Chlorella sp. for Bioactive Compound Detection and Synthesis of Biochar, it's Application and Characterizations

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CANDIDATE'S DECLARATION

I hereby certify that the work which is presented in the research work entitled ***"Chlorella sp. for Bioactive Compound Detection and Synthesis of Biochar, it's Application and Characterizations"*** in fulfilment of the requirement for the award of Degree of Masters of Technology in Biotechnology and submitted to the Department of Biotechnology, Delhi Technological University, Delhi is an authentic record of my own work, carried during a period from 2-Jan-2023 to 18-May-2023, under the supervision of Dr. Navneeta Bharadvaja. The matter presented in this thesis has not been submitted by me for the award of any other degree of this or any other University. The work has been published and communicated in various journal under my name with the guide.

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Date: 31 May, 2023

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CERTIFICATE

This is to certify that the Project dissertation titled **"*Chlorella sp. for Bioactive Compound Detection and Synthesis of Biochar, it's Application and Characterizations*"** which is submitted by **Vandana Joshi, 2K21/IBT/19**, 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 for the project work carried out by the student under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to this University or elsewhere.

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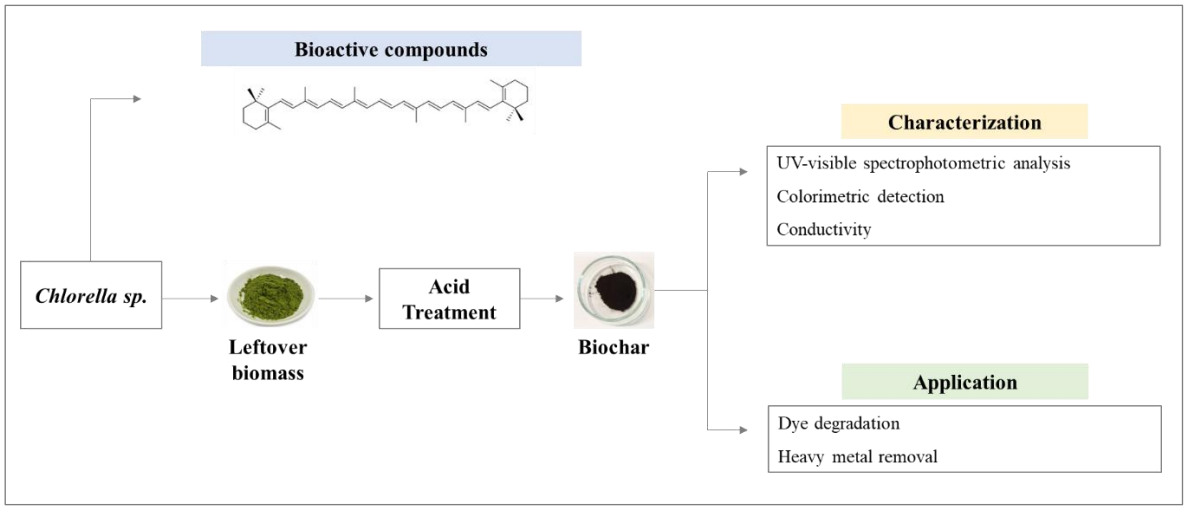
ABSTRACT

Marine vegetation offers a plethora of plant species which can not only impart bioactive compounds but also have environmental remediation potential. Numerous plants have been screened till date out of which algae are the most promising source for medicines, food, feed, bio-stimulant, additives, colloids, environmental remediation solutions etc. A variety of applications are possible from algae only because of the compounds present in algae species such as pigments (β-carotene, fucoxanthin, lutein, zeaxanthin, astaxanthin, phycocyanin, phycoerythrin); polysaccharides (porphyran, fucoidan, laminarin, and carrageenan); fatty acids (hexadecenoic acid, fucosterol, saringosterol eicosapentaenoic acid, docosahexaenoic acid); and some miscellaneous secondary metabolites (eckol, phloroglucinol, dieckol, cryptophycin, borophycin, lyngbyastatin, dolastatin 10, dolastatin 15, coibamide A, and apratoxin A). In our study we have focused on a green algae species C*hlorella sp.* which acts a functional food due to the presence of different bioactive compounds including pigments like β-carotene, fatty acids, phenols, etc. The algal species have been tested as a source of nutraceutical by β-carotene extraction. Numerous methodologies have been conducted to conclude the highest amount of β-carotene extraction methodology. Additionally, the leftover biomass has been used to generate biochar, a carbonaceous substance which acts as an adsorbent and remediate pollutants from waste water. In our study we have studied the potential of *Chlorella sp.* derived biochar for the remediation of methylene blue, an organic pollutant as well as chromium, an inorganic pollutant. UV-visible spectrophotometric analysis was done to analyse the biochar.

GRAPHICAL ABSTRACT



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LIST OF ABBREVIATIONS

DNA: Deoxyribonucleic acid

MB: Methylene Blue

Chlorella sp.

BG11: Blue Green 11

BBM: Bold's Basal Medium

UV-Vis: Ultra violet visible spectroscopy

FE-SEM: Field emission scanning electron microscopy

XRD: X-ray diffraction

NADPH: Nicotinamide adenine dinucleotide phosphate- hydrogen

SEM- Scanning electron microscopy

RT: Residence time

TEM: Transmission electron microscopy

SEM-EDX: Scanning electron microscopy -energy dispersive X-ray spectroscopy

EDS: Energy dispersive spectroscopy

SA: Surface area

THF: Tetrahydrofuran

$HgCl_2$: Mercuric chloride

KI: Potassium iodide

NMR: Nuclear magnetic resonance spec

FTIR: Fourier transform infrared spectroscopy

CuO: Copper oxide

ROS: Reactive oxygen species

Cr: Chromium

Pb: Lead

As: Arsenic

Hg: Mercury

Cu: Copper

Zn: Zinc

KI: Potassium Iodide

HCl: Hydrochloric acid

PPM: Particles per million

Rpm: rotations per minute

OD: Optical density

MB: Methylene blue

H_2SO_4: Sulphuric acid

CHAPTER 1: INTRODUCTION

1.1 Background

Algae are a primitive photosynthetic microorganism which are either micro/macro on the basis of size as well as morphology. They majorly contribute to 61% of the global manufacturing including food products (36%); food related uses like nutraceutical, additives, and hydrocolloids (15%), and animal feed (10%). While, other less significant uses of algae are in industries like cosmetics (17%), bio-stimulants, and fertilizers (11%). So far, approximately 20,000 therapeutic metabolites were isolated from aquatic environment including microalgae. They are phytoplankton existing in either marine or freshwater environment. Freshwater habitat is defined by any slightly wet area wherein species like cyanobacteria, chlorophyta, and charophyta are widely present. While a marine water habitat comprises of saline bodies including oceans and estuaries growing phaeophyta (brown algae), pyrrophyta (golden-brown algae), and rhodophyta (red algae) specie [1].

Apart from being a raw material for the bio-fuel industry. Algae also serves as a source to extract metabolites for pharmaceutical industries and are termed as functional foods or nutraceuticals. Numerous bioactive compounds such as have been studied for therapeutic effect [2]. Out which β-carotene, a provitamin A has been studied as a great source of antioxidant with a potential to treat different diseases like anticancer, anti-allergy, anti-viral, and anti-bacterial. The natural sources are useful as well as less toxic which makes them a perfect raw material for pharmaceutical interventions [3].

Globally, water pollution is a great havoc and still there are not many cost-effective measures to treat hazardous materials such as heavy metals like chromium, lead, arsenic etc. and dyes such as methylene blue, eosin etc. from industrial waste water. Such water is not only toxic to humans but also to the animals. With the increase in anthropogenic activities like inappropriate textile disposal, fertilizers, pharma, and lead industries leading to the release of non-biodegradable wastes such as heavy and dyes (methylene blue, crystal violet, eosin Y, and rhodamine B) are left untreated in effluent discharge. Lack of strict environmental policies implementation and sufficient waste water treatment plant setups have enhanced the adverse effects of such contaminants in a country like India. To remediate these toxins in not only eco-friendly but also cost-effective manner biochar has been utilized as a successful adsorbent over other traditional physiochemical methodologies [4].

1.2 Importance of study

To date approximately 175 small molecules are approved to treat cancer and other diseases out of which 49% of the drugs are derived from natural sources. However, more

than 95% of the natural sources still remain unexplored. Out of which oceans contain approximately 80% of the world's flora and fauna with a huge diversity of organisms. One of the most desired groups of marine ecosystems are algae (micro and macro), a group of non-terrestrial photosynthetic organisms that can rapidly proliferate and produce medicinally important metabolites [5][6]. They can grow individually or as a symbiont with fungi and can bear extreme weathers. Based on cellular organization, it is either prokaryotic or eukaryotic with pigments like chlorophylls, carbohydrates, proteins, vitamins, fatty acids, polysaccharides and release oxygen as a by-product. Furthermore, these organisms produce functional metabolites demonstrating anti-viral, anticancer, antioxidant, and antibacterial activities. Several algal species including, Phaeophyceae, Chlorophyta, Rhodophyta, Cyanophyta, and Diatoms have been investigated for the biosynthesis of compounds [7].

Apart from the presence of bioactive compounds, algae can also be used to treat environmental pollution which is another significant topic of concern for most of the cities in India. A great havoc has been caused by metals (heavy) which are mainly defined as elements comprising of larger than 20 (atomic number) and more than 5 g cm^{-3} (an atomic density) [1]. Such high density possessing metals are hazardous even at ppb levels [2].Currently, heavy metals are mutual toxicants found in the lakes, ponds, and rivers[3]. Metals are streamed in to the environment via both anthropogenic as well as natural sources such as ore refining, tanning of leather, synthesis of pigments, dying of textiles, electroplating of the metals, fertilizers, as well as fungicide industries [4]. Since they are non-biodegradable so they accumulate in the water body as well as human beings. Moreover, most of them are potential carcinogens possessing free radicle scavenging activity. Apart from this, many chronic degenerative diseases including, mental disorder, muscle and joint pain, gastro-intestinal problems, vision blurring, fatigue, and skin issues are also caused due to the prolonged exposure to such heavy metal. There are numerous conventional techniques including from waste water. However, these methodologies are not successful when the concentration of heavy metal is less than 100 ppm level in the water. Also, they are not cost-efficient for small scale remediations. So, a need for natural product is required which is not only eco-friendly but also cheap for large scale remediations [8].

From an economic perspective, the market share of algae-based products was estimated approximately 10 billion USD in 2021 and is growing at a rate of nearly 8% (2020-2026) [9]. According to the studies, high downstream process cost, difficulty in metabolites isolation, lack of mass cultivation strategies and low concentration of accumulated metabolites makes it difficult to scale up the research and production of such metabolites. Incorporating latest tools and technologies will make will make algae economically feasible for not only environment products but also pharmaceutical interventions.

1.3 Objectives of Study

- Analysis of bioactive compounds from *Chlorella sp.*
- Synthesis parameter optimization and characterization of biochar from *Chlorella sp.*
- To study *Chlorella sp.* derived biochar mediated remediation of Chromium

- To study *Chlorella sp.* derived biochar mediated abatement of dye methylene blue

CHAPTER 2: LITERATURE REVIEW

2.1 Algal Bioactive compounds

Pigments are the chemical composites usually found in thylakoid membrane of chloroplast which reflect a particular wavelength of visible light. Carotenoids and xanthophylls are the major lipophilic pigments which give characteristic colours to plants as well as phytoplanktons. Light is absorbed at a discrete wavelength to impart a corresponding colour for a particular species. According to the scientific investigations, most of the carotenoids possess a C-40 backbone (isoprene units) called terpenoids which are subdivided into carotenes and xanthophylls [10]. Currently, pigments showcasing market potential are either carotene or xanthophyll. All of them can present cis (Z)-trans (E) isomerism and are termed as geometric isomers [5][6]. The pigments help in chemo preventive mechanisms and are involve in altering signalling cascade, immune modulation, change in growth factor as well as cell cycle progression, cell differentiation, and apoptosis. While, as we know nearly 45% of the photosynthesis is performed by aquatic plants possessing light harvesting complexes termed as phycobilisomes (PBS) [7]. They are multiprotein complexes present on the thylakoid membrane and absorb green-yellow light in the 450–660 nm wavelength which is later transferred to the chlorophyll molecules. Each phycobilin possess conjugated double bonds that give it a spectroscopic character which impart different colour. Red algae and cyanobacteria are the main organisms possessing four types of bilin which impart specific colour at different spectral wavelengths [8].Some of the health benefits of algal compounds have been listed in the table below.

Table 1: Different class of algae derived bioactive compounds and their health benefits

Phytocompound Class	Phytocompound	Health benefits	Reference
Algal Pigments	β-carotene	Antioxidant, antibacterial, antiviral, anticancer, anti-inflammatory immunomodulant, prevent cystic fibrosis, bronchiectasis, and prevent cardiovascular diseases	[9]
	Fucoxanthin	Anti-inflammatory, antioxidant, anticancer, antiobesity, anti-	[10]

		diabetic, hepatoprotective, antiallergic, antimalarial	
	Lutein	Protection of eyes from UV radiation, antioxidant, anticancer, prevent uveitis, retinitis pigmentosa, scleritis, cataracts, glaucoma, retinal ischemia, and choroideremia	[11]
	Zeaxanthin	Protection of eyes from UV radiation, antioxidant, anticancer, prevent uveitis, retinitis pigmentosa, scleritis, cataracts, glaucoma, retinal ischemia, and choroideremia	[11]
	Astaxanthin	Antioxidant, antiallergic, anticancer, neuroprotective	[12]
	Phycobiliproteins	Anti-inflammatory, immunomodulatory, antioxidant, anticancer, antiobesity, wound healing, antidiabetic	[13]
Polysaccharides	Laminarin	Anti-inflammatory, immunomodulatory, antioxidant, anticancer	[14][15]
	Carrageenan	Anti-inflammatory, antimicrobial, anti-cancer, antioxidant	[16]
	Porphyran	Anti-inflammatory, immunomodulatory, anticancer, antioxidant, anticoagulant	[13][17]
	Fucoidan	Anti-inflammatory, antimicrobial, anti-thrombotic, antiviral, anticancer	[18]
Fatty Acids	Hexadecenoic acid	Antioxidant, anticancer	[19]
	Saringosterol	Antidepressant, antitubercular, anticancer	[20]
	Fucosterol	Anti-inflammatory, antioxidant, neuroprotective, antiadipogenic, antiobesity, antidiabetic, antiosteoarthritic,	[20]

		immunomodulatory, prevent skin-aging, antibacterial/viral/fungal/protozoan, anticancer	
	Eicosapentaenoic acid (EPA)	Anti-inflammatory, antioxidant, antiviral, anticancer, prevent osteoarthritis, cardiovascular diseases, obesity	[21][22]
	Docosahexaenoic acid (DHA)	Anti-inflammatory, antioxidant, antiviral, anticancer, prevent osteoarthritis, cardiovascular diseases, obesity	[23]
Other Secondary Metabolites	Eckol	Anti-inflammatory, antioxidant, antidiabetic, neuroprotective, anticancer, anticoagulant, antiobesity, antibacterial, antiviral, antiphotoaging radioprotective, antihyperlipidemic	[24]
	Phloroglucinol	Antioxidant, antitumor	[25]
	Dieckol	Antibacterial/viral/fungal, anticancer, antioxidant, antiaging, antidiabetic, antihepatic, antiobesity, antihyperlipidemic neuroprotective, antithrombotic	[26]
	Cryptophycin	Prevent cell proliferation, anticancer	[27]
	Borophycin	Cytotoxic, antitumor	[28]
	Lyngbyastatin	Anticancer, antiproliferative	[29]
	Dolastatin 10	Antiproliferative, antiprotozoal, antiproliferative, Osteogenic	[30]
	Dolastatin 15	Anticancer	[31]
	Coibamide A	Anticancer	[32]

	Apratoxin A	Cytotoxic, anticancer	[27]

2.1.2 Xanthophylls

A class of lipid soluble pigment are xanthophylls. They are oxygen abundant hydrophilic pigments possessing hydroxyl group at their general structure. The presence of oxygen atoms makes them more polar than their counterpart carotenoids. The carbon backbone of xanthophyll comprises of double bonds such as allenic bond (C=C=C), and triple bond like acetylene (C≡C) which permit free electron movement and easy absorbance of light in the blue–violet region of the visible spectrum (300–600 nm) [33] [10]. While hydroxyl and epoxide groups at the β-rings contribute with the antioxidant scavenging activity. Overall, they can act as (i) (ROS); (ii) (RNS); (iii) efficient chain-breaking antioxidants. Also, due to improved polarity they are able to quench dissolved singlet oxygen more efficiently than carotenoids which are non-polar. Xanthophylls with potent antioxidant/anticancer activity include fucoxanthin, lutein, zeaxanthin, and astaxanthin [34].

