WASTE WATER TREAMENT AND PRODUCTION OF BIODIESEL FROM *Scenedesmus obliquus* & NATIVE ISOLATED MICROALGAE

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By

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in dissertation entitled **"Waste Water Treatment & Production of Biodiesel from Scenedesmus obliquus & Native Isolated"** submitted in partial fulfilment of the requirements for the award of degree of Master of Technology in Bioprocess Engineering, Indian Institute of Technology Roorkee, is an authentic record of my work carried out under the supervision of Dr. P.Mondal, Associate Professor, Department of Chemical Engineering, IIT Roorkee and Dr. Saurav Datta, Assistant Professor, Department of Biotechnology, IIT Roorkee . The matter embodied in this has not been submitted by me for the award of any other degree.

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ABSTRACT

Excessive use of fossil fuels and environmental degradation has led us to generate efficient and environment friendly sources of energy. Third generation algal biofuels are considered to be the most promising alternative source of energy as compared to first and second generation biofuels. Microalgal strain *Scenedesmus obliquus* was grown in N-11 media under different nutrient starvation conditions to enhance lipid content of the cells. Among all the nutrient deficient conditions, Phosphorous deficient was found to be more suitable where 2.58 g/L biomass was achieved on 15th day against 1.56 g/L biomass in nutrient rich. The Lipid Content in *Scenedesmus obliquus* was observed maximum in Phosphorous-Nitrogen-Potassium deficient N-11 media on 20th day i.e. 52.941 % against 41.46% in nutrient rich. The maximum biomass and lipid content can be obtained at condition (5 g N deficient, 13.41 g P deficient and 5 g K deficient) i.e. 52 g/L and 49.35% as suggested by Response Surface Methodology (RSM). It was also observed that *Scenedesmus obliquus* has the tremendous capability to treat waste water. After treatment of grey water nearly the range of drinking water or clean water was achieved. Further, different algal strains were isolated from the local water bodies to explore their potential for waste water treatment and biodiesel production. Among them most suitable microalgal strain was identified as *Chlorella sirokiana*. Hence the integration of waste water treatment with biodiesel production process will be cost effective, carbon neutral, environment friendly and will also provide clean water.

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ABBREVIATIONS AND SYMBOLS

%	Percentage
µg	Microgram
µL	Micro litre
°C	Degree Celsius
CO_2	Carbon dioxide
L	Litre
m	Mass
mg	Milligram
mL	Millilitre
N	Nitrogen
O_2	Oxygen
PPM	Parts per million
P	Phosphorous
K	Potassium
Ca	Calcium
Ca-Fe	Calcium-Iron
NP	Nitrogen-Phosphorous
PK	Phosphorous-Potassium
NK	Nitrogen-Potassium
NPK	Nitrogen-Phosphorous-Potassium
TDS	Total Dissolved Solids
H_2O	Water
(W/W)	Weight by weight

CHAPTER 1
INTRODUCTION

Due to the over use of fossil fuels and disastrous climate changes which is due to depletion of non- renewable resources, the need for the search of effective, efficient and eco-friendly fuels such as biodiesel which can replace crude oil is required. [1] Different types of biomass sources such as corn, soybean, sugarcane, jatropa seeds, edible as well as non-edible oil, lignocelluloses biomass and macro and microalgae has been used for the biofuel and bio oil production so far. But microalgae are the most preferred option for the production of biodiesel as they are having rich biomass and lipid content, photosynthetic efficiency, can fix CO_2, and easily grow on non-cultivated lands which are not suitable for food crops. Microalgae can be grown in race ponds and photobioreactors very effectively.

Energy is a basic prerequisite for any general public; the high vitality request in the industrialized world and in addition in the residential segment; expanding populace and contamination issues caused because of the far reaching utilization of fossils energizes makes it progressively important to investigate sustainable power source asset of boundless term which is condition inviting and effective to utilize. At present, all sustainable power sources, i.e. hydroelectric, sunlight based, wind, tidal and geothermal focus on the power showcase, while energizes make up a substantially bigger offer of worldwide vitality request. One conceivable contrasting option to petroleum derivative is the utilization of vegetable oils. This elective diesel fuel can be named as biodiesel. It has developed as the most capable other option to oil diesel fuel inferable from its eco-accommodating qualities, biodegradability and inexhaustibility. [2] Bio ethanol and biodiesel are the elective fluid energizes for sustainable development. [3] Biomass is the standout amongst the most encouraging inexhaustible asset used to produce diverse sorts of biofuels, for example, biodiesel [4] and bio-ethanol [5]. With-the over utilization of the non-renewable energy sources and their hurtful impact on atmosphere; the look for sustainable and condition benevolent biofuels is important and biodiesel can supplant oil based powers viably.

Biofuels are referred to solid, liquid or gaseous fuels derived from organic matter. They are for the most part partitioned into essential and auxiliary biofuels. Essential biofuels, for example, fuel wood are utilized as a part of a natural frame fundamentally to heat, cooking or power creation. Auxiliary biofuels, for example, bioethanol and biodiesel are delivered by preparing

biomass and can be utilized as a part of vehicles and different modern procedures. The optional biofuels can be arranged into three stages viz. initially, second and third based on various parameters, for example, the sort of handling innovation, kind of feedstock or their level of advancement [6].

The original biofuels incorporate diverse powers acquired from trim plants. As of now, bio-ethanol from, for instance, corn starch, sugar stick or sugar beet, and biodiesel from oil crops, are the most broadly accessible types of biofuels.

Be that as it may, there are two noteworthy issues over the manageability of the first age biofuels. Right off the bat, these harvests would vie for arable land with nourishment crops. Furthermore, the general reserve funds in vitality and ozone harming substance discharges over the lifecycle of the biofuels might be not as much as expected, for instance for biodiesel from oilseed assault and soya the contribution of vitality required over the life-cycle is half of the vitality contained in the fuel [7].

The second era biofuels are created from non nourishment harvests, for example, bioethanol got from the lingo cellulosic materials viz. horticultural deposits, straw, grass and wood and biodiesel got from non eatable oil plant like Jatropa, cassava or Miscanthus. In any case, changing over the woody biomass into fermentable sugars requires expensive advances including pre-treatment with unique chemicals, implying that second era biofuels can't yet be created monetarily on a substantial scale.

