Impact of different media on biomass yield and lipid yield of *Chlorella sp.* and assessment of decolorization of azo dye

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Submitted by

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CERTIFICATE

This is to certify that the dissertation entitled **Impact of different media on biomass yield and lipid yield of *Chlorella sp.* and assessment of decolorization of azo dye** submitted by **Deepti Bhardwaj (2K19/IBT/01)** in the partial fulfilment of the requirements for the reward of the degree of Masters of Technology, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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DECLARATION

This is to certify that the thesis of Major Project entitled “**Impact of different media on biomass yield and lipid yield of *Chlorella sp.* and assessment of decolorization of azo dye**” submitted by Deepti Bhardwaj (2KI9/IBT/01) in the partial fulfilment of the requirements for the reward of the degree of Master of Technology, Delhi Technological University (Formerly Delhi college of Engineering, University of Delhi), is an authentic record of my own work carried out under the guidance of my project supervisor **Dr. Navneeta Bharadvaja**, Assistant Professor, Department of Biotechnology, DTU. The information and data enclosed in this thesis is original and has not been submitted elsewhere for honouring of any other degree.

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Abbreviations

°C- degree Celsius

L – litre

mg- milligram

ml- millilitre

µl- microlitre

mg/l- milligram per litre

lx- lux(illuminance)

nm- nanometre

ppm- parts per million

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ABSTRACT

Green algae are photosynthetic organisms which acts as carbon sink and play a crucial role in aquatic ecosystem. It has been consumed by animals and humans and useful to produce different value-added products such as biochar, biocomposites, carbon quantum dots, biopolythene. These are used by different nutraceutical and cosmetics industries as they have richness of lipids, proteins, chlorophyll, carotenoids and carbohydrates. Algae culturing brings prospects to phycoremediation of industrial effluents, removal of excess nutrients, resource recovery and algal biofuel production. The species of green algae with high yield and biomass characteristics is desirable to improve the efficiency of algal economies of the world. Industrialization and urbanization results in rise of wastewater containing dyes and also decline in reserves of fossil fuels. The major objective of this study is to explore different media, bioremediation of synthetic dyes such as azo dyes and analyze yield of biomass obtained from algae in order to enhance lipid yield. The present work offers wide uses of green algae rich in phycocompounds for polluted water, biooil, nutraceuticals, food and animal feed industries. This work unveiled that among the 5 different media studies, BG-11 media offers maximum biomass and lipid yield as 782 mg/l and 198 mg/l respectively. The effective decolorization of methylene blue dye found to be more than 90%, achieved in concentration range of 0.2-1mg. This study reveals the significance of green algae and enables to provide sustainable solution to the problems of energy crisis, water pollution and malnutrition.

INTRODUCTION

1.1 Introduction

Biomass is an essential resource for humans in the form of food, fodder and firewood. Initially plants were major source of biomass and later agriculture played important role to meet the rising needs of human civilization [1]. The use of forest and open lands for firewood collection and other purposes has caused exploitation of land resources due to its over utilization [2]. During industrialisation the clearing of forests for cultivation of crops and fuels has pushed humans for other alternatives. This leads the quest for energy resources like coal, petroleum and later unconventional sources of energy. But excessive consumption of finite fossil fuels has caused environmental calamity such as more greenhouse gas emission, global warming, pollution and climate change of our ecosystem [3][4]. The survival of humans now requires economically accessible and environment friendly energy resources [5]. Biomass appears as better alternative source for trapping unlimited solar power at less cost. The conventional biomass (land-based) leads to huge environmental deterioration in terms of flora and fauna. The biofuels produced from plants and other crops has raised the conflict between food and fuels where hunger exists as a problem in various nations. It is also not economically viable and requires intensive cultivation of land for crops like soyabean and jatropa etc. which causes decline of water resources and ultimately degrade the ecosystem. Therefore, imbalance between food and fuel stimulates humans to search for easily and fast producible biomass such as algal biomass [6][7]. Algae are investigated to be one of the best sources of biomass with multiple applications [8].

Algae since its inception has played a major role in food chain as algal biomass acts as primary producer and works in symbiosis with corals like aquatic/marine organisms. Algae are natural carbon sequestration resource [9] and are fastest growing photosynthetic organisms. They contribute approximately 50% of total organic carbon, produced through photosynthesis on the earth [10]. Algae being found largely in water bodies, occupies about 67% of earth’s surface. Climate change is causing rise in temperature which negatively impacts all organisms but its impact is less on photorespiration of algal cells. This fact also becomes one of the significant reasons for high yield and productivity of algae. Algal cells have higher photosynthetic efficiency as well as oil productivity in comparison to other biomass and oil producing flora [11][12]. Diversity in algae has adapted to grow in various extreme as well as habitable environmental conditions. This makes them suitable to cultivate commercially in almost all spheres of earth without the need of arable land used for other different purposes [13]. The capability of algae to grow in wastewater makes them suitable candidate for cultivation, as it does not strive for water resources useful for various other crop cultivation [14][15]. Industrial wastewater comprises of organic and inorganic pollutants along with synthetic dyes. Dyes are widely utilized by different industries or manufacturing units such as textile, pharmaceuticals, food and beverages, cosmetics etc. due to their aesthetic nature [16]. The synthetic dyes are responsible for adverse impacts like teratogenicity, carcinogenicity, bronchitis, mutagenicity and ecotoxicity [17]. It consists of toxins like benzene, pyrene, P-nitrophenol which are reported to be carcinogenic towards cells. Certain dyes are found to hinders infiltration of sunlight into water bodies which reduces photosynthetic activity and hence deficiency of oxygen for the survival of ecosystem [18][19][20]. The presence of dyes with other pollutants makes then less prone to remediation and therefore exist into the environment for longer period of time [21][22]. The remediation of such effluents containing dyes becomes economically unviable when physico-chemical methods are used. While bioremediation is the natural treatment of such effluents through biological components like plants, algae, fungi and bacteria [23][24]. The phycoremediation however allows treatment along with utilization of excess nutrients from wastewater containing dyes for algal biomass growth [25]. The production of algal bio-oil is a cost-effective economic process. The organic compounds are stored in the algal cells as food reserve or for other cellular activities. These diverse chemical compounds are accountable for the expansion of food and fuel industries based on algae [26][27][28]. Algae are useful as feed for fishes, shrimps, shellfishes and coral reefs which constitute more biodiversity than amazon forest [29]. Certain algae are significant natural food colorants and rich source of omega-3 fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and omega-6 fatty acids which includes linoleic acid (LA), arachidonic acid (AA) etc. [30]. They are thus potential candidate for the production of eco-friendly and renewable liquid fuel, which offers algae as ‘green gold’ of future biofuel economy [31]. Many green algae species naturally accumulate substantial amount of triacylglycerols, approximately 30- 60% of its dry weight [32]. The bio-oil produced by green algae are believed to be clean, sustainable and environment friendly substitute for currently used non-renewable and unsustainable energy fuels [33]. This is due to the fact that burning of fossil fuels emits greenhouse gases, while cultivation of green algae works as CO_2 sequestration chamber to combat global warming [34]. The less cost of maintenance, easy cultivation conditions and methods along with abundance in aquatic environment present green algae as a highly significant source of biomass. While green algae are one of the blooming species present in eutrophicated aquatic system. But it can be used as a good resource for nutrient recovery which is one of the prominent reasons of eutrophication [35][36]. The eutrophicated water provides an opportunity to bioremediate such waters and investigate the influence of such environmental conditions on biomass productivity and oil yield of algae. These experimentations are helpful to identify the ideal environment necessary for optimum growth of algal biomass and bioactive compounds present in it.

Algal biotechnology now focuses upon synergising higher biomass yield, phycoremediation of wastewater containing dyes, enhanced lipid efficiency to produce biofuels as sustainable development option. Unconventionally, the advancement in algae-based industries looking for improved species or strain which may avail diversity essential for artificial recombination of desirable specifications in the suitable species. Algae being a cosmopolitan resource requires identification of high biomass yielding species anywhere across the globe to utilize it at maximum potential. While, such information on novel species provides prospects for intensive research in different biotechnological application of algae. Consequently, this study enables us to evaluate the industrial potential of our algal resources.

1.2 Microalgae *Chlorella sp.*

Algae embodies a set of photosynthetic lower plants from unicellular to multicellular and gigantic marine kelps. The presence of photosynthetic pigment in them enables to classify into various types such as Chlorophytes (green), Cyanophytes (blue-green), Xanthophytes (yellow), Chrysophytes (golden-brown), Phaeophytes (brown) and Rhodophytes which are red in colour. The most common algae such as blue green algae are prokaryotic organisms, while diatoms are largest biomass producing species on the earth [37]. Eukaryotic algae comprise of three major groups Rhodophyceae (red), Phaeophyceae (brown) and Chlorophyceae (green). Algae such as blue-greens, golden brown and diatoms are unicellular while red and green algae contains both unicellular and multi-cellular forms. Unicellular microalgae such as *Chlorella sp.* are cosmopolitan in distribution and fastest growing photosynthetic species, containing photosynthetic pigments like chlorophyll a and chlorophyll b. The unicellular algae are amenable to large scale production technologies in bioreactor systems and can be efficiently modified genetically according to required strains using biotechnological procedures[38]. In comparison to terrestrial plants and other aquatic macrophytes, microalgae survive at higher rate of production. Several variety of unicellular algae (*Chlorella sp.*) are capable to reproduce and multiply its biomass two-fold within 3.5 hours to one day [39]. The algae are fastest producers in terms of biomass per unit time, space and required nutrient sources. They have simple life cycle as well as metabolic pathways like higher plants. Conventionally algal biomass are majorly sea weeds. The algal biomass of macroalgae such as phaeyophytes and rhodophytes are used to produce agar, alginate, carrageenans, phyco-colloids and fucerallans etc. for different purposes [40].