2.1.3 Carotenoids

A primary class of lipid-soluble pigments are carotenoids. They are oxygen deficient hydrophilic pigments (only hydrocarbons) comprising of polyene chain having 9-11 conjugated double bonds in their general structure and form an extended π-electron system which makes both ultraviolet (UV) radiation and visible light absorption possible. Comprise of conjugated double bonds in the C-40 backbone and cyclic moieties, in addition to oxygenated modification, lead to diverse colour range of these pigments [35][36]. They can (i) quench single oxygen molecule; (ii) convert hydroperoxides into stable compound; (iii) prevent free radicle formation by inhibiting oxidation of free radicle as well as auto-oxidation of chain reaction; (iv) prevent metal pro-oxidation by converting iron and copper into harmless molecule and act as metal chelators. Due to the involvement of carotenoids in physiological mechanism as well as their harmful effects on human body a balance between pro-oxidant and antioxidant molecule is maintained. Oxidative stress arises due to the presence of an imbalance between free radicle formation as well as their neutralization. Therefore, reducing oxidative stress directly reduce the chance of cancer development [37]. Carotenoids are also sensitive to light, heat, and oxygen which increase storage difficulty. Since human beings cannot synthesize carotenoids naturally, it can be given in the form of supplements to treat disorders including degenerative diseases (age-related), cardiovascular problems, cancer and stimulates immunity. A major carotenoid with potent antioxidant/anticancer activity includes β-carotene [38].

2.2 β-carotene

β-carotene, a vitamin A precursor is the most abundant cyclic carotene comprising of a C-40 backbone containing two β-unsaturated rings at both ends of the molecule with an isoprenoid chain attached. They are apolar and found inside the hydrophobic core of bio-membranes. Due to the presence of therapeutic potential in it has been recognised as the potential nutraceutical. It is also involved in different biochemical functions such as a hormone, antioxidant, cell signaling mediator, tissue growth regulator, and cell differentiation inductor [10]. They are required in the micro quantity by the human body. [39].Which means 28 mg of β-carotene can only be converted to 1mg of retinol. Therefore, this creates a potential threat of developing vitamin A deficiency in humans. To date not many adverse effects other than 'carotenodermia', a skin discolouration condition which happen due to elevated concentrations of carotenoids in the body. Additionally, it is not even mutagenic, carcinogenic, or teratogenic in any of the clinical studies. Moreover, it has been studied that long-term exposure to β-carotene may not enhance serum retinol level in people having sufficient amount of vitamin A level. It should be administered with fats for increased serum and tissue accumulation. Mostly β-carotene is converted by the microbes present in the gut to some unknown compounds and the rest is excreted out of the body [40].

2.3 β-carotene Extraction Methodologies

There are numerous methodologies used to extract carotenoid from algae. However, most of the carotenoids are hydrophobic in nature so the easiest way to extract them is with the help of organic solvents (non-polar) including hexane, petroleum ether, and tetrahydrofuran (THF). Whereas, hydrophilic carotenoids are extracted using organic solvents (polar) including acetone, ethanol, and ethyl acetate [34]. There are various methods that are used in the extraction of β-carotene including

(i) Atmospheric liquid extraction with maceration
(ii) Soxhlet extraction
(iii)Super critical fluid extraction
(iv)Ultrasound assisted extraction
(v) Microwave assisted extraction
(vi)Pressurized liquid extraction
(vii)Pulse electric field assisted extraction
(viii)Enzyme assisted extraction
(ix)Green extraction

All the above methods differ by difference in cell disruption technique. Additionally, there is a variation in temperature, pressure for the extraction. However, more recently the techniques have been improved with rapid cost-effective extraction such as using organic solvents for carotene extraction is more common these days as it is not only environmentally and non-toxic but also cost effective.

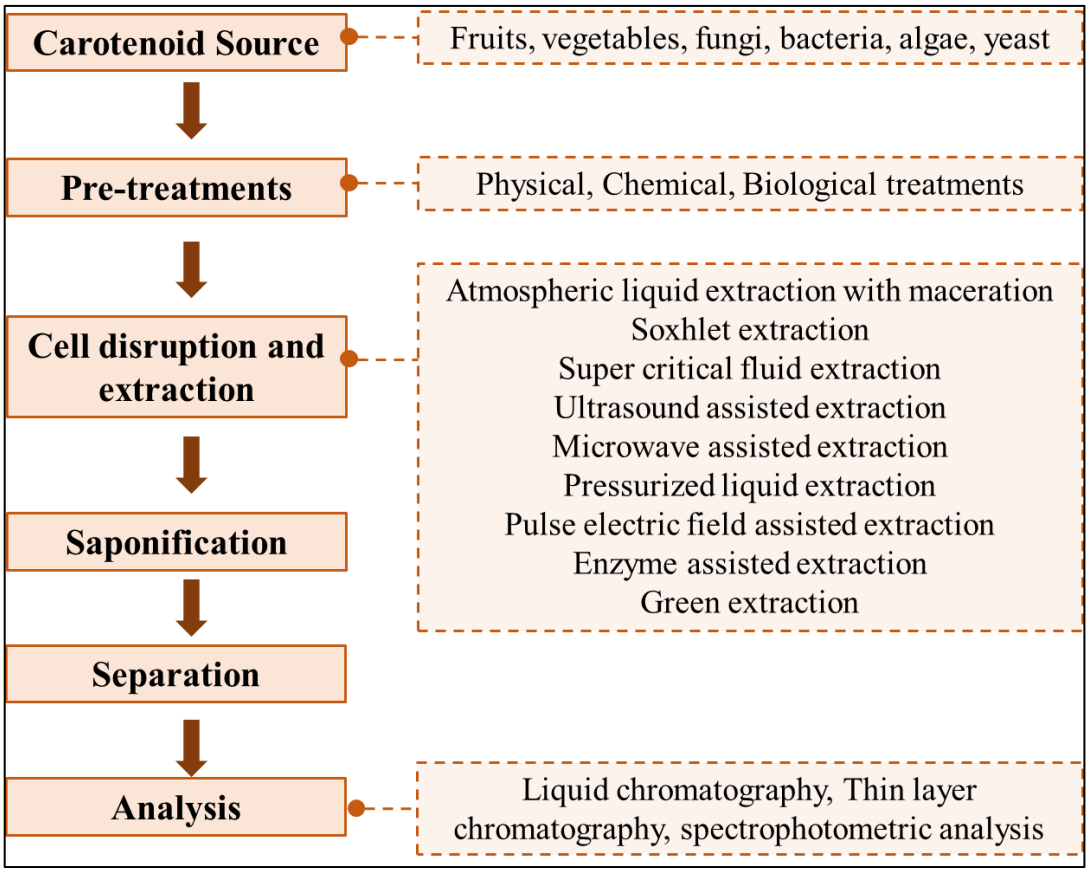

Fig 1: Summary of β-carotene extraction methodologies

2.4 Biochar

A biochar is a carbonaceous porous substance formulated via thermal treatment of waste biomasses in the oxygen deficient environment as per European Biochar Certificate (EBC) [6]. A prominent advantage of utilizing biochar is that it can be created from a variety of wastes including agricultural waste, kitchen leftover, forest, industrial, and sewage sludge. It has been targeted as great source for not only waste management but also improving soil fertility, production of energy, mitigation of climate, heavy metal and dyes from waste-water. The main reason for their remarkable result is its adsorption capacity which is available due to the presence of functional groups (surface) of char along with alkaline pH, improved SA, porosity, desired carbon/nutrition/ash content, and water holding capacity [4].

2.5 Biochar Synthesis Methodologies

The physiochemical characteristic of a biochar totally depends on the methodology with which it has been prepared. Currently, the major biochar fabrication techniques involve thermal treatment including carbonization (hydrothermal), torrefaction, and pyrolysis. For the high yield product appropriate technique, biomass selection, and rate of heating, temp., residence time shall be on point. Such conditions not only affect the physical but also the chemical state of biochar. Numerous methods have been summarised in **table 2** below.

2.5.1 Pyrolysis

Decomposition of waste primarily organic matter in an O_2 deficient situation in the]250 - 900°C is called pyrolysis. This method can also be used as the process to convert biomass (waste) into a value-added product including biochar, syngas, and bio-oil [41]. Moreover, within the process cellulose, hemicellulose, and lignin are also depolymerised and fragmented into solid, liquid, or gaseous state. [42]. The nature of biochar mainly depends on the type as well as the nature of biomass used under optimum temperature conditions. Temperature increase is not directly proportional to yield of biochar. Higher the temperature more the amount of syngas is produced and vice versa. Furthermore, on heating rate basis, residence of time, and temperature [43].

Fast Pyrolysis: It can make biomass (solid) into a liquid oil under high heating conditions (400-600°C). Characteristics of fast pyrolysis are:

i. Quick warming of biomass particles (less than 100°C/min)
ii. Treatment temperature of 400-600°C
iii. Shorter duration at higher temperature leading to fumes at (0.5-2 s)

Slow Pyrolysis: Heating rate less than 5-7°C/min with a residence time of more than 1 hour. In comparison to rest of the methods, slow pyrolysis produces the best quality of char [44].

2.5.2 Carbonization (Hydrothermal)

This process can be done at a temperature range of 180-250°C and hence is considered to be the most cost-efficient methodology. The produce derived from this technique is termed as hydrocar. However, a pressure range of 2-10 MPa is maintained overall. Biomass is mixed with water first and then heated to be converted into biochar [45]. Different products are obtained at varying temperatures such as at 250°C biochar is produced. While between 250-400°C hydrothermal liquification leads to bio-oil extraction. At 400 °C hydrothermal gasification occurs which release gaseous products including CO, CO2, H2 and CH4 are released [46]. According to the studies the carbonaceous material produced via this process is more in quantity. The hydrophilic part of the char comprises of particles with aromatic rings enclosing carbonyl, hydroxyl, ester, phenolic, and carboxylic group. Therefore, it is a great solution to remediate waste water contaminants. The process of adsorption can be improved further by treating it with surfactants such as acids or alkalis [42].

2.5.3 Gasification

It is methodology mainly comprises of thermochemical reaction which changes carbon comprising material to product mainly syngas (CO, CO2, CH4, H2). The temperature is directly promotional to the syngas production that is as the temperature increase the amount of CO also increased while hydrocarbons decreased. However, the main product obtained is syngas and a by-product is also produced which is char [47]. Gasification has 2 main steps

Drying: It evaporates all the moisture from biomass without recovering any energy. The moisture is dependent on variety of raw materials (biomass). This is done separately only when the biomass comprises of elevated content of moisture [43].

Oxidation: Oxidation is a prominent step for gasification. The agents react with species that are combustible in a gasifier to release CO2, H2O, and CO [48].

2.5.4 Torrefaction

The most recent technique developed to produce biochar. Since it involves low rate of heating. Therefore, is a mild pyrolysis. The air, oxygen, and CO2 are eliminated from the biomass via different decomposition processes and the char is produced by heating biomass in an inert environment at 200-300^{o}C for less than 30 minutes. The rate of heating is less than 50 This process can not only modify the biomass but also improve particle size, moisture, rate of heating is less than 50^{o}C/min, and energy density. The process of torrefaction involves a few steps to produce the output (biochar) [49] [50].

Heating

The process involves heating biomass till the moisture is completely evaporated and the temperature of drying is maintained.

Pre-heating

The process arises at nearly 100^{o}C till the moisture content of biomass is majorly evaporated

After drying

The temperature raises upto 200^{o}C to evaporate water completely. This also leads to loss in the char mass

Torrefaction method

A major step wherein temperature is maintained at 200^{o}C to evaporate water completely

Cooling method

Once the product is formed, the temp. is brought down to normal (room temperature) for taking the product out

On the basis of medium used to heat biomass torrefaction can be of three types:

Steam torrefaction: Biomass is heated at around 260^{o}C for 10 minutes with the help of steam only. This method is defined as steam torrefaction

Wet torrefaction: Biomass is heated with the help of water at a temperature range of 180-260^{o}C for around 5-240 min. This method is alternatively defined as hydrothermal carbonization [51].

Oxidative torrefaction: Biomass is heated using gases which are oxidizing to generate heat energy [49].

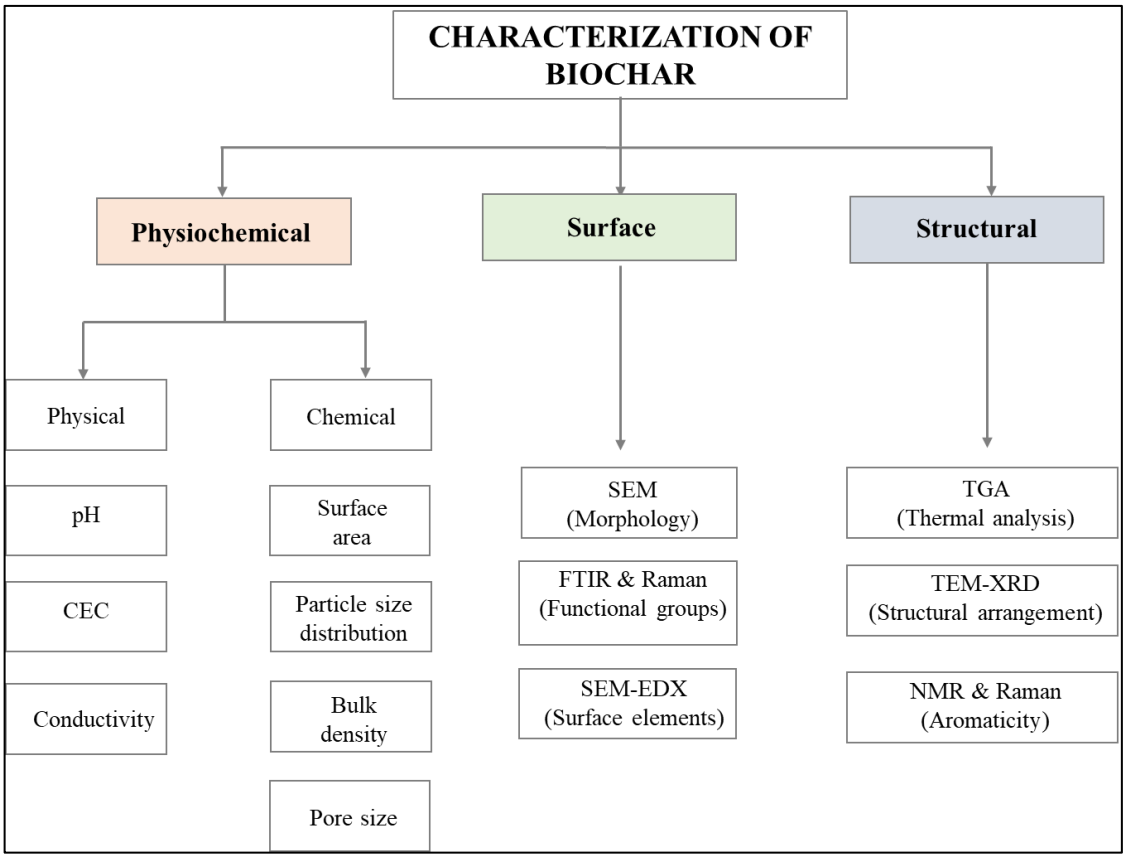

Fig 2: Biochar characterization techniques

Table 2: Biochar synthesis methodologies summary

Technique	Temperature (°C)	Residence Time	Biochar Yield (%)	Bio-oil Yield (%)	Syngas Production
Slow pyrolysis	300-700	<2 s	35	30	35
Fast pyrolysis	500-1000	Hour-day	12	75	13
Hydrothermal Carbonization	180-300	1.6 h	50-80	5-20	2-5
Gasification	750-900	10-20 s	10	5	85
Torrefaction	290	10-60 min	80	0	20
Flash Carbonization	300-600	<30 min	37	-	-

2.6 Factors Affecting Properties of Biochar

The major reason responsible for biochar production is pyrolysis. While the factors which can alter the biochar yield are temperature, feedstock (raw material), particle size, and rate of heating. In order to determine the biochar application an analysis on its properties is required [45]. Most common raw materials for the production of biochar are plant materials, agricultural residue, wooden biomass, sewage waste etc. According to the

studies, biochar produced via pyrolysis in the rate of 400–1000°C from animal waste is more in quantitative terms as compared to the wood derived biochar or agricultural residue derived. Common factors affecting char quality are detailed below [52].

