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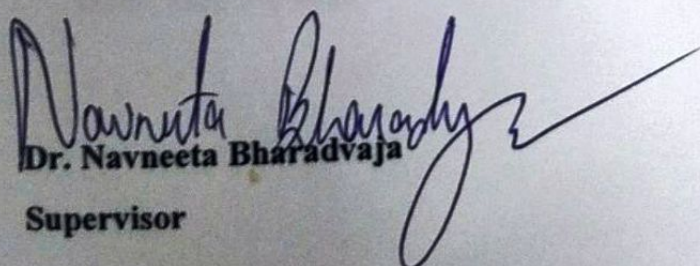


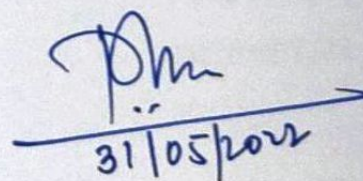
CERTIFICATE

This is to certify that the Project dissertation titled “**Bioplastic production from *Chlorella* sp., and synthesis of biochar from residual biomass, characterization and its application**” which is submitted by **Neha Nanda, 2K20/IBT/07**, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision. To the best of our knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

Place: Delhi

Date: 30 May 2022


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Supervisor


31/05/2022

Prof. Pravir Kumar

Head of Department

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CANDIDATE'S DECLARATION

I, **Neha Nanda, 2K20/IBT/07** student of M.Tech (Industrial Biotechnology), hereby declare that the project Dissertation titled "**Bioplastic production from *Chlorella* sp., and synthesis of biochar from residual biomass, characterization and its application**" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associate-ship, Fellowship or other similar title or recognition.

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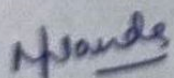
Date: May 30, 2022

Neha Nanda

2K20/IBT/07

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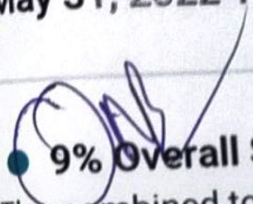
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**M. Tech
(Industrial Biotechnology)**

Neha Nanda

2022

**Bioplastic production from *Chlorella* sp., and synthesis of
biochar from residual biomass, characterization and its
application**

A PROJECT REPORT

SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF TECHNOLOGY
IN
INDUSTRIAL BIOTECHNOLOGY

Submitted by:

Neha Nanda
2K20/IBT/07

Under the supervision of

DR. NAVNEETA BHARADVAJA



DEPARTMENT OF BIOTECHNOLOGY

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MAY, 2022

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Neha Nanda
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ABSTRACT

Wastewater and plastic pollution have been persistent global issues of concern for years. Development of a cheap, efficient and eco-friendly remediating agent like biochar can be a step forward in water treatment. Hence in this study, biochar was synthesised from *Chlorella sorokiniana* and *Nephrolepsis auriculata*. A comparison of their physico-chemical properties was drawn and the algal biochar was tested for remediation of the Methylene blue and heavy metal chromium from synthetic wastewater solutions. Moreover, various parameters of the adsorption experiments like pH, biochar dosage and initial pollutant concentration were optimized. It was concluded that algal biochar could completely remediate the organic cationic dye within 30 minutes but could only remove 75% chromium even after 3 hours of contact time. In continuation with a lookout for greener alternatives to plastic, the bioplastic polyhydroxybutyrate was extracted from *Chlorella minutissima* as well as *Chlorella sorokiniana*. Algae are better alternatives than plants and microbes due to their higher lipid content, non-competitiveness with food and excellent growth rate even on wastewaters. It was observed that the species *minutissima* accumulated higher amounts of PHB intracellularly, than the *sorokiniana* species. The project aims to highlight the potential of microalgae as a solution to two of the major environmental problems: water pollution and plastics.

Keywords: Pollutant, Biochar, Chlorella, polyhydroxybutyrate, wastewater remediation

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LIST OF SYMBOLS AND ABBREVIATIONS

h	Hour
MB	Methylene Blue
RE	Removal Efficiency
<i>Cm</i>	<i>Chlorella minutissima</i>
<i>Cs</i>	<i>Chlorella sorokiniana</i>
ddH ₂ O	Double distilled water
MABC	Microalgal biochar
RT	Room temperature
PHB	Polyhydroxybutyrate
LAF hood	Laminar Air Flow Hood
HTC	Hydrothermal Carbonisation
EBC	European Biochar Certificate
°C	Degrees Celcius
temp	Temperature

CHAPTER 1- INTRODUCTION

1.1 Background

Water pollution is a worldwide issue that has only intensified over time due to anthropogenic activities and improper waste disposal. Textile, fertilizer, pharmaceutical and leather industries release non- biodegradable heavy metals and dyes into water. These include nickel, mercury, chromium, lead, cadmium, zinc etc. Dyes like crystal violet and methylene blue are also commonly present in untreated effluent discharge. Lack of implementation of stringent environmental norms and wastewater treatment plants has magnified the adverse effects of these contaminants in India [1]. To remediate them from wastewater in a cost effective and eco-friendly manner, biochar has been explored as a superior adsorbent over other physico-chemical wastewater treatment strategies. Plastics are undebatably a hot-topic of discussion across international forums and global summits due to their huge ecological footprint. Bioplastics produced from renewable sources are a result of lookout for sustainable alternatives. The successful commercial exploitation of microalgae for bioplastic production requires identification of the most promising and efficient species and taking into consideration the physiochemical factors that affect the production pathways. (García et al. 2021). The *Chlorella* species is a freshwater green alga comprising 51 to 58% protein of the total dry cell weight. Its thick cell wall imparts it better crack resistance and thermal stability than *Spirulina*. *C. sorokiniana* is widely used in making starch blends because of its high gelatinization temperature of 110°C. There are many studies on these two microalgae due to their small cell size, which makes them desirable in film and fiber market where particle size is a crucial determinant. In addition, their small size and high protein content makes it easier for them to be converted into bioplastic on a large scale, in a cost-effective manner without generating much waste in pre-treatment. *Chlorella* and *Spirulina* have the potential to remediate the wastewater they grow in and recycle the CO₂ bubbled into the growth medium in the form of higher biomass productivity. Bioplastics made wholly of *Chlorella vulgaris* (CV) are brittle

irrespective of the species used, but when plasticized, CV bioplastics exhibit better plastic-like behavior (yielding and initial modulus) than *Spirulina*.

1.2 Importance of Study

Textile dyes are recalcitrant organic compounds due to their photostability, resistance to aerobic digestion and oxidants. Methylene blue dye has been targeted in this study. This toxic dye is harmful for humans and plants alike. Its exposure leads to nausea, skin diseases, rhinitis and disturbed blood pressure [2][3]. In plants, it reduces the photosynthetic efficiency, growth and induces oxidative stress [4]. It also mutates microbes and reduces bacterial luminescence. Thus it disrupts the food chain and contribute to global warming by affecting the carbon sequestering ability of phytoplanktons [5][6]. But this dye can be abated efficiently by using microalgae derived biochar. The heavy metal contamination of water bodies and soil is attributed to the discharge of untreated water from industries. Heavy metals like chromium persist in the ecosystem to cause adverse health effects like liver and kidney damage, pulmonary edema, cancer, eye and skin irritation. Among plants, Cr reduces the transpiration rate and photosynthetic pigments, damages root cells and affects nitrogen uptake [7]. These problems can be mitigated by using a cheap eco-friendly adsorbent like biochar. Conventional methods of contaminant removal like filtration, flocculation, solvent extraction, chemical precipitation and electrochemical treatment suffer from inefficiency at lower adsorbate concentrations. They are unable to completely transform the pollutants into less harmful or biodegradable form. They are also complex, energy demanding, polluting and expensive to maintain. On the contrary, biochar reduces these inorganic and organic pollutants and even pathogenic organisms by restricting their mobility, bioavailability and toxicity. It can be produced from agricultural residues, animal manure and algae, that are otherwise left unutilized. Its high carbon content, surface area, porosity cation exchange capacity and stability renders it as a efficient treatment option [8][9][10].

The second part of this research addresses the burning issue of global plastic pollution. Population explosion has led to an ever increasing demand for plastic commodities. On an average, an annual demand of 140 million tons of plastic requires 150 million tons of fossil fuel as an input. If this trend continues, estimates are that by 2050, plastic will outweigh all the fish in the sea [11]. Petroleum based plastics have long been used and offer convenience, versatility, durability and

affordability due to well established processes and mature technology [12]. But they are a threat to the ecosystem as they consequently accumulate in the environment, being non-biodegradable. They are recalcitrant and leach into water bodies to form hazardous chemicals. Micro plastics are becoming a matter of growing concern due to their ubiquitous presence in water, food and air samples as well as inside the human body. About 8 million tons of plastic leak into the oceans annually. This can be alleviated by redesigning packaging materials. Plastic debris in the oceans and seas affect aquatic species through ingestion, entanglement and suffocation, eventually leading to death in many cases [11]. Plastic waste is piling up at an annual rate of 25×10^6 tonnes globally, completely jeopardizing the solid waste management system. Hence it is rightly termed as ‘White pollution’ and it will not be an exaggeration to say that we live in a plastisphere [13]. Many countries have recently banned single-use plastics (SUP) like disposable bottles, straws and polybags. While crude oil prices are at an all time high and our fossil reserves are fast depleting, need of the hour is to look for sustainable and greener alternatives. Extensive research efforts are being carried out in this direction. As a result, biopolymers like Polyhydroxyalkanoates (PHA) have recently gained popularity. They offer the dual advantage of being biodegradable and at par with the synthetic plastics in mechanical and physical properties. They decompose about 60% or more into CO₂, water and compost by the surrounding microbes within 3 months while petrochemical-based plastics take 1000 years for the same . They have reduced carbon footprint and managed end-of- life [14]. This study explores algae as a source of bioplastic production over plants and microbes because it does not compete with food or feed, requires less attention and minimal nutritional requirements. It can thrive on wastewaters with an excellent growth rate and photosynthetic efficiency. Moreover it has high lipid accumulation capacity and utilizes cheap carbon substrate, in contrast to microbes.

1.3 Objectives of study

The study aims to achieve following objectives:

- Synthesis and Characterisation of Biochar from *Chlorella sorokiniana* and *Nephrolepsis auriculata*
- To study microalgal biochar mediated Chromium remediation
- To study microalgal biochar mediated abatement of Methylene blue dye

- Screening of *Chlorella minutissima* and *Chlorella sorokiniana* for PHB production
- Extraction and estimation of PHB from the *Chlorella* species

CHAPTER 2- LITERATURE REVIEW

PART 1

2.1 Biochar

According to the guidelines mentioned for the European Biochar Certificate (EBC), 'Biochar is a porous, carbonaceous material that is produced by pyrolysis of biomass and is applied in such a way that the contained carbon remains stored as a long-term C sink or replaces fossil carbon in industrial manufacturing. It is not made to be burnt for energy generation [15].' Biochar can be made from a wide variety of feedstock including manure, industrial waste, algae, agricultural residues etc. It has exceptional adsorption capacity attributed to its high porosity, surface area and abundant surface functional groups that function as binding sites [16]. Biochar synergistically helps in waste management, combating climate change, environmental pollution reduction and cheap energy production.