Chlorella is rich source of fatty acids (mono/polyunsaturated fatty acids) and have about 30%–60% of it. But the quantity may get affected by environmental conditions where they grow like under nitrogen stress conditions the lipid content get increased in them [41]. Carotenoids are the natural pigment, not being synthesised by humans, are present in *Chlorella sp.* up to 0.2% [42]. The carotenoids such as astaxanthin, lutein, β-carotene and zeaxanthin are found in *Chlorella*. These are also industrially produced by food, nutraceuticals and cosmetics production units. *Chlorella vulgaris* is a rich source of ascorbic acid, tocopherol, vitamins, minerals, protein and polysaccharides [43]. While the presence of carotenoids and chlorophylls such as lutein, chlorophyll a and b, and pheophytin, enables it to have antioxidant capacity [44]. In the 20th century microalgae was commercialized as a nutritional food source [45]. *Chlorella sp.* were incorporated as human food (natural food or food supplements) in various forms such as powders, tablets, capsules, or extracts [46]. While these are also used as food additives (pasta, biscuits), dyes, emulsifiers and beneficial to enhance the nutritive characteristics of these products. *Chlorella pyrenoidosa* when used as dietary supplement, it improved quality of life by reducing high blood pressure and acting against fibromyalgia, hypertension, and ulcerative colitis [47]. While they are investigated as chemo-preventive agent to combat inflammation and cancer [48]. The violaxanthin(carotenoid) extracted from *Chlorella ellipsoidea* was has anti-inflammatory effects [49]. The astaxanthin isolated from *Chlorella zofingiensis* and lutein from *Chlorella sorokiniana* are potentially active as antitumour, antioxidant, and anti-inflammatory effects[50][51][41]. Sulphated extrapolysaccharides (EPSs) are biopolymers also known as exopolysaccharides present in microbial cells. The *Chlorella* is rich in exopolysaccharides and has approximately 8% of these compounds [15]. Sulphated polysaccharide β-glucan (*Chlorella regulis*) was reported to have antitumour effect [52]. *C. stigmatophora* examined to have anti-inflammatory activity against paw edema test as well as have an immunosuppressive effect [53]. Research suggest that these compounds have beneficial effects such antimicrobial, anti-inflammatory, antioxidant and anticoagulant etc. The secondary metabolites produced from *C. vulgaris* was researched to have antifungal activities and antibiotic action to counter Gram-positive and Gram-negative bacteria [54][55]. The presence of high levels of chlorophyll in comparison to other microalgal species makes them effective to treat ulcers in the liver [56].

The countries such as China, Germany, Japan and other Asian countries use *Chlorella sp.* biomass in traditional medicine and health foods due to its richness in protein, carotenoids, vitamins, and minerals [57]. While *Chlorella sp.* is reported to possess abundant nutritional value, which makes them diathetic substitutes along with anti-inflammatory property among other. Its outdoor cultivation began during late 1940s in the countries such as USA, Germany, and Japan. They are cultivated by using various systems which are phototrophic, heterotrophic and mixotrophic performed in open-culture or closed-culture systems [58][59]. Moreover, the development in algal technology due to existence of several high value bioactive compounds such as carotenoids, antioxidants, vitamins, polysaccharides, proteins, fatty acids, among others allowed them to be used as food substitute in medical research and biofuel industries.

1.3 Importance of algal oil

Fuels are energy yielding resources serve as feedstock of various industrial hubs and for household purposes. Fossils are the conventional source of energy which include coal and petroleum. Urbanisation, population growth and industrialisation have drastically increased the need for fuels in our day today life. There are various unconventional sources of energy such as thermal power, nuclear power, hydel and tidal energy. But due to high cost of raw material and non-renewable in nature, input cost, lack of infrastructure and facilities these are not harnessed to its full potential [60]. The increase in global demand for energy and limited reserves of fuels mismatch the demand-supply. It is speculated that the usage of non-renewable fossil fuels would go above 86% of the present consumption by 2035 [61]. While the major concern is that these conventional energy fuels are the major reason for global warming, acid rains, air pollution, oil spilling, radiation fall-outs and climate change. These environment impacts are likely to induce more health threatening diseases or ill-health effects. It would also impact agricultural fields and change in monsoon pattern, rise in sea level, warming of oceans, variation in culture conditions of crops as well as massive destruction to the global environment stability [62][63]. This all inflicting towards other sources of fuel which can contribute to sustainable development. The search for stable, economically viable and environment friendly source of alternative fuels has led to extensive research and development focussing on the conversion of various feed stocks into biofuels. Biomass is a traditional form of energy in the form of fire-wood and other biomass-based fuels like bio-ethanol and bio-oils/biodiesels. Biodiesel is considered as a potential form of another energy resource, amenable to be used for aviation fuel purposes. The Biofuels are biomass derived fuels [64], which comprise solid biomass, liquid fuels and biogas produced from decomposition of biomass.

Biodiesel is one of the kinds of biofuel, which consist of concoction of monoalkyl esters derivative of fatty acids (lipids). The oil required for biodiesel production can be obtained from traditional oil-crops or algae. The biodegradable and nontoxic nature of biodiesel makes them eco-friendly. While the heating of biodiesel does not emit unburnt hydrocarbons, carbon monoxide, sulphates, nitrates and other polycyclic aromatic hydrocarbons into environment thus it is an environmentally sustainable source of energy [65]. The practice of biofuel production on agricultural lands and extraction from food crops may cause ethical concerns [66]. This is due to malnourishment in 60% or more of the world human populations that need food grains, moreover cultivation of such non-edible oil crops can cause serious environment threats to marginal lands[67]. Consequently, the research on biomass to harness energy need focus towards alternative means and sources for bio-energy. Lately algae are emerged as a substantial alternative to produce bioenergy. They have abundance of lipids, carbohydrates and proteins which can be converted into biofuels. *Botryococcus brounii* produces hydrocarbons, which are similar to those present in petroleum [68][34]. While oil isolated from green algae resembles those extracted from oil-seeds. However, research suggests that various kinds of fatty acids isolated from algae as methyl esters by transesterification process can be used for the production of biodiesel. The carbohydrate present in algae is generally used to produce ethanol through fermentation process, which is also considered as a biofuel. The Algal biomass can be considered as one of the easiest ways to produce renewable and eco-friendly biofuels [69]. The algal oil (lipid) in comparison to lipids obtained from land plant biomass, possess high caloric value with less viscosity and density. These specifications make algae more appropriate source of biofuel than the conventional biofuel materials such as oil crops or oil produced by pyrolysis of wood [70]. The species diversity available in algal biomass enables algae for testing the viability of production of bio-oils. Algae thus offer variety of biofuels such as biodiesel, jet fuels, aviation fuel, gasoline and bioethanol based on their energy producing capacity [71][72]. However, the success of algal biofuel industry needs identification of high lipid yielding species, optimization of culture conditions for mass production and standardization of efficient lipid extraction process [73]. The algal biofuels are now becoming a critical element of national and international bodies to combat global greenhouse gas emissions associated with rising demands for energy. Certainly, in the era of global warming, algal bio-oil/biofuels must be regarded as effective panacea to alternative biofuel.

1.4 Necessity to remediate synthetic dyes

Annually, 0.4-0.5 million tons of effluents are being discharged into the water bodies, consist of dyes and its derivatives responsible to cause ecotoxicity. Organic compounds in general do not display various colors like dyes. Dyes are widely used by different manufacturing sectors and industries like food and beverages, textile, leather, paper, stationery, cosmetics and pharmaceutical etc. [22]. In some cases, these are used as aesthetic agent and as an identifier in other instances. Dyes have one or more color bearing groups called chromophores which makes them colorful, while alternate single and double bonds present in its structure provides them conjugate structure and stabilizing property [74]. In case of synthetic dyes, an insignificant amount of it yields bright color while natural dyes provide limited range of colors with less durability. Although the rising population demands heavily depends upon synthetic dyes due to its inexpensive affordability and other characteristics like less colorfastness. The synthetic dyes consist of chemical compounds such as amines, naphthalene, toluene, benzene, sulphonic and carboxylic acid groups [75]. The metallic mordants present in such dyes increases the dyeing time but cause health hazards. The global organic textile standard (GOTS) recommends judicious usage of dyes not beyond permissible limits without destroying its nature [76].