Along these lines, third era biofuels got from microalgae are thought to be a suitable elective vitality asset that is without the real downsides related with first and second era biofuels.

Microalgae have so far gotten thought as the most reassuring alternative for biodiesel age in light of its photosynthetic capability, high cell biomass and lipid content, carbon dioxide fixation and advancement on non-arable territories which are unsuitable for improvement). Microalgae can make 15– 300 times more oil for biodiesel age than regular items on a region introduce. Other than differentiated and conventional item plants which are typically gathered all over a year, microalgae have a short assembling cycle, allowing various or predictable harvests with out and out extended yields [8]. Splendid plant arrive isn't required to build up the biomass [9]. Green development advancement does not require herbicides or pesticides. Green development can be

produced in waste water, made water, or saline water on non-arable land, in this way decreasing contention with arable land, confined freshwater and supplements used for conventional agriculture [10]. Microalgae sequester CO_2 from pipe gases transmitted from oil subordinate let go control plants and diverse sources, in this way diminishing releases of a significant ozone hurting substances. Dependent upon the microalgae species distinctive blends may moreover be evacuated, with noteworthy applications in different current sections, including a broad extent of fine chemicals and mass things, for instance, polyunsaturated unsaturated fats, standard hues, polysaccharides, shades, tumor avoidance operators, high-regard bioactive blends, and proteins. Green development can create on wastewater media so potential wellspring of insignificant exertion lipids for age of liquid bio fuels [11]. Near everything green development can bioremediate misuse water by ejection of NH_{4+}, $NO_{3-,}$ and $(PO_4)_3$-from a variety of wastewater sources (e.g. agricultural run-off, concentrated animal feed exercises, and mechanical and city wastewaters).

The fundamental central purposes of biodiesel are that it is a champion among the most unlimited forces and moreover non-deadly and biodegradable [12]. It devours in standard diesel engines with or without any changes while diminishing pollutions (100% less sulfur dioxide, 37% less unburned hydrocarbons, 46% less carbon monoxide, and 84% less particulate issue) interestingly with the conventional diesel fuel [13]. The microalgal lipids are basically unprejudiced lipids with a higher degree of splashed and mono unsaturated fats, likewise making it a potential exchange for oil subordinates. Moreover, the oil content in algal strains are overall significantly higher than interchange harvests which are starting at now used for production of biodiesel. Microalgae have risen as a potential manageable biomass asset on account of their lack of bias towards regular habitat [14, 15] and adaptable development. Contrasted with earthly plants, low lipid microalgae show speedier development rate [16], and the photosynthetic effectiveness of microalgae can possibly surpass 10%, which is 10– 50 times more noteworthy than that of earthbound plants [17, 18]. Under troublesome ecological conditions, microalgae can aggregate a lot of lipid [19, 20] which is reasonable for biodiesel creation through transesterification [21, 22].

It has been demonstrated that the appropriation of biofuel could lessen carbon discharges, and may expand vitality security [23, 24]. Be that as it may, high lipid focus is typically contrarily corresponded with biomass profitability and subsequently lipid efficiency. In the event that the high development rate and lipid content are joined, microalgae would turn into a promising feedstock for biofuel generation, particularly for biodiesel.

Contrasted with plant-based biofuel crops, microalgae can likewise be adjust to a more extensive assortment of water sources (crisp, bitter, saline and wastewater) [25, 26], and possibly reuse other supplement squander streams [27]. The use of non-consumable water adds to bring down water impression (WF) of microalgae based biofuel generation. Since microalgae are fit for settling surrounding CO_2 [28, 24] and using it as carbon source to develop and recreate [29], the creation of fuel from microalgae gives a promising other option to regular carbon catch and capacity advances (CCS). The settled carbon is consolidated into sugars and lipids and along these lines stores vitality, produces chemicals, and nourishments [30, 27].

There are various species of microalgae available and to develop a competitive biodiesel production process the choice of the microalgal species selected is important. The capability of the microalgae to adopt the local environment, accumulate lipid and to grow rapidly makes a microalgae appropriate for the biodiesel production process. Microalgae are not only having biodiesel producing capabilities but they are capable of treating waste water.

Profiting from its plenitude and improvement of supplements, wastewater can be utilized as a minimal effort supplement hotspot for microalgae development. Microalgae can possibly evacuate supplements from wastewater and biomass for biofuel creation. Algal treatment of wastewater offers a less expensive and more productive intends to expel supplements and metals from wastewater than ordinary tertiary treatment [31, 32]. For instance, most business methodologies of phosphorus expulsion try not to reuse it as a completely supportable item, as it is recovered alongside different other waste items, some of which are lethal [33]. Be that as it may, these supplements can be joined into green growth biomass [34] and hence expelled from wastewater by algal treatment [35, 36]. Moreover, the use of microalgae wipes out the slop treatment, which is strategically testing.

Refined of microalgae in wastewater likewise generously lessens the need of substance manures and their related weight on life cycle [37, 38]. Through the usage of wastewater, the zero-squander idea is additionally actualized, and in this way invigorates a more manageable practice for the microalgae biofuel industry. It has even been suggested that incorporated phyco-remediation and biofuel innovation has all the earmarks of being the main wellspring of economical generation of biofuels [39].

Bio-treatment of waste water using microalgae is beneficial because of their capability of photosynthesis, converting solar energy into biomass and incorporating nutrients which leads to eutrophication such as nitrogen and phosphorous. [40] The algal system can treat human sewage, livestock feed wastes, agro-agricultural waste, industrial waste, dairy waste, and grey waters. Grey water is basically any domestic waste water produced, excluding sewage. Grey water have less organic loading as compared to sewage.