2.2 Synthesis Techniques

The physiochemical properties of biochar vary depending on the production technique employed [17]. There various methods of biochar synthesis are summarized in **Table 2.1**.

2.2.1 Pyrolysis

It is the commonest method employed for biochar production. It is the thermal decomposition and carbonization of the lignin, cellulosic and hemicellulosic content in the raw material, in absence of oxygen. During this process, oxygen and hydrogen get eliminated as gases or volatiles, causing a dip in H/C and O/C atomic ratios [18]. Three resultant fractions are formed, all in different states: The solid biochar, the condensable liquid bio-oil and the non condensable syngas (H_2 , CO_2 , CH_4 , CO). The nature and distribution of these biproducts is influenced by the pyrolysis temperature with the corresponding heating rate, residence time of the feedstock and the nitrogen flow rate [19][20]. Based on the operating temperature, pressure and heating rate, pyrolysis has been classified s fast, moderate and slow. Pyrolysis temperature beyond $500^\circ C$ imparts stability, hydrophobicity and higher porosity to the biochar which

makes it highly desirable for environmental remediation [21][22]. A higher rate of heating also yields lesser biochar and more syngas [23].

2.2.2 Hydrothermal Carbonisation

It is a technique of conversion of wet biomass into hydrochar with high efficiency. It saves energy by operating at lower temperatures and skipping the energy intensive drying process. But a pressure of 2-10 MPa is persistent throughout synthesis [24]. Water/biomass ratio profoundly impacts the nature of products since water serves as the reaction medium to enhance the hydrolysis process. Production of organic acids during this process is associated with a concomitant drop in pH [25]. The carbonaceous material is produced in far greater amount than CO₂ and bio-oil. The spherical solid particles have the aromatic ring enclosing the oxygen rich surface functional groups (carbonyl, hydroxyl, ester, phenolic, carboxylic) in their hydrophilic part. So it is promising for yielding biochars that are amenable for quenching wastewater contaminants. The adsorption potential may be further magnified by modification of biochar using surfactants and acids or alkalis [26][27]. Many studies have conducted HTC based biochar production from various feedstocks like cassava, banana, switchgrass and poultry litter [28][29][30][31].

Table 2.1: Summary of biochar synthesis methods, process conditions and product distribution [32][26]

Synthesis method	Temp(°C)	Heating rate	Residence time	By product yield (%)		
				Biochar	Bio-oil	Syngas
Flash pyrolysis	775-1025	Very fast	Very short ($\leq 2s$)	10-15	70-80	5-20
Fast pyrolysis	400-1000	fast	Short (seconds)	12	75	13
Intermediate pyrolysis	Low moderate	Moderate	Moderate	25	50	25
Slow pyrolysis	350-980	Slow ($< 10^{\circ}/min$)	Long (1 h)	27-37	19-29	25-41
HTC	200-350	Slow	Long(1-16 h)	50-80	5-20	2-5
Gasification	700-1500	Moderate-fast	Short (seconds)	10	5	85
Torrefaction	200-300	Slow ($< 10^{\circ}/min$)	10-60 min	80	0	20

2.2.3 Gasification

It is a thermochemical conversion process of organic raw material into mainly syngas. The solid (char) and liquid fractions (tar) are undesirable fractions of this method. It is a less researched method for biochar synthesis with studies producing contrasting results. The gasifying agent is one of the decisive factors of the biochar properties along with the type of feedstock, temperature and reactor design. According to a study by Hernandez et al. HTC produced biochar exhibit lower

surface area [33]. This is in disagreement with the results of Galhetas et al. who produced high yield good quality hydrochar with enormous surface area (1500 m²/g) and adsorption capacity, using HTC [34].

2.2.4 Torrefaction

It is a simple conventional method of homogenizing the feedstock and improving its thermochemical properties by slow heating, under limited oxygen environment and atmospheric pressure. This boosts the energy density and physical characteristics of the products [35]. Torrefaction char showcased better methylene blue removal with an adsorption capacity of >350 mg/g compared to 193 mg/g for biochar produced by pyrolysis [36]. The lower operating temperature supports the formation of more oxygen-containing groups on its surface that adsorb the organic contaminant and toxic heavy metals [37].

2.3 Characterisation of Biochar

There are various methods of physical and chemical characterization of biochar. Some of them are briefly discussed here. Proximate analysis involves determining the moisture content, ash content and the volatile matter content in biochar. The elemental or CHNS analysis gives the carbon, hydrogen, oxygen, sulphur and nitrogen concentrations in the char along with the associated ratios. The hydrogen to carbon and oxygen to carbon ratios indicate the degree of aromatic content and maturation of the biochar sample. The extent of process completion and oxidative ability of biochar can be reliably estimated by the C/H, O/C and O/H ratios [54]. BET (Brunauer, Emmett, and Teller) analysis give information about the pore size distribution, pore volume and the total surface area of biochar sample [38]. Scanning Electron Microscopy (SEM) reveals the morphology of the adsorbent. The micropores or macropores formed are a reflection of the starting material and water holding capacity [39]. X-ray diffraction (XRD) analysis gives the crystallographic structure of bio-char. The zeta potential reveals the surface charge of biochar which is inversely proportional to the pyrolysis temperature. Synthesis at higher temperatures gives relatively positive charges to the biochar surface [16]. During the conversion of raw material to biochar, it passes through four phases of dehydration (water loss), thermal decomposition, graphene nucleation and carbonization. Fourier

Transform Infrared (FTIR) spectra is used for identification of organic functional groups on the biochar surface [40]. Higher synthesis temperature translates to decrease in peaks attributed to C=C or C=O stretching (1740– 1600 cm^{-1}), O-H (3600–3100 cm^{-1}), C-O-C stretching (1097 cm^{-1}) and alkene deformation modes (1500–1100 cm^{-1}). This analysis helps in estimating the mechanism of contaminant adsorption by directly influencing the cation exchange capacity of biochar [17].

2.4 Applications

One of the well studied area of biochar application is its excellent potential in amending soil. Addition of biochar to the soil prevents its aggregation and enriches its organic content. It leads to better aeration and porosity of the soil while maintaining a healthy pH at the same time. Biochar adds to its water retention and ion exchange capacity. Moreover it prevents the emission of greenhouse gases from the soil and leaching of vital nutrients like phosphorus, nitrogen etc. Lastly, the presence of biochar in soil affects the bioavailability and transformation of contaminants [41]. Apart from this two noticeable areas where the potential of biochar has been researched as a bioremediating agent includes removal of organic (dyes) and inorganic pollutants (heavy metals) from industrial effluents and wastewater (**Fig 2.1**). However this depends to a large extent on the physiochemical properties of the biochar like ion exchange potential, surface charge, elemental and pore size distribution which are decided by the feedstock, synthesis method and conditions [42].

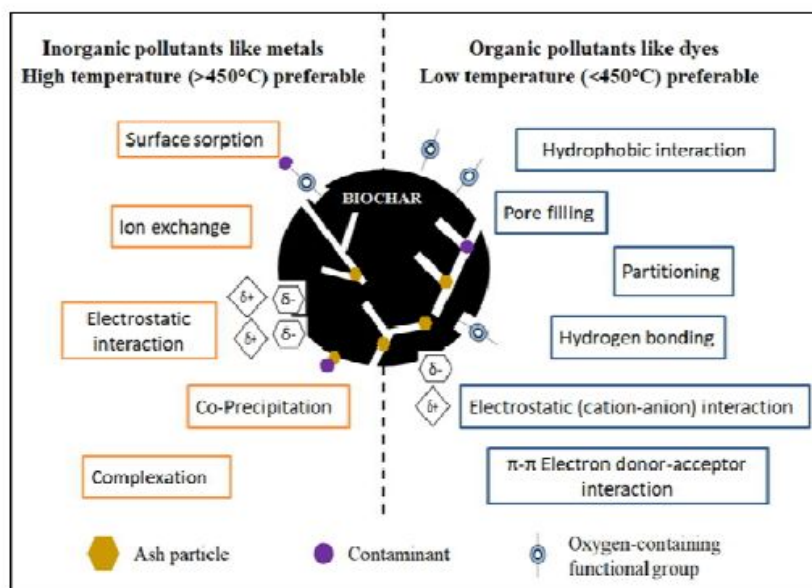


Fig 2.1: Mechanism of removal of organic and inorganic pollutants by biochar

2.4.1 Abatement of organic pollutants

Synthetic dyes like congo red, malachite green and crystal violet are used in textile industries for dyeing of fabrics. The effluents from such industries, when discharged into the environment without proper treatment pollutes the water bodies. Such organic pollutants like pigment containing dyes, surfactants, esters, hydrocarbons, atrazine, solvents etc.. can also be abated using biochar via mechanisms elaborated henceforth. Biochar has two parts, the carbonized one and the non-carbonized one which may be amorphous or crystalline in nature. The non-carbonised part can adsorb the organic pollutant diffused into its pores [43]. This partitioning mechanism works efficiently when the adsorbent is rich in volatile matter and high adsorbate concentration [22]. Pore filling of mesoporous (2-50 nm) and microporous (<2 nm) biochar by the organic contaminant also results in their abatement. It is largely dependent on the type and nature of adsorbent as well as the contaminant polarity. As opposed to partitioning, it is facilitated at low adsorbate concentration and lesser volatile matter content in the biochar [43]. The net charge on biochar surface is responsible for attracting or repelling ionizable organic compounds. At higher pH of the effluent, biochar has a negatively charged surface and vice versa. So the efficiency of electrostatic interaction between the pollutant and biochar is dependent on the charge on the organic pollutant and ionic strength of the effluent, alike [44].

Electron donor-acceptor interaction is a method that is specific to aromatic contaminants and grapheme like structured biochar. When the pyrolysis temperature is below 500°C, the biochar is the electron accepting component amongst the two while when produced at higher temperatures it becomes the electron donating partner due to change in its electron density [45]. Hydrophobic interaction is a less-energy intensive process than partitioning, when the abatement of neutral or hydrophobic organic compounds like chlorobenzene and benzoic acid is concerned [46].

2.4.2 Heavy Metal Remediation

Heavy metals like lead, chromium, mercury, cadmium, zinc etc can be removed using biochar by different mechanisms. Surface sorption is a reversible process where metal ions diffuse in the pores of biochar. The higher the carbonization temperature, the higher is the surface sorption due to greater pore volume and surface area [47]. Biochar also restricts the mobility of metal ions by electrostatic attraction onto its charged surface. This mechanism functions better when biochar is synthesized at a temperature above 400°C [44]. Metal ion immobilization however is affected by the pH of the wastewater and isoelectric point of the biochar [48]. Bivalent metal ions (cations) can be exchanged with the functional groups on the biochar surface leading to their removal by adsorption [49]. The cation exchange capacity increases when the temperature rises above 350°C and if the raw material used in its preparation is iron rich [50]. Precipitation is the primary removal mechanism when the starting material is plant biomass high in cellulosic and hemicellulosic content. Biochars prepared at a temperature above 300°C are alkaline in nature and form mineral precipitates with the inorganic pollutant either in the solution or on its surface [51]. Biochars produced at low temperatures possess oxygen containing functional groups (carboxyl, hydroxyl, phenols) that bind to heavy metal ions to form a complex. This process is termed as metal complexation. Modification by surface oxidation of the adsorbent enhances it [33][52]. Plant biochars exhibit higher ion exchange and complexation capacity. However, Fourier transform infrared spectroscopy and (FTIR) and X-ray photoelectron spectroscopy can give better insights into these mechanisms [53].