The improper transfer of dye to substrate, inept dyeing measures and poor treatment of effluents containing dyes when discharged by different industries leads to environment pollution after its mixing with aquatic bodies and surroundings. It has adverse effects on human health like bronchitis, skin problems, occupational risks like mutagenicity as well as carcinogenicity and disruption into the biosphere [77][78]. Synthetic dyes have toxic and carcinogenic effects, reduce the light intensity available to waster bodies. This hinders the photosynthesis action of phytoplankton and leads to decline in dissolved oxygen levels which is an important water quality parameter[19]. Studies suggest that 4-aminobiphenyl dye shows incidence of carcinogenicity in bladder and hepatocellular carcinoma in mice. In humans it induces angiosarcoma, teratogenicity, chromosomal instability and initiates damage to lymphocytes [79][80]. The “International Agency for Research on Cancer” gave direction on use and adverse impacts of dyes for instance, benzidine reflects carcinogenicity toward mammals and humans. Inhalation of reactive dyes, can cause bronchial problems, bladder cancer and occupational diseases. Sunset yellow dye leads to gastro-intestinal problems, while itching, sneezing and hyperactivity in children were observed in case of quinoline yellow dye [81][82]. Methylene blue was studied to have effects like headache, convulsions, imbalance in blood pressure levels and vomiting. The toxic chemicals present in the dyes induces health complications such as nausea, rhinitis, bleeding, skin problems, ulcers, and cardiac risks [83]. The dyes are likely to cause oxidative stress in plants due to chromium (textile industry), decline the pace of photosynthesis and regeneration in them [84]. The microbial world is not left unimpacted by dyes, here its impacts lead to reduce bacterial luminescence. Azo dyes are researched to slow down algal growth and may bring mutagenic effects on several microbes [85][86]. The change in color of water reservoirs when impacted by dyes, decrease the rate of photosynthesis among algae and phytoplankton. This becomes trigger factor to cause disruption in food chain, less carbon sequestration and more global warming [87][88][89]. Therefore, it becomes necessary to regulate the use of dyes and the effluents discharged by the industries based upon it. Though various countries took measures to prevent the detrimental effects of dyes. Amongst the European nations Germany was the first to proscribe the production and utilization of specific azo-dyes. India and the Netherlands along with a few more nations have restricted use of synthetic dyes moreover, these are used in abundance by various industries [90][91]. While the synthetic dyes cannot be replaced by natural dyes completely but treatment procedures at the discharge unit can reduce its adverse impacts. Different physical and chemical methods are available which can be used to treat effluents containing dye. However, bioremediation is preferred over such process as far as environment impacts are concerned. Algal biomass is found to be an effective alternative, owing to the existence of several functional groups as active sites for its remediation. While reductase enzymes present in them are beneficial to decolorize the effluents. They can be reused and various value-added products can be obtained using the same algal biomass. The recalcitrant nature of synthetic dyes in extreme environmental conditions requires further investigation to make them a viable option for consumption.

1.5 Strategies involved in culture of microalgae

Algal biomass productivity and accretion of various bioactive compounds in algae majorly depends upon their growth conditions and media composition [92]. There are various environmental conditions such as sunlight, temperature, relative humidity, land topography, availability of nutrient, carbon source and water quality which influence the growth and biomass productivities of algae [93]. These parameters limit the extent of diversity in algae in specific locations of a country. The bioactive compounds such as oil and pigment, varies with the composition of media [94] and pH [95]. Hence, before upscaling the cultivation of algal species, experimental study in specific growth media under different nutrient and other environmental regimes is required. It would provide diverse cultivation methods, essential to standardize the production and harvesting of desirable algal species. The algae culture isolated from a water body can be cultivated in standard algal growth media such as BG-11, K&C medium, Fog medium, Bold’s Basal Medium (BBM), Chu medium and Bristol etc. to test the suitability of the species to grow in artificial media [69]. While algal biomass productivity and quality of lipid present can also be evaluated at this juncture. The algal species are required to be grow in suitable medium for long duration for further estimation of dye degradation efficiency by the sub culturing process. While, biomass productivity of different culture media may be evaluated in diverse ways.

The assessment at laboratory level can be performed in 100 mL to 1 L conical flask. The culture media are required to be controlled at specific pH, light, and temperature conditions following suitable protocols. Approximately 20-30 days of incubation period is enough to harvest the biomass from the culture medium by centrifugation, in order to quantify the biomass for further evaluation. As the algae are photosynthetic in nature, so assimilation of inorganic carbon depends on the light source under normal prevailing conditions. The light energy directly or indirectly affects the remediation efficacy towards dyes, biomass and lipid productivity of algae [96][97]. The P^H is a substantial factor of culture medium that affects the biomass productivity, dye degradation efficiency, fatty acids and its composition in algae [98]. At extreme low and high P^H, the biomass as well as lipid yields are less, but at optimum range of 7-9 the yield are high. Such variation in P^H value also affects the rate of phycoremediation as high amount of biomass would decolorize dyes fast. Consequently, the P^H of the culture medium can be optimized and may standardize the same for productive conditions. Aeration in the culture medium and stirring of the same is essential to avoid the sedimentation of algae. In a medium, aeration confirms that the cells present in a culture medium are uniformly exposed to the light conditions and nutrients [99][100]. Therefore, cultivation of algal species can be developed towards bioremediation of wastewater containing dyes and the biomass and bio-fuel yield as an additional monetary advantage [101][102]. Such arrangement enhances the composition of phyco-compounds, as the nutrients present in the effluents containing dyes would be exploited by algae for its growth and results in phycoremediation. *Chlorella* species used for waste water treatments stated to be effective in the elimination of nutrients and can also help to combat the problem of eutrophication [103] [104]. Nowadays, the potential of wastewaters is investigated as culture media and its impacts on different compounds. While standardization of culture conditions will enable, development of suitable approaches for application of apt species or strains towards standardized industrial effluents treatment procedures. As a consequence, the strategies adopted in the experimental culture of *Chlorella sp.* are viable to became successful to solve the problems of the 21st century and would serve to achieve sustainable development goals.

1.6 Objective of study

Chlorella sp. is considered as most vital source of food being rich in proteins, carbohydrates, iron, antioxidants, PUFA, carotenoids, anti-inflammatory, vitamins such as Vit B12, Vit C and minerals [49][47]. Among diverse algal species *Chlorella* have high potential to produce algal oil and other valuable products. Local availability of it enables, less prone to demand-supply gap if replace the fossil fuels [33]. The presence of different functional groups in *Chlorella* and enzymes like reductase offers various binding sites as well as catalytic activity to remediate pollutants such as dyes and heavy metals [22]. The application of lipid extracted biomass and use of algae proceeding phycoremediation of dye containing effluents besides a natural source of carbon sink, entitle them as a sustainable solution for environmental problems like water pollution, energy crisis and global warming.

The objective of the study includes:

- Culture of *Chlorella sp.*in five different invitro media.
- Quantitative estimation of the biomass yield of the *Chlorella sp.* cultured in different invitro medium.
- Assessment of dye remediating activity of *Chlorella sp.* cultured in BG-11 media.
- Comparative analysis of lipid yield under different culture media.

2. Review of literature

Algae are the first formed, highly diverse autotrophic organisms. It is known that, for millions of years algae exist as the phototrophic organisms [105]. The huge amount of algal biomass which got buried into the deep depths during primitive stages of earth, underwent biological development got transformed into petroleum under intense pressure and temperature that later serve as fuel resource for humans. While land biomass got buried and converted into coal in later stages of earth evolution. Humans for centuries extracting these fossil fuels from the earth and their burning has caused a secondary impact. Increased consumption of these fuels leads to high amount of greenhouse gas emission in the atmosphere, which when exceeded the thresholds became a menace in the form of global warming. These facts elucidate the importance and the role played by algae since the era of origin, development, evolution and sustainability of earth's ecosystems along with human civilization on the earth. In modern times, humans are now becoming more dependent on algae as alternative to biomass and as bio-fuel to combat global warming [106]. The algal biomass now proposed unique ways to resolve the vital issues of present day related to food security, energy, bioremediation and environment crisis. Ecologically, algae are the natural, phototrophic and vital primary producer of all categories of wet ecosystems on the earth, particularly the oceans, which constitute 70% of the earth's surface. Algae being present as primary producers, the major source of biomass for all aquatic systems. They act as a crucial element of the aquatic as well as marine food chain. In the aquatic system small crustaceans and other zooplanktons consume the algae, which in turn are consumed by larger animals. Therefore, the variety of algae became one of the reasons of diversity in microbial and macrobial organisms of the aquatic system [107]. Specific algae like green algae (*Chlorella sp.*) are thus, vital components of aquatic systems. In wet terrestrial ecosystem algae have unique role like on wet surfaces of rocks, soils, tree trunks, and on the skin of certain wild animals found in the rain forests. We can now say that apart from being a biomass contributor, it plays explicit roles in various ecosystems. In all the ecosystems algae are significant in supporting other organisms by direct or indirect means for the sustainability of such natural ecosystems.