2.6.1 Raw Material

Biomass also referred to as feedstock is the raw material which is either derived from living or from non-living organisms. Depending on the nature of material it can be woody (involves tree and forest residues)) [53]. Amongst all the properties moisture content is the most prominent attribute for good quality biomass. It can if solid, liquid, or gas form. Higher the moisture reduced is the char quantity. Therefore, a low moisture biomass is the preferred feedstock for biochar production [54].

2.6.2 Temperature for Carbonization

Biochar is prepared via pyrolysis, a thermochemical process done in an oxygen deficient environment. On the basis of temperature range pyrolysis is grouped into 3 types that is (i) slow pyrolysis: <300°C temperature range, (ii) moderate pyrolysis: 300-500°C temperature range, (iii) fast pyrolysis: more than 500°C temperature range. Pyrolysis not only influence the physiochemical properties but also the structure size of pore, SA, and different functional groups on biochar surface [55] [56].

2.6.3 Residence Time

The time required to heat the char at a particular temperature is referred as residence time. Decreasing the temp. of pyrolysis decreased the yield of bio-char. While expanding residence time at increased temperature created a no impact on yield of char.

2.6.4 Pre-treatment of Biomass

The char characteristics are influenced by pre-treatment. The most common pre-treatment method is to reside the raw material in a solution. For instance, pine-wood when immersed in dilute acidic acid solution resulted in high char yield. While nitrogen and metal doping can also influence the char yield. Soaking and steaming may change the elemental competition. Baking can change the carbon content and remove moisture content of biochar [28].

2.7 Biochar Characterization

Characterization of biochar is done to understand it’s potential to remove pollutants as well as for other applications. Biochar generally works as an excellent adsorbent to remove soil and water pollutants. Therefore, the elemental composition of biochar helps to predict its environmental impact [50]. The most common characterization methodologies are based on structure [57].

2.7.1 Functional groups

The prominent functional groups which enhance the sorption properties of char are carboxylic, hydroxyl, amine, amide, and well as lactonic groups. These groups are present on the char surface are dependent on the biomass as well as temperature conditions [58]. While if the pH, surface area, and porosity of the char is increased then

there is a function group reduction on the char surface. Such groups can be identified via FTIR. Additionally, NMR is also capable to determine the presence of functional groups.

2.7.2 Surface area and porosity

A char possessing more SA and increased pores is successful as an adsorbent. The porous cavities are formed during pyrolysis. That is at high temperature water is evaporated creating dehydration and formation of mesh like structure in the heated biomass [55]. The size of these pores can range from 2 – 50 nm. Also, a biochar with reduced pore size is uncapable to adsorb pesticides. This can be determined via SEM. SA is responsible for biochar adsorption property while temperature determines its accurate formation. According to the studies activation creates a more porous structure. Therefore, a physical or chemical activation shall be fused when producing a biochar [59].

2.7.3 SEM

Structural surface of a biochar is determined via scanning electron microscopy. It helps to identify that different temperature may alter surface morphology of the particle. Images formed after SEM provide a detailed description of meso as well as micro-pore in the biochar. While SEM-EDX is used to predict the elements (contaminants) adsorbed on the surface of the biochar. The only disadvantage of SEM-EDX is that is it not suitable for organic contaminants [60].

2.7.4 FTIR

FTIR is used to determine the FG available on the bio-char's surface. As temp is changed during pyrolysis the functional groups on the surface also alter. Therefore, the increase in temperature enhanced the functional groups on surface. The major reason behind his decrease is rapid evaporation of volatile compounds [58]

Table 3: FTIR peak analysis interpretation [58]

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-0 stretch, aryl-alkyl ether

2.7.5 XRD

XRD technique used to understand bio-char's structure. The XRD graph implicate attributes of various materials which have been created over 350°C. The crystalline structure formed can be analysed as they are resembled by sharp XRD peaks. Therefore,

XRD produces char which is not only high in quality but is also made quickly in a non-destructive manner resulting in high sorption [57].

2.7.6 NMR

NMR examines the structural composition of the char. Their main role to find aromatic rings and aliphatic groups. The only drawback of NMR is that is cannot work properly in the presence of ferromagnetic minerals. Because they interrupt NMR signals at high-temperature leading to low signal-to-noise ratio [61].

2.7.7 CHNS Analysis

The amount of oxygen present in the biochar can be determined using CHNS distribution. According to the studies the sum total of all these (C, H, N, and S) should be 100. In most of the studies, lower aromaticity and higher oxidative state demonstrate that a decrease in N content on the char surface is due to the replacement of Cr or MB instead of N on the char surface.

Table 4: CHNS analysis of *Chlorella sp.* derived biochar

Parameter	Biochar (Reference values)
N%	11.32
C%	41.95
H%	2.45
S%	7.01
C/N Ratio	3.7
C/H Ratio	17.11

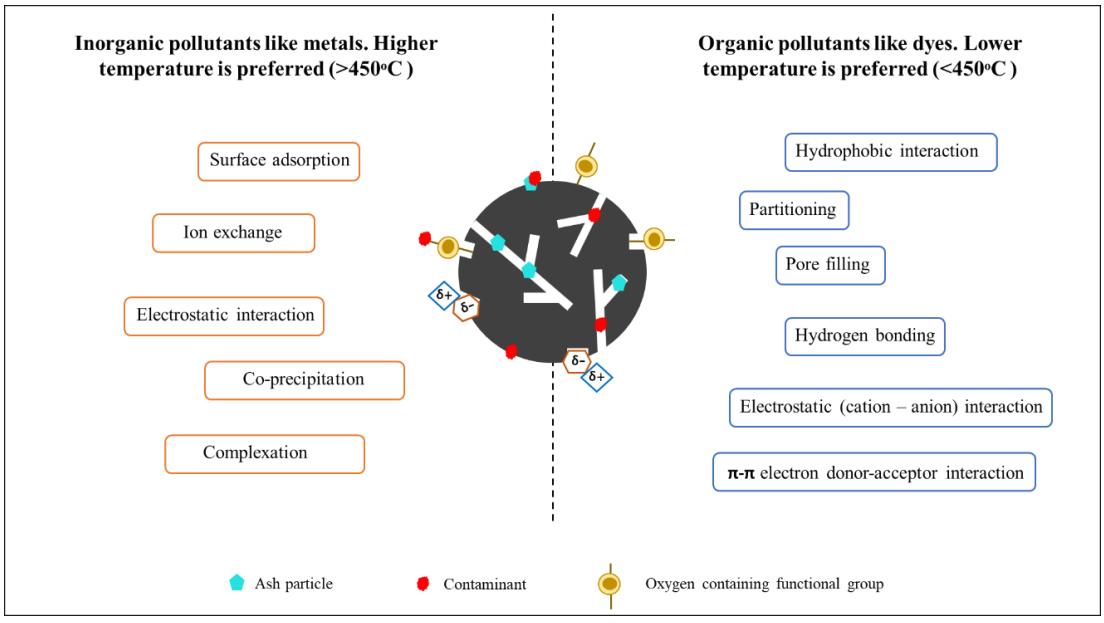

Fig 3: Mechanism of pollutants removal via biochar (organic/inorganic)

2.8 Biochar Applications

The synthesis of biochar is not only eco-friendly, but also inexpensive. It can be easily prepared from a variety of biomasses and water as well as soil [9]. Carbon enriched char produced during higher temperature possess more removal efficiency due to porosity, improved SA, pH, as well as hydrophobicity. While, biochar synthesized at low heat involves functional groups (oxygen), organic carbon (dissolvable), low pores. Therefore, such chars are used to remove inorganic pollutants from any environment. Some of the other unconventional uses of biochar are it can act as a catalyst, can treat waste-water, used while composting, for storing energy, and for sequestering carbon, and soil amendment [45].

2.8.1 Heavy Metal Removal

Metals including toxicants are detected in ricer, ponds, and lakes which can be removed via biochar. Higher the carbonization temperature more (< 400^{o}C) is the surface sorption area and volume of the pore. Biochar attaches metal to its surface via electrostatic attraction. However, immobilization of a metal ion is dependent on pH as well as isoelectric point of char [62]. Cations which are also called as bivalent metal ions can be exchanged with the functional groups on char surface. This efficiency of exchanging cations can be enhanced if the biochar is produced at higher temperature (<350^{o}C) with iron used in the raw material. If the char is produced from plant then it is initially precipitated as plant biomass comprises on not only cellulose but also hemicellulose. Such biochars are alkaline form mineral precipitate from inorganic pollutants. However, biochar produced at lower temperature will possess more of functional groups having oxygen including carboxyl, hydroxyl, and phenols to bind heavy metals. Plant derived biochars encompass enhanced ion exchange capacity [63].

2.8.2 Organic pollutants abatement

Synthetic dye is used in fabric dying industries. The effluents from such industries pollute water bodies. These pollutants containing pigments can be abated using biochar. Biochar can be either amorphous or crystalline in nature. The non-carbonised part adsorbs organic pollutants. The portioning mechanism works well in volatile matter. Mesopore and micropore biochar results in abatement. The net charge on biochar leads to attracting ionizable compounds. Higher the pH more is the electrostatic strength of attraction of pollutants [51] [58].

Table 5: Summary of different application of biochar

Application	Aim	Advantage	Limitation
Catalyst	Act as supporting material for catalysis	Cheap, more functional groups, increased surface area	Less efficiency
Energy storage	Utilize electrode as material	Cheap, highly porous, increased surface area	Low in performance
Soil amendment	Enhance soil fertility, quality, do carbon sequestration	Low cost, reduced greenhouse emission, help to retain nutrients and water, control nutrient loss	Comprise of heavy metal, aromatic hydrocarbons,
Adsorbent	Removal of organic as well as inorganic pollutants from soil or aqueous medium	Low cost, more oxygen groups, enhanced adsorption	Removal efficiency of pollutant is undetermined and heavy metal retains
Composting	Improve structure of microbial population and carbon mineralization	Porous, reduced greenhouse emission, increased surface area, and retain nutrients	Chances of heavy metal and other contaminants invading into soil

2.9 Factors affecting removal efficiency (Biochar)

2.9.1 pH of solution

pH of waste water possessing heavy metals as well as dyes (organic) is the main factor to influence sorption of biochar [64]. The overall charge on the char was totally dependent on wastewater pH. With higher pH such functional groups are de-protonated thus enhancing the sorption capacity of char. While at acidic pH similar functional groups gets protonated which favours Cr (VI) [65]. However, at the pH lower than zero-point charge (ZPC) biochar is charged positively which repels metal ions as protons. Cr removed at lower pH form an (negatively charged). Due to increased redox potential Cr (VI) gets oxidized to Cr (III). As the pH is increased less amount of chromium was adsorbed to the char surface. This is mainly due to a binding competition between chromate and hydroxyl ions [42].

2.9.2 Dose for adsorbent

The adsorbent amount is linked directly to the removal efficiency of metals and pollutants (organic/inorganic) [66]. Therefore, knowing the optimum quantity of biochar which is not only cost effective but also provide maximum remediation is necessary. The amount of char is directly promotional to the removal efficiency as it provides more surface area and pores for sorption. However, a very dose of biochar leads to declined sorption capacity. This may occur due to aggregation of biochar particles that leads to unsaturated sites for adsorption in long run [43].

2.9.3 Initial contaminant concentration

The concentration of contaminant initially may also affect the adsorption. Increased contaminant concentration can lead to reduced removal efficiency. This is possibly due to limited adsorbent offering few sites for adsorption. At increased concentration such sites get saturated leading to sustained adsorption [47].

CHAPTER 3- MATERIALS AND METHODOLOGY

C*hlorella sp.* was provided by Dr. Lakhan Kumar, Plant Biotechnology Laboratory, Delhi Technological University. All the chemicals required to prepare BG-11 media, dyes, and metal salts were obtained from the same lab. All the glassware, tubes, and flasks were thoroughly washed from laboline and treated with very dilute sulphuric acid followed by deionised water. Finally, all the glassware was dried in oven before using in experiment. Milli-Q water was taken for all the solution preparation required in the experiment.

3.1 Algal cultivation and growth optimization

Growth medium optimization for *Chlorella sp.*

- 10% algal strain (*Chlorella sp.)* was inoculated in BG-11 media under aseptic conditions.

- The culture flasks incubated at 16:8 hours at 28^{o}C for 30 days. 2mL aliquots were used to take spectrophotometer reading (690 nm) at an interval for 2 days.

- A semi-log graph having number of days on X-axis and on Y-axis absorbance was plotted for different mediums for a comparison.

pH optimization of *Chlorella sp.*

- 10% algal strain (*Chlorella sp.)* was inoculated at 5 different pH conditions including pH-6, 7, 8, 9, and 10 under aseptic conditions

- The culture flasks were incubated for 16:8 hours light dark cycle at 28^{o}C for 30 days. 2mL aliquots were used to take spectrophotometer reading (690 nm) at an interval for 2 days.

- A semi-log graph having number of days on X-axis and on Y-axis absorbance was plotted for different mediums for a comparison.

Carbon source optimization of *Chlorella sp.*

- 10% algal strain (*Chlorella sp.)* was inoculated with 5 different carbon sources including glucose, sucrose, fructose, glycerol

- The culture flasks were incubated for 16:8 hours light dark cycle at 28^{o}C for 30 days. 2mL aliquots were used to take spectrophotometer reading (690 nm) at an interval for 2 days.

Nitrogen source optimization of *Chlorella sp.*

- 10% algal strain (*Chlorella sp.)* was inoculated with 4 different nitrogen sources including ammonium chloride, sodium nitrate, urea, glycine

- The culture flasks were incubated for 16:8 hours light dark cycle at 28°C for 30 days. 2mL aliquots were used to take spectrophotometer reading (690 nm) at an interval for 2 days.

Phosphorous source optimization of *Chlorella sp.*

- 10% algal strain (*Chlorella sp.)* was inoculated with 3 different phosphorous sources including potassium nitrate, monopotassium phosphate, potassium dihydrogen phosphate.

- The culture flasks incubated at 16:8 hours at 28°C for 30 days. 2mL aliquots were used to take spectrophotometer reading (690 nm) at an interval for 2 days.

Biomass Measurement: Take 1.5 mL centrifuge tube and fill them with the culture solution of *Chlorella sp*. Centrifuge at 10,000 rpm for 5 mins. Only pellets were taken as supernatant. Collected pellets were cleaned with water (distilled water) nearly two times via followed by 40°C in the oven. The weight of the pellet was measured using weighing balance and added in the formula below to calculate the biomass productivity.

$$Biomass\ productivity = \frac{Weight\ (mg)}{Volume\ of\ culture\ (L) \times No.\ of\ cultivation\ days} \quad 3.1$$

$$Biomass\ concentration = \frac{Weight\ (mg)}{Volume\ of\ culture\ (L)} \quad 3.2$$

3.2 Qualitative test for bioactive compounds in *Chlorella sp.*

Test for Alkaloids

- Mayer's reagent preparation

Add 1.36 gm of $HgCl_2$ to 5 gm KI make volume upto 100 mL distilled water.

- Sample preparation

1 gm of dried algae biomass is mixed in 10 mL solution (70% methanol and 70% acetone). The above was macerated at 50 rpm for 24 hrs.

- Mayer's test procedure

The samples were centrifuged 10,000 rpm for nearly 9 mins. 1 N HCl added to sample and the mixture was filtered. Finally, Mayer's reagent added to the filtrate and incubated at RT for nearly 1 hour.

A white precipitate at the bottom of the test tube indicated the presence of alkaloids in our sample algal species (*Chlorella sp.*)

Test for Carotenoids

1 gm of algal powder was added in a ratio of 4:1 (Acetone: water) making a total volume of 10 mL solution.

Solution centrifuged at 12,000 rpm, 2 mins.

Supernatant extracted, analysed with via spectrophotometer at 470 nm to confirm the presence of carotenoid (β-carotene).

Test for Flavonoids

- Prepare 2mL algal extract and add 2-3 drops of 2% sodium hydroxide (NaOH) to it. Initially a deep yellow orange colour will appear.

- Now add dilute HCl (1N) to the mixture which resulted in making the solution colourless.

Test for Phenols

- Neutral Ferric chloride ($Fecl_3$) preparation

1N NaOH is mixed to 1% $FeCl_3$ dropwise to form coloured precipitate (brown). Precipitate was filtered and supernatant was collected (neutral $FeCl_3$)

- Neutral $FeCl_3$ Test

Solution of neutral $FeCl_3$ was added dropwise to the algae samples and the colour was noted (green).