2.5 Factors affecting the removal efficiency of biochar

2.5.1 Initial pH of the solution

The pH of the wastewater containing the dissolved heavy metals and organic dyes is one of the key factors influencing the sorption efficiency of biochar. The net charge on the biochar surface and ionization of oxygen containing functional groups that it harbours is dependent on the pH of wastewater. At basic or higher pH, these functional groups get de-protonated, enhancing the sorption of biochar for cationic metals. On the other hand, at acidic or lower pH, the same functional groups get protonated, favouring the sorption of anions like chromium (VI). At pH lower than the zero point charge (ZPC) of biochar its positively charged, both the protons and metal cations repel each other and compete for the binding sites on biochar, leading to lesser adsorption [54].

Zhang et. al reported the same trend. They noticed that more Cr(VI) was removed at lower pH owing to the electrostatic attraction between the positively charged biochar surface functional groups and the negatively charged chromate ion. The Cr(VI) gets rapidly oxidized to lesser toxic Cr(III) due to its higher redox potential [55]. On increasing the pH, lesser chromium was adsorbed due to competition between the chromate and hydroxyl ions for the active sites. Similar results were observed by Shang and colleagues in their research [56]. The adsorption capacity of organic contaminants like dyes onto biochar is directly related to the pH of the medium. According to a study by Parshetti et al. alkaline pH facilitated the electrostatic interaction between the cationic dyes like methylene blue and negatively charged biochar surface. At pH below 3.8, this dye is positively charged, while above it, its negatively charged. Likewise biochar is positively charged below or upto a pH of 5 and negatively charged above it. However at lower pH electrostatic repulsion and competition between the hydrogen ions and positively charged dyes for the active sites, reduced their removal [57][58]. If the zero point charge of the biochar is higher than the pH of the wastewater, MB is adsorbed onto biochar by π - π bond while if the case is opposite, electrostatic interactions also come into play [59].

2.5.2 Adsorbent dosage

The amount of adsorbent, here, biochar is directly linked to the removal efficiency of toxic metals and organic pollutants. Thus, finding out the optimum biochar

concentration for achieving maximum remediation is vital for the cost-effective large scale application of this strategy. More amount of biochar generates more number of binding sites on its surface with a simultaneous increase in its surface area and pores for sorption [54]. Similar conclusions were made by Wang et al. in their study where they observed higher removal of methylene blue dye with biochar concentration upto a certain concentration [60]. It is interesting to note that higher biochar dosage resulted in a decline in adsorption capacity. The possible explanation may be the aggregation of adsorbent particles when present in excess, which provided unsaturated sites for adsorption and prolonged the diffusion path length [61].

2.5.3 Initial contaminant concentration

The initial contaminant concentration also affects the extent of heavy metal and dye abatement. Increasing concentration led to reduction in its removal percentage. This is because there is a fixed amount of adsorbent in the wastewater offering limited number of binding sites for the pollutant. At higher concentrations these sites get saturated with the pollutant and so there is no further adsorption that could occur [62][63]. However, increase in initial Cr concentration was coupled to increased adsorption capacity of biochar due to lesser mass transfer resistance of ions from liquid to solid phase [64].

PART 2

2.1 Bioplastic: Plastic of the future

Bioplastics can be classified as either biodegradable, bio-based or both. Being biobased means that it has originated from renewable biomass like plants. However, the property of biodegradability is decided by the chemical composition of a polymer and not by its source. Likewise, the time of biodegradation is decided by the environmental factors like temperature and microbial consortium in the vicinity. There are three major classes of bioplastics, i) Fossil fuels based plastics but biodegradable. Eg: Polybutylene Adipate Terephthalate (PBAT), ii) Bio based but non-biodegradable (Drop-ins). Eg: Bio based Polyethylene, Polypropylene, Polyethylene-terephthalate iii) Bio-based as well as biodegradable plastics. Eg: Polylactic acid (PLA), Polyhydroxyalkanoate (PHA), Polybutylene succinate (PBS) [65]. This review is focuses on bioplastics that are both, biobased and biodegradable.

Polyhydroxybutyrate (PHB) is a biopolymer synthesised by prokaryotes in the form of intracellular storage compound and belongs to the PHA family. It is majorly composed of 3-hydroxyvalerate (3HV) along with 3-hydroxyhexanoate (3HH) as minor components. It exists as an amorphous fluid intracellularly but becomes crystalline upon extraction. PHB was first discovered by a microbiologist named Lemoigne in *Bacillus megaterium* in 1966 by chloroform extraction. Its tensile strength and thermal properties are similar to synthetic plastics. It is an environment-friendly option and suitable replacement of petroleum based plastics due to its thermoplastic processibility, hydrophobicity, high degree of crystallinity, optical purity, gas barrier properties and high melting temperature of 175°C. It can degrade under home-composting conditions unlike other bioplastics that require industrial composting facility. PHB fibers are elastic and hard but have low elongation at break (EAB) which can be improved by blending it with PHBV or polyhydroxyvalerate (PHV) to form a co-polymer, either biologically or chemically [14][66]

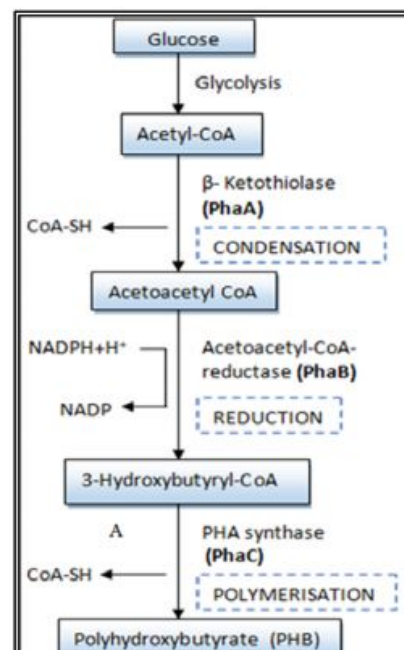


Fig 2.2: (a) Polyhydroxybutyrate (PHB) synthesis pathway from glucose. [13][67].

When microalgae are encountered with nitrogen or phosphorus deficiency, the intracellular accumulation of PHB is favored due to the high NADPH pool. ATP production, electron transfer and protein synthesis are downregulated. Certain

proteins (like alkaline phosphatase enzyme) are synthesised that assist in acclimation of the cell to such starvation conditions. The entire pathway of PHB synthesis has been illustrated in **Figure 2.2**. It occurs with the help of three critical enzymes and involves three PHB precursors. It starts with glucose being converted to two molecules of acetyl CoA via glycolysis. Then the phaA encoded enzyme, β -Ketothiolase condenses them into a single molecule of acetoacetyl-coA which is further reduced by NADPH dependent acetoacetyl-coA-reductase (encoded by the gene phaB) to D-3-hydroxybutyryl CoA. Finally the third enzyme PHA synthase (encoded by the gene phaC) links D-3-hydroxybutyryl CoA molecules via ester bonds to form the biopolymer, Polyhydroxybutyrate (PHB) [13][67].

2.2 Production technologies

Microalgae are very small in size and are highly sensitive to moisture. So, mixing them with materials that complement their properties, makes them cheaper, highly processible and widely applicable. Acetone and sodium sulphite are solvents used for washing the biomass before the blending process [68]. Plasticizers are non-volatile organic compounds that are mixed with bioplastics to improve their stretchability, processability, biodegradability and thermoplasticity. Compatibilizers are compounds that are added while making of polymer blends to enhance the consolidation, mechanical and physiochemical properties. One portion binds to the biopolymer while other binds to the synthetic polymer. Some of its examples are diethyl succinate, poly(ethylene-co-glycidyl) meth acryloyl carbamate and maleic anhydride. The choice of compatibilizer is governed by the polymer blend in consideration [66][69]. Genetic engineering is easy to perform on simple, single celled phototrophs like microalgae and cyanobacteria due to no cell differentiation as in complex plants. So, cyanobacteria and microalgae can be genetically modified by various ways. Random mutagenesis using UV radiations or chemical mutagens like ethidiumbromide (EtBr) is one such way [70]. Metabolic engineering of algal and cyanobacterial strains has been attempted to introduce PHB production pathways, enhance the PHB yield, produce new PHBs and to utilize cheaper and a variety of substrates to achieve economies of scale. CRISPR-Cas9 system of gene editing can be used in future to overexpress PHB biosynthetic genes. It is already being analysed in *Chlamydomonas reinhardtii* [71].

2.3 Screening and Extraction of PHB from algae

Intracellular PHB can be visualized by various methods for screening of PHB positive cells. Sudan Black B staining, Nile red and Nile blue staining are some of the known screening methods. Nile blue staining involves heat fixing the cells and their exposure to 1% of the staining solution for ten minutes. Slide can then be viewed at an excitation wavelength of 460 nm. In Nile red method, the slide is visualized at an excitation wavelength of 465 nm. PHB positive cells take up the stain and the lipid droplets in them fluoresce in deep red. Similarly Sudan black B stains the lipid rich PHB fractions of the cell as black. The slide in this case, can be viewed under a light microscope instead of a fluorescent microscope for the other two methods [72][73].

The solvent extraction method is one of the widely accepted methods for PHB isolation from microalgae. Sodium hypochlorite is a cell disrupting agent. Addition of chloroform following cell lysis releases the intracellular PHB into the solvent with minimal damage to other macromolecules. This method results in quick and efficient PHB extraction with lesser time of exposure of PHB to sodium hypochlorite. Xu et al. extracted PHB using this method from *Acidiphilium cryptum* DX1-1 with an extraction rate of 73% and 92% purity [74]. For quantification of extracted PHB, it is first treated with sulphuric acid at 100°C and then subjected to UV-visible spectroscopy for characterization. The reaction with acid fragments the cell membrane and digests PHB to convert it to crotonic acid. Crotonic acid displays a sharp peak in the range of 230 and 240 nm, specifically 235 nm to confirm the presence of PHB in the unknown sample. The absorbance value and standard graph help in knowing the concentration of extracted biopolymer [75][76].

CHAPTER 3- MATERIALS AND METHODOLOGY

Chlorella sorokiniana and *Chlorella minutissima* cultures were gifted by PhD student in Plant Biotechnology Laboratory, Mr Lakhan Kumar. Chemicals required for the preparation of BG-11 and dyes/heavy metal salts for checking the application of biochar were obtained from the same lab. Pure Poly[(R)-3-hydroxybutyric acid (Sigma-Aldrich) was procured from outside. Stains and solvents for algal staining and bioplastic extraction were obtained from the same lab. All the glasswares and centrifuge tubes were thoroughly washed with laboline and treated with sulfuric acid, then washed with deionized double distilled water and finally dried in oven prior to use. Milli-Q water was used for the preparation of required solutions.