Scenedesmus obliquus consist of palmitic, oleic and stearic acid as major fatty acid which are useful for biodiesel production & is also an excellent candidate for the combination of biodiesel production and waste water treatment. [41] *S. obliquus* is a freshwater microalga that can develop in modern wastewaters of various beginnings appearing great adjustment capacity [42] and it is an extremely adaptable microalgae as crude material for biofuels creation [43].It is likewise viewed as a standout amongst other possibility for biodiesel creation among a few microalgae animal varieties [44] with a lipid content running in the vicinity of 18.8 and 29.3 % dwt for a supplement loaded medium and up to 42 % dwt for a supplement inadequate medium [45]. Additionally, the ideal temperature run for the S. obliquus development is generally more extensive, since its development rates change little in the vicinity of 14 and 30 °C [46]. This finding is particularly applicable for the outside development of this microalga, as temperature is one of the major ecological elements restricting the microalgae efficiency [47], which regularly changes among sweltering or frosty climate and day or evening temperatures.

In addition, microalgae are rich in oil, which could surpass 80% (of the dry weight) as detailed in a few animal categories, though being very normal oil levels of 20– half. The unsaturated fat synthesis in the diverse microalgae species is likewise extremely fundamental, in light of the fact that the sort and measure of unsaturated fats in lipids can specifically impact the nature of the accomplished biodiesel. Algal oil contains soaked, monounsaturated and polyunsaturated unsaturated fats with12– 22 carbon ions. The nature of biodiesel is to a great extent controlled by the proportion of soaked to unsaturated fats. Immersed unsaturated fats (SFAs) are impervious to debasement what's more, autoxidation, and thusly, increment the capacity to long capacity. Unsaturated fats (UFAs), then again, upgrade cool stream qualities. It is for the most part considered that microalgae with high extent of soaked and monounsaturated unsaturated fat is alluring to get a superior biodiesel quality. Such constituents give a tasteful balance between cold flow and oxidative solidness and additionally between kinematic thickness and cetane number [48] .Microalgae developed under pressure conditions can modify their biomass synthesis and a mass lipids and starches which could be utilized for biofuel generation, consequently their

capability to be utilized as biofuel feedstock builds. Under ideal states of development, green growth incorporate unsaturated fats to deliver film polar lipids, as glycolipids what's more, phospholipids, while under pressure conditions numerous green growths change their lipid amalgamation pathways and collect unbiased lipids, principally as triacylglycerols. In the algal biodiesel production process Fatty Acid Methyl ester (FAME), chemical composition of biodiesel are prepared by transesterification of using algal oil either by alkali or acid as a catalyst.[49]

Transesterification is a reversible response and continues basically by blending of the reactants. The procedure of transesterification is influenced by different elements like molar proportion of liquor to oil, kind of liquor, sort and measure of impetus, response time and temperature, and immaculateness of reactants.

The essential points of interest of biodiesel are that it is a standout amongst the most sustainable energizes and furthermore non-dangerous and biodegradable. It consumes in ordinary diesel motors with or with no changes while decreasing contaminations (100% less sulfur dioxide, 37% less unburned hydrocarbons, 46% less carbon monoxide, and 84% less particulate issue) in contrast with the customary diesel fuel [50]. The microalgal lipids are essentially unbiased lipids with a higher extent of immersed and mono unsaturated fats, subsequently making it a potential swap for petroleum products Moreover, the oil content in algal strains are for the most part considerably higher than alternate yields which are right now utilized for generation of biodiesel.

Enhancement of high-thickness cell development conditions is significant for amplifying modern efficiency of microalgae. Amid the enhancement procedure, culture conditions for the maximal development rate are controlled by rehashing tests. The procedure can be encouraged by a Response Surface Methodology (RSM), which can decide the improved qualities with a set number of analyses and less time. Also, relationship among the factors can be analyzed from the strategy.

The aim of this study is to check biomass Production and lipid content of the microalgal strain *Scenedesmus obliquus* for the production of biodiesel by evaluating their growth at different stress conditions by making it nutrient deficient. Further, we have transesterify lipid for the production of biodiesel followed by FAME analysis. Also, *S. obliquus* was examined as a potent source for biodiesel production by obtaining the optimum growth conditions which influence the lipid yield by using Response Surface Methodology (RSM). To develop an integrated process for

real waste water treatment and biodiesel production, in this study we have treated grey water collected from Indian Institute of Technology Roorkee (IITR) hostel Mess and washing areas. Test for the reduction of chemical as well as biochemical oxygen demand, removal of N and P, etc was performed. To obtain the microalgal strain which can easily grow in the local environment conditions; collection, screening and isolation of microalgal species was done from Yamuna river water.

Objectives

The objectives of this study are:-.

- To enhance the production of lipid from ***Scenedesmus obliquus*** in N-11 media under various stress conditions.
- To transesterify lipids for the production of biodiesel and FAME analysis.
- Isolation, Screening and Identification of microalgal strain from the Yamuna River water.
- To test the potential of ***Scenedesmus obliquus*** in synthetic waste water treatment.
- Optimisation of the different stress conditions to enhance lipid content using Response Surface Methodology (RSM).
- To develop an integrated process for waste water treatment & biodiesel production.

CHAPTER 2
REVIEW OF LITERATURE

The present study was conducted to produce biodiesel from *Scenedesmus obliquus* and to treat the grey water. Also, this study was done to isolate microalgal species from Yamuna river water. Therefore, an attempt was made to review the literature related to the biomass production, lipid production and biodiesel production from microalgae. Also, to get knowledge about methods for treatment of waste water using microalgae.