3.3 Quantitative test: β-Carotene

The estimation of β-carotene from *Chlorella sp.* is done via sonication (10 minutes), heat treatment, and bead milling in tetrahydrofuran (THF). A wet biomass of *Chlorella sp.* was taken to disrupt the cell via above techniques. Finally, UV-Vis spectrophotometry was performed to estimate the amount obtained. According to the literature, a peak to estimate β-carotene is obtained at 450 nm. Following formula is used to estimate the quantity of β-carotene obtained.

$$\text{Total β-carotene (μg/ml)} = 25.2 * A450 \quad (3.3)$$

Finally, for the extraction methodology *Chlorella sp.* was cultivated in BG-11 media along with sucrose as a supplement to produce enhanced growth. At different disruption techniques β-carotene was extracted to find the maximum content.

3.4 Microalgae harvesting for biochar synthesis

- A month-old microalgae culture was taken in a centrifuge tube for centrifugation 10,000 rpm for nearly 10 mins at room temperature.
- The pellets obtained were washed in double distilled water 2-3 times
- Wet biomass obtained was dried at 60^{o}C for 24 hours (in oven).
- Pellets of dried biomass was further grinded in mortar and pestle into fine particles.

Fig 4: *Chlorella sp.* cultivation in growth chamber

3.5 Biochar synthesis methodology

- Powdered algal sample nearly 1 gm was taken in a small beaker (50ml) and a conc. H_2SO_4 was added dropwise to it in a ratio of 1:1.8 (means in 1 g algae powder 1.8 ml H_2SO_4 was added)
- The acid treated samples were heated in a hot plate at nearly 160-200^{o}C for 6-8 hours for the formation of black coarse biochar.
- The biochar was stores at RT
- Dried black coarse biochar was collected followed by grinding into a fine powder followed by sieving through 90 µm.
- The sieved sample was then washed using double distilled water by centrifuging at 10,000 rpm nearly 5-6 times till a pH 7 is achieved.
- The washed samples having pH 7 were then dried at 60^{o}C for 3-4 hours in order to remove water from it.
- The biochar obtained was weighed, characterized, and stored at room temperature for testing its applications.

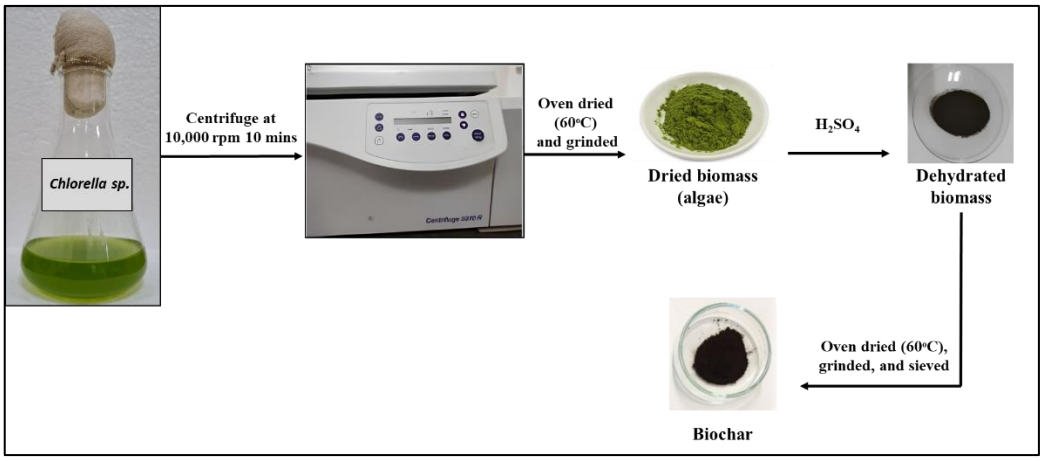

Fig 5: Summery of synthesis of biochar from *Chlorella sp.*

Note: *Precautions such as gloves, mask, and protective eyewear were used during the synthesis of biochar to prevent any human body damage from acid*

3.6 Biochar Yield

Yield of biochar was calculated by the formula as follows:

$$Yield\ (\%) = \frac{Mass\ of\ biochar\ (gm)}{mass\ of\ raw\ material\ (gm)} * 100 \qquad 3.4$$

Here, raw material is dried microalgae powder (in grams)

3.7 Characterization of biochar

Chlorella sp. derived biochar was characterised visually as the colour changed from green to coarse black. Additionally, other techniques including UV-Vis spectroscopy was used to confirm biochar production. While, FE-SEM was used to determine not only shape but also surface morphology. To determine crystalline structure X-ray diffraction can be used. Additionally, to analyse functional groups which acts as binding sites is done by FTIR. For determining the size zeta-potential is used.

3.7.1 Visual Characterization Method

The primary characterization methodology is confirmed visually during the synthesis of char. As the H_2SO_4 is added to the dry algal powder or plant powder the colour changes from dark green to grey-black.

3.7.2 UV-Visible Spectroscopy Method

A suspension mixture of biochar after its synthesis was made at 0.5 g/L. Conversion of dried biomass into char was analysed using a UV-visible spectrophotometer (200-700 nm) against deionised water as the blank. All the scans were done in triplicates.

3.7.3 Biochar Conductivity

250 mg of biochar was added to distilled water (50 mL). The solution was left undisturbed for more than an hour after which its conductivity was measured using a conductivity measuring probe.

3.7.4 Biochar pH

1% solution of biochar was prepared in double distilled H_2O.The solution was stirred restlessly for the time interval of 5 minutes after which pH was determined using pH meter along with gentle swirling of the solution.

3.8 Biochar applications

3.8.1 Heavy metal removal

Biochar synthesized from *Chlorella sp.* was used to remove heavy metal majorly Cr from synthetic waste water

- **Preparation of standard curve (Cr):** The freshly synthesized biochar has been utilized to remediate chromium (Cr) from the waste water prepared synthetically in the Plant biotechnology laboratory, DTU. The analytical grade potassium dichromate ($K_2Cr_2O_7$) was used to prepare waste water. A 30-ppm stock solution was made in double distilled water with altering chromium concentrations. A graph was made with concentration of chromium on X-axis as well as absorbance on Y-axis (350nm).

- **Study of adsorption:** Experiments of adsorption were performed in REMI shaker with the 120-rpm rotation speed at 25^{o}C (RT).

- **Adsorbent dosage:** Different amount of biochar was used in varying concentrations to determine adsorbent effect on chromium removal. Amounts including 0.1, 0.3, 0.6, 0.9, 1.2, 1.5 g/L were mixed to 50 mL chromium solution having 30 ppm metal concentration at 25^{o}C (RT).

- **pH effect:** Cr metal solution's pH was varied between 2-10 at 30 ppm concentration of metal salt having biochar dosage 1.5 g/L. Additionally, and was adjusted using HNO_3 and KOH.

- **Chromium removal:** The biochar synthesized from *Chlorella sp.* has been used to remove heavy metal chromium.

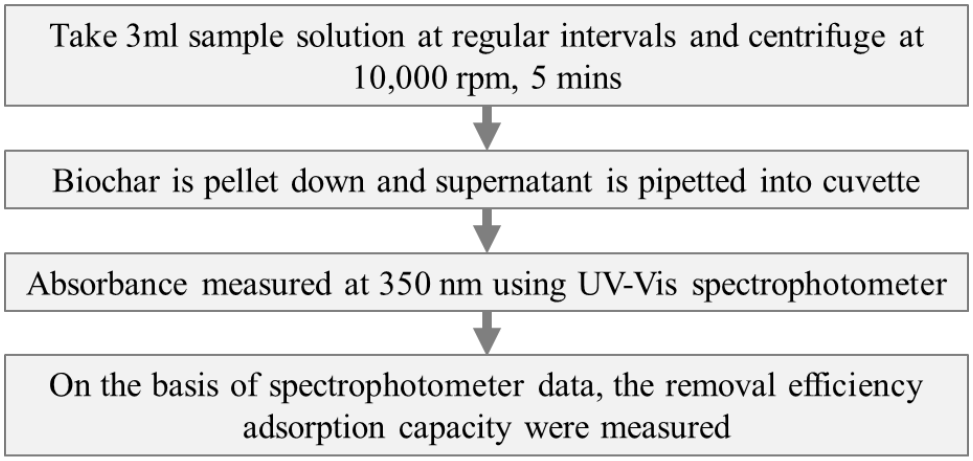

Fig 6: Steps for Chromium remediation via biochar

Formula to calculate removal efficiency (RE) and adsorption capacity (Q_e) of Methylene Blue is:

$$RE = \frac{Initial\ concentration\ (Ci) - Final\ concentration\ (Cf)}{initial\ concentration\ (Ci)} * 100 \quad 3.5$$

$$Qe = Initial\ concentration - Final\ concentration * \frac{Total\ volume\ (v)}{Amount\ of\ adsorbent(w)} \quad 3.6$$

Where,

Ci: Initial MB concentration (mg/L)
Cf: Final MB concentration (mg/L)
v: Total volume of dye solution tested for degradation (mL)
w: Amount of adsorbent (g)

3.8.2 Dye abatement

The biochar synthesized was used for remediating the dye Methylene Blue (MB), an organic pollutant usually discarded from textile industries.

- **MB standard curve:** The biochar prepared was used to remediated the dye MB. A 30-ppm solution called as stock solution was prepared in MiliQ water and was diluted to at varying concentrations. A graph was plotted X-axis comprising of methylene blue and Y-axis possessing absorbance at 665nm.

- **Adsorption:** Adsorption experiments were carried out using *Chlorella sp.* derived biochar for MB removal. The samples were rotated/agitated at 120-rpm.

- **Adsorbent dosage:** In 5 mL synthetic dye solution biochar was mixed in different concentrations ranging as (0.5, 0.75, 1, 1.5, 2, and 5 g/L). Initial concentration was 30 ppm at natural pH until equilibrium was attained.

- **pH effect:** At 30 ppm solution concentration and 10 mg/L dosage of adsorbent the pH was varied from 2 – 10. The pH was adjusted using HNO_3 and NaOH.

- **Dye concentration effect:** Biochar adsorption effect was analysed at different concentrations of dye solution including 10,20,30,50, and 100 ppm until an equilibrium condition was achieved.

- **Methylene blue removal:** The *Chlorella sp.* derived biochar was utilized to remove dye from waste water. Removal efficiency was calculated by altering pH, dye concentration, and dose of adsorbent.

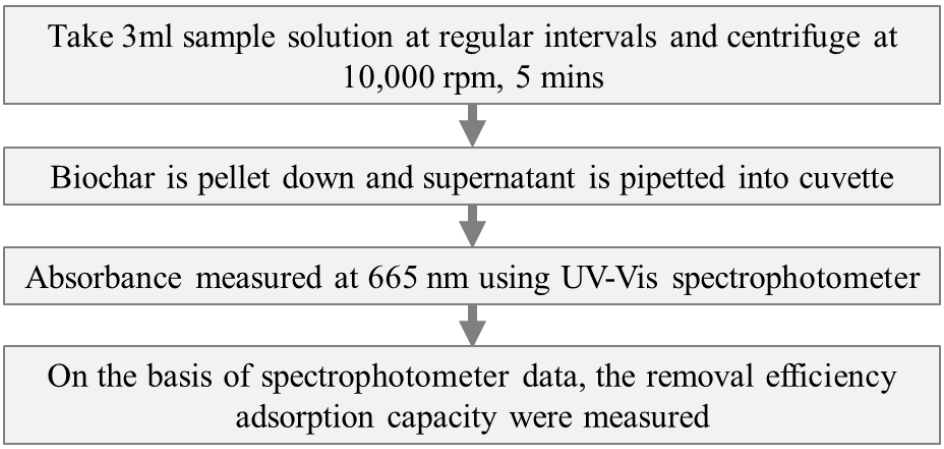

Fig 7: Steps for methylene blue remediation via biochar

Formula to calculate removal efficiency (RE) and adsorption capacity (Q_e) of Methylene Blue is:

$$RE = \frac{Initial\ concentration\ (Ci) - Final\ concentration\ (Cf)}{initial\ concentration\ (Ci)} * 100 \quad 3.5$$

$$Qe = Initial\ concentration - Final\ concentration * \frac{Total\ volume\ (v)}{Amount\ of\ adsorbent(w)} \quad 3.6$$

Where,

Ci: Initial MB concentration (mg/L)
Cf: Final MB concentration (mg/L)
v: Total volume of dye solution tested for degradation (mL)
w: Amount of adsorbent (g)

CHAPTER-4 RESULT AND DISCUSSION

4.1 Algae cultivation for biomass production

Cultivation of *Chlorella sp.* in RO water at different pH conditions

At 690nm, absorbance of *Chlorella sp.* was recorded for different pH conditions including pH- 6,7,8,9, and 10 for a period of 40 days.

Cultivation of *Chlorella sp.* in MiliQ water at different pH conditions

At 690nm, absorbance of *Chlorella sp.* was recorded for different pH conditions including pH- 6,7,8,9, and 10 for a period of 40 days.

Table 6: Optical density of *Chlorella sp.* at different pH recorded in 3 days interval at 690 nm

pH	0	3	6	9	12	15	18	21	24	27	30	33	36	39
PH-6	0.0732	0.3578	0.8938	1.2114	1.4186	1.7043	1.9687	2.1678	2.3067	2.5474	2.6663	2.7658	2.9239	3.047
PH-7	0.0692	0.2574	0.7673	1.2225	1.2155	1.5481	1.7341	1.8692	2.0502	2.1769	2.3485	2.4415	2.5324	2.6154
PH-8	0.0347	0.3121	0.9078	1.5726	1.6734	2.0069	2.2536	2.4085	2.6233	2.7001	2.8602	2.9946	3.0474	3.097
PH-9	0.1096	0.335	0.9121	1.4597	1.5531	1.7524	1.8932	1.8979	2.2175	2.3205	2.5123	2.6404	2.6819	2.8097
PH-10	0.1092	0.4428	0.9813	1.5358	1.6373	1.7265	1.8517	1.8863	2.2601	2.4291	2.599	2.7098	2.7525	2.803

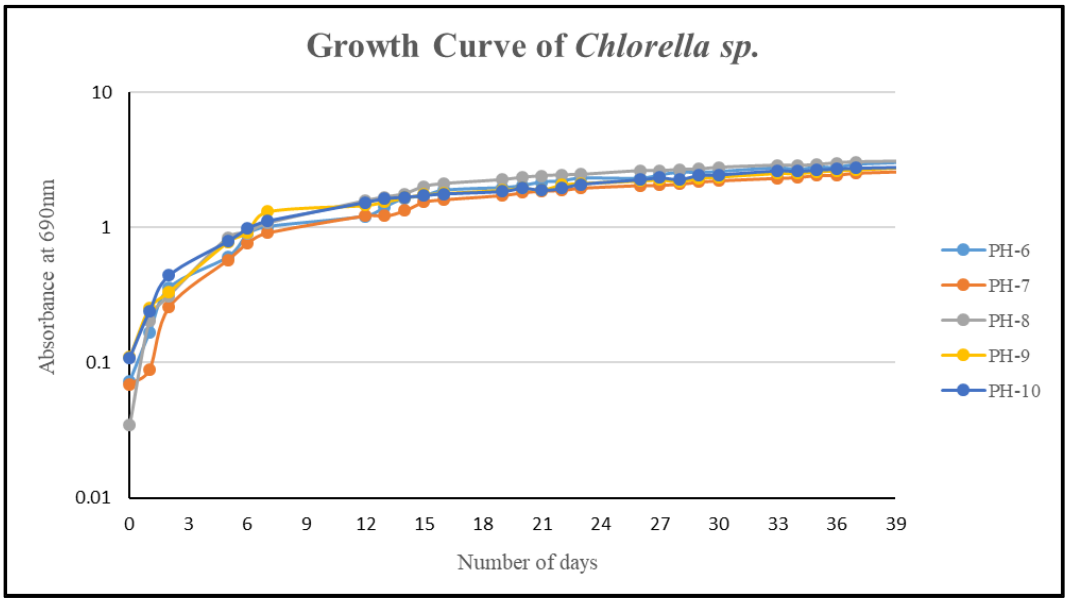

Fig 8: Cultivation of *Chlorella sp.* in different pH

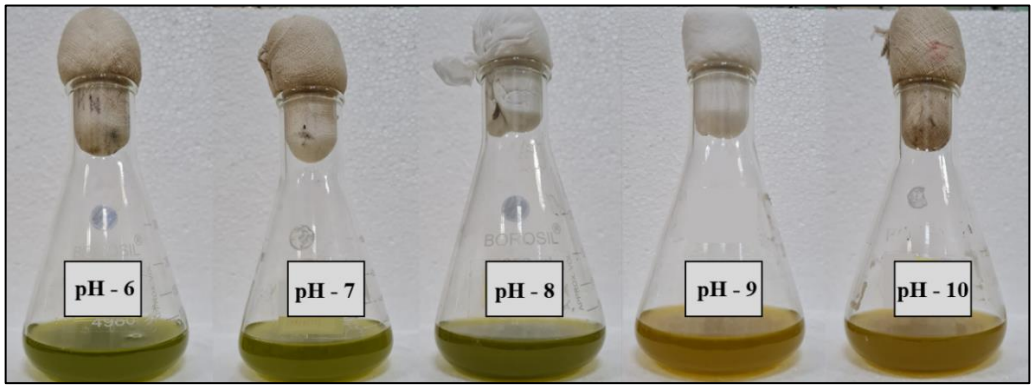

Fig 9: Day 25 picture of *Chlorella sp.* in different pH medium

Growth of *Chlorella sp.* was tested at different pH conditions including pH- 6,7,8, and 9. A pH can be different for different algae strains depending on the types (freshwater or marine water) the requirement of pH varies. In our study it was analysed that at pH-8 the *Chlorella sp.* showed the best growth. The prominent reason behind this is algae requires CO_2 for its growth and pH can affects its availability.