3.1 Microalgae Cultivation

- BG-11 stock solutions were prepared in double distilled water, as mentioned in the appendix. BG-11 media was prepared in a Erlenmeyer flasks and the pH was adjusted between 8 using 1M HCl or 1N NaOH for optimum algal growth upon inoculation.
- It was autoclaved and sterilized media was aseptically inoculated with *Chlorella minutissima* (5% of the media volume).
- The flasks were incubated at a temperature of $25\pm 2^{\circ}\text{C}$, light/dark cycle of 16h and 8h respectively and illumination of 2500 lux.



Fig 3.1: Microalgae cultured in BG-11 medium are incubated under optimum growth conditions in the algal growth chamber

3.2 Microalgae Harvesting for Preparation of Growth Curve

- For the preparation of growth curve, 2ml of the culture volume was withdrawn at regular 3 days interval and centrifuged in aliquots.
- Supernatant was withdrawn and absorbance was measured at 690 nm
- A semi-log graph was plotted taking number of days on the X-axis and absorbance value on the Y-axis

3.3 Microalgae Harvesting for Biochar Synthesis

- Microalgal biomass was collected by centrifuging fully grown, 24- days old culture of *C. sorokiniana* was at 10000 rpm for 10 minutes at room temperature in 50ml centrifuge tubes
- The pellet was washed with ddH₂O for 2-3 times
- Wet biomass was dried in glass plates in hot air oven at 60°C for one day.
- This dry biomass was collected and converted into fine powder by grinding in mortar and pestle.

3.4 Plant Harvesting for Biochar Synthesis

- Plant samples of *Nephrolepis auriculata* were obtained from Ms Roopal Pal, Plant Biotechnology Laboratory, DTU, Delhi.
- Plant leaves were oven-dried and grinded using mortar and pestle into a fine powder.

3.5 Synthesis of biochar from plant/microalgae

- Dried algal/ plant powder samples were taken in two separate beakers and conc. H₂SO₄ was added dropwise to them in the ratio of 1:1.8.
- Samples were heated on a hot plate at 160°C for 6 h to release black, coarse biochar (BC)
- Biochar was allowed to cool and dried overnight in hot air oven at around 70°C
- The dried coarse biochar was ground in a mortar pestle and passed through 90 µm sieve to obtain only fine BC particles
- The sieved samples were then washed with ddH₂O by repeated centrifugation at 10000 rpm for 5 minutes until pH between 7.0 -8.0 was attained.
- The washed biochar samples were oven dried at 110°C for 3-4 h until complete moisture removal

•The dried BC samples hence obtained were weighed, characterized and stored at room temperature in air tight containers until testing of further applications

Note: Precautions were taken while performing the synthesis. Gloves, mask and protective eyewear were used to prevent skin from protect eyes and skin from exposure of acid and to prevent inhalation of any generated fumes



Fig 3.3: Synthesis of *Nephrolepis auriculata* biochar from dried plant leaves powder

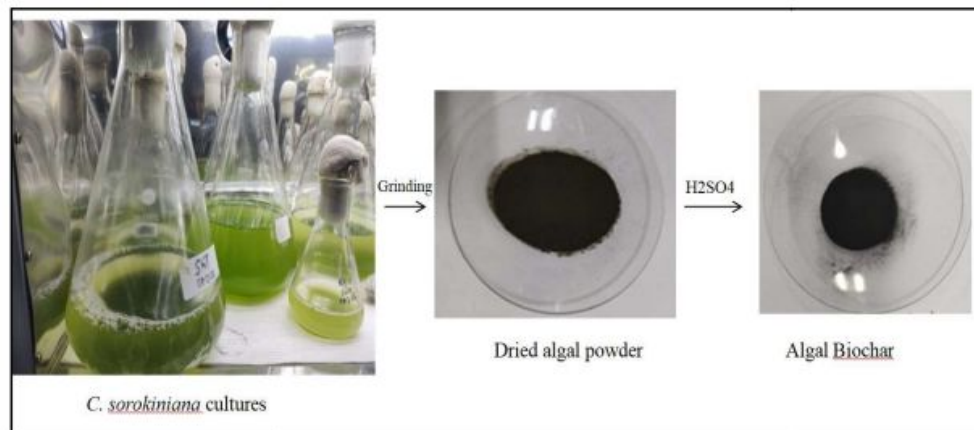


Fig 3.2: Synthesis of *Chlorella sorokiniana* biochar from dried algal powder

- **Biochar yield**

Yield of bio char was calculated according to the following formula.

$$\text{Yield (\%)} = (M_B/M_{RM}) * 100$$

Where, M_B = Mass of biochar (in grams)

M_{RM} = Mass of raw material, here microalgae or plant powder (in grams)

3.6 Characterisation of plant and microalgal biochar

Chlorella sorokiniana biochar was visually characterised through color change along with other techniques like UV-Visible spectrophotometry (for confirmation of biochar formation), Field Emission Scanning Electron Microscopy FE-SEM (for shape and surface morphology), X-ray diffraction (XRD) analysis (for crystallinity determination, Fourier-transform infrared spectroscopy FTIR (for identification of surface functional groups acting as binding sites for compounds) and Zeta-potential (for size determination). Similarly, *Nephrolepsis auriculata* biochar was characterised visually, and by FTIR, SEM Zeta potential and UV-Vis spectroscopy.

3.6.1 Visual Characterisation

This is the first characterisation method to confirm biochar synthesis. Color change is observed after heat treatment and addition of sulphuric acid to the plant and microalgae powder.

3.6.2 UV-Visible Spectroscopy

A biochar suspension of 0.5g/L was prepared. The thermochemical conversion of biomass into biochar was recorded using a full scan in the UV-visible range (200-700 nm) against distilled water as blank. The scans were taken in triplicates.

3.6.3 Conductivity of biochar

500mg of biochar was mixed with 50 ml distilled water in a beaker. It was then left undisturbed for about 1 h. Conductivity was measured using a conductivity meter.

3.6.4 pH of biochar

Biochar pH was determined by preparing 1% solution of biochar in ddH₂O in a flask. The flask was heated at 90°C for 20 minutes with constant mixing on a magnetic stirrer to dissolve its soluble components in water. After cooling down to RT, pH of the solution was determined while gentle swirling.



Fig 3.4: Determining the pH of plant and algal biochar

3.7 Application of microalgal biochar

3.7.1 Dye abatement

Synthesised biochar was used for degrading the dye Methylene Blue (MB).

- **Standard curve of MB:** The synthesized biochar was tested for the degradation of Methylene Blue (MB) dye. A stock solution of 30 ppm was made, which was diluted with ddH₂O to prepare different dilutions having varying MB concentrations. Standard graph was plotted with MB concentration in ppm on the X-axis and corresponding absorbance values at 665nm on the Y-axis.

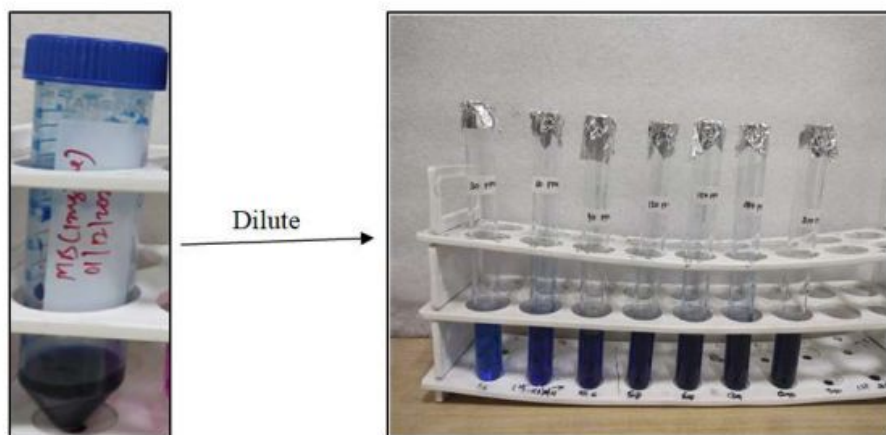


Fig 3.5: Methylene blue dilutions made from MB stock solution for preparation of standard graph



Fig 3.6: Test solutions put for continuous shaking on a mechanical shaker to assess remediation over time

Adsorption study: Batch mode adsorption experiments for MB removal were carried out using *Cs* biochar as the adsorbent. The experiment was performed using orbital shaker at an agitation rate of 150 rpm at RT in various conical flasks.

- **Effect of adsorbent dosage:** Algal biochar was taken in different amounts (0.5, 0.75, 1, 1.5, 2, 5 and 10 g/L) and mixed with 5 ml of dye solution and initial concentration of 30 ppm of MB at RT and natural pH until attainment of equilibrium.
- **Effect of initial pH**
- pH of 30 ppm of initial dye concentration was varied from 2 to 10 under adsorbent dosage of 10 g/L until attainment of equilibrium at RT. The initial pH of test solutions was adjusted with 0.1 M HNO₃ or 0.1 M NaOH

➤ **Effect of initial dye concentration:**

The effects of initial MB concentration were determined at different initial concentrations of 10, 20, 30, 50 and 100 ppm using the optimum adsorbent dosage and pH condition until equilibrium.



Fig 3.7: Test solutions for determining the effect of initial dye concentration on removal efficiency

- **Removal of Methylene Blue**

The synthesized microalgal biochar was tested for removal efficiency from synthetic wastewater containing Methylene blue dye.



Fig 3.8: Procedure for calculating the removal efficiency of biochar

- About 3 mL of sample solution was collected at regular intervals and centrifuged at 10,000 rpm for 5 min.
- The biochar got pelleted down and supernatant was pipetted out into a cuvette
- Absorbance was measured at 665 nm using a UV-Vis spectrophotometer.
- Based on the spectrophotometer data, the Removal efficiency (RE) of dye and adsorption capacity (Q_e) of microalgal biochar were calculated by using the following formulae:

$$\text{RE (\%)} = \left\{ \frac{C_i - C_f}{C_i} \right\} * 100$$

$$Q_e \text{ (mg/g)} = (C_i - C_f)V/W$$

where,

C_i: Initial concentration of MB (mg/L)

C_f: Final concentration of MB (mg/L)

V=Total volume of dye solution tested for degradation (ml)

W= Amount of adsorbent (g)

3.7.2 Heavy metal remediation

Synthesised biochar was employed for the degradation of heavy metal chromium.

- **Preparation of Standard curve for Cr:** The synthesized biochar was tested for chromium removal efficiency from synthetic wastewater prepared by using analytical grade K₂Cr₂O₇. A stock solution of 30 ppm was prepared, which was diluted with ddH₂O to prepare different dilutions having varying Cr concentrations. Standard graph was made with Cr concentration in ppm on the X-axis and corresponding absorbance values at 350 nm on the Y-axis
- **Adsorption study:** Batch mode adsorption experiments for Cr removal were carried out using Cs biochar as the adsorbent. The experiment was performed using a mechanical shaker (REMI) under at the agitation rate of 150 rpm at RT in a series of beakers.
- **Effect of adsorbent (MABC) dosage:** To determine the effect of adsorbent dosage different amounts of biochar (0.1, 0.3, 0.6, 0.9, 1.2 and 1.5 g/L) were mixed with a fixed volume (5 ml) of chromium solution of initial

concentration 30 ppm under natural pH until equilibrium state at room temperature.