2.1. Review for Biodiesel Production

Green growth, especially green unicellular microalgae have been proposed for quite a while as a potential sustainable fuel source [51] . Microalgae has the possibility to produce critical amounts of biomass and oil reasonable for transformation to biodiesel. Microalgae have been evaluated to have higher biomass profitability than plant edits in terms of land zone required for development, are anticipated to have bring down cost per yield, and can possibly diminish GHG discharges through the substitution of petroleum derivatives (for surveys and further investigation see [52,53,54]. Similarly as with plant-inferred feed stocks, algal feed stocks can be used straightforwardly or handled into fluid powers and gas by an assortment of biochemical transformation or thermo chemical change forms [55]. Dried algal biomass might be utilized to produce vitality by coordinate ignition [56] yet this is most likely the slightest appealing use for algal biomass. Theromchemical transformation techniques incorporate gasification, pyrolysis, hydrogenation furthermore, liquefaction of the algal biomass to yield gas-or oil-based biofuels [57]. Biochemical transformation forms incorporate maturation and anaerobic absorption of the biomass to yield bioethanol or methane. Moreover, hydrogen can be delivered from green growth by bio-photolysis [58]. At long last, lipids, mainly triacylglycerol lipids can be isolated and disconnected from collected microalgae and after that changed over to biodiesel by transesterification [59, 60]. This last procedure, the utilization of microalgae for biodiesel creation has pulled in a lot of intrigue. Research as part of the Aquatic Species Program subsidized by the US Department of Vitality broadly investigated the oil generation abilities of microalgae and recommended that potential profitability of oil from microalgae might be essentially more prominent than oilseed harvests such as soybean [60]. This and ensuing examination has focused on recognizing microalgae strains that are prepared to do integrating huge amounts of lipids and in distinguishing development conditions that will give the best lipid productivities [61].

Numerous examinations have focused on distinguishing conditions that initiate high amassing of impartial lipids (especially triacylglycerol) in the microalgae cells, similar to a supplement pressure, for example, nitrogen (N) or phosphorus (P) impediment [62]. Be that as it may, a noteworthy restriction of this approach is that in spite of instigating high lipid yield, biomass profitability of the cells is frequently low thus lipid profitability won't be high. Development conditions that emphasis on giving high biomass efficiency rather may at last be more helpful and may be a more effective methods for expanding all out lipid efficiency [63]. Moreover, with substantial amounts of algal biomass it might be all the more financially suitable to produce vitality through the creation of alternate sorts of biofuel.

The biodiesel got from microalgae and different microorganisms are thought to be the third age biodiesel [64]. Microalgae are considered as the most encouraging feedstock for third era biodiesel creation because of their points of interest over other earthly products, similar to basic development prerequisites, the capacity to be developed basically anyplace with enough daylight, high photosynthetic capacity and short recovery time [65] . Also, microalgae are rich in oil, which could surpass 80% (of the dry weight) as revealed in a few animal groups, while being very normal oil levels of 20– half [66].

Biodiesel is an alluring vitality asset for a few reasons:

(1) It is an inexhaustible fuel that could be reasonably provided;

(2) It is profoundly biodegradable and has insignificant poisonous quality;

(3) It seems to cause critical change of provincial financial potential [67];

(4) It is earth well disposed, bringing about low figure discharge and no net expanded arrival of carbon dioxide, fragrant mixes or other concoction substances that are destructive to the earth [68];

(5) It is superior to anything oil based diesel as far as its lower burning outflow profile, and it doesn't add to an unnatural weather change as a result of its shut carbon cycle;

(6) It diminishes reliance on remote unrefined petroleum;

(7) It can be utilized as a part of existing diesel motors with almost no adjustment [71] and with great motor execution;

(8) It can be mixed in any proportion with conventional oil based diesel fuel in a diesel motor [72];

(9) When added to customary diesel fuel in a measure of 1– 2%, it can change over fuel with poor greasing up properties into an adequate fuel [73] and

(10) It can give enhanced ignition over oil based diesel on account of its high oxygen content.

To upgrade the financial attainability of utilizing algal oil for biodiesel generation, the microalgal biomass efficiency, lipid cell substance, and general lipid profitability are the three key parameters that should be progressed. The perfect procedure is that the microalgae can create lipid at the most astounding profitability and with the most astounding lipid cell content. Lamentably, this isn't generally achievable, on the grounds that high lipid substance are normally created under natural pressure, ordinarily supplement impediment, which is regularly connected with moderately low biomass profitability and, along these lines, low general lipid efficiency. The lipid substance of microalgae could be expanded by different development methodologies, for example, nitrogen exhaustion [74], phosphate restriction [75], high saltiness [76], and high iron fixation [77].

The demand for biofuel is extremely strong and currently rising; therefore, the production of algae for biofuel and/or CO_2 capture requires extremely large-scale culturing [78]. With this in mind, the outdoor cultivation systems seem to be the most suitable and commercialized, as they would require lower initial capital costs [79].

The utilization of microalgae for biofuels production can also serve other purposes. Some possibilities currently being considered are listed below.

• Removal of CO_2 from industrial flue gases by algae bio-fixation, reducing the GHG emissions of a company or process while producing biodiesel.

• Wastewater treatment by removal of NH_4+, NO_3-, $(PO_4)_3-$, making algae to grow using these water contaminants as nutrients [80]

• After oil extraction the resulting algae biomass can be processed into ethanol, methane, livestock feed, used as organic fertilizer due to its high N:P ratio, or simply burned for energy co-generation (electricity and heat);

• Combined with their ability to grow under harsher conditions, and their reduced needs for nutrients, they can be grown in areas unsuitable for agricultural purposes independently of the seasonal weather changes, thus not competing for arable land use, and can use wastewaters as the culture medium, not requiring the use of freshwater.

• Depending on the microalgae species other compounds may also be extracted, with valuable applications in different industrial sectors, including a large range of fine chemicals and bulk products, such as fats, polyunsaturated fatty acids, oil, natural dyes, sugars, pigments, antioxidants, high-value bioactive compounds, and other fine chemicals and biomass [81].

• Because of this variety of high-value biological derivatives, with many possible commercial applications, microalgae can potentially revolutionize a large number of biotechnology areas including biofuels, cosmetics, pharmaceuticals, nutrition and food additives, aquaculture, and pollution prevention [82].

The primary critical advance in building up the microalgal procedure is to choose a proper species. Choice of a proper microalgae species can bring about single-cell plants that create 40–half oil by weight.