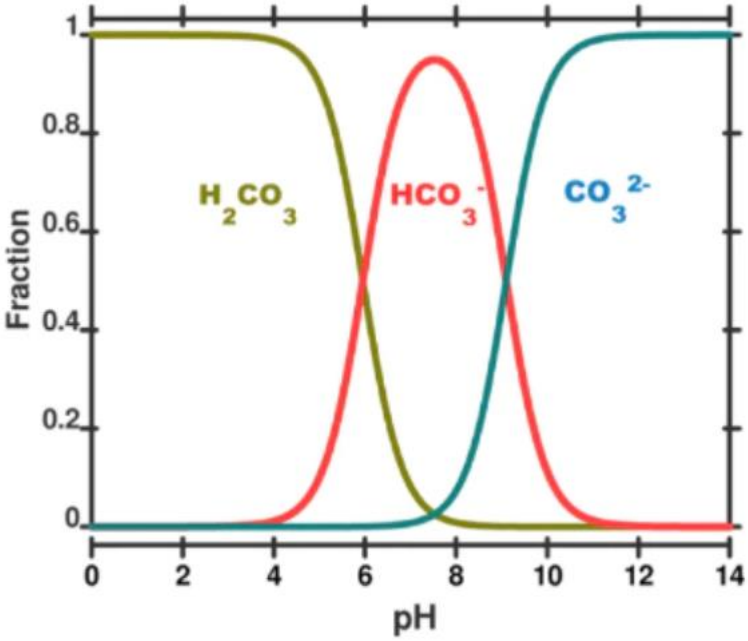

Fig 10: Different forms of Carbon in water at varying pH

Cultivation of *Chlorella sp.* in different carbon sources

At 690nm, absorbance of *Chlorella sp.* was recorded for different carbon sources including glucose, sucrose, fructose, glycerol

Table 7: Optical density of *Chlorella sp.* at different carbon sources

DAY	0	1	2	4	7	8	11	14	15	16	17	21	23	24	28
Glucose	0.1554	0.4538	0.7279	1.1195	1.4113	1.5487	1.7685	2.0043	2.1125	2.1921	2.2145	2.4159	2.5679	2.6655	2.8098
Sucrose	0.1401	0.3363	0.4413	0.7645	1.1009	1.2281	1.4766	1.68	1.7231	1.8344	1.8819	2.0697	2.2144	2.3015	2.3236
Fructose	0.1419	0.449	0.7832	1.2078	1.5841	1.6755	2.0075	2.2061	2.2719	2	2.4743	2.6194	2.808	2.816	2.9465
Glycerol	0.1512	0.3657	0.4912	0.9895	1.3141	1.2431	1.687	1.686	1.652	1.618	1.584	1.55	1.516	1.482	1.448

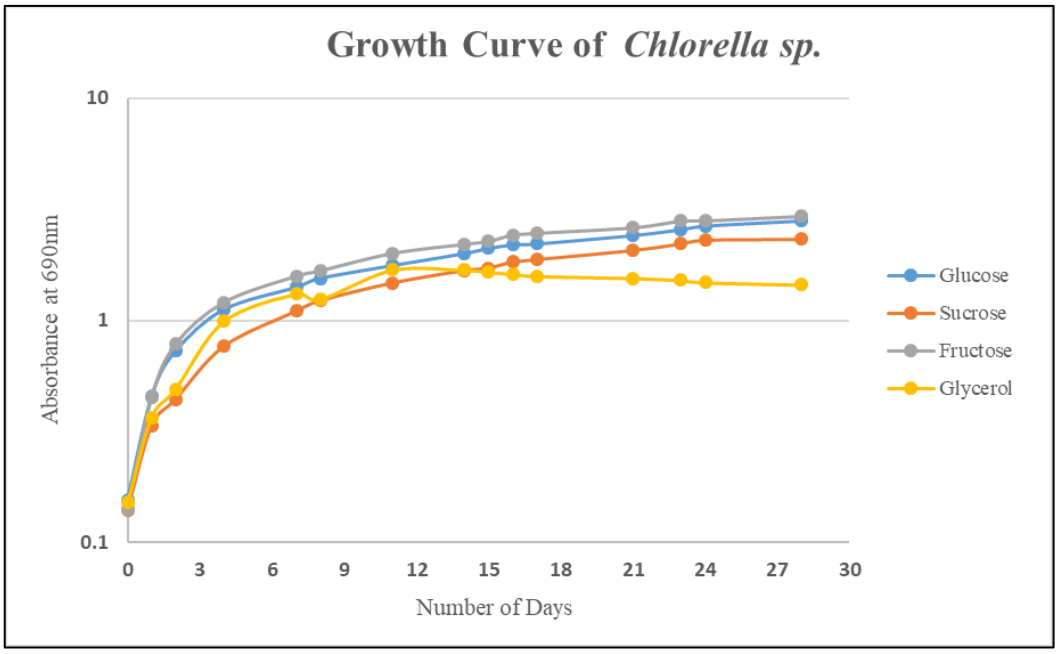

Fig 11: Growth of *Chlorella sp.* in different carbon sources

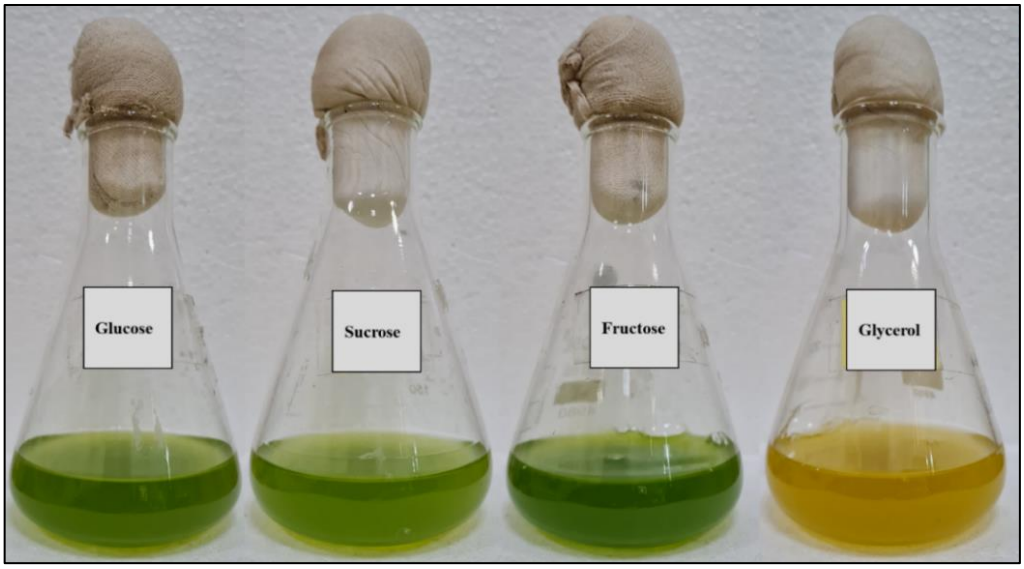

Fig 12: Day 25 picture of *Chlorella sp.* grown in different carbon sources

Growth of *Chlorella sp.* was tested at different carbon sources including glucose, sucrose, fructose, and glycerol. According to the scientific research Glucose is the widely used source of carbon for microalgae cultivation as it is not only organic but also produce more energy per mole. In our study, the species *Chlorella sp.* could show the best growth in sucrose when added externally to the medium (BG11).

Cultivation of *Chlorella sp*. on different Nitrogen sources

At 690nm, absorbance of *Chlorella sp.* was recorded for different nitrogen sources including ammonium chloride, sodium nitrate, urea, and glycine**.** A growth curve was plotted for all of them. The curve was used to select the most suitable medium for the growth of *Chlorella sp.*

Table 8: Optical density of *Chlorella sp.* in different nitrogen sources recorded at 2 days interval at 690 nm

DAY	0	1	2	4	7	8	11	14	15	16	17	21	23	24	28
Ammonium chloride	0.1609	0.3513	0.4555	0.6307	0.6353	0.6402	0.7236	0.777	0.8479	0.8733	0.9294	1.0634	1.1398	1.195	1.4329
Sodium nitrate	0.1614	0.3424	0.4227	0.6011	0.8442	0.9536	1.1133	1.2212	1.293	1.3629	1.4201	1.5301	1.6066	1.7373	1.7978
Urea	0.1568	0.358	0.4823	0.5569	0.7728	0.7799	1.0906	1.2883	1.4485	1	1.6838	1.82	2.0222	2.0726	2.3566
Glycine	0.1617	0.3645	0.6139	0.8954	0.9124	0.8603	0.8463	0.8451	0.8422	0.832	0.723	0.71	0.6432	0.532	0.51

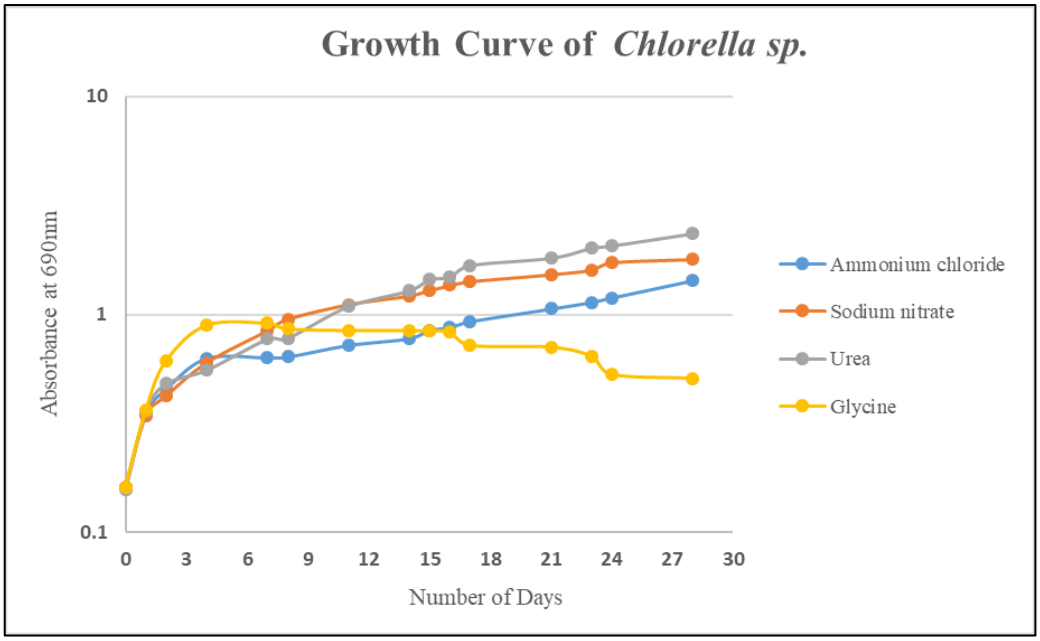

Fig 13: Day 25 picture of *Chlorella sp.* growth curve in different nitrogen sources

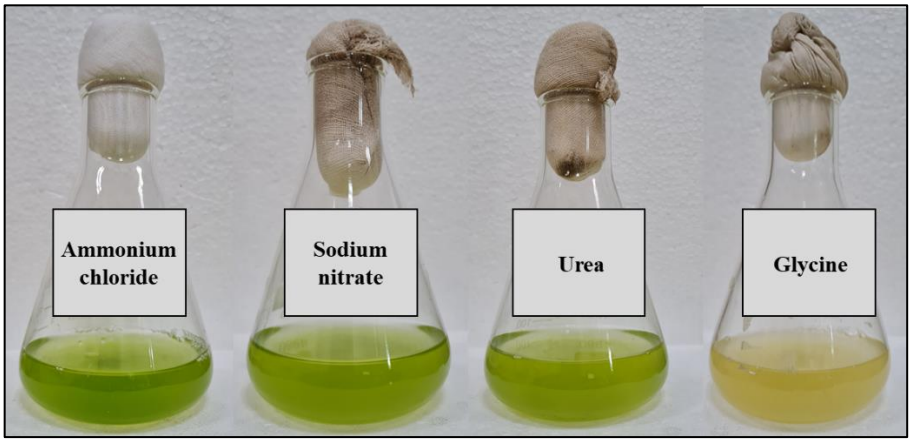

Fig 14: Day 25 picture of *Chlorella sp.* grown in different nitrogen sources

Growth of *Chlorella sp.* was tested at different nitrogen sources including, ammonium chloride, sodium nitrate, urea, and glycine. According to our research urea is the widely used source of carbon for microalgae cultivation. One of the reasons behind this can be due to the fact that algae can break urea into ammonium and bicarbonate ions via enzyme urease. Additionally, we know that urea degradation requires 2 main enzymes called

urease and amydolayase. Such enzymes are not easily available in the direct metabolite synthesis pathway of algae. Therefore, urea degradation is done externally via acid reaction which hydrolyse urea via extracellular algal secretions. In our study, the species *Chlorella sp.* could show the best growth in urea when added externally to the medium (BG11).