Fig 3.9: Test solutions containing varying concentrations of biochar

- **Effect of initial pH:** The pH was varied from 2 to 10 under 30 ppm of initial concentration with the adsorbent dosage of 1.5 g/L until attainment of equilibrium at RT. The initial pHs of the HM solutions were adjusted using 0.1 M HNO₃ or 0.1 M KOH. Throughout the experiment, the pH values of solutions were not maintained nor monitored until equilibrium.
- **Effect of initial heavy metal (Cr) concentration:** The effects of initial HM concentration were determined at initial concentrations ranging from 20 to 80 ppm using the optimum adsorbent dosage and pH condition until equilibrium.
- **Chromium Remediation:** The synthesized microalgal biochar was tested for removal efficiency from synthetic wastewater containing the heavy metal chromium.
 - About 3 mL of sample solution was collected at regular intervals and centrifuged at 10,000 rpm for 5 min.
 - The biochar got pelleted down and supernatant was pipetted out into a cuvette
 - Absorbance was taken at 350 nm using a UV-Vis spectrophotometer.
 - Based on the spectrophotometer data, the Removal efficiency (RE) of metal ions and adsorption capacity (Q_e) of microalgal biochar were calculated by using the following formulae:

$$RE (\%) = \left\{ (C_i - C_f) / C_i \right\} * 100$$

$$Q_e (\text{mg/g}) = (C_i - C_f)V/W$$

where,

C_i : Initial chromium Cr (VI) concentration (mg/L or ppm)

C_f : Final chromium concentration of in the solution (mg/L)

V = Volume of heavy metal solution tested for remediation (ml)

W = Amount of adsorbent (g)

CHAPTER 4- RESULT AND DISCUSSION

4.1 Microalgae growth curve

Absorbance at 690 nm of *Chlorella sorokiniana* and *Chlorella minutissima* cultivated in BG-11 medium were monitored to plot a growth curve. The plotted growth curve was used for determining the harvesting day with optimum microalgae growth for subsequent experiment of biochar synthesis and PHB extraction so as to obtain maximum yield.

Table 4.1: Optical density of *Chlorella sorokiniana* and *Chlorella minutissima* on BG-11 medium noted at a 3-day interval at 690 nm

Strain/Days	Day 1	Day 3	Day 6	Day 9	Day 12	Day 18	Day 24
<i>C. minutissima</i>	0.0041	0.1237	0.2652	0.4906	0.6699	0.8274	1.2334
<i>C. sorokiniana</i>	0.003	0.108	0.1908	0.4527	0.6274	0.8398	1.1908

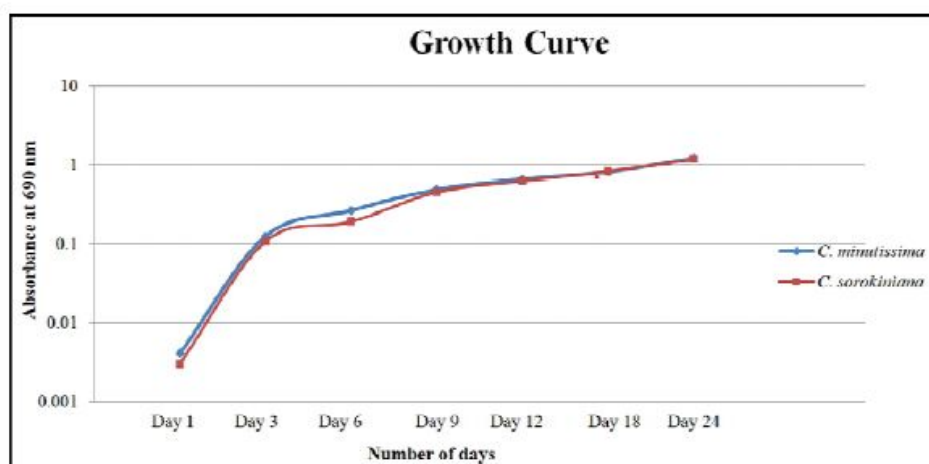


Fig 4.1: Growth curve of *Chlorella sorokiniana* and *Chlorella minutissima* cultivated on BG-11 medium.

Growth of both the algal species was monitored on BG-11 medium (pH 8). It was observed that growth of both the species was optimum at day 24 of cultivation. Following which, the growth would start declining due to cell death in nutrient limiting conditions or stressed environment.

4.2 Biochar synthesis from plant/microalgae

When sulphuric acid was added to Cs powder an exothermic reaction took place with the generation of fumes. Upon heating on a hot plate for 6h, charring and carbonisation of microalgal/plant biomass occurred which was visually confirmed. Gases with pungent odour were released during the synthesis process.

4.3 Characterisation of plant and microalgal biochar

4.3.1 Visual characterisation

In this, a color change is observed upon completion of reaction of sulphuric acid and the biomass. The green color of Cs powder and the light brown color of the dried plant powder was converted to jet black. This occurred due to dehydration (water loss), thermal decomposition, graphene nucleation and carbonization of the biomass resulting in its charring. Similar results were reported by Nupur et al. using the plant *Bacopa monieri* [60].

4.3.2 UV-Visible Spectroscopy

The thick black layer of biochar blocks the visible light. Pure biochar exhibits adsorption peak in the range around 200-300 nm due to the black color of the adsorbent. Here, 10 mg plant and algal biochar were dissolved in ddH₂O and kept on mechanical shaking for some time. When the scan was run, peak absorbance of 0.1303 and 0.2941 were observed around 210 nm for microalgal and plant biochar respectively.

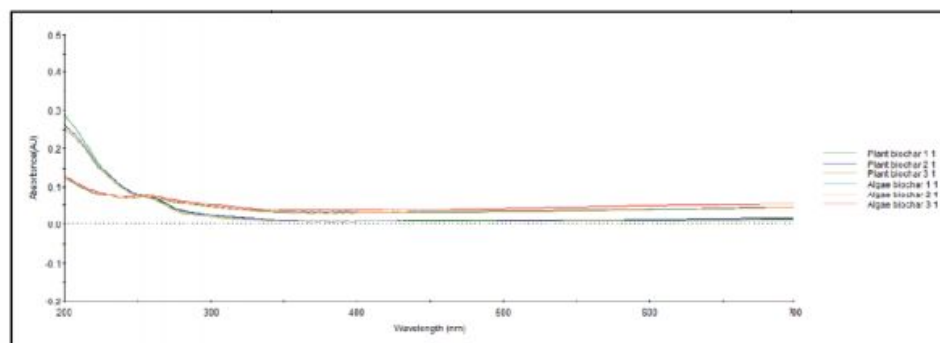


Fig 4.2: Absorbance scan of biochar solution.

4.3.3 Yield of biochar

In case of microalgae, 2.08 grams of dried biochar was produced from 4.1 grams of Cs powder giving a biochar yield of 50.7%. In case of plant, 0.88 grams of dried biochar was produced from 2.31 grams of the dried leaves powder giving a biochar yield of 38.09%. Thus this method proved to be more efficient in biochar production when microalgae was used as a feedstock due to the higher lignocellulosic content in plants.

4.3.4 pH of biochar

The pH of Cs biochar was 8.2 while that of plant biochar was 7.9. Plant biochar tend to possess higher ash content and slightly acidic pH due to greater ash content attributed to the lignin, cellulosic and hemicellulosic fractions. Similar results were reported by Kanyaporn et al. in their study [77].

4.3.5 Conductivity of biochar

Conductivity of Cs biochar was 884 μ S/cm and that of plant biochar was 1.2 mS/cm. Greater conductivity, post synthesis reaction is attributed to particle size variation and elimination of oxygen. Abundance of free electrons on the negatively charged biochar surface may also be a reason behind enhanced conductivity. Similar observations were made by Seth et al [78].

4.3.6 Other physico-chemical characterisation

FE-SEM analysis of synthesized biochar revealed a spherical and microporous morphology with large surface area. It revealed several pores and surface fragmentation and irregularities that can serve as excellent binding sites for dye and chromium. The elimination of volatile organic compounds formed deep channels in the biochar [79]. Similar observations were made by Gope and Saha in their research [80]. Plant biochar exhibited structures resembling honey-comb due to tubular shape of the plant cell which served as its starting material.

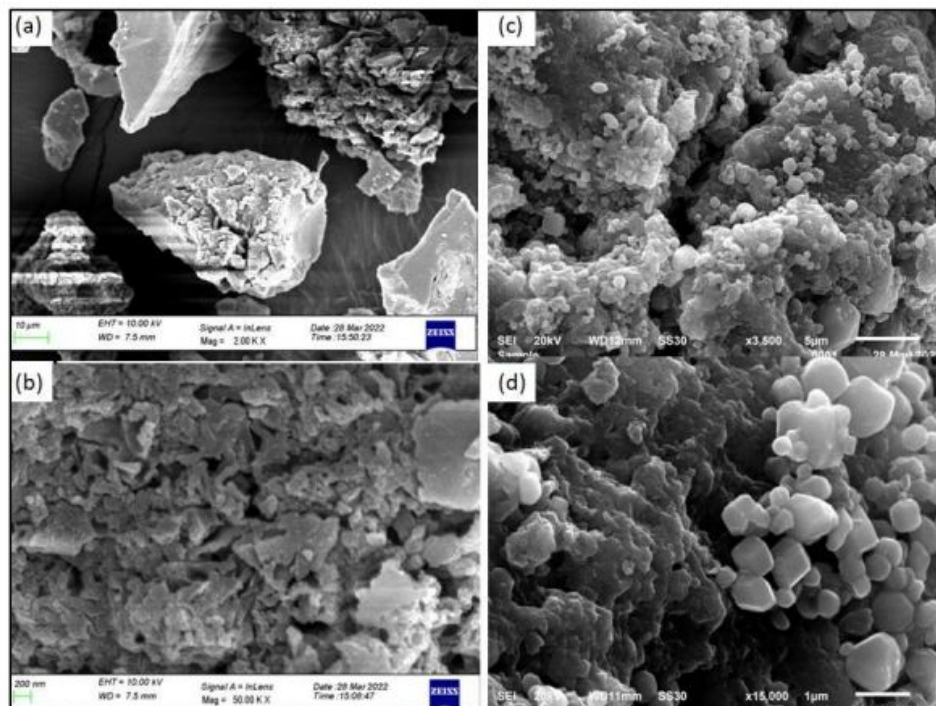


Fig 4.3: SEM micrographs of algal (a,b) and plant biochar (c,d) for morphology analysis

A summary of FTIR analysis of algal biochar is presented in **Table**. The spectrum in **Fig** shows peaks in agreement with those reported in several other studies [81][82][83].