The role of algal biomass is quite unique, which has received high prominence in the recent time due to many unavoidable reasons. Algal industries are now becoming a crucial part of global economy due to the nutrient richness in its biomass. In bio-based industries, one of the major problems is the partial dependence of these industries on conventional sources of energy and raw materials, which leads to pollution and other environmental hazards [108]. While the sustainability of biomass-based industries depends on the restricted use of fossil fuel and chemical additives in the manufacturing process or products. In order to substitute the conventional raw material, algal biomass becomes seamless sources of both fuel and raw materials which has latent potential for industries to overcome the limitations of former categories of biomass industries. Algal industries are emerging as an economy comprising vast expanse such as energy, foods, feeds, fine organic chemicals, health care products and organic fertilizers [56]. The algal biomass has been a source of both animal feed and human food. The diversity in algae species is acknowledged for different varieties of nutrient contents present in their cells. The applications of biomass obtained from algae as a source of biofuel and its conversion into various other forms of energy like biodiesel, biohydrogen, biomethane biogas are gaining immense attention in the present day of energy and environmental crisis[109]. Algal biomass was used as a significant source of food in many parts of the world has a long history, particularly in coastal populations. While further research and development unearth diverse applications based on algal biomass in several fields of industry. Algae are later recognized as rich spring of phyco-compounds such as carbohydrates, proteins, lipids, pigments and mineral nutrients. Progressively, the rising human needs and demands has compelled to develop large-scale commercial cultivation of valuable algae species for numerous purposes. The cultivation procedures evolved from the initial natural algal farms to the construction of advanced bioreactors[94]. The development of biotechnological and metabolic engineering approaches to modify specific species for the large-scale production of desired biomass has changed the scenario of unicellular algal biomass production systems. At present, research and technological innovations are conducted throughout the world towards production of algal biomass which use least energy sources synergised with the solution of environmental problems [110]. This study has exhaustive scrutiny of all significant researches carried out in the arena of algal biomass exploitation, discovery of potential algal resources, in vitro culture and productivity of algae. It provides information about mass production of algae, oil yield of specific species, application of the algal oil components in medicines or other fields of utilities, as well as problems associated with the research and algal industry.

2.1 Algal biomass research

The biomass extracted from algae such as green algae *Chlorella sp.* has wide range of applications in different arenas which is shown in figure 1 and summarized here.

Figure 1: Different applications of algal biomass

2.1.1 Algal biomass as a source of bio fuel

Algal oil was used as an alternative fuel resource in the late 1970s under an initiative of the United States in their aquatic species program. Consequently, approximately 3000 algal species of fresh and marine water were found appropriate for the biofuel production [3]. The US Department of Energy has investigated that algal biomass is efficient to produce thirty times extra oil per unit area of space when resources are compared to conventionally used terrestrial oil crops for biofuel applications. They presented the technical feasibility of low-priced biodiesel obtained from algal oil and highlighted the necessity of research and development to isolate better strains in order to achieve sustainable production of algal biofuel. Algal biomass has been examined to produce various kinds of biofuels including biodiesel, biohydrogen, syngas, bioethanol and biogas[111][112]. At present, algae-based biodiesel is one of the most researched biofuels which received immense consideration. These are carbon neutral and the raw material requirement can be met by easily cultivable high yielding algae. Fuels based on petroleum began in 1905 onwards [113] and subsequently the biodiesel-based engines were modified to petroleum-based engines. Different countries have their domestic biodiesel standards and guidelines like the United States of America, Brazil, South Africa, Australia and Europe have for the testing of biodiesel and its applications [114][115]. Further attempts were made to develop model systems for the characterization of biodiesel based on chemical composition of fatty acids [116]. Research suggest that chemical components of the fatty acids present in algal lipids has significant role in the production of enhanced quality of biodiesel. The environmental conditions and media composition also impact the composition of fatty acid in different algal species [117]. Algal biomass of *Scenedesmus* sp. ISTGA1 has provided 452 mg/L lipid, while *Chlorella vulgaris* yielded 84.01 % biofuel [118][119]. Different species of green algae such as *Chlorella vulgaris*, *Scenedesmus* sp., *Spirogyra* sp., and *Chlorophyceae* etc. are investigated to produce biofuels [120]. While, the lipid extracted biomass of *Chlorella sp.* has reported to further produce bio-oil by the application of pyrolysis [121]. Biotechnological approaches such as genetic engineering and metabolic engineering are applied to enhance the fatty acid metabolism in algal technology to improve quality of lipids suitable for biodiesel production [122][123]. Research is now conducted in the molecular characterization of potent oleaginous algae for biodiesel production, using both marine and fresh water environments.

2.1.2 Algal biomass for animal feed

Algal biomass, particularly the biomass of seaweeds such as *Ulva, Enteromorpha, Gracilaria, Hypnea, Sargassum* has been used as animal feeds since thousands of years in ancient Greece and Iceland [124]. Research studies on nutritional quality of algal feed supplements has potential to enhance growth rate in rats and chicks. *Spirulina* have been used as a protein rich feed or food for both humans and animals [125]. In countries like Japan and China, seaweeds were used as fodder for domestic animals for decades. The use of algal biomass of *Chlorella* and *Scenedesmus* as protein supplementation to ruminant animals and pigs shown positive impacts. Biomass of *Schizochytrium sp. and Chlorella sp*. has revealed better yield and improved quality of meat in animals like pigs, rabbits, poultry and ruminants. While *Chlorella vulgaris* supplementation to the feedstock of *Capra aegagrus hircus,* yielded 10-15% improvement in feed intake and digestibility [126]. The feeding of algal suspension than oil cakes in milking animals, improved the fibre digestibility and feed efficiency utilization. The use of alga as feed supplement like *Porphyridium sp*. has positive impact on metabolism of chicken, which leads to increase in nutritional value of the meat [127]. Oil rich algal biomass provided as livestock feed improves the amount of polyunsaturated fatty acids (PUFA) in milk, specifically the amount of docosahexaenoic acid (DHA) [128]. The biomass of *Chlorella and Ulva* (green algae) and others such as *Porphyra* (Red algae), *Laminaria*, *Undaria*, and *Hizikia fusiforme* (Brown algae), are recognized as safe for consumption which are of high nutraceutical value[129]. Among diverse species of algae available in animal feed industry, *Chorella sp.* has received great attention in recent times. Various experimental studies discovered that, *Chlorella* provide nutrient-rich high yield biomass, which can be economically transformed into animal feed [130]. In addition to this, *Chlorella* is widely known fresh water species rich in pigments, antioxidants, vitamins, immune-stimulants and some Chlorella-Growth-Factor compounds [131].

2.1.3 Algal biomass as source of human health

Algae has been a segment of human diet since prehistoric period, particularly in coastal countries like China, Japan and south Korea. Ashes of brown algae were used since 1750s by Asian physicians to treat iodine deficiency disease goitre (Rao, 1970). Initially, seaweeds biomass was used commercially as food or feeds and fertilizer components. The presence of phycocolloids in algae and its industrial and food application has caused the harvesting of algal since 1950s. During 20th century, commercial cultivation started at large scale for food and feeds [133]. In this period, algal cultivation of species such as *Chlorella, Scenedesmus, Spirulina* has begun for single cell proteins, health food and β-carotene in different parts of the world like USA, European countries like Germany, France and in Asian nations like Israel, Japan, Thailand [134]. Algal species such as *Porphyridium cruentum, Nannochloropsis sp., Phaeodactylum tricornutum, Monodus subterraneus* were researched and well recognized as source for the production of eicosapentaenoic acids. While *Crypthecodinium cohnii* and *Chroomonas salina* reported significant amount of docosahexaenoic acid in them [135]. Research suggest that PUFA extracted from algal biomass are beneficial in preventing CVDs (cardiovascular diseases), cancer [136] and inflammatory diseases [137]. *Porphyridium sp.* (polysaccharides) has anti-inflammatory effects on skin [51], monosaccharides (mannitol, glucose) extract of *Isochrysis galbana* and *Nannochloropsis oculate* presented anticancer and antimicrobial activities [138]. *Nannochloropsis* are rich source of carotenoid such as astaxanthin, β-carotene, lutein/zeaxanthin, canthaxanthin, which currently has potential to grow as market of natural additives in food and feed. Green algae such as *Chlorella* and *Scenedesmus* has rich composition of carbohydrate (primary neutral monomers) like glucose and galactose. Polysaccharides like starch and cellulose, present in *Chlorella sp.* and higher plants are similar in properties. It is investigated that storage polysaccharides (glucomannan and arabinomannan) are present in *Chlorella* which can be used as food additives and in pharmaceuticals as well as textiles upon fermentation [139]. Algae are also researched in clinical applications such as microcapsules and microspheres for drug delivery [140], recombinant proteins, blood-clotting factors, immune regulators etc. [141]. While studies on the developments in metabolic pathway and algal transgenics are carried out for understanding of regulation of product formation and the role of several biocomponents [142].

2.1.4 Algal biomass for environment solutions

An integration of algal cultivation for biomass and subsequently used for wastewater treatments has provided wider scope of algal biomass utilization. This strategy enables retrieval of lost nutrients present in wastewater while purifying the same. Across various parts of the globe like Israel, India, Thailand and the USA [143][144][145], this synergistic approach has been adopted. The remediation of polluted water in this way provides one of the best of application for tertiary wastewater treatment. The algal biomass thus cultivated and lipids yielded will provide further economic benefit [102]. The chlorophytic microalgae like *Chlorella* are the globally used for wastewater treatments with or without the presence of dye derivates in them. These are found to be very effective in the removal of nutrients from wastewater [122][146]. In the wastewater treatment, the impacts on quantity and quality of lipids extracted from algae are widely researched. While the lipids obtained from such biomass are specifically used for biofuel productions [147][148]. *Chlorella vulgaris* when investigated to bioremediate textile waste effluent (WE), results change in Chemical Oxygen Demand (COD) by 69.25% and colour removal at 75.68% [149]. The use of wastewater such as industrial, municipal and agricultural wastewater for *Chlorella sp.* cultivation provides high yields. Thus, it can be considered as remarkable species appropriate for the wastewater related cultivation of biomass and lipid production [150][151]. Therefore, the tendency of microalgae for the remediation of nitrogen and phosphorus from waste waters should be studied more extensively to tackle the problem of water pollution and achieve sustainable development goal (SDG-6) as Clean Water and Sanitation.