Cultivation of *Chlorella sp.* at different Phosphorous sources

At 690nm,s absorbance of *Chlorella sp.* was recorded for different phosphorous sources including potassium nitrate, monopotassium phosphate, and potassium diphosphate**.** A growth curve was plotted for all of them. The curve was used to select the most suitable medium for the growth of *Chlorella sp.*

Table 9: Optical density of *Chlorella sp.* in different phosphorous sources recorded at 2 days interval at 690 nm

DAY	0	1	2	4	7	8	11	14	15	16	17	21	23	24	28	30	32	34	36
Potassium nitrate	0.1551	0.3415	0.4084	0.654	0.9665	1.0297	1.1877	1.3426	1.4363	1.46	1.5333	1.6355	1.7876	1.8175	1.9711	2.0123	2.1245	2.2264	2.2346
monopotassium phosphate	0.1834	0.3747	0.4029	0.5752	0.5923	0.5622	0.6777	0.8156	0.891	0.9251	0.9957	1.2255	1.3413	1.4086	1.7038	1.7345	1.8023	1.8345	1.8789
Potassium diphosphate	0.1455	0.4085	0.4669	0.5949	0.7125	0.7619	0.8888	1.3633	1.5854	1.698	1.7363	1.8781	1.9891	1.9965	2.2771	2.3771	2.609	2.8091	2.8271

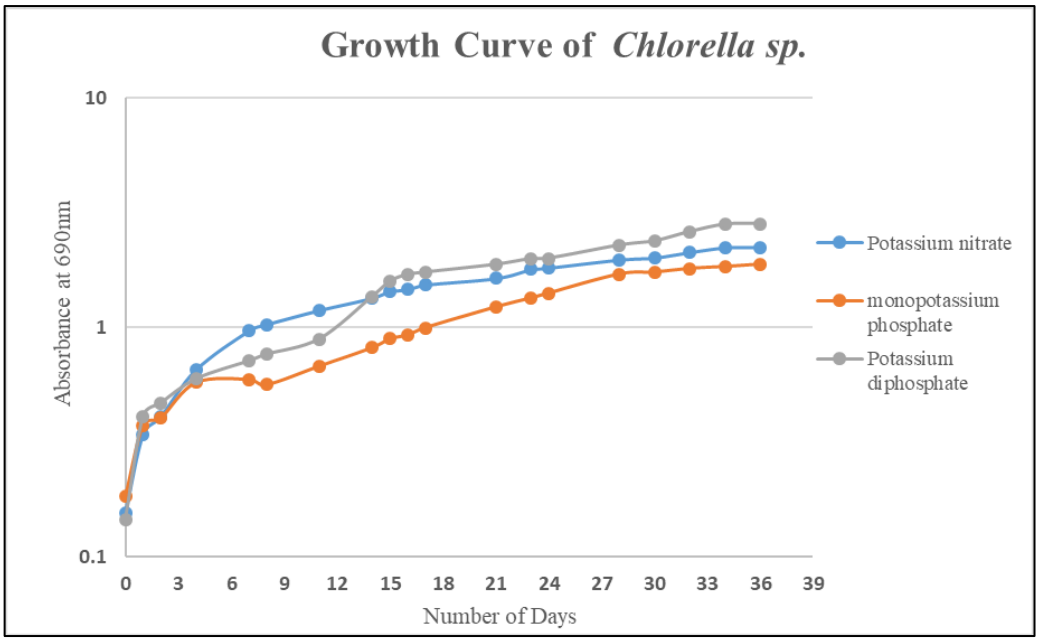

Fig 15: *Chlorella sp.* growth curve in different phosphorous sources

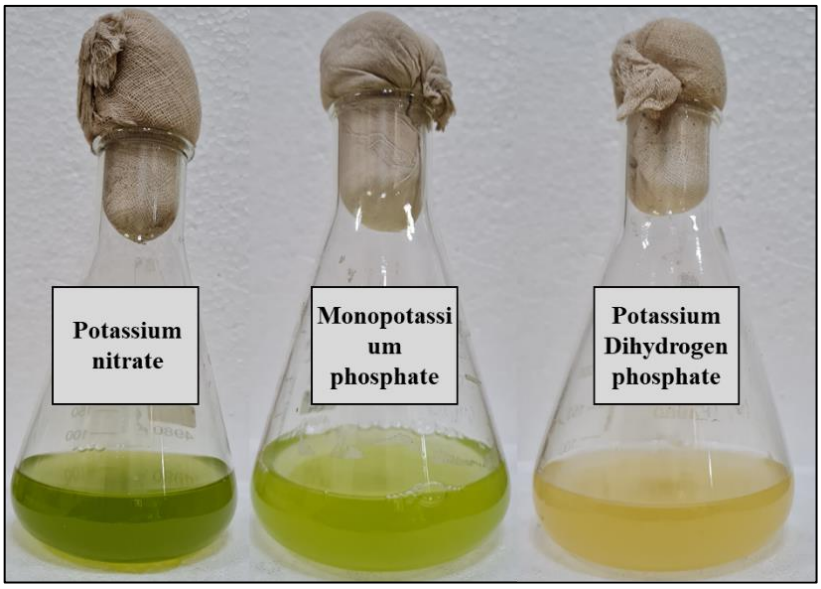

Fig 16: Day 25 picture of *Chlorella sp.* grown in different phosphorous sources

Growth of *Chlorella sp.* was tested at different phosphorous sources including potassium nitrate, monopotassium phosphate, and potassium dihydrogen phosphate. According to our study it is clear that the algae when grown only in potassium showed growth inhibition. One of the possible reasons behind this is that high dose of phosphorous leads to access accumulation of phosphates within each cell. This leads to binding of the phosphorous to cellular components (intracellular). Though phosphorous is an important nutrient but excess of it leads to cell death. According to our results the best growth was observed in potassium nitrate.

4.2 Quantitative Analysis of bioactive compounds in *Chlorella sp.*

After doing a variety of tests on *Chlorella sp.*, it was analysed that algae species comprise of many bioactive compounds including alkaloids, phenols, flavonoids, and carotenoids.

Table 10: Confirmation of Presence of different bioactive compounds in *Chlorella sp.*

Bioactive Compound	Result	Inference
Alkaloids	A white precipitate is formed	Alkaloids were detected in the algal sample
Carotenoids	$OD_{470} - 0.8178$	Carotenoids were detected in the algal sample
Flavonoids	Yellow colour solution formed	Flavonoids were detected in algal sample
Phenols	A green solution formed	Phenols were detected in algal sample

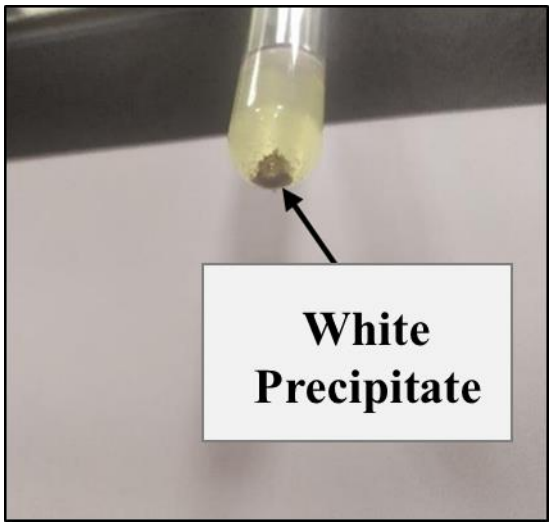

Fig 17: White precipitate confirms the presence of alkaloid in *Chlorella sp*.

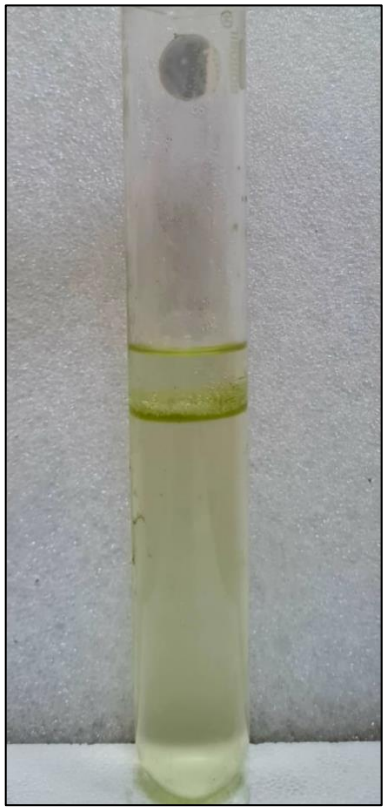

Fig 18: Upper layer comprises the presence of β-Carotene in *Chlorella sp.*

Fig 19: The faded yellow confirms the presence of flavonoids in *Chlorella sp.*

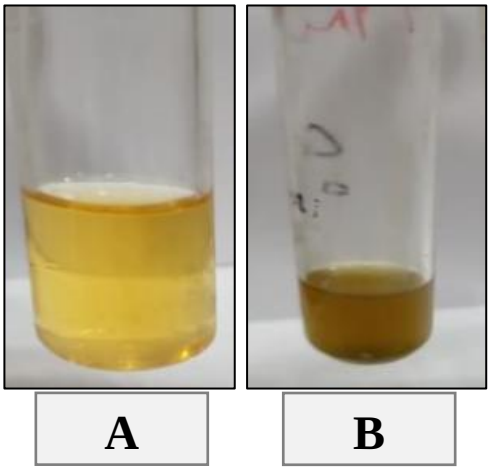

Fig 20: A: Neutral $FeCl_3$ solution; B: Formation of green colour – confirms the presence of phenol in *Chlorella sp.*

4.3 Quantitative detection: β-Carotene

The β-Carotene (a carotenoid) amount was found to be much higher when *Chlorella sp.* was posed under the stressful environment. Such as when the pH of the BG-11 medium was between 9-10 the good amount of β-Carotene was detected. A good amount of β-Carotene was extracted using THF (tetrahydrofuran), a solvent, and heat treatment.

Table 11: Amount of β-Carotene from wet biomass detected in different cell disruption techniques

Samples	Amount of β-Carotene (µg/mL)
Chlorelal sp. cultivated in BG11	11.52
Chlorelal sp. cultivated in BG11 (THF + Sonicated)	13.14
Chlorelal sp. cultivated in BG11 (THF + Heat treatment)	15.26
Chlorelal sp. cultivated in BG11 (THF + Bead milling)	12.84

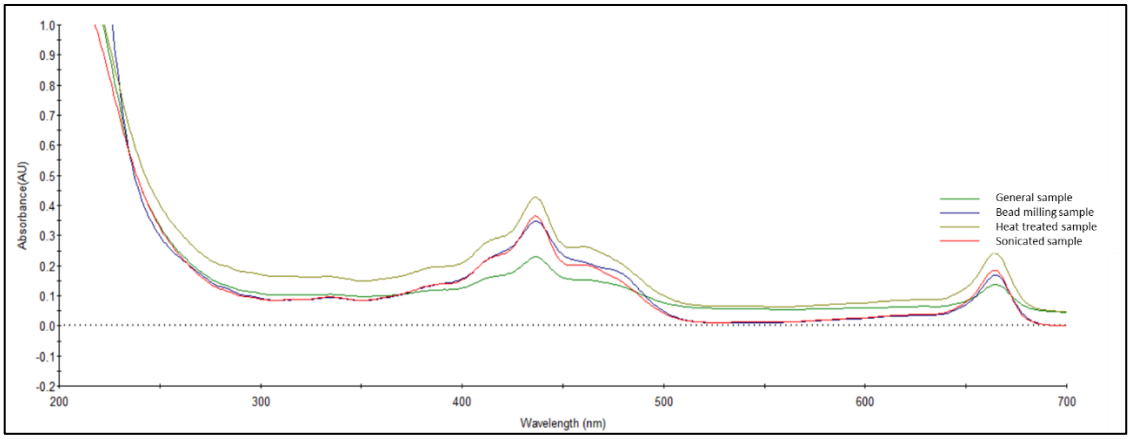

Fig 21: Spectrophotometric detection of β-Carotene

4.4 Biochar Analysis

4.4.1 Synthesis of biochar from *Chlorella sp.*

Sulphuric acid was initially added to the dried algal biomass which not only lead to the change in the colour of biomass but also released pungent fumes. The mixture was heated at 200^{o}C for 6 hours leading to charring. The presence of carbonization was visible due to change in the colour.

4.5 Characterization of *Chlorella sp.* derived Biochar

4.5.1 Characterization (visual)

A change in colour was observed after the reaction of sulphuric acid with the dried algae biomass. The green coloured biomass was converted to jet black char. This change of colour was due to the occurrence of dehydration (loss of water), thermal decomposition, nucleation of graphene, and carbonization of algal biomass resulting in charring. Relatable analysis were documented from Roy *et.al* in the plant *Plumbago zeylanica* [67].

4.5.2 Spectroscopic Analysis (UV-Vis)

The coarse black colour blocks the natural light. Crude biochar displays the adsorption around 200-300 nm due to dark colour of the adsorbate. In the current study 10mg dried algal biochar was dissolved in 100 mL ddH_2O and kept for shaking on a mechanical shaker for different time intervals. When scanned the absorbance peaks were noted at 0.1303 and 0.2941 respectively at around 210 nm for the algal samples.

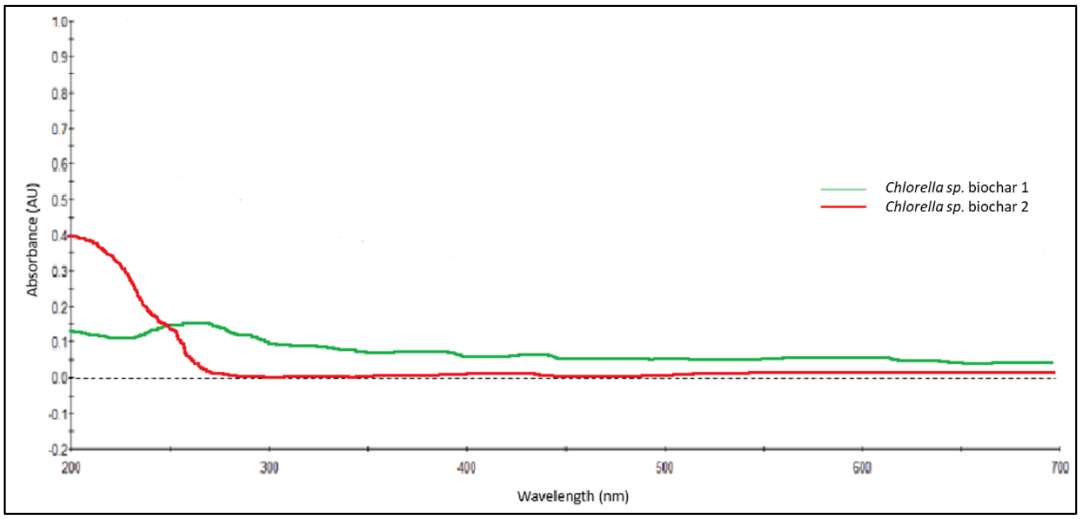

Fig 22: Absorbance of *Chlorella sp.* derived biochar

4.5.3 Biochar yield

From the *Chlorella sp.* species taken 4.1 gm as raw biomass nearly 2.08 gm of biochar was produced as a final product. Therefore, giving a yield of 50%. While according to the literature the yield of a plant derived biochar is less due to the presence of lignocellulosic biomass. Therefore, microalgae have proved to be an efficient feedstock for the production of biochar.

4.5.2 Biochar pH

pH of *Chlorella sp.* species derived biochar was 8.2. Due to the less ash content and absence of lignin, cellulose, and hemicellulose fractions. Therefore, preparing algal biochar is quite beneficial.

4.5.4 Biochar conductivity

Conductivity of *Chlorella sp.* species derived biochar was nearly 884 µS/cm. More conductivity after biochars synthesis is inferred by reduction in particle size and elimination of oxygen (reduced O_2 content in char). Additionally, presence of free electrons on a negatively surface of biochar is another reason for increased conductivity. Similar observations were attributed by Seth et.al. [45].

4.6 Parameters optimization for Cr remediation

4.6.1 Preparation for standard curve

A standard curve was plotted at 350nm for different chromium concentrations (10, 20, 30, 50, 100) made via dilutions. The graph obtained indicated a linear line with a correlation coefficient 1.

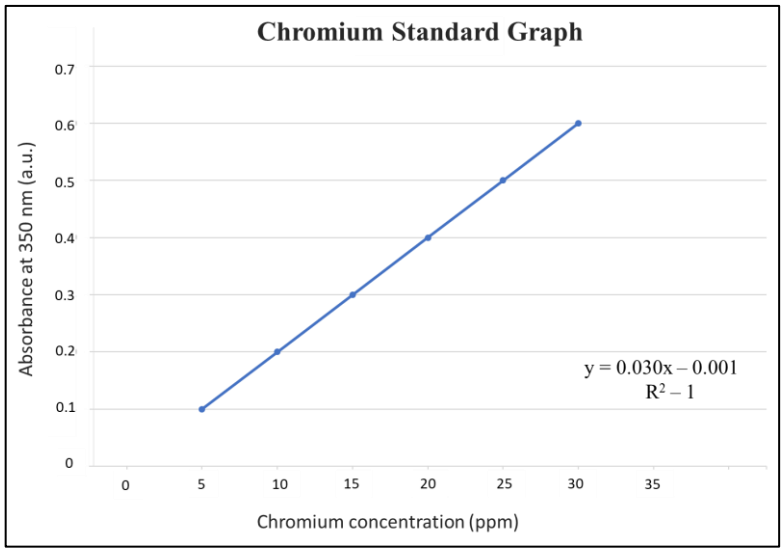

Fig 23: Chromium solution standard curve

4.6.2 Varying biochar dosage

From the zeta potential analysis, it is evident that the surface of the char comprises of negatively charged functional groups. Therefore, charged chromate ion removal was a little less effective in comparison to the dye. This occurs due to the electrostatic repulsion between char and the contaminant (Cr). Therefore, addition of more amount of char lead to the increase in remediation. Therefore, maximum removal efficient obtained was only 75% after 60 minutes with 1.5 g/L adsorbent dosage and no pH adjusted. Additionally, above 1.2 g/L the Cr adsorption was reduced and was not that significant. large blocks may lead to reduced surface area available for Cr ions removal.

