDATIONS

- Continue exploring and cataloguing the diversity of algae, both in terms of species and strains, particularly in various ecological niches such as extreme environments, freshwater, marine, and wastewater systems.
- Emphasize the identification and isolation of novel algal species with unique characteristics, including high growth rates, resistance to environmental stressors, and valuable biochemical profiles.
- Develop advanced cultivation techniques to optimize the growth and productivity of specific algal strains. Explore innovative photobioreactor designs, growth media formulations, and cultivation strategies to enhance biomass yield, lipid production (for biofuel), and other valuable metabolites.
- Implement high-throughput screening methods for rapid assessment of algal strains with desirable traits, such as high lipid content, nutrient uptake efficiency, and tolerance to environmental stressors. Use automated systems to accelerate the identification and selection of promising strains for various applications.
- Explore and identify algal strains with enhanced heavy metal tolerance and accumulation capabilities.
- To properly utilize microalgae in heavy metal phycoremediation, further research is needed in genetic engineering, immobilization techniques, pretreatment techniques, and integrate with other technologies.
- Until now, complete heavy metal remediation has been rare. This can be overcome by using genetic engineering, metabolic engineering, or protein engineering, which allow for modifications to genes, proteins, or metabolic pathways, resulting in overexpression of the protein used for remediation.
- By using transgenics, one can also enhance the productivity of the by-product like pigments, proteins andvitamins, etc.
- More research is needed to understand how algae tolerate heavy metals and their resistance mechanisms. More research is needed to turn it into a system that can detect and remove heavy metals from the environment.
- Extend research on scaling up algae-based bioremediation methods from lab to field conditions. Evaluate the economic viability and practicality of large-scale algae-based bioremediation projects.

- Create cost-effective tools for monitoring and evaluating the effectiveness of algae-based bioremediation in practical applications. In situ monitoring of metal concentrations and algal biomass can be accomplished using modern sensor methods.
- In future, the expression analysis of different genes can be studied by using the transcriptome data (in Chapter 5).
- Transcriptome analysis of different strains of Family Scenedesmaceae can be carried out to know about the upregulation and downregulation of genes by different environmental stressor.
- Whole genome sequencing of different strains can be conducted in future.

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APPENDICES

Composition of Bold and basal media (Bischoff and bold, 1963) is as follow:

Appendix 1

Stock solution	Components	Concentration gm./l H_2O	Volume
Macronutrients:			
SL1	$NaNO_3$	25	10 ml
SL 2	$CaCl_2.2H_2O$	2.5	10 ml
SL 3	NaCl	2.5	10 ml
SL 4	K_2HPO_4	7.5	10 ml
SL 5	KH_2PO_4	17.5	10 ml
SL 6	$MgSO_4.7H_2O$	7.5	10 ml
Trace elements solution:			1 ml
SL 7	$ZnSO_4.7H_2O$	8.82	
	$MnCl_2.4H_2O$	1.44	
	MoO_3	0.71	
	$CuSO_4.5H_2O$	1.57	
	$Co(NO_3)_2.6H_2O$	0.49	
Boron solution			1 ml
SL 8	H_3BO_3	11.42	
Alkaline EDTA solution:			1ml
SL 9	EDTA	50	
	KOH	31	
Acidified ferric solution:			1 ml
SL 10	$FeSO_4.7H_2O$	4.98	
	H_2SO_4	1 ml	

APPENDICES

Appendix 2

Composition of BG11 media

Stock solution	Components	Concentration gm./l H_2O	Volume
SL 1	$Na_2MgEDTA$	0.1	10 ml
	Ferric ammoniumcitrate	0.6	10 ml
	Citric acid	0.6	10 ml
	$CaCl_2.2H_2O$	3.6	10 ml
SL 2	$MgSO_4.7H_2O$	7.5	10 ml
SL 3	$K_2HPO_4.3H_2O$	4.0	10 ml

Appendix 3

Micronutrients

			1 ml
SL 4	H_3BO_3	2.86	
	$MnCl_2.4H_2O$	1.81	
	$ZnSO_4.7H_2O$	0.22	
	$CuSO_4.5H_2O$	0.079	
	$NaMoO_4.2H_2O$	0.391	
	$CoCL_2.6H_2O$	0.050	

APPENDICES

Appendix 4

CTAB Buffer (pH: 8)

Components	Amount (gm./100 ml)
CTAB	2.0
NaCl	8.19
Tris HCL	1.2
EDTA	0.58

1 M Tris HCL:

Dissolve 6.05 grams of Tris base to 45 ml of distilled water. Adjust pH to 8 using HCL. Autoclave before use.

Appendix 5

10X CTAB:

Components	Amount (gm./ml)
NaCl	4.1
CTAB	10
Distilled water	100 ml

Appendix 6

10X TBE Buffer (pH: 8)

Components	Amount (gm./ml)
Tris Base	108
0.5 M EDTA	40
Boric acid	55
Distilled water	1 L

APPENDICES

0.5 M EDTA:

Add 7.306 gram of EDTA in 50 ml of distilled water. Adjust pH to 8.0 and autoclave before use.

Appendix 7

1% Agarose gel:

Components	Amount (gm./ml)
1X TBE	10 ml
Agarose	1 gm.
Ethidium bromide	2 µl
Distilled water	90 ml

Appendix 8

Chloroform isomyl-alcohol (24:1):

Components	Amount
Chloroform	24 ml
Isomyl alcohol	1 ml

Appendix 9

T.E Buffer:

Components	Amount (ml)
1 mM Tris HCL	0.5
0.5 M EDTA	0.1

Add in 50 ml of water and adjust the pH to 8.0.

Appendix 10

Nutrient agar

Components	Amount
Nutrient broth	8 ml
Agar	14 gm.

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