2.1.5 Algal biomass as source of nutraceuticals

Microalgal biomass is now emphasis on the commercial production of valuable nutraceutical compounds. In comparison to macroalgae, microalgae consist of efficient cellular system to harvest solar energy in order to produce various organic compounds [57]. *Chlorella, Spirulina, Isochrysis, Dunaliella* and *Hametococcus* are widely used to produce bioactive compounds of nutraceutical importance. *Chlorella sp.* is economically significant species known for valuable food-grade metabolites such as carbohydrates, proteins and lipids. *Chlorella* is a valuable species due to its high biomass productivity and secondary metabolite yielding properties [152]. Species consisting pigments are cultivated to produce natural food colouring substances. *Dunaliella sp.* is a rich source of β-carotenes, whereas *Porphyridium* and *Synechococcus* are reported to be rich sources of phycobiliprotein pigments [153]. Lycopene, β-carotene, xanthophylls are types of carotenoids extracted from *Chlorella* [39][40]. *Dunaliella sp.* has significant quantity of β-carotenes which revealed properties like antioxidant, anticancer, cellular growth induction, cardiovascular protection and pharmecodynamic distribution [154]. Proteins extracted from algal biomass are of superior quality in comparison to other plant extracted proteins [155]. Essential fatty acids such as Docosahexaenoic acid (DHA), Arachidonic acid, Eicosapentaenoic acid (EPA), gamma-Linolenic acid (GLA), and Linoleic acid (LA) are present in algal lipids which are immensely important for nutraceutical industry. *Nanochloropsis, Porphyridium, Isochrysis, Phaeodactylum* were reported to have essential fatty acid rich lipid extract [137][156]. Sulphated poly saccharides found in marine algae, reported effects like antioxidant, anti-allergic, anti-HIV, anticancer and anticoagulant activities on human along with certain nutraceutical benefits [157]. *Spirulina sp.* being rich in essential and non-essential amino acids is proved to be beneficial in improving human body weight [158]. *Chlamydomonas reinhardtii* (green algae) is studied to be potential strain, extensively used and cultivated by many nutraceutical industries for the production of diverse kinds of nutraceutical compounds through genetic engineering process [159][160]. Therefore, algal biomass of some species is unique due to the occurrence of biochemical compounds such as polyunsaturated fatty acids (PUFA), minerals like sodium, potassium, calcium, magnesium, iron, zinc and other trace elements, vitamins like riboflavin, thiamine, carotenes and folic acids which has immense role in nutraceutical industries.

2.2 Prospects of algal biomass and bio-oil

In India mass cultivation of micro algae for animal feeds and foods as a source of protein was first introduced by Becker and Venkataraman [161]. A project of Central Food Technological Research Institute, Mysore was the first venture setup for algal biomass cultivation work in India [162]. *Spirulina* and *Scenedesmus acutus* cultured using rural wastewater were used for animal feed. Extensive research worked on to utilize seaweeds as manure for higher plants, for food and nutraceuticals during the 1990s [163]. Institutions and laboratories such as National Institute of Oceanography, Andhra University, Central Marine Research Institutes (CMFRI), Central Drug Research Institute (CDRI), Indian Institute of Technology (Mumbai and Delhi), Indian Institute of Chemical Technology (IICT), Central Salt and Marine Chemical Research Institute (CSMCRI) are involved in marine based algal natural product researches [164]. Strategic designing of algae was developed to achieve sustainable production of valuable phycocompounds. Indian institutions have contributed remarkably in the field of algal biotechnology. In Indian waters *Botryococcus braunii* was investigated as hydrocarbon producing algae [165]. Sundarbans fresh water resources were explored for algal diversity as feedstock for bio-diesel [166], procedure for algal biofuel technology was established to treat industrial effluents and waste water [167], enhanced biomass as well as lipid production and biofuel production possibilities for biogas, bio ethanol and bio diesel [168].

Algal biomass is investigated to be used for bioelectricity generation where microbes such as bacteria and algae use organic molecules present in wastewater or dye containing effluents for electricity generation [25]. Research found that algae-based nanoparticles called phyconanoparticles possess properties like antibacterial and antifungal action, antitumor, antioxidant as well as anti-inflammatory activities [169]. Spherical nanoparticles (NPs) were reported to be produced from *Chlorella vulgaris* having antimicrobial property [43]. Anti-pathogenic activity was shown by self-assembled spherical gold nanoparticles formed from *Chlorella vulgaris* [170]. Phyconanoparticles have application in wastewater treatment as well such as methylene blue dye reported to be remediated by AgNPs based on *Chlorella vulgaris* [171], and *Chlorella pyrenoidosa* [172]. The lipid extracted biomass can be used to synthesize phyconanoparticles like carbon nanotubes (CNTs) and carbon quantum dots (QDs) [173]. A number of technologies are developed for the extraction of lipid from algal biomass as bio-oil. The cultivated algal biomass is harvesting by using various methods like centrifugation, gravity sedimentation, electromagnetic separation, filtration, flocculation and electrophoresis [174][175]. Algae based industries are now directed to be sustainable as far as phycocompounds extracted biomass is concerned [130]. Now a days lipid extracted biomass (LEB) is used for numerous applications. Bioplastic (Poly-β-hydroxybutyrate / PHB) was reported to be produced from *Chlamydomonas reinhardtii* [176] and *Phaeodactylum tricornutum. Hydrodictyon reticulum* [177] produced biodegradable plastic as polylactic acid (PLA). LEB based Biochar in case of *Chlorella sp.*, are reported with compact surface area and highly capable of cation exchange [178]. Such biomass are also reported to be useful in adsorption of dyes and heavy metals [179]. The LEB of *Chlorococcum sp.* produced bioethanol, yields 60-70% more ethanol compared to original biomass [1]. The production of biogas from LEB of *Nannochloropsis sp.*, *Tetraselmis sp.* and *Scenedesmus sp.* [180] revealed one of its application which enabled algal industries a sustainable sector in future.

2.3 Invitro culture and production of biomass from algae

Biomass production and standardization of cultivation systems becomes one of the crucial fields of study to establish in-vitro culture to obtain enhanced algal biomass and lipid/biofuels production systems. This approach started with invitro culture of *Haematococcus pluvialis,* while successful cultivation completed using simple inorganic medium for green algae *Chlorococcum sp.* and *Protococcus sp.* [181]. Unialgal culture of *Chlorella* was developed for physiological experimental purposes in 1890s. Algal cultivation system at small-scale and large-scale began in early 1950s, *Chlorella sp.* was used for research and pilot scale studies. The successful application for algal production using *Chlorella pyrenoidosa* demonstrated with large volume photobioreactors [134]. Algal biomass cultivation system synergised with waste water treatments was established in 1960s. Environment conditions like temperature and nutrient requirements of algal growth was setup in marine open pond culture systems [182]. The nutritional valuation of *Chlorella* for industrial potential were carried out in Japan. *Haematococcus* was cultured for the isolation of astaxanthin using reactors in Israel [183]. *Crypthecodinium cohnii* was industrially produced for essential fatty acids in USA [184]. World’s largest tubular photo bioreactor was established to culture *Chlorella sp.*in Germany, having 500 kilometer glass tube length system and 700 metric cube volume [185]. *Chlamydomonas reinhardtii* produced hydrogen gas using outdoor flat-vertical photo bioreactors [186]. Different methods of cultivation developed for algal biomass includes phototropic cultivation, heterotrophic method (aerobic in nature), mixotrophic cultivation (presence of light uses both inorganic CO_2 and organic carbon sources) and photoheterotrophic cultivation [29][187]. The invitro cultivation of algae requires optimization of different growing conditions in order to produce high yield of algal biomass and to isolate enhanced quantity of target phyco-compound. The factors considered during cultivation of algal biomass are discussed below.

a) Light: Intensity of light and duration affects the rate of photosynthesis in algae and thus influence biomass yield and biochemical composition [188]. Increase in light, increases the rate of photosynthesis to maximum point and stabilize when rate of photosynthesis balanced by photorespiration and photoinhibition. A certain duration of light/dark periods is essential as light is responsible for ATP and NADPH synthesis, while darkness produce carbon skeletons [189]. About 200–400 μM photons/m^2 /s is optimum level of light intensities for algal growth. *Chlorella vulgaris* attain maximum growth rate and lipid production at 5000–7000 lux [190].

b) Temperature: Climatic variation in species cause difference in growth temperature. An exponential algal growth takes place with rise in temperature up to an optimum range, but variation beyond the optimal range retards algae growth [191]. Research reports that optimum range of temperature occurs 20 to 30 °C for almost all species of algae. *Chlorella vulgaris* produce additional carbohydrates and lipids when temperature changed from 30 °C to 25 °C [192].

c) Nutrients: Nitrogen, phosphorus, and carbon are referred as macronutrients, vital for growth thus form the backbone of microalgae species [98]. The requirement of nitrogen and phosphorus differs from one species to another. Lack of nitrogen in a culture media may reduce algal growth, while it increases production of carbohydrates and lipids like in case of *Chlorella vulgaris* [69].

d) Mixing/Aeration: It offers uniform circulation of nutrients, air, and CO_2 in culture media. This allows infiltration and even transfer of light in the culture, which prevents settling of biomass [193]. Biomass productivity significantly decline due to uneven mixing. Uniform mixing in a photo-bioreactor allows nutrient dissolution, penetration of light and efficient gaseous exchange [194].

e) P^H and Salinity: Different microalgae species requires different P^H as per their environmental conditions. Variation in culture media needs different P^H value and the P^H range varies from 6 to 8.76 for almost all algal species. Algae are prone to variation in P^H values, very less are adapted to it like *C. vulgaris* can endure broad range [195][196]. Research suggest *C. vulgaris* achieve high rate of growth and biomass productivity at P^H 9–10. Increase in P^H of a culture media causes increase in salinity, which is not suitable for algae cells.