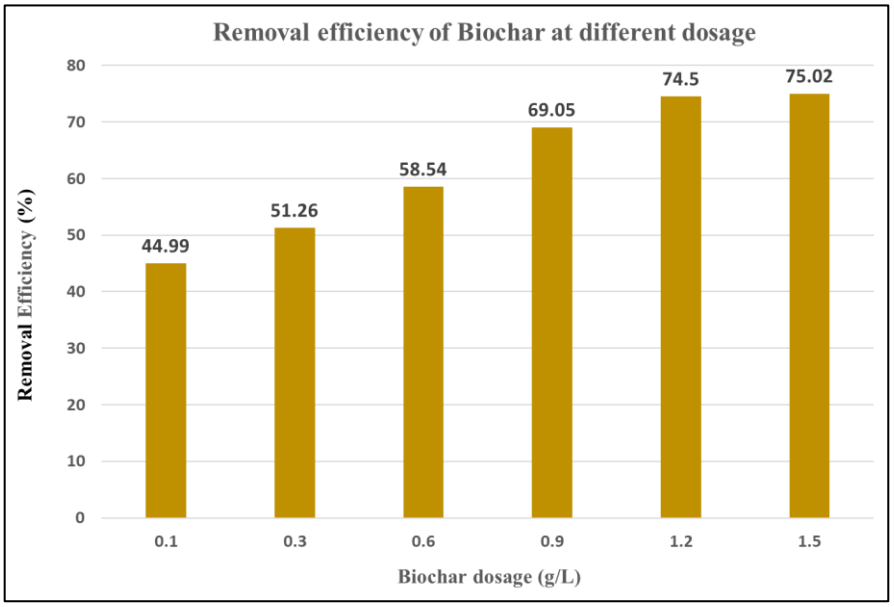

Fig 24: Effect of *Chlorella sp.* derived biochar dosage on Cr

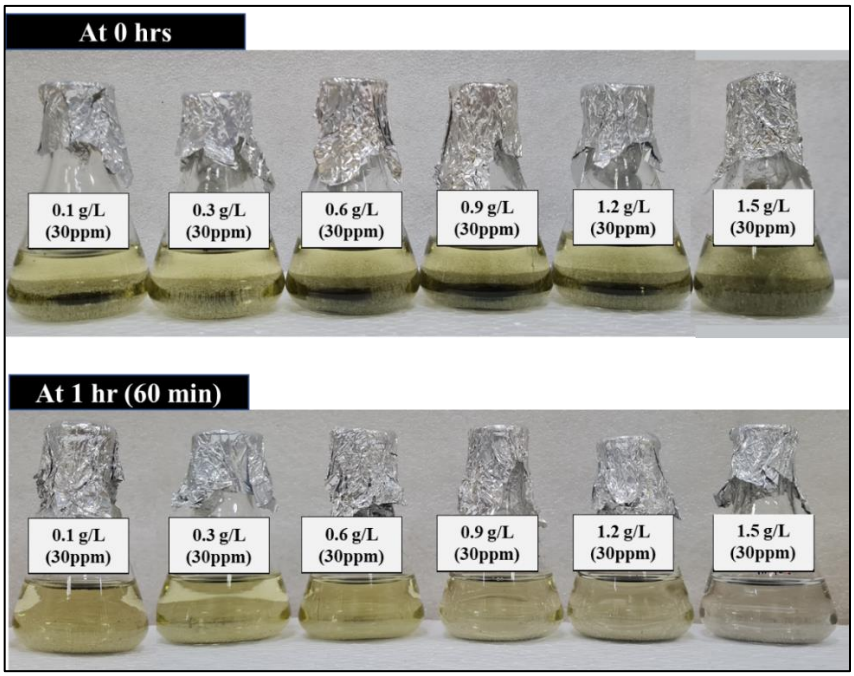

Fig 25: Visual remediation of *Chlorella sp.* derived biochar dosage on Cr

4.6.3 Varying pH

Effect of pH on biochar is very much. As the pH of the solution concentration switched from 2,3,4,5,6,7,8,9, and 10 the Cr sorption decreased. This because lower the pH higher is the redox potential of chromate ions which transform Cr (VI) into Cr (III) which has reduced toxicity. Therefore, highest removal efficiency (88.2%) in 60 minutes and was observed at pH-2. Additionally, the colour of reduced from bright yellow to nearly transparent upon remediation.

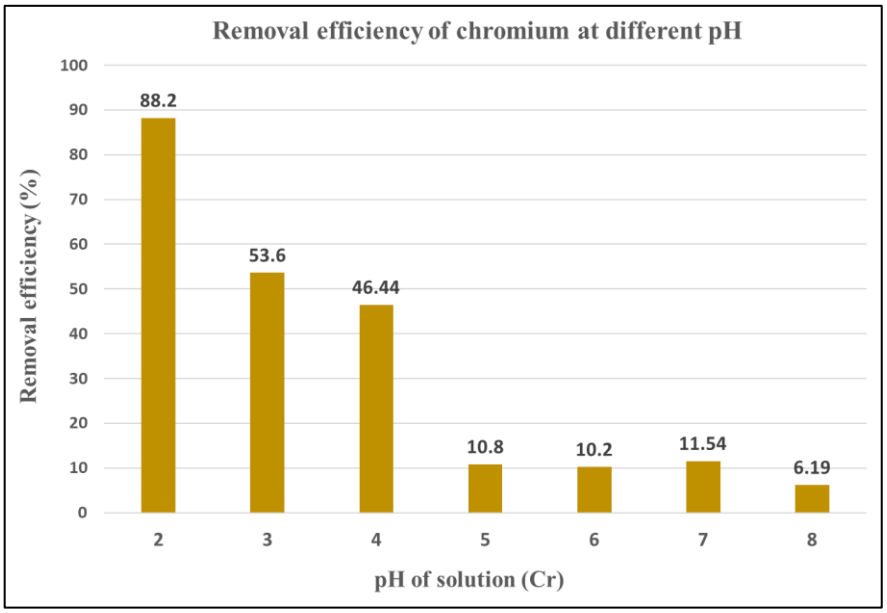

Fig 26: Effect of pH on adsorption

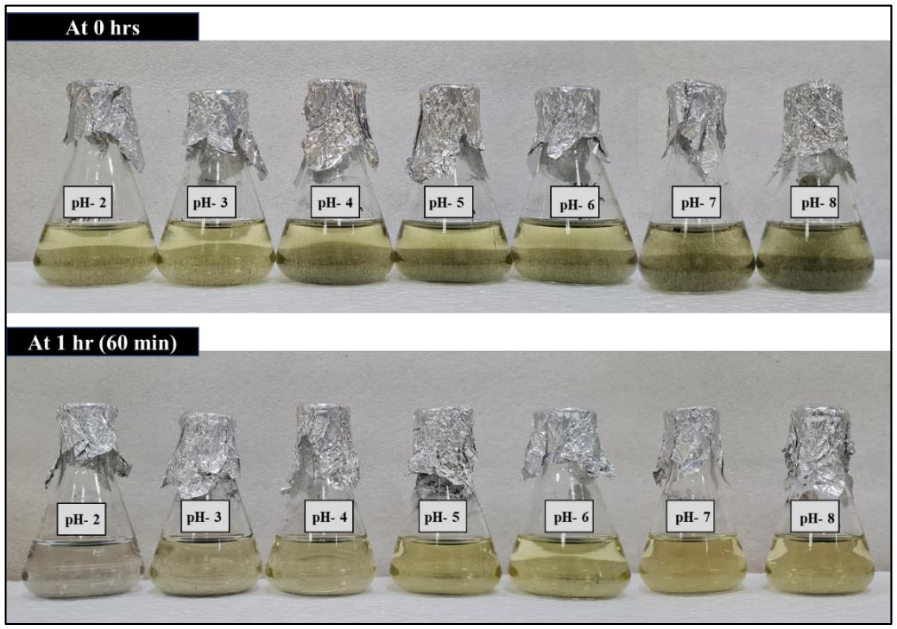

Fig 27: Visual remediation of *Chlorella sp.* derived biochar at varying pH

4.6.4 Varying Cr concentration

As concentration of Cr (heavy metal) increased the remediation efficiency reduced drastically. This is due to the limited sites on biochar that can adsorb the Cromate ions. Therefore, highest removal was 76.55% in 60 minutes at 20 ppm Cr concentration.

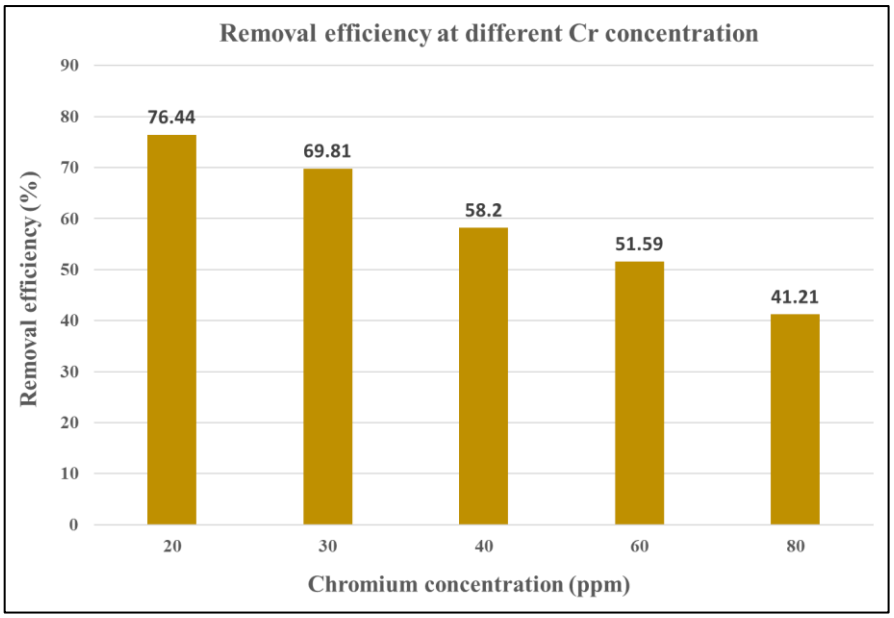

Fig 28: Effect of Cr solution concentration on biochar

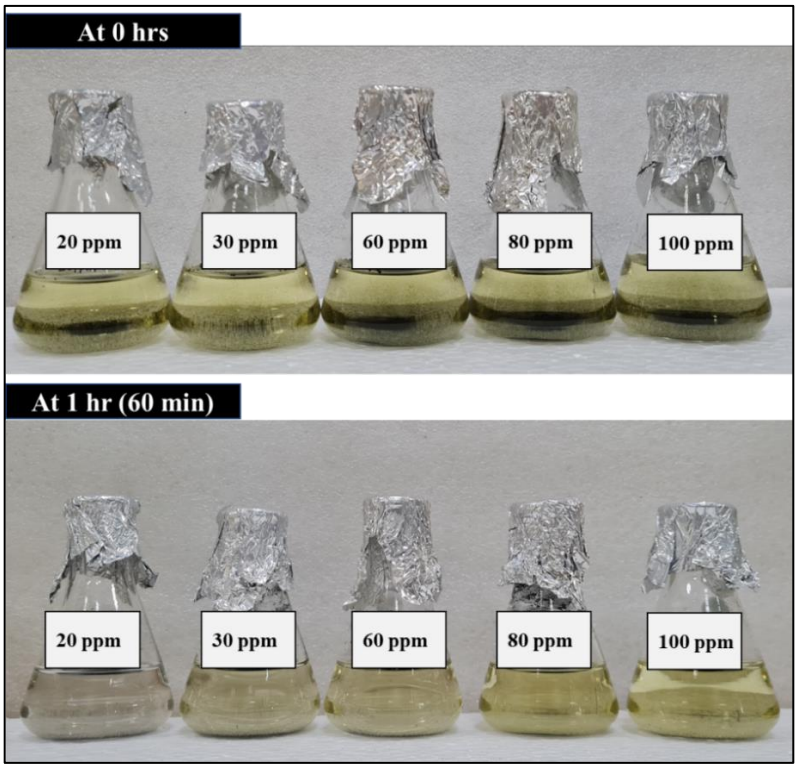

Fig 29: Visual remediation of *Chlorella sp.* derived biochar at varying Cr concentration

4.7 Parameters optimization for methylene blue adsorption

A standard curve was made at (665nm) for different dilutions of methylene blue stating a linear trend and a correlation coefficient of 0.998.

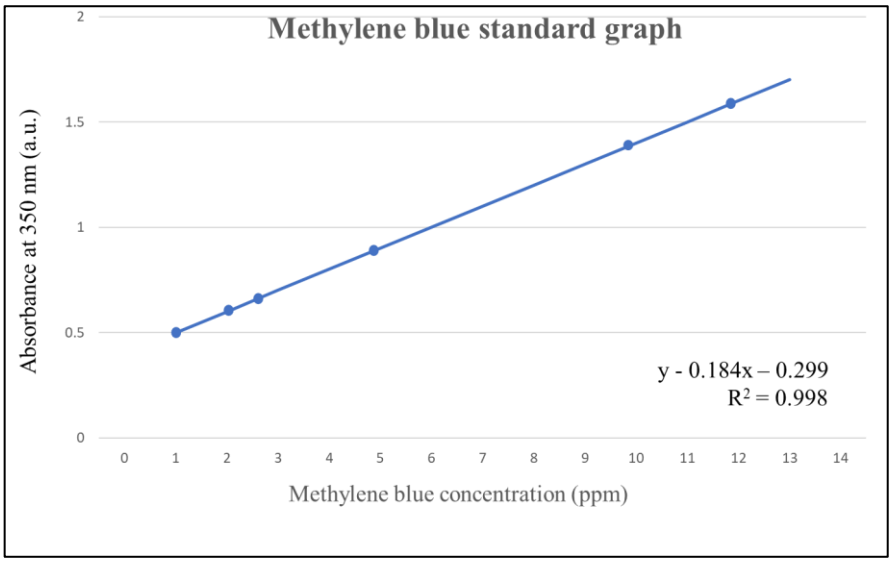

Fig 30: Methylene blue standard curve

4.7.1 Varying adsorbent dosage

It is clear that as we increase the amount of char dose the availability of the biosorption sites increase leading to better removal. The dye abatement of 97.85% was recorded after 60 mins with the biochar dosage at 10 g/L. But above this value the particles tend to form aggregations followed by reduced remediation.

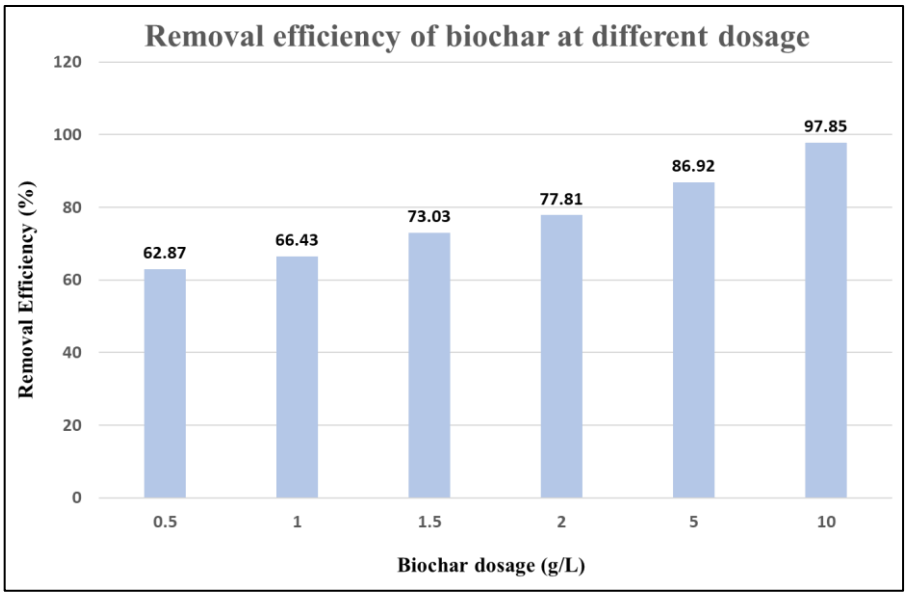

Fig 31: Effect of *Chlorella sp.* derived biochar dosage on methylene blue

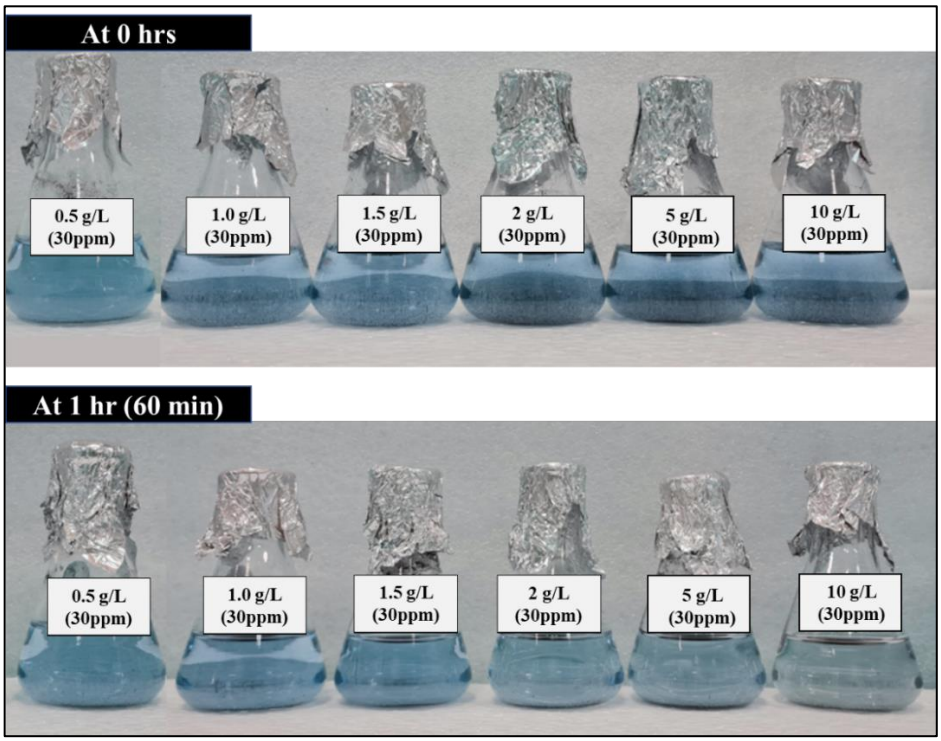

Fig 32: Visual results of *Chlorella sp.* derived biochar dosage on methylene blue

4.7.2 Varying pH

As we increase the solution's pH it favours removal efficiency of dye methylene blue (Cationic). A maximum of 99.56% of removal was observed at pH 10. Adsorption was visible from the solution. As the remediation happened the solution started becoming transparent like water. Negligible removal was observed at pH-2 because below pH 3.8 dye is negatively charged and it repels functional groups which are anionic on the char surface.