Table 4.2: Analysis of peaks in the FTIR spectrum of *C. sorokiniana* biochar

Peak	Wave number Range	Interpretation
3345	3100-3600	-OH bond stretch of carboxylic group
2127,2111		Alkynes
1988		C-C-C stretch, Alkene
1705		C-C and C-O stretching
1182,1139,1022	1740-1600	Fingerprint region
740,680		Alkene
1558		-COOMe
1488	1400-1500	Alkane, bending oscillation from CH ₂ , C-C stretching
1488,1083,1022		Fingerprint region, C-O stretch, aryl-alkyl ether

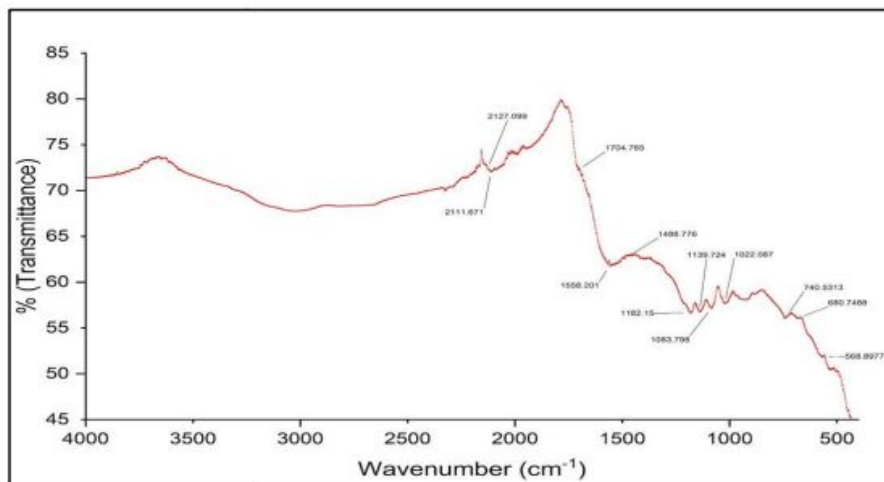


Fig 4.4: FTIR Spectrum of *C. sorokiniana* biochar

XRD patterns of biochar show sharp peaks throughout due to the presence of miscellaneous inorganic constituents like magnesium oxide, silicon dioxide and calcium oxide. In the XRD pattern of *Cs* biochar, a sharp peak at 25.92° is attributed to crystalline silicon dioxide and confirms heterogeneity in the biochar surface. It reveals that there is layering of aromatic functional groups in biochar. This diffraction peak is associated with the (002) crystal plane while the peak at 46.04° corresponds to the (101) plane of disordered carbon. These two peaks are also present in the XRD patterns of graphite [83]. Similar XRD patterns were reported by Zhu and Zou in their work [84].

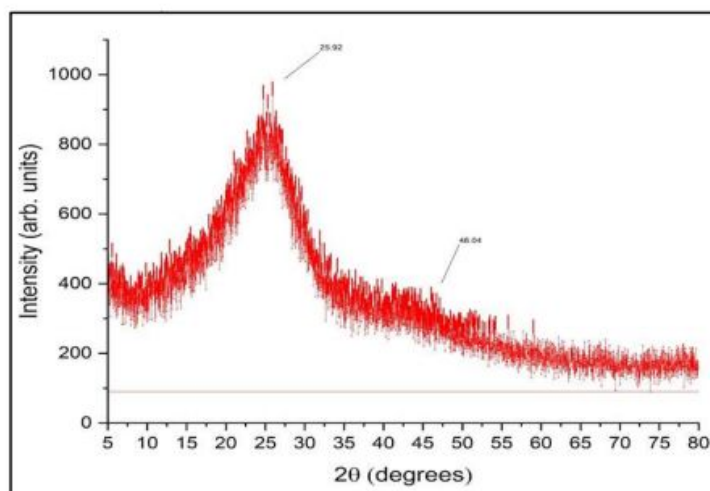


Fig 4.5: XRD pattern of algal biochar

Zeta Potential results of algal biochar suggested an average diameter of 4326 nm with a Pdl value of 0.643 indicating moderately uniform particle size distribution. They possessed a zeta potential value of -15.7. Zeta Potential results of plant biochar suggested an average diameter of 3239 nm with a Pdl value of 0.147 indicating highly uniform particle size distribution. They possessed a zeta potential value of -4.56. Hence we can conclude that the biochar surface has a net negative charge due to the negatively charged functional groups. This is in agreement with adsorption studies further where the biochar was able to better remediate the cationic dye MB as opposed to the Cr(VI) anion.

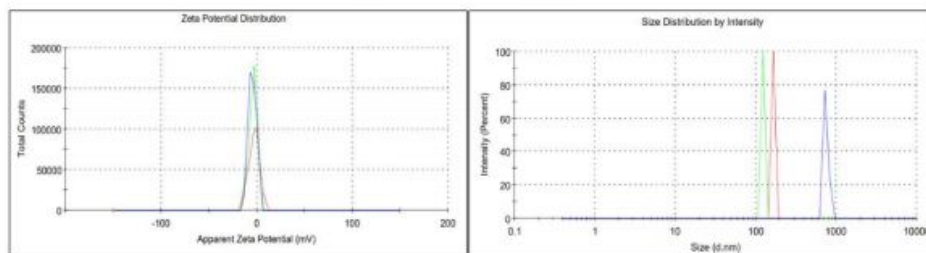


Fig 4.6: Size distribution and zeta potential distribution of *C. sorokiniana* biochar

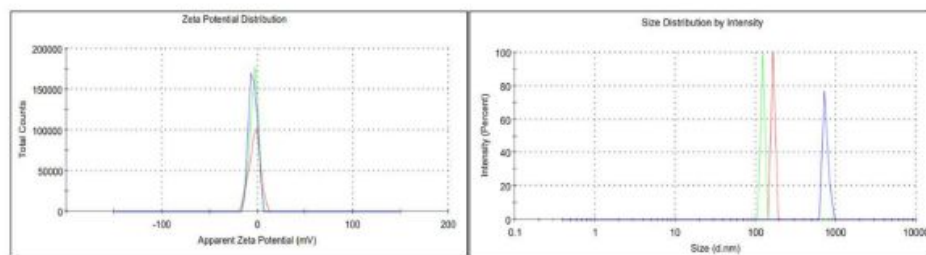


Fig 4.7: Size distribution and zeta potential distribution of *N. cordipholia* biochar

Oxygen content in the synthesised biochars can be calculated by their CHNS concentrations. The sum total of C,H,N and ash component must be 100. The results indicate lower aromaticity and higher oxidative ability of plant biochar when compared to algal biochar. These results are supported by the research of Shaheen et al. [85].

Table 4.3: CHNS analysis of *C. sorokiniana* and *N. cordipholia* biochar

Parameter	Algal Biochar	Plant Biochar
N%	11.32	2.77
C%	41.95	19.08
H%	2.45	3.31
S%	7.01	15.17
C/N ratio	3.7	6.88
C/H ratio	17.11	5.75

4.4 Parameter optimisation for dye abatement

- **Preparation of Standard curve for MB:** The standard curve plotted by taking absorbance values (665 nm) of different MB dilutions indicated a linear trend. It was accurate with a correlation coefficient of 0.998.

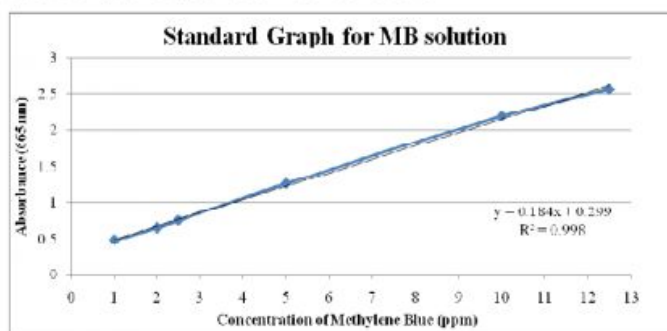


Fig 4.8: Standard curve for Methylene blue solution

- **Effect of adsorbent dosage:** It is evident from figure 4.9 that increasing the biochar concentration led to increased availability of sorption sites for MB, hence enhancing its removal. The best dye abatement of 97.85% was reported with a biochar dosage of 10g/L. However beyond a certain biochar dosage, the particles tend to aggregate resulting in decreased remediation.

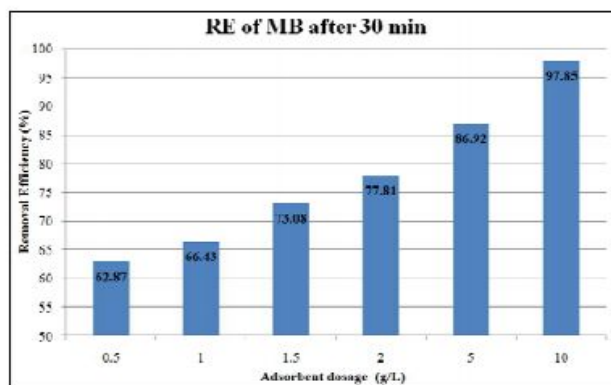


Fig 4.9: Effect of algal biochar dosage on MB removal

- **Effect of initial pH:** It is evident from figure 4.10 that increasing the pH of the test solution favors the removal of cationic dye MB. A maximum removal efficiency of 99.56% was reported at pH 10. Upon remediation the intense blue color of the test solution became clear as water. Minimum removal was reported at pH 2 because at a

pH < 3.8, the dye is negatively charged and repels the anionic functional groups on the biochar surface.

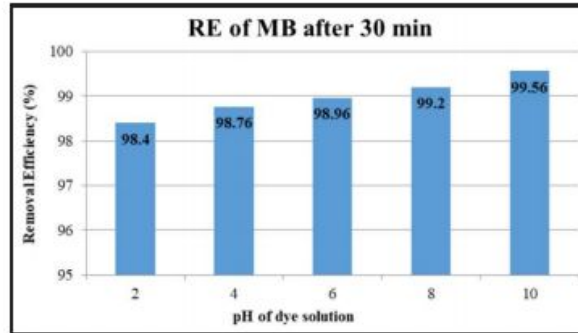


Fig 4.10: Effect of initial solution pH on MB removal

- **Effect of initial dye concentration:** It is evident from figure 4.11 that increasing dye concentration reduced the removal efficiency of biochar and saturated its active sites. Hence optimal RE of 99.71% was observed at a dye concentration of 10 ppm while the lowest removal of 91.37% was seen at 100 ppm concentration of MB.

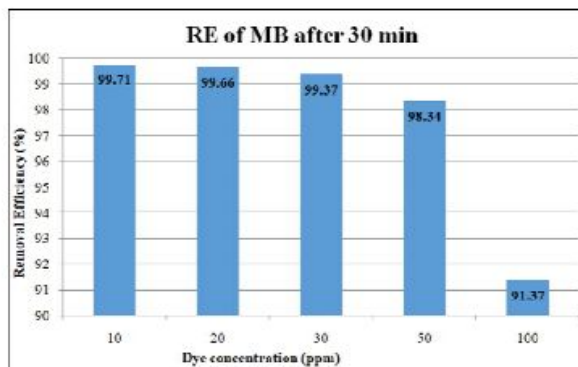


Fig 4.11: Effect of initial MB concentrations on removal efficiency of algal biochar

4.5 Parameter optimization for heavy metal remediation

- **Preparation of Standard curve for Cr:** The standard curve plotted by taking absorbance values (350 nm) of different chromium concentrations dilutions indicated a linear trend. It was highly precise with a correlation coefficient of 1.