A few specialists have concentrated on the Chlorella sp.[83], which has all the earmarks of being a decent alternative for biodiesel creation since they are promptly accessible and effectively refined in the research center [84] endeavored to build the lipid content in microalgae by shifting the temperature and nitrogen focus amid the way of life of *Nannochloropsis oculata* and *Chlorella vulgaris* and reasoned that variety of temperature and nitrogen fixation firmly affected the lipid substance of the microalgae [85] utilized techniques including autoclaving, dab beating, microwave, sonication and a 10% NaCl answer for distinguish the best strategy for lipid extraction from *Botryococcus sp*., *C. vulgaris* and *Scenedesmus sp.* They reasoned that the microwave broiler strategy was the most basic and compelling intends to remove the lipids from microalgae. Furthermore, they found that the *Botryococcus sp*. created the most elevated lipid content contrasted with different species. In any case, Griffiths and Harrison [86] have discovered that the lipids created by *Botryococcus braunii* are unsatisfactory for use in biodiesel on the grounds that the hydrocarbons delivered by *B. braunii* have a chain length of more noteworthy than 30 carbons.

Lipid gathering in *Scenedesmus obliquus* was considered by [87] under different culture conditions. They reasoned that the biodiesel from S. obliquus contains primarily immersed and mono unsaturated fats, which gives it high oxidative strength. In this way, *S. obliquus* could be viewed as a potential life form for biodiesel creation [88] screened six sorts of microalgae, *C. vulgaris, Spirulina maxima, Nannochloropsis sp., Neochloris oleoabundans, S. obliquus and Dunaliella tertiolecta* to decide the amount and nature of the oil to choose the best oil hotspot for biodiesel creation. Of the species tried in their work, *N. oleoabundans* (freshwater microalgae)

and *Nannochloropsis sp.* (marine microalgae) were resolved to be appropriate as crude materials for biofuel generation, due to their high oil content (29.0% and 28.7%, separately).

For creation of biodiesel from microalgae the initial step is the determination of a fitting species with the important properties for the particular culture conditions and items [89]. The way of life conditions, including light, temperature, pH, air (carbon dioxide) and supplement fixation, must be considered. Microalgae can be collected utilizing micro screens, sedimentation, centrifugation, flocculation or film filtration. The collected biomass is then dried under vacuum to discharge water until the point when it achieves a consistent weight. The dried biomass is pummeled with a mortar and pestle before the oil is separated. There are three surely understood strategies to separate the oil from microalgae: (1) expeller/squeeze, (2) dissolvable extraction utilizing chemicals and (3) supercritical liquid extraction [90]. The most prevalent extraction strategy is Soxhlet extraction utilizing hexane as a dissolvable and an extraction time of 4 h. Different solvents, for example, oil ether, ethanol or a hexane– ethanol blend, can be utilized. After extraction, the oils are changed over to biodiesel utilizing one of the four essential strategies: (1) coordinate utilize and mixing of crude oils, (2) micro emulsions (3) warm splitting (pyrolysis) and (4) transesterification. Finish portrayals and correlations of these techniques have been given by Ma and Hanna [91] and Leung et al. [92]. The most generally utilized strategy is transesterification, likewise called alcoholysis, on the grounds that transesterification of the oil to its comparing greasy ester (biodiesel) is the most encouraging answer for the high consistency issue [93]. In the transesterification procedure, triglycerides are first changed over to triglycerides, at that point the triglycerides are changed over to monoglycerides, and the monoglycerides are then changed over to esters (biodiesel) and glycerol (by-items).

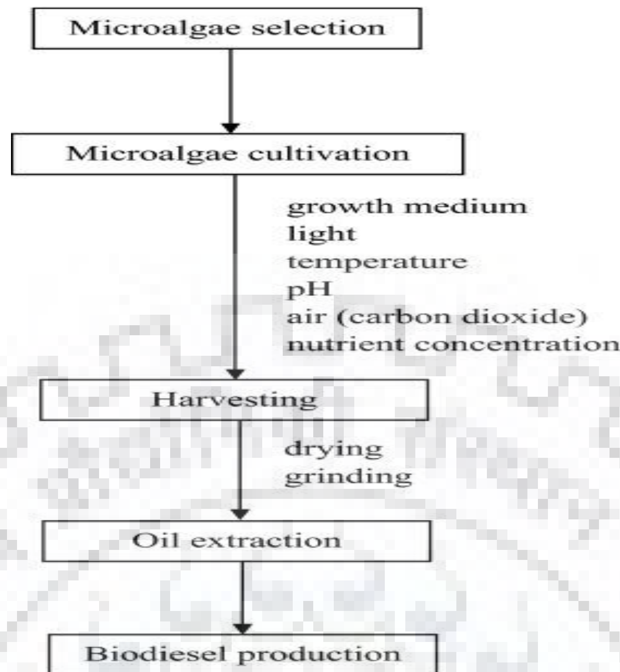

Fig 1: Flow chart to show the steps involved in the conversion of biodiesel from microalgal strain

Every species of microalga produces diverse proportions of lipids, sugars, and proteins. All things considered, these little living beings can control their digestion through basic controls of the concoction piece of the way of life medium [94], along these lines high lipid profitability can be accomplished. For instance, supplementation of glucose to the development medium under nitrate impediment was found to raise the unrefined lipid content up to 55% of the dry cell weight (dcw, against 15% under control condition) in *Chlorella protothecoides* [95]. In *Chlorella vulgaris* lipid substance of 40% and 56.6% (dcw) were recorded, when developed individually in low nitrogen-and iron-supplemented medium [96] . Consequently, oil substance of 20– 30% is anything but difficult to incite in a few microalgal animal groups [97]. Astoundingly, an oil substance of 86% (dcw) was accounted for in the darker resting state provinces of *Botryococcus braunii* [98].In any case, the significant impediment in centering *B. braunii* as the modern creature for biodiesel generation is its poor development rate [99].

2.2. Review for waste water treatment from microalgae

Notwithstanding biofuel and other bioproduct applications, expansive scale techniques for creating and reaping green growth have utilizes as a part of wastewater treatment [100]. Without legitimate treatment, overabundance nitrogen and phosphorus in released wastewaters can prompt downstream eutrophication and biological system harm [101]. The negative impacts of

such supplement over-burdening of recipient frameworks incorporate disturbance green growth, low broke down oxygen focuses and angle executes, unwanted pH shifts, and cyanotoxin generation. While concoction and physical based advancements are accessible to expel these supplements, they devour noteworthy measures of vitality and chemicals, making them expensive procedures. Concoction treatment regularly prompts auxiliary sullying of the slime result also, making extra issues of safe transfer. The vitality and cost required for tertiary treatment of wastewater remain an issue for businesses and districts.