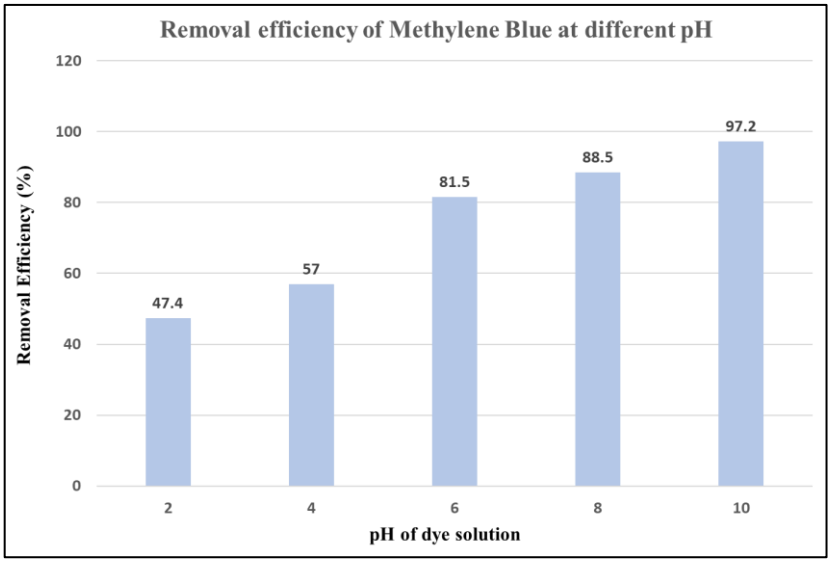

Fig 33: Effect of pH on adsorption

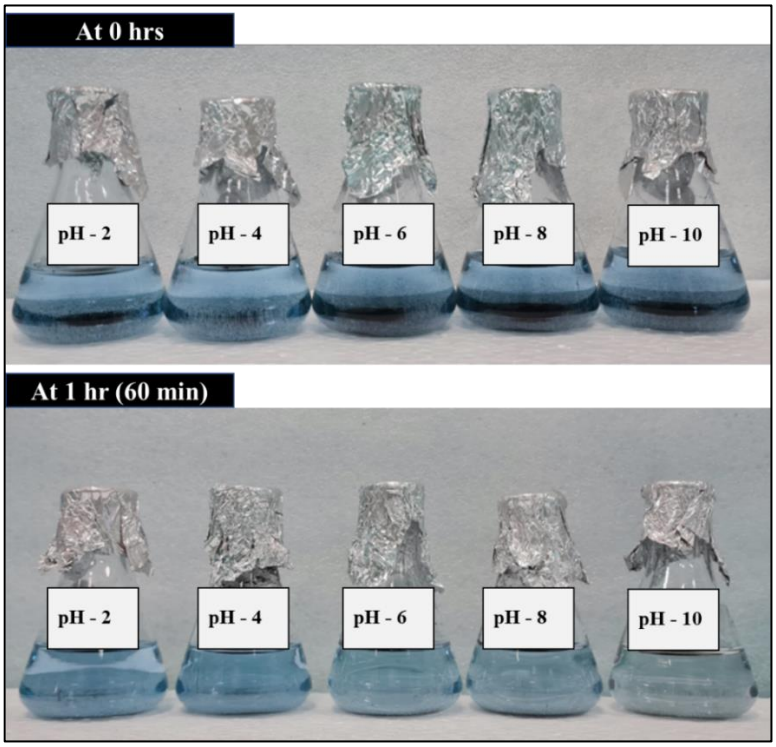

Fig 34: Visual results of pH on adsorption

4.7.3 Varying dye concentration

As concentration of (MB) dye reduced then the efficiency of removal increased. Therefore, efficiency of removal was reported 97.71% at the solution concentration of 10 ppm. While lowest was at 100ppm 90.1%.

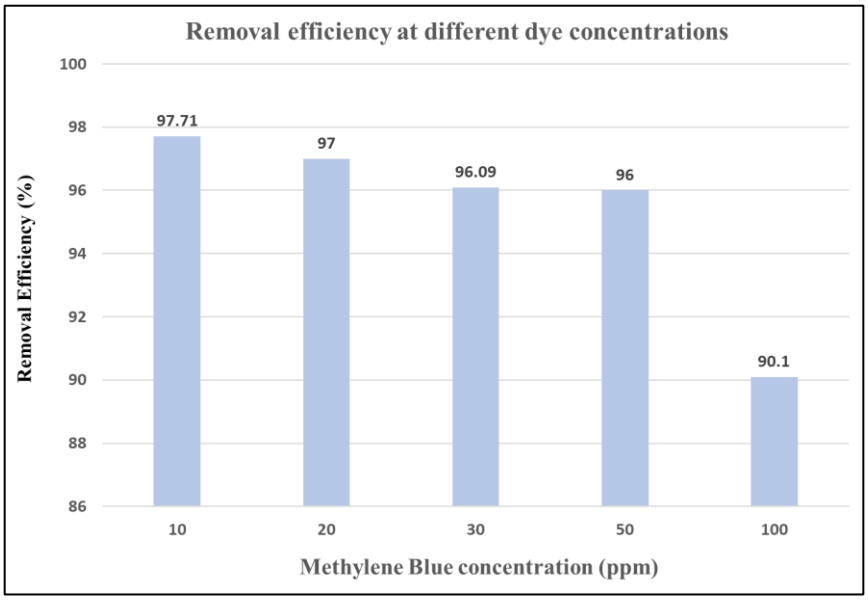

Fig 35: Effect of biochar adsorption on methylene blue concentration

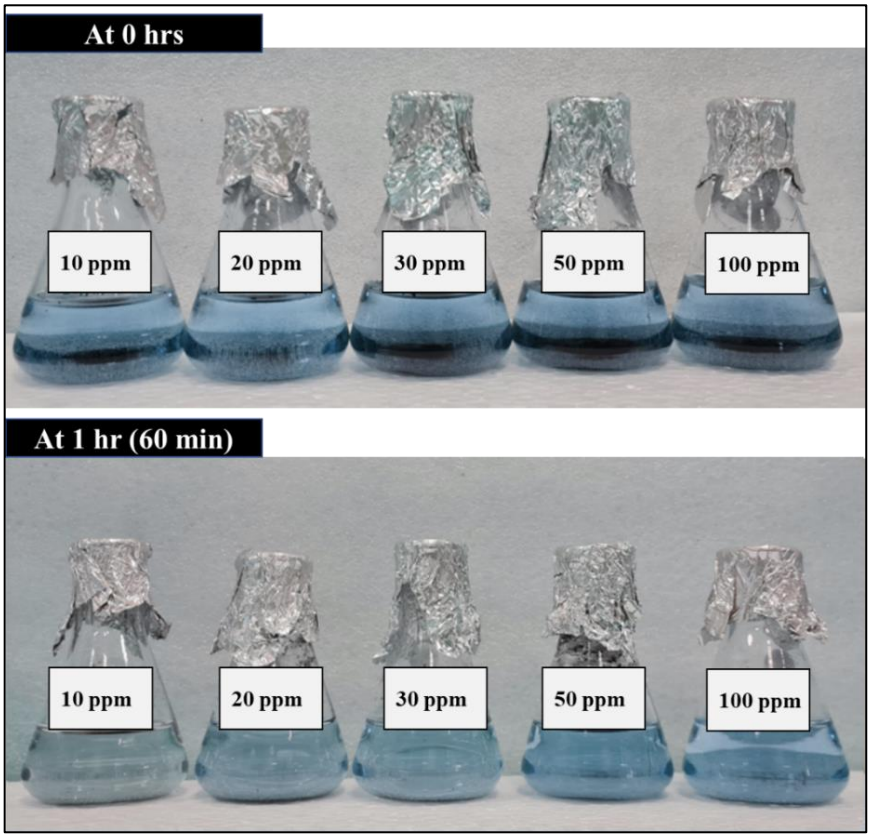

Fig 36: Visual results of biochar adsorption on methylene blue concentration

CONCLUSION AND FUTURE PROSPECTS

This study explored about C*hlorella sp.,* a green-algae as a source for β-carotene extraction. According to the literature review β-carotene is a bioactive compound which acts as a nutraceutical and possess numerous health benefits including anticancer, antibacterial, antiviral, immunomodulant etc. There are a variety of extraction techniques which are used to extract the β-carotene including organic solvents, atmospheric liquid extraction with maceration, soxhlet extraction, super critical fluid extraction, ultrasound assisted extraction, microwave assisted extraction, pressurized liquid extraction, pulse electric field assisted extraction, enzyme assisted extraction followed by cell disruption. Our study found out that treating the algal cells with Tetrahydrofuran ($(CH_2)_4O$) followed by heat treatment produced the highest amount of β-carotene extract valued 15.26 from C*hlorella sp.* The amount of β-carotene extracted was analysed using the spectrophotometric detection. We used the PerkinElmer Lambda 365 UV-Vis spectrophotometer to detect the absorbance of the carotene at 450nm range. Additionally, after the carotenoid extraction the leftover cells (biomass) were preserved to be dried and used for biochar generation. Biochar is a carbonaceous substance used as an adsorbent to remediate waste water and soil. To date a variety of studies are present which state about the promising qualities of biochar as an adsorbent. Studies confer that it can not only adsorb organic substances (heavy metals) but also inorganic pollutants (dye). Our study resulted in understanding the characteristic of biochar on three major parameters mainly (i) char dosage, (ii) adsorption pH, and (iii) pollutant concentration. So, the remediation can vary according to the above parameters. In our study the biochar was tested for dye abatement and heavy metal remediation. Out of which the successful results were observed at 1.5 g/L, 10pH, and 30 ppm methylene blue concentration. Whereas for the chromium remediation it was observed that at 1.5 g/l, 2pH, and 30 ppm chromium concentration the best remediation results were observed. However, there is still a need to develop modification in the biochar to enhance its adsorption characteristics. In our study modification has been done via acid. Acid dehydrates the physical structure of the algae cells which creates deep rooted pores in the cellular surface. Therefore, a simple change in the preparation enhanced the adsorption of the char in comparison to the previous studies. This biochar is referred to as activated char. However, a more understanding is required to improve the char dosage and make it economically feasible at commercial level. In order for it to be a good remediating agent it has to be cost effective on not only laboratory but also commercial scale. Therefore, optimizing its production and dosage makes it efficient and economically feasible.

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APPENDIX

Media Composition

1. BG-11 Medium (pH-8)

Stock 1

Chemical Compound	Concentration (g/L)
Na-MGEDTA	0.1
Ferric ammonium citrate	0.6
Citric acid.H_2O	0.6
$CaCl_2$.2 H_2O	3.6

Stock 2

Chemical Compound	Concentration (g/L)
$MgSO_4$	7.5

Stock 3

Chemical Compound	Concentration (g/L)
$K_2HPO_4.3H_2O$	4
Or K_2HPO_4	3.05

Stock 4

Chemical Compound	Concentration (g/L)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.5H_2O$	0.222
$CuSO_4.5H_2O$	0.079
$CoCl_2.6H_2O$	0.050
$NaMoO_4.2H_2O$	0.018

Final Composition

Stock solution	Amount (per Liter)
Stock 1	10 mL
Stock 2	10 mL
Stock 3	10 mL
Stock 4	1 mL
Na_2CO_3	0.02
$NaNO_3$	1.5

LIST OF PUBLICATION

1. Joshi, V., Bharadvaja, N. Current Prospects and Clinical Status of Microalgae Derived Chemotherapeutics. *Rev. Bras. Farmacogn.* **33**, 445–470 (2023). https://doi.org/10.1007/s43450-023-00384-9 **(SCI, IF-2.4)**

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Review | Published: 22 March 2023

Current Prospects and Clinical Status of Microalgae Derived Chemotherapeutics

Vandana Joshi & Navneeta Bharadvaja

Revista Brasileira de Farmacognosia **33**, 445–470 (2023) | Cite this article

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Abstract

Globally, despite clinical advancements made over a decade, cancer remains a prominent cause of death. Numerous drugs have been chemically synthesized to cure this disorder. However, a need for effective remedy to not only treat the disease but also address its root cause with minimal side effects remains a concern. Marine vegetation offers a plethora of bioactive chemicals holding anticancer activities with no side effects. Out of which, algae, a photosynthetic eukaryote, is a rich source of these functional metabolites for antineoplastic development. Previously, many studies have discussed about algae derived compounds and their efficacy as an anticancer drug. However, a very few have explored about their apoptotic mechanism targeting components of a signaling pathway. Several new investigations reveal

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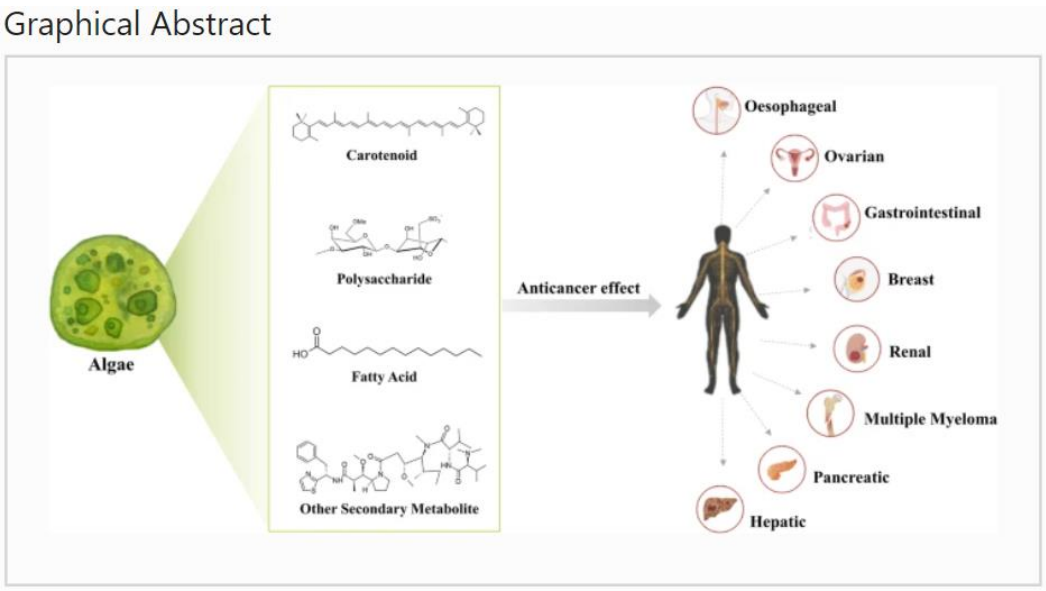

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