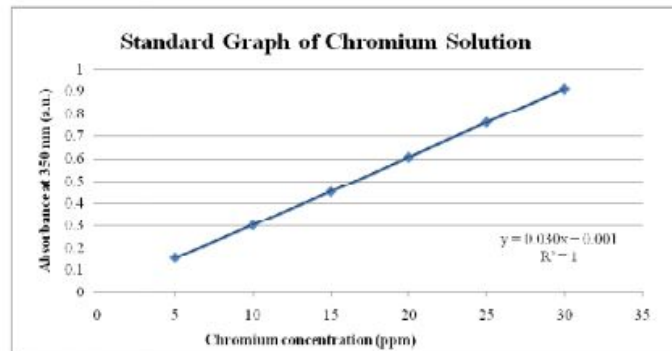


Fig 4.12: Standard curve for Chromium solution

- Effect of adsorbent dosage:** It is evident from zeta potential analysis of algal biochar that it has negatively charged functional groups on its surface. Hence the removal of negatively charged chromate ion Cr (VI) was not as effective as the dye due to electrostatic repulsions between the adsorbent and the adsorbate. **Fig 4.13** showcases a similar trend as in MB removal. Here also, more biochar addition led to greater HM remediation. The maximum removal efficiency of 75% was reported at the optimum adsorbent dosage of 1.5g/L. Infact, beyond a dose of 1.2g/L there was negligible rise in chromium removal.

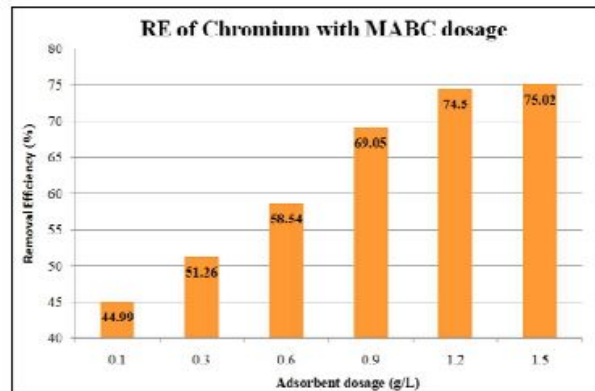


Fig 4.13: Effect of algal biochar dosage on chromium removal

- Effect of initial pH:** It is clearly indicated in **Fig 4.14** that rise in pH from 2 to 10 reduced the chromium sorption onto the biochar surface. This is attributed to the fact that at lower pH the chromate ion having higher redox potential gets transformed into its lesser toxic form of Cr(III). So the highest removal of 76.61% was reported at an acidic pH of 2. The color of the solution faded from the original bright orange color upon remediation.

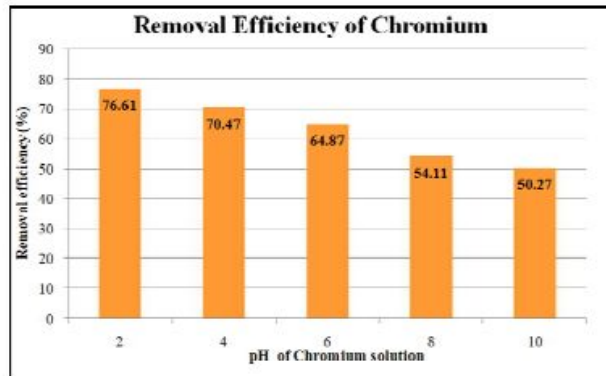


Fig 4.14:Effect of initial solution pH on chromium removal

- **Effect of initial dye concentration:** It is clearly indicated in **Fig 4.15** that increasing chromium ion concentration in the solution significantly reduces the remediation potential of biochar due to presence of limited adsorption sites in a limited amount of biochar in the solution.

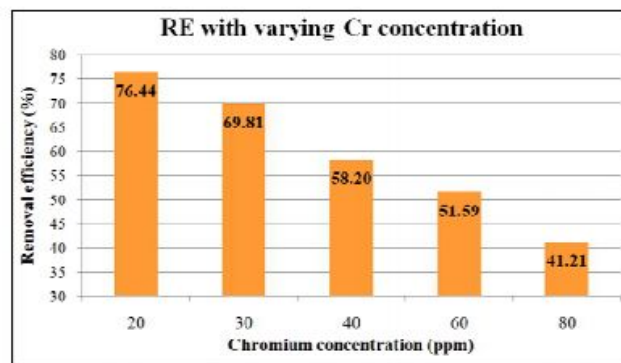


Fig 4.15: Effect of initial chromium concentration on removal efficiency of algal biochar

PART 2: Extraction of Algal Bioplastic

CHAPTER 3- MATERIALS AND METHODOLOGY

3.1 Microalgae cultivation:

Two species of microalgae *Chlorella*, namely, *C. vulgaris* and *sorokiniana* were tested for their PHB production ability. They were cultivated and maintained as described in section 3.1

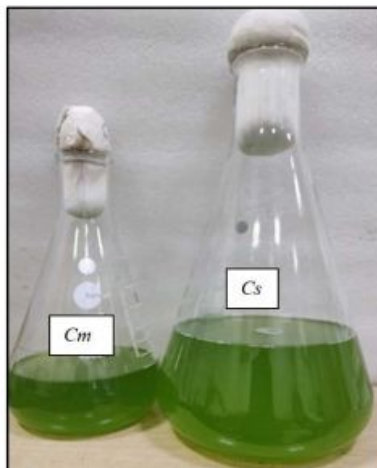


Fig 3.10: Algal broth cultures of *C. minutissima* and *C.sorokiniana*

3.2 Screening of PHB positive strain

Both the species were subjected to Sudan Black B staining to assess for the presence of PHB in the algal cells.

- Two clean glass slides were taken. A drop of algal broth was taken in an inoculating loop from the culture flasks of each respective species to make a circle of 1 cm radius on the slide.
- The slides were allowed to air dry and heat fixed by passing over the flame for 2-3 times.
- The above steps of preparing algal smear were performed in a LAF hood to maintain aseptic conditions
- The slides were flooded with 3% (w/w) sudan black solution in 70% ethanol
- The slides were left undisturbed at RT for 10-15 minutes and then immersed in xylene until complete decolorisation (10-15 seconds)
- They were counterstained with 0.5% safranin solution for 10 seconds.
- Finally, the slides were rinsed with water and allowed to air dry completely
- Slides were visualized under light microscope (1000X)

3.3 Preparation of Standard Curve

A standard curve of PHB was prepared using Pure PHB (Sigma, USA) to determine the amount of PHB in the samples under study.

- 10 mg of standard (Pure PHB, Sigma) was dissolved in 10 mL of conc. H₂SO₄ taken in a capped glass tube
- The mixture was heated in a water bath maintained at 100°C for 20 min
- During this reaction, PHB was completely converted into Crotonic acid to give a stock solution of 1mg/mL concentration of Crotonic acid.
- The above stock was diluted with H₂SO₄ to give working standard solutions of varying concentrations. Absorbance was noted for each dilution at 235 nm taking H₂SO₄ as blank.



Fig 3.11: Pure Poly[(R)-3-hydroxybutyric acid (Sigma-Aldrich)

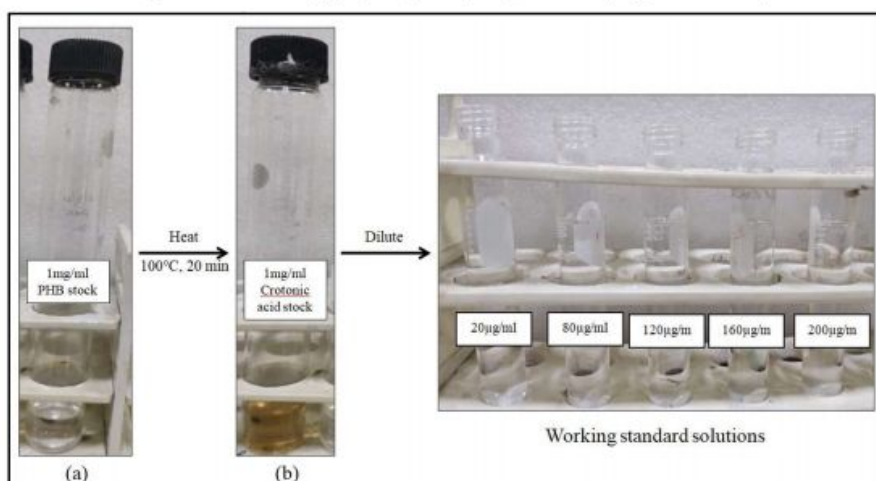


Fig 3.12: Preparation of crotonic acid dilutions for plotting PHB standard curve

3.4 Extraction of PHB

- Culture flasks containing *C. minutissima* and *C. sorokiniana* were taken. A broth volume of 50 ml was withdrawn from each flask and centrifuged at 10000 rpm for 10 min.
- The pellet so obtained, was dissolved in chloroform and 4% sodium hypochlorite (1:1) mixture.
- Afterwards, the suspension was centrifuged at 8000 rpm for 10 min at RT. This results in formation of three phases: The upper phase has the sodium hypochlorite solution. The middle phase contained all the undisrupted cells and Non-polymeric cell materials (NPCM). The bottom phase was a mixture of PHB in chloroform.
- So the upper phase was removed by pipetting and the bottom phase was obtained by pipetting after centrifugation
- Now, PHB was recovered from this chloroform phase by the non-solvent precipitation method. The non-solvent employed was a mixture of water and acetone (1:1) and its volume was five times the volume of the recovered chloroform phase.
- The polymer separated from the solvent in the form of a thin film or layer

3.5. Quantification and characterisation of PHB

For quantifying and characterizing the extracted PHB, the sulphuric acid digestion method was used. The crotonic acid released was a direct measure of PHB extracted from the microalgae.

- A small volume of the formed PHB film was pipetted out
- It was mixed with 10 ml H₂SO₄, and heated in a water bath maintained at 100°C for 20 min
- After the reaction, the sample was suitably diluted and absorbance was taken at 235 nm with H₂SO₄, as blank.

CHAPTER 4- RESULTS AND DISCUSSION

4.1 Screening of PHB positive strain: The Sudan Black B staining results of *C. minutissima* (Fig 4.16) and *C. sorokiniana* (Fig 4.17) reveal that both the strains are PHB positive. Both of them took up the stain due to which the lipid rich PHB fractions in the cell appeared as black. Mostly, black rings were observed along the intracellular boundaries against a reddish background due to safranin counterstain. Screening suggested that *C. minutissima* may accumulate higher amount of PHB than *C. sorokiniana* due to more cells with the distinct black color.

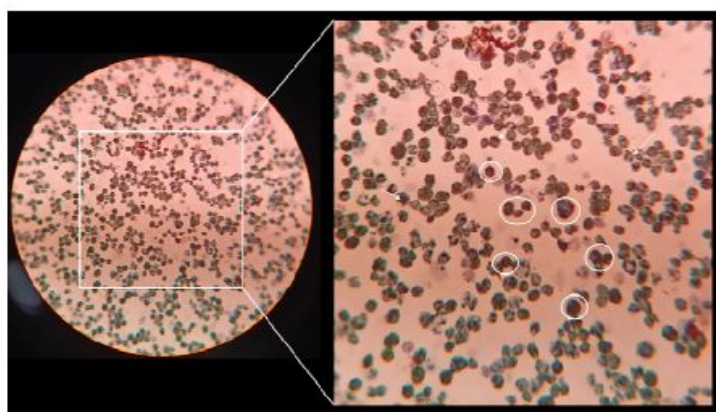


Fig 4.16: Sudan black B staining of *C. minutissima*. Encircled and marked cells indicate PHB rich cells.