Contrasted with physical and substance treatment forms, green growth based treatment can possibly accomplish supplement expulsion in a more affordable and biologically more secure route with the additional advantages of asset recuperation and reusing [102]. Basic nitrogen evacuation techniques, for example, bacterial nitrification/denitrification expel most of the nitrogen as N_2 gas, while algal treatment holds valuable nitrogen mixes in the biomass. Despite these advantages, adequate supplement levels in the profluent can't be accomplished without adequate generation and gathering of the green growth edit. Sadly, no present approach has been shown to be basic and sufficiently modest for practical substantial scale use with biomass.

The U.S. Branch of Energy has perceived the potential cooperative energy of wastewater treatment and biofuel creation from green growth, expressing that "Unavoidably, wastewater treatment and reusing must be fused with green growth biofuel generation (U.S. DOE, 2010)." Because a great part of the foundation is as of now set up, green growth based wastewater treatment can be sent moderately soon. The utilization of wastewater can balance the cost of business manures generally required for the generation of green growth, and wastewater treatment incomes can balance green growth creation costs. It is clear that defeating the ebb and flow difficulties to the generation and reaping of green growth will be gainful for both wastewater treatment and for the creation of biofuels and bio products.

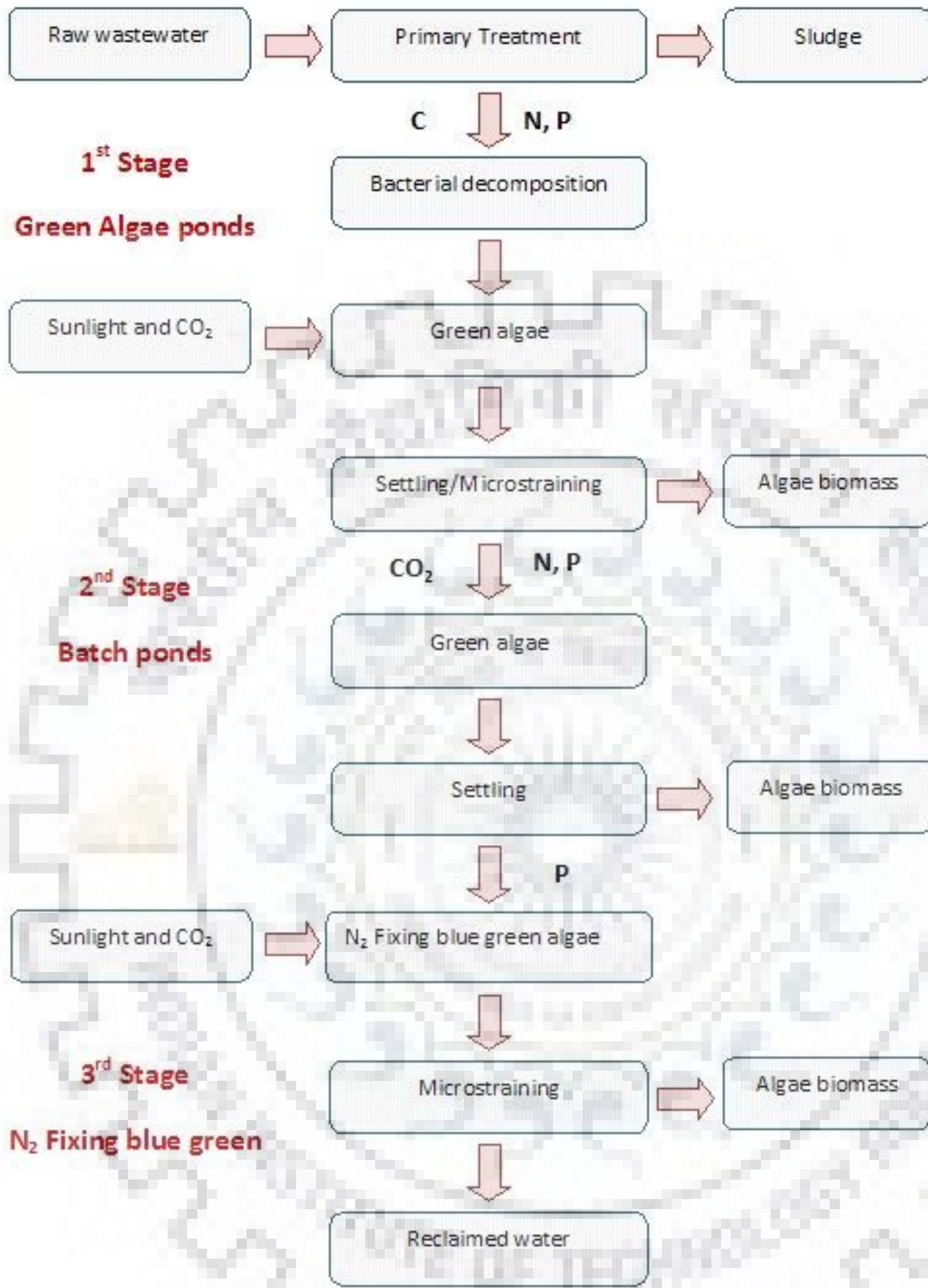

Fig 2: Steps showcasing waste water treatment from microalgae

Worldwide yearly freshwater utilization was assessed at 3,908.3 billion m^3 amid 2009, and a large portion of the expended water transformed into wastewater. Besides, add up to N and P fixations in wastewater can be as high as 10– 100 mg/L in civil wastewater and significantly in excess of 1000 mg/L in rural emanating [104]. Taking the city of Tianjin of China (populace of around 14 million out of 2012) for instance, the yearly N and P discharge in wastewater in 2011 was 36,700 t and 4700 t, individually, comparing to 7.18 and 0.92 g/d per capita. Without

appropriate treatment, the arrival of N and P would prompt eutrophication and environment harm in downstream watersheds [105] .Profiting from its plenitude and advancement of supplements; wastewater can be utilized as a minimal effort supplement hotspot for microalgae development. Microalgae can possibly expel supplements from wastewater and to gather biomass for biofuel generation [106]. Algal treatment of wastewater offers a less expensive and more productive intends to evacuate supplements and metals from wastewater than traditional tertiary treatment [107]