2.4 Phycoremediation of synthetic dyes

Photodegradation is a natural process of remediation for effluents containing dyes. Natural processes are not enough to treat such wastewater. Physicochemical methods are available but chemicals requirement, skilled manpower, maintenance cost and process regulations makes it less sustainable [197][198][199]. Phycoremediation is the treatment of effluents in presence of algae. The dyes present in the effluents used by algae as a nutrient source and decolorize them by converting them into simpler carbon compounds. Adsorption, biosorption, bioconversion and biodegradation are diverse mechanism used by algae for the remediation of dyes [146]. The process of biosorption involves cell wall properties of algae and electrostatic attraction to decolorize dyes. There are functional groups present in the microalgae including carboxylate, phosphate, carbonyl, amino and hydroxyl acts as binding site for dyes [25]. The functional groups or ligands offers large surface area and high binding affinity to interact with dye ions and immobilize them [200]. Algal cells can be used living or dead for decolorization of dyes. Live algal cells are capable for self-replenishment and can continuously uptake metallic ions, while dead cells may grow without essential nutrients [149]. Mechanism like physical adsorption (surface phenomenon involves process of chemisorption) and physisorption (consequence of weak van der Waals forces) can occurs in both live or dead algal cells [201][81].

Chlamydomona reinhardtii reported decolorization of Reactive Blue 221 and Remazol Black 5 dyes [202]. Methylene blue was decolorized up to 98% by the application of *Desmodesmus sp.* (Al-Fawwaz and Abdullah 2016). It was researched that *Chlorella vulgaris* has high affinity to remediate dyes such as Basic Fuschin, Basic cationic, Reactive Orange II, Methyl Red, Remazol Black B, Remazol Red RR, Remazol Golden Yellow RNL, Congo Red and Tectilon yellow 2G [203] [204] [205]. *Chlorella pyrenoidosa* decolorize methylene blue dye [206] and *Stoechospermum marginatum* discolourised different dyes like Acid Blue 25, Acid Orange 7 and Acid Black 1 present in textile wastewater [207]. Phycoremediated effluents containing dyes can be used to extract and isolate such microalgae for further remediation. Study reveals artificial neural network (ANN) can be used in such instances like algal consortia of *Chlorella, Cosmarium* and *Euglena* species can be used to decolorize triphenylmethane dye and Malachite Green (MG) [208]. Algae based nanoparticles (NPs) are not synthesised from conventional physicochemical methods, but biogenically and thus environmentally sustainable [209]. It was reported that presence of bioactive compounds like pigments, lipids and other secondary metabolites makes them suitable to synthesize NPs [210]. Golds based NPs (AUNPs) produced using algae are efficient to remediate dyes like Sulforhodamine 101 and Rhodamine B. While photocatalytic degradation of methyl orange was observed when AgNPs fabricated with *Ulva Lactuca* are used [211][212]. *Caulerpa racemose* based AgNPs have reported remediation of methylene blue [213]. Reactive Blue 221 and Remazol Black 5 dyes remediated using polysulphone nanofibrous web made up of *Chlamydomona reinhardtii* exposes application of nanotechnology in dye remediation [202]. Phyconanotechnology, which provides an amalgamation of phycology and nanotechnology to produce NPs has significant role in treatment of effluents containing not only dyes but also heavy metals, organic-inorganic pollutants. Thus, algae play crucial role in cleaning of environment along with carbon dioxide sequestration performed in marine ecosystem.

2.5 Algal oil extraction

The development of algal bio-oil industry depends upon reliable extraction method and purification of oil and fatty acids from algal biomass. The extraction of algal oils can be performed by several methods such as physical, chemical and mechanical [214]. The standard method developed for lipid/oil extraction from plant and animal cells are beneficial to extract lipids from algal biomass. Folch's method is one of the oldest, rapid and easy process for the extraction of lipids from microalgae [215]. In this method chloroform-methanol (2:1 v/v) solvent mixture is used. While, Bligh and Dyer method is considered as efficient lipid extraction option from algae [216]. Later, Bligh and Dyer methods has been adopted with modifications by researchers so as to improve the basic method [217]. Modification in the Bligh and Dyer method was made by the addition of acetic acid- water phase in the existing solvent combination, useful in increasing the recovery of acidic phospholipid content of algae [218]. Thermo chemical liquefaction technology used *Dunaliella tertiolecta* to extract the total oils. Solvent based extraction of oils uses chemicals, which is one of the major environmental concern [70]. Toxicity to the extracted oil, easiness to handle it, environmental safety and cost of manufacturing are various factors considered during selection of a solvent in oil extraction.

Microwave assisted method is safe and economically feasible, useful for the lipid extraction without dewatering of algal biomass [219]. Bio-oil extracted from algae now employs various other lipid extraction techniques like bead milling, electroporation, enzymatic treatment, homogenization, osmotic shock, chemical hydrolysis and supercritical carbon dioxide extraction ($SCCO_2$) [220][221][167][222][223]. High oil recovery with efficient method of lipid extraction becomes the major focus of algal oil refineries. Despite the environmental cost solvent extraction method is frequently used to extract lipid from microalgae. Therefore, minimal use of solvents, better oil recovery and less toxicity or environment damage has emerged as major prerequisite in oil extraction process. Extraction process is the major hinderance in commercialization and cost-effective production of algal oil. Hence, more research studies are needed for developing better methods for the same.

3. Materials and methods

The general procedures to be followed are shown in figure 2, which comprises of 4 major steps proceeded by collection of microalgae.

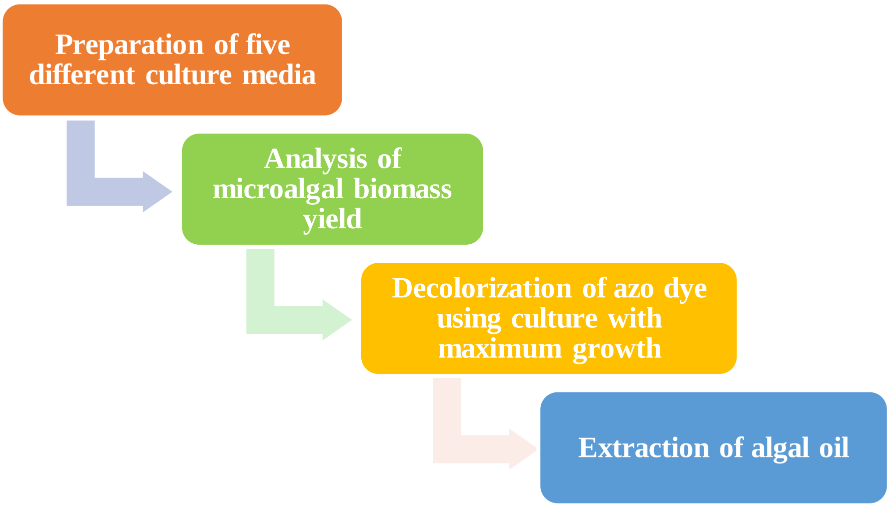

Figure 2: Flow chart of general study design

3.1 Algal culture

Chlorella species was collected from Plant Biotechnology Laboratory, Department of Biotechnology, Delhi Technological University, Delhi (India).

3.2 Different media preparation

Culture media for the growth of *Chlorella sp.* is a mixture of different macro and micro nutrients. Stock solution of 0.1g/l were prepared for all the chemicals required in various 5 media such as BG-11 medium [224], BBM medium [225], K&C medium [226], Bristol medium (modified) [227] and Fog medium [40]. Different media were prepared by mixing all constituents given in table 1, into 500 ml distilled water and then P^H was adjusted at 9 in all 5 media using 1N NaOH.

Table 1: Chemical composition of different culture media used for growth of Chlorella sp.

Chemicals required	BG-11 medium	Bristol medium (modified)	BBM medium	K&C medium	Fog medium
$NaNo_3$	15 ml	250 mg	250 mg	--	--
$K_2HPO_4.3H_2O$	400 µl	75 mg	75 mg	--	200 mg
$MgSO_4.7H_2O$	750 µl	75 mg	75 mg	250 mg	200 mg
$CaCl_2.2H_2O$	360 µl	25 mg	25 mg	15 mg	100 mg
Citric acid	60 µl	--	--	--	--

Ammonium ferrous citrate	60 µl	--	--	--	--
$Na_2EDTA.H_2O$	10 µl	--	--	--	--
Na_2CO_3	200 µl	--	--	--	--
KH_2PO_4	--	175 mg	175 mg	--	--
NaCl	--	25 mg	25 mg	470 mg	--
EDTA.Fe	--	1 ml	--	--	5 ml
EDTA(Disodium)	--	--	1.57 mg	8 mg	--
H_3BO_3	28.6 ml	--	11.42 mg	0.5mg	2860 mg
$ZnSO_4.7H_2O$	2.2 ml	--	8.82 mg	0.2 mg	220 mg
$MnCl_2.4H_2O$	18.1 ml	--	1.44 mg	0.5 mg	1810 mg
MoO_3	--	--	0.71 mg	--	--
$CuSO_4.5H_2O$	0.8 ml	--	1.57 mg	--	80 mg
$Co(NO_3)$	0.5 ml	--	0.49 mg	--	--
KNO_3	--	--	--	810 mg	--
$NaH_2PO_4.H_2O$	--	--	--	470 mg	--
$Na_2HPO_4.H_2O$	--	--	--	360 mg	--
$(NH_4)_6Mo_7O_{24}.H_2O$	--	--	--	0.2 mg	--
$FeSO_4.7H_2O$	--	--	--	6 mg	--
$Na_2MoO_4.2H_2O$	3.9 ml	--	--	--	390 mg

After sometime final volume was made up to 700 ml in 1 litre flask with distilled water. The media were then sterilized using autoclave at 121℃ temperature and pressure at 15 psi. Afterward all the media were cooled at room temperature and inoculation was done with strain of *Chlorella sp.* (1% of media volume).