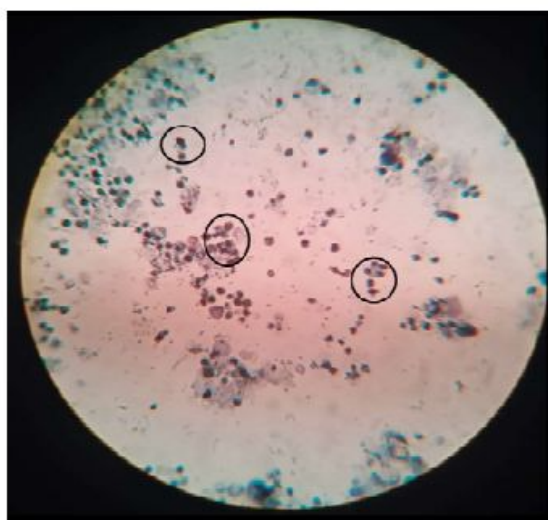


Fig 4.17: Sudan black B staining of *C. sorokiniana*. Encircled cells indicate PHB rich cells.

4.2 Preparation of Standard Curve of PHB: The standard curve plotted by taking absorbance values (235 nm) of different crotonic acid (PHB) dilutions indicated a linear trend. It was accurate with a correlation coefficient of 0.9917.

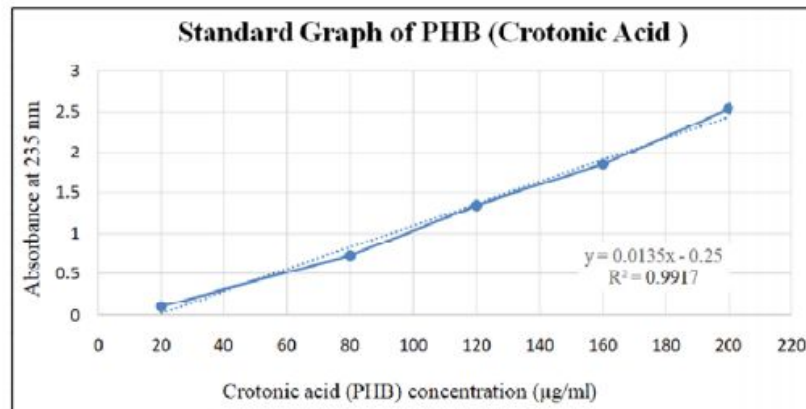


Fig 4.18: Standard curve of polyhydroxybutyrate (converted to crotonic acid)

4.3 Extraction of PHB: PHB was present in the bottom chloroform phase. When separated from the solvent it was visible as a thin hydrophobic layer (like that of oil over water) as shown in **Fig 4.19**. A small volume of this layer was carefully pipette for quantification.

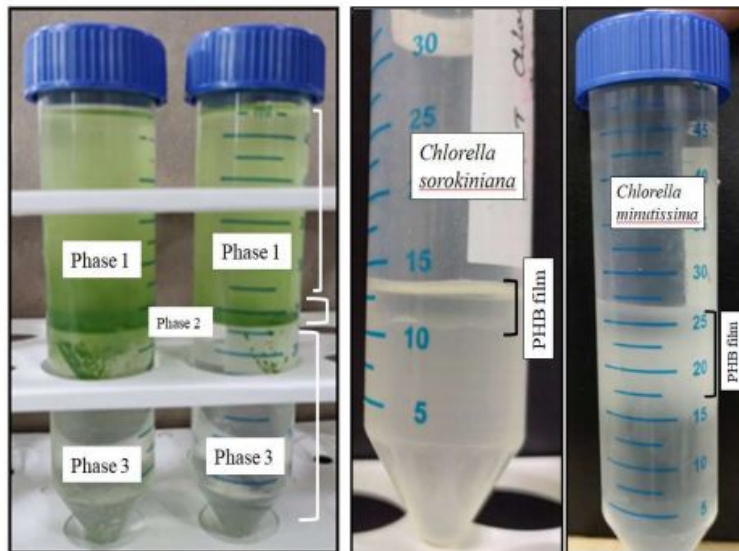


Fig 4.19: Presence of biofilm layer, separated from chloroform

4.4. Quantification and characterisation of PHB: PHB was quantified using the standard graph. The separated lipid rich PHB containing layer was subjected to sulphuric acid digestion. **Fig 4.20** shows the bioplastic layer over sulphuric acid, before the start of acid digestion. PHB extracted was characterized by UV-Visible spectroscopy to give optimum absorbance at 235 nm, corresponding to crotonic acid (**Fig**). The crotonic acid (brown colour) concentration in the unknown samples was corresponding to the respective PHB concentrations extracted from the two species. Peak of *Chlorella minutissima* was observed at 240 nm with an absorbance value of 4.7696 as shown in **Fig 4.21** while the peak of *Chlorella sorokiniana* was observed at 235.65 nm with an absorbance value of 4.6983 as shown in **Fig 4.22**. Thus bioplastic was produced in slightly higher concentration in *Chlorella minutissima* as compared to *Chlorella sorokiniana*

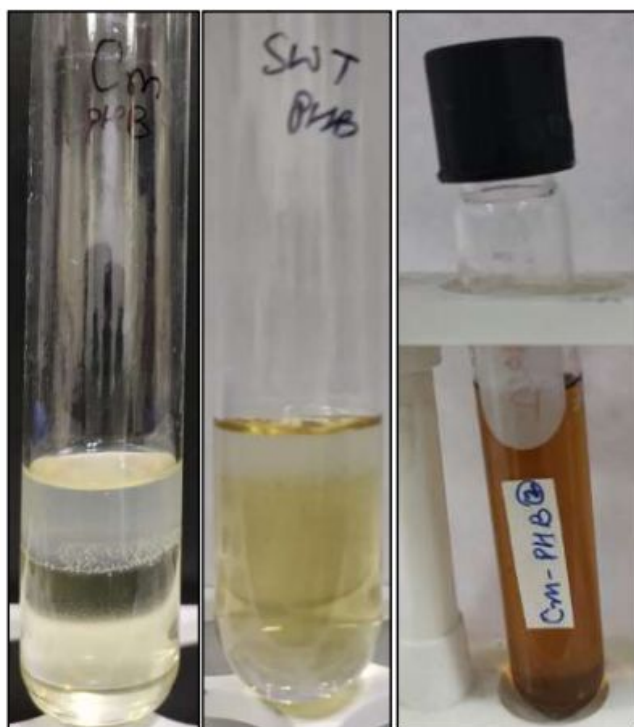


Fig 4.20: PHB Biofilm forms a layer over sulphuric acid (prior to reaction)

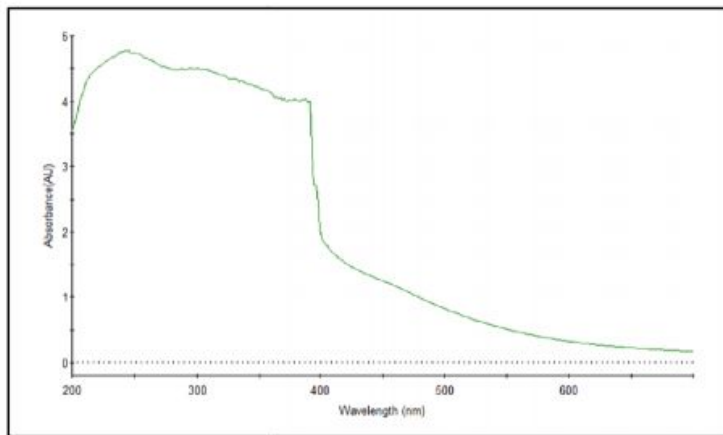


Fig 4.21: UV-Vis spectra of PHB derived from *Chlorella minutissima*

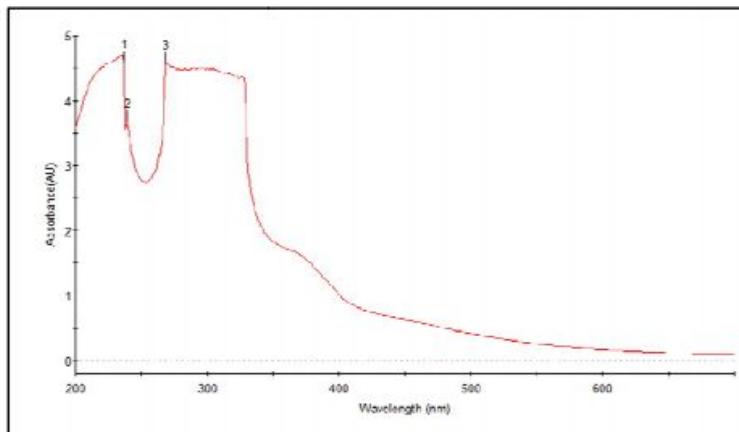


Fig 4.22: UV-Vis spectra of PHB derived from *Chlorella sorokiniana*

CONCLUSION AND FUTURE PROSPECTS

This study has explored *Chlorella* species for its environmental application in terms of wastewater remediation (biochar production) and plastic pollution management (PHB extraction). There is enough research evidence in support of biochar being a promising adsorbent for environmental remediation of organic and inorganic pollutants in industrial effluents. However, there are lesser studies exploring the dimension of biochar modification for better adsorption capacity. Application-specific activators that modify the biochar surface functional groups and porosity need to be searched for. Development of models for the modified adsorption process can prove to be insightful for understanding surface oxidation, amination, sulfonation and porosity modification of selective biochars. Activated biochars exhibit superior removal efficiency in comparison to the conventional ones. However, their adsorption mechanisms need to be elucidated. As discussed in this study, biochar production is an energy intensive procedure. To be able to utilize it as a remediating agent on a large scale, its production needs to be cost effective on the commercial scale. So deciding the optimum production route and biochar dosage for effective removal is crucial to make it economically feasible [43].

Bioplastic, rightly called as the 'Plastics of future' has the power to relieve the overburdened waste management system. But some hurdles need to be overcome, in order to make this platform widely accessible and economically feasible. Extensive research efforts are needed in the direction of bioengineering, improved downstream processing and network modelling of PHA production pathways. The most attractive approach in this regard is a microalgal biorefinery that integrates multiple value-added product streams (food, feed, pharma products, biofuel) and wastewater remediation so as to extract maximum benefit from a single batch of biomass. It improves the techno-economics of microalgae cultivation on a commercial level. However, ways to minimize the use of additives in the process need to be further investigated since they render the product unsuitable for application in the medical and food packaging sectors. Various options like nutrient limitation, precursor supplementation and parameter optimization are being explored to get maximum PHB yield from cyanobacteria. In addition, innovative, cheaper and more efficient conversion technologies need to be researched. For instance, PHB purification from microalgae and carbon source are two significant cost drivers in the production process. Conclusively, the current market trend and technology improvisations in the area suggest a bright future for startups in the algal bioplastic avenue. It is now only a matter of a few years that they will soon hit the market and become a popular choice among people within no time.

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