For instance, most business methodologies of phosphorus evacuation try not to reuse it as a completely practical item, as it is recovered alongside different other waste items, some of which are poisonous. Be that as it may, these supplements can be consolidated into green growth biomass [108] and consequently expelled from wastewater by algal treatment. Furthermore, the utilization of microalgae dispenses with the ooze treatment, which is strategically testing [109]. Refined of microalgae in wastewater likewise generously diminishes the need of compound composts and their related weight on life cycle. Through the usage of wastewater, the zero-squander idea is additionally executed, and along these lines invigorates a more economical practice for the microalgae biofuel industry. It has even been suggested that incorporated phyco-remediation and biofuel innovation gives off an impression of being the main wellspring of manageable generation of biofuels [110].

Profiting from its wealth and enhancement of supplements, wastewater can be utilized as a minimal effort supplement hotspot for microalgae development. Microalgae can possibly evacuate supplements from wastewater and to aggregate biomass for biofuel creation [111]. Algal treatment of wastewater offers a less expensive and more proficient intends to evacuate supplements and metals from wastewater than traditional tertiary treatment [112]

For instance, most business methodologies of phosphorus expulsion try not to reuse it as a completely manageable item, as it is recovered alongside different other waste items, some of which are harmful. Be that as it may, these supplements can be joined into green growth biomass and in this way expelled from wastewater by algal treatment [113]. Also, the utilization of microalgae wipes out the slime treatment, which is strategically testing [114]. Refined of microalgae in wastewater additionally considerably decreases the need of synthetic manures and their related weight on life cycle. Through the usage of wastewater, the zero-squander idea is additionally executed, and consequently invigorates a more reasonable practice for the microalgae biofuel industry. It has even been recommended that coordinated phyco-remediation

and biofuel innovation seems, by all accounts, to be the main wellspring of practical creation of biofuels [115].

Concerning the utilization of this microalga to wastewaters treatment few examinations still exist. An illustration is the Hodaifa et al. in 2008 examined that utilized wash water from the olive-oil extraction industry for developing microalga *S. obliquus*, demonstrating that despite the fact that this wastewater is N lacking, the most noteworthy level of mono and poly unsaturated fats in the biomass' lipids part was achieved when 100 % wash water was utilized as culture medium. McGinn et al. in 2012 additionally investigated the likelihood of utilizing microalga *Scenedesmus sp*. for a metropolitan wastewater treatment in bunch and persistent mode.

Previous studies have demonstrated successful treatment with microalgae of municipal wastewater, which was rich in nitrogen and phosphorus [116], even at low light intensity [117]. Only few species of the family of *Chlorella*, such as *Chlorella pyrenoidosa* [118], and *Chlorella vulgaris* [119] have been employed for wastewater treatment. However even though these species have shown excellent efficiency for nutrient removal from wastewater, the concentration of algal biomass and lipids content were not satisfactory for biofuel production [120,121,122]. The organic matter and nutrients content of the secondary effluent, as well as the presence of other heterotrophic microorganisms affect the growth of microalgae. The selection of microalgae for potential biofuel production should take into consideration the high algal cell density, and high lipids content. The use of wastewater in algal cultivation could have a double role, the reduction of the wastewater pollution load, and the utilization of microalgae for biomass and energy production.

CHAPTER 3
MATERIAL AND METHODS

3.1. For Biodiesel Production

3.1.1. Materials

Laboratory grade chemicals of Himedia Laboratories Pvt. Ltd. (Mumbai, India) were used. The glass-wares used were of Borosil.

Instruments used: Vertical Autoclave; Centrifuge; Incubator Shaker; Laminar Air Flow; Spectrophotometer; Vaccum Oven; PCR; Gel electrophoresis; GC-MS; etc.

3.1.2. Organism and Growth Conditions

Scenedesmus obliquus was obtained from NCIM, National Chemical Laboratory, Pune and was maintained in N-11 media. Cultures were incubated in Incubator Shaker at 27^0 C for further growth. The organisms were grown in 1000 ml Erlenmeyer flasks containing 500 ml of the specific medium as mentioned in Table 1.

Table 1: Composition of N-11 medium

Component	Concentration (mg/L)	SAZ stock	Concentration (mg/L)
KNO_3	1500	$MnCl_2.4H_2O$	99
$Na_2HPO_4.H_2O$	83	$NiSO_4.6H_2O$	23.6
KH_2PO_4	52	$ZnSO_4.7H_2O$	63
$MgSO_4.7H_2O$	50	$CuSO_4.5H_2O$	5
$CaCl_2.H_2O$	10	$CoSO_4.7H_2O$	2.8
Fe-EDTA stock (10g chelate per liter)	1 ml	NH_4VO_3	2.9
SAZ stock	1 ml	$(NH_4)_6Mo_7O_{24}.4H_2O$	1.8

The media was distributed into 1000 ml conical flasks, closed with cotton plugs and sterilized by autoclaving at 121ºC for 20 min and allowed to cool at room temperature before inoculation. Inoculation was carried out under aseptic conditions inside the laminar air flow.

3.1.3 Estimation of dry cell weight (dcw)

Dry cell weight (dcw) of the microalgal strain was determined gravimetrically according to Rai et al. (1991). 50ml of algal culture was centrifuged at the speed of 7500 rpm for 10 min and the harvested biomass was collected in a vial and dried at 50 ^{0}C in the vaccum oven at 700psi till to reach a constant weight. Biomass weight was measured and expressed in g/L.