3.3 Analysis of algal biomass productivity

Cultures were carried out in 1litre flask containing 5 media in duplicate. All the culture flask were allowed to incubate under controlled light (2500 Lux and 12:12 h, light:dark) and temperature (25±2℃) conditions. The algal cultures were allowed under manual shaking to avoid accretion of algal cell on the wall of flask. OD was taken at 670 nm for all media and after 27 days of algal growth biomass yield was evaluated. The biomass was collected on completion of incubation period by centrifugation, executed at 5000rpm for 10 minutes at 25℃ and the left biomass was then oven dried at 66℃ for one day, until constant weight was attained. The algal biomass productivity was calculated by the formula stated below [228].

$$\textit{Biomass productivity} = (C_2 - C_1)/(T_2 - T_1)$$

Where, C_1 and C_2= Concentrations of biomass (g/L) and

T_1 and T_2 = Initial and final sampling time

3.4 Decolorization of synthetic dyes

A known concentration of (0.2, 0.4, 0.6, 0.8, 1, 2, 4 mg/l) Methylene blue (azo dye) was prepared. The BG-11 media was used after incubated for 20 days and treated with different dye concentration. Control experiments were observed under light and dark conditions in the absence of *Chlorella species*. The effect of light on degradation was evaluated in presence of light source. The degradation ratio was assessed after 12 days of incubation by analysing the absorbance of the cell free supernatant of the sample and each dye solution at 665 nm. The decolorization percentage was evaluated with the help of below equation [229].

Decolorization (%) = (Initial absorbance -Final absorbance) x Initial absorbance/100

3.5 Extraction of bio-oil from algae

The lipid extraction from the dried biomass of microalgae was carried out on the basis of Bligh & Dyer method [216]. The biomass was collected after 27 days of incubation by centrifugation at 5000rpm for 10 minutes at 25℃ and the solid biomass was further oven dried at 66℃ for 24 hours. Dried algal biomass then mixed with 1N HCl and heated for 30 minutes at 60℃. The mixture is then treated with solution of chloroform and methanol mixed in the ratio of 1:2 (v:v) and agitated for 20 minutes. The mixture was afterwards centrifuged at 7000 rpm, at 4°C for 5 minutes and impurities left in the supernatant were discarded. The supernatant was vortexed for 10 minutes preceded by washing with 0.9% NaCl. The upper phase was discarded and chloroform containing lower phase of lipid was oven dried at 80℃ using pre-weighed weighing bottles. The formula used to calculate lipid productivity shown below [230].

Lipid productivity=BP x lipid content (% dry cell weight basis)

Where, BP = Biomass productivity

4. Results and discussion

The microalgae grown in 5 different media was studied to evaluate biomass yield of all the media. BG-11 medium was observed with high yield and used to analyze the effective dye concentration for decolorization, among various known amount of methylene blue dye. While, lipid yield of all the media was estimated, to find out the potential media for Biooil production.

4.1 Biomass yield and Lipid yield of microalgae

In the screening of most efficient growth media for *Chlorella sp.*, BG-11 resulted with maximum biomass growth as shown in figure 2. Nitrogen and zinc are crucial nutrients responsible for increased microalgal growth. It can be implied that among different culture media, the presence of nitrogen and zinc might lead to high growth of microalgae.

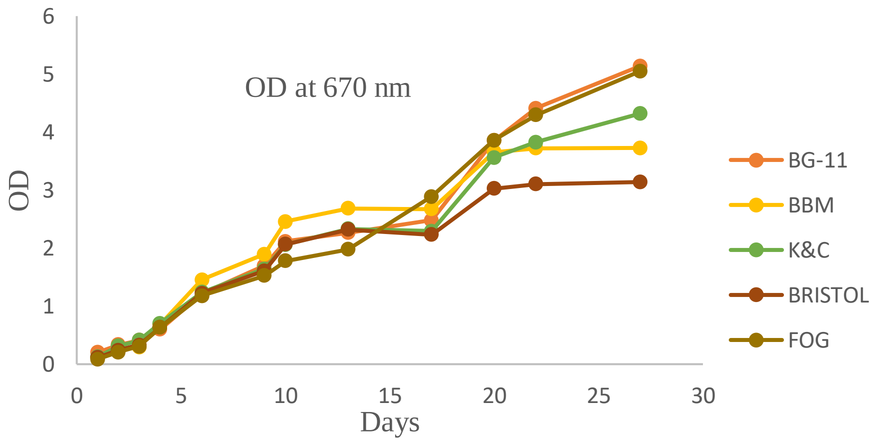

Figure 3: Microalgal growth in 5 different media

The assessment of these media produced maximum biomass yields of 782 mg/l for BG-11 medium and 780 mg/l for Fog medium (figure 4). The lowest biomass yield was achieved in Bristol medium with 570 mg/l.

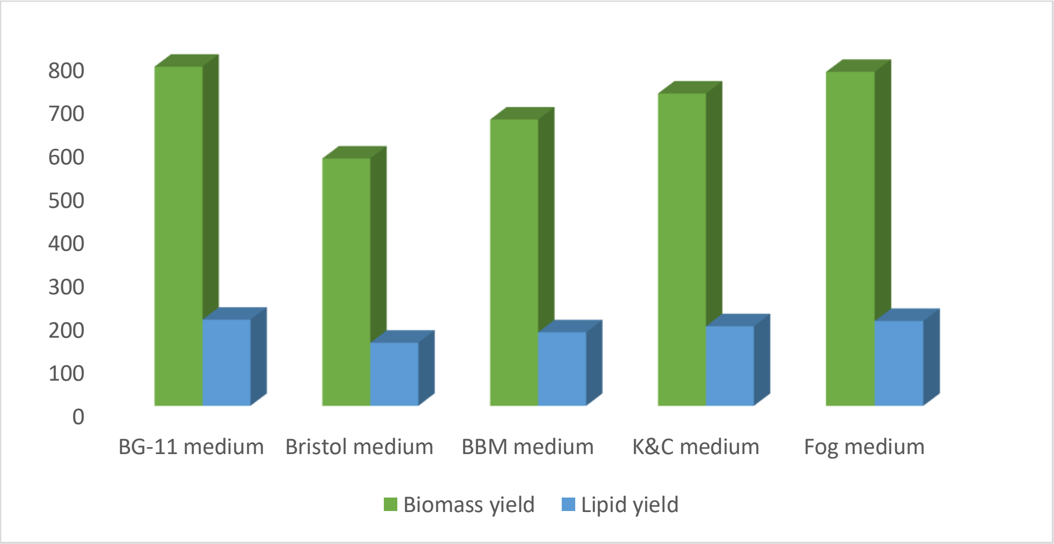

Figure 4: Biomass yield and Lipid yield of microalgae in 5 different media

Nitrogen is known to be vital nutrient for the production of proteins and nucleic acids in a cell. 7-20% of dry cell weight is constituted by it. The dearth of nitrogen promotes protein translation and synthesis of more lipid [231]. When concentration of nitrogen increased up to 300 mg/L, lipid yield of 579.86 ± 0.076 mg/l, lipid productivity of 48.32 ± 0.0063 mg/l/day and improved lipid content of 50.39 ± 0.018% were attained [230]. In microalgae, deficiency of iron can decrease microalgal growth while abundance of iron can cause oxidative stress and physiological changes [232]. Increase in the yield of biomass and lipid along with lipid content were obtained with surge in concentration of iron from 10 to 50 mg/l. When iron concentration of 50 mg/l was used, lipid yield of 446.9 ± 0.42 mg/l, lipid productivity of 37.24 ± 0.035 mg/l/ day and lipid content of 45.2 ± 0.077% was obtained. In case of *Botryococcus braunii* KMITL 2, when iron concentration of 27 mg/L was used, high lipid content of 34.93 ± 1.89% and lipid yield of 0.08 g/L were attained [231].