3.1.4. Extraction and estimation of lipids

Extraction of lipids was done by considering binary solvent system viz. chloroform - methanol from the dried biomass of a single batch cultures. To a glass vial containing known amount of dry biomass, 2 ml methanol and 1 ml chloroform were added and kept at room temperature for 24 h [123] .The mixture was then agitated in a vortex for 2 min. After that, 1ml of chloroform was again added and the mixture was shaken vigorously for 1 min. After this, 1.8 ml of millipore water was added and the mixture was vortexed again for 2 min. The layers were separated by centrifugation at 2000 rpm for 10 min. The supernatant was filtered through Whatmann No. 1 filter paper into a previously weighed clean vial (W_1). Evaporation was carried on in a water bath and the residue was further dried at 104 $^{\circ}C$ for 30 min. The weight of the vial was again recorded (W_2).

3.1.5. Preparation of starved media

To study the effects of nitrate, potassium, calcium, phosphate and combination of Calcium-Iron, Phosphate-Potassium, Nitrate-Potassium, Potassium-Phosphate, Nitrate-Phosphate-Potassium starvation on the accumulation of microalgal biomass and lipid, the selected microalgal strain *Scenedesmus obliquus* was grown in the above mentioned starved media. In N-11 medium, nitrogen starvation was achieved by substituting nitrogen source (KNO_3) of the medium with equimolar concentrations of KCl. For phosphate starvation cultures were transferred to the mineral salt medium, where $Na_2HPO_4.H_2O$ and KH_2PO_4 were replaced by equimolar concentrations of Na_2SO_4 and KCl, respectively. Similarly, Potassium starvation was achieved by substituting KNO_3 and KH_2PO_4 with equimolar concentration of $NaNO_3$ and NaH_2PO_4 respectively. Calcium starvation was obtained by replacing $CaCl_2.H_2O$ by equimolar concentration of NaCl. Similarly, the combinations were prepared by replacing the salts with the equimolar concentration of similar salts.

3.1.6. Transesterification

Transesterification was done by adding 3.4 ml methanol, 0.6 ml sulfuric acid and 4 ml chloroform in the samples having algal oil and then it is heated at 90°C for 40 mins. Then, mix the samples thoroughly and let them cool down at room temperature. After that add 2 ml of distilled water to the samples to allow phase separation. Lower phase which contained (FAME) was collected and transferred to a pre-weighed glass vial. The solvent was evaporated using nitrogen and the mass of biodiesel was determined gravimetrically.

3.1.7. Gas Chromatography Mass spectroscopy

The fatty acid profile was determined by gas chromatography–mass spectrometry (GC–MS) analysis, carried out in an Agilent 7890, a gas chromatograph equipped with an Agilent 5975B mass selective detector. Compounds were profiled on a 32 m × 0.5 mm ID × 1 µm film Agilent DB-5 capillary column. The temperature of the injector was maintained at 240°C. Helium was used as carrier gas and the ions were generated by 70eV electron beam and the mass range scanned was 50-550 m/z at a rate of 2 scan s^{-1}. The oven temperature for FAME analysis was initially maintained at 50^{0} C followed by a temperature rate of 25°C min^{-1} to 180 °C min^{-1} and then held for 10 °C min^{-1} ramp to 220 °C min^{-1} and then held 15 °C min^{-1} ramp to 250 °C for 15min. Ionization voltage was 70 eV and sample injection volume was 1 µl. GC analysis was carried out by matching the mass spectra of each compound with the mass spectral library.

3.1.8. Experimental Modeling

DOE (Design-Expert 11) is a tool which is used to manage and optimize number of experiments. Response surface methodology (RSM), with central composite design (CCD), was used to investigate the impact factors on the yield and develop a model [126]. Three variables Nitrogen deficient, phosphorous deficient and potassium deficient were investigated. In this study, three center point replicate experiments were performed, for a total of 20 experiments.

- **Statistical Analysis.** Central composite design was first introduced by Box-Wilson (1951) and they now days are the most popular second order designs especially for using chemical engineering [127]. Experimental results were determined using analysis of variance (ANOVA) and standard least-squares regression modeling. Quadratic models were developed to predict yield(Oil, Char) using the following power-second order polynomials as per CCD methodology[128]:

$$\boldsymbol{Y_k = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4}\sum_{j=1}^{4} \beta_{ij} X_i X_j + \sum_{j=1}^{4} \beta_{ii} X_i^2 \qquad K = 1, 2, 3} \qquad (1)$$

Where Y represents product yield and K represents number of factors. $\beta_0, \beta_i, \beta_{ij}$ and β_{ii} are constant and obtained by fitting a model, and X_i and X_j are the factors being studied (eg., temperature and residence time).

The factors are normalized to vary between +1 and -1, according to the equation 2:

$$\boldsymbol{X_i = \frac{x_i - x_i^0}{\Delta x_i}} \qquad (2)$$

Where x_i^0 is the mid point, x_i is the real value, Δx_i is the real value, Δx_i is the half range Δx_i is the half range, and X_i is the coded value which varies from −1 to +1. The model coefficients (β_0, β_i, β_{ij}, and β_{ii}) were obtained by using the following equations:

$$\boldsymbol{\beta_0 = a_1 \sum_{u=1}^{n} Y_u + a_2 \sum_{i=1}^{k}\sum_{u=1}^{n} Y_u X_{iu}^2} \qquad (3)$$

$$\boldsymbol{\beta_i = a_3 \sum_{u=1}^{n} X_{iu} Y_u} \qquad (4)$$

$$\boldsymbol{\beta_{ij} = a_4 \sum_{u=1}^{n} X_{iu} X_{ju} Y_u} \qquad (5)$$

$$\boldsymbol{\beta_{ii} = a_5 \sum_{u=1}^{n} Y_u X_{iu}^2 + a_6 \sum_{i=1}^{k}\sum_{u=1}^{n} Y_u X_{iu}^2 - a_7} \qquad (6)$$

where a_1, a_2, a_3, a_4, a_5, a_6, and a_7 are determined from the literature. After calculating the regression coefficients, the impact on the final yield was estimated. From this data, the factor-based model was developed.

3.2. For Waste Water Treatment

3.2.1. Collection of sample

Grey water samples were collected from Cautley bhawan, IIT Roorkee Campus, Roorkee mess and washing area on Saturday and Sunday around 4 pm thrice. Samples were collected and stored at $4^{o}C$ for further use.

3.2.2. pH

pH of the raw and treated grey water sample was measured