Phosphorus is another abiotic macronutrient crucial for the growth of microalgae. It is essential for synthesis of protein, nucleic acids, and development of cellular framework. While its deficiency can cause lack of chlorophyll and meagre protein content [118]. Increase in its concentration leads to improved algal yield up to 350 mg/l from 103 mg/l. When phosphorus concentration of 250 mg/l was used, lipid yield of 537.79 ± 0.27 mg/l and lipid content of 49.31 ± 0.024% were obtained. At high phosphorus concentration of 350 mg/l, lipid yield of 572.78 ± 0.45 mg/l, lipid productivity of 44.81 ± 0.023 mg/l/day and lipid content of 48.30 ± 0.023% was achieved. This implies that increase in phosphorus concentration give rise to high biomass and lower lipid content. Inefficient consumption of high phosphorus content by microalgae might be the reason for less lipid yield. Zinc is a micronutrient crucial for growth of microalgae. Deficiency of Zinc may cause decline in microalgal growth. Increase in zinc concentration would increase biomass yield. *Chlorella vulgaris* has reported zinc tolerance limit up to 600 mg/L [233] while, under microscopic observations deformity was observed in cell morphology [234]. Temperature can change the physiological process of a microalgae. Lipid content in case of *Chlorella vulgaris* reduced from 15% to 2% when temperature increased from 25°C to 30°C [235].

4.2 Effect of dye on microalgae

Decolorization of various dye concentration of methylene blue (MB) was observed in growth media for 20 days. It was observed that more than 90% decolorization by *Chlorella sp.* was achieved in 0.2, 0.4, 0.6, 0.8, 1 mg concentration of methylene blue dye. While in 2 mg concentration up to 70% decolorization and less than 70% was obtained in 4 mg as implicit by figure 5.

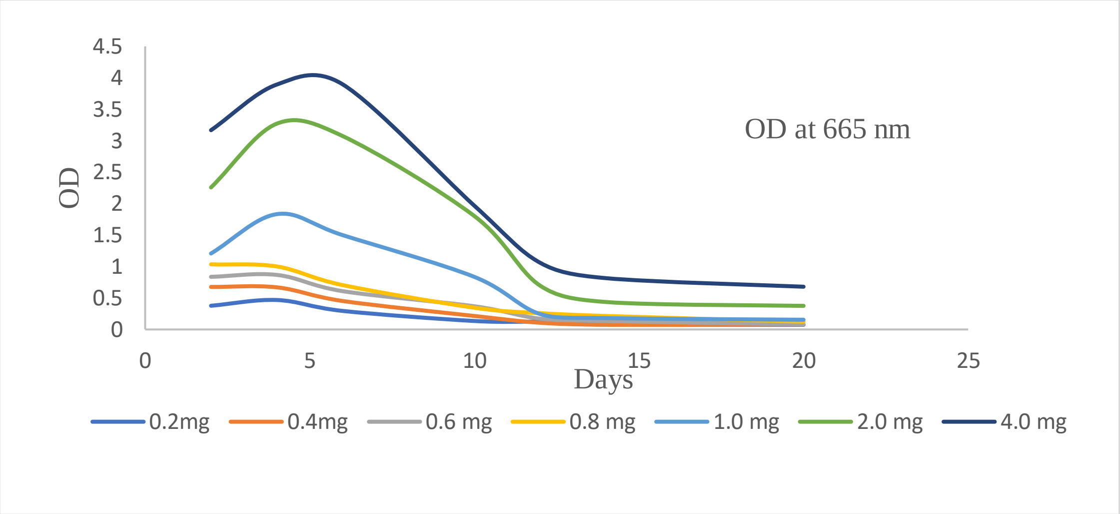

Figure 5: Absorption spectra of Chlorella sp. in different concentration of Methylene Blue

The *Chlorella sp.* is efficient to remediate MB when concentration of dye was low (0.2-1 mg), while higher dye concentration reduces the dye removal efficiency of microalgae. Dye concentration and algal biomass are important factors that directly affect the dye decolorization rate. Decolorization of MB by *Chlorella sp.* rises with algal growth. Here, in 3 days of incubation 70% was degraded, after 12 days it increased more than 90%. Basic fuchsin was decolorised from 78.3% to 91.2% with increase in incubation period of *Chlorella vulgaris* from 3 days to 7 days. *Oscillatoria rubescens and Elkatothrix viridi* achieved 94.79% and 93.25% respectively for basic fuchsin after 3 days [236]. The increased concentration of MB at 2 mg and 4mg attained decolorization up to 40% by *Chlorella sp.* after 5 days. Orange II (20 ppm) was 43.67% and 43.68% decolorized after 5 and 7 days of incubation respectively by *Chlorella vulgaris*. Though, dye removal and initial dye concentration were reported with negative relation for decolorization of acid yellow 199 dye in presence of *Shewanella oneidensis* (Yang et al., 2011). The decolorization percentage of MB dyes was observed to be 99% in presence of *Chlorella* sp., while less than 90% was achieved by *Chlorella pyrenoidosa* [237].

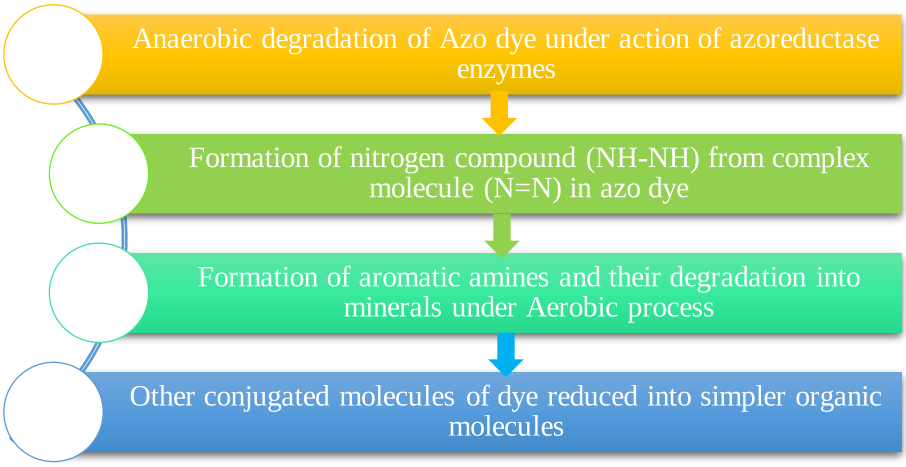

Figure 6: Phycoremediation of azo dyes under enzymatic action

Temperature has direct impact on dye degradation, decolorization percentage of dye increase with rise in temperature in range of 20-40℃. Further increase in temperature up to 50℃ lowers decolorization activity. The loss of cell viability or inactivation of reductase enzymes at high temperature might be the reason for dye degradation [238]. Azo reductase enzymes are responsible for degradation of dyes like methylene blue [236]. The enzymatic activity cause cleavage of azo bonds into simpler organic compounds as shown in figure 6. Selection of dye concentration for effective decolorization is based on maximum decolorization achieved, P^H and temperature conditions. Photodegradation occurs in presence of light conditions improves degradation while, under dark conditions there is no report of dye decolorization.

5. Conclusion

Deforestation of major areas of the earth like amazon forest for harvesting biomass to meet the demands of rising population has put a large pressure on our natural resources. The extraction of fossil fuels has caused deterioration of terrestrial and aquatic ecosystem. While their consumption has caused pollution, global warming and climate change which reveals the inception of ecological imbalance. The current scenario of earth's ecology and crisis for fuel as well as food reveals algal resources as indispensable to sustainable development. It needs an important approach to harness algal wealth across the world. The microalgae are unique due to its capacity to grow fast and presence of valuable phyco-compounds. Lipid-rich algal biomass is beneficial both as nutritional supplement and energy resource. Though, the success of utilization of algal resource depends on the presence of local strains and their biomass yield and lipid productivity. The presence of different functional groups and enzyme activity enables microalgae as potential candidate for remediation of dyes and its derivatives present in effluents. However, the lipid content as well as its action on dyes depends upon on presence of algal biomass and dye concentration in case of phycoremediation.

Among the diverse species of fast-growing algae, green algae are significant in terms of its biomass potential. Several species of green algae have richness of oil and other valuable products. The low cost of maintenance and simple culture techniques allow cost effective harvesting and utilization of green algae. Therefore, measures to assess biomass and lipid yield as well as the decolorization action are crucial for the development of algae-based industries for solution oriented towards environment, energy and food supplements. Consequently, algal cultivation can be accomplished as a double-edged technology for simultaneous cleaning of wastewater containing dyes as well as a source of lipid rich biomass for bio-oil production. Despite of biomass potential green algae have applications in nanotechnology, biomaterials, bioremediation of polluted water, medical arena and feedstock for animals and aquaculture.

The review of literature has revealed the significance of exploring the diversity of green algal wealth. This has also demonstrated that knowledge of each species from their natural environment to laboratory cultivation stage has high significance. It helps in standardization of the cultivation protocol of each individual species and provide insights into the effects on growth when cultured in different media towards diverse industrial applications. The study is beneficial for the evaluation of remediating activity of green algae in various concentration of dye towards treatment of effluents containing heavy metals and other compounds. Such comprehensive investigations are essential for identification and economic utilization of the algal wealth. The present study revealed biomass and lipid yield in BG-11, Bristol, BBM, K&C and Fog media and decolorization of MB dye in BG-11 media which offers maximum algal growth of *Chlorella* species. The biomass yield of different media ranges between 780 mg/l to 570 mg/l, while lipid yield varies from 198 to 145 mg/l. Methylene blue dye attained maximum decolorization of more than 90% when dye concentration kept low as 0.2- 1mg. This depicts sufficient dye degradation potential of microalgae. The present study empowers to unravel the green algal wealth of the fresh water systems as potential industrial resource for future food, fuel and other ecotechnological or biotechnological applications

6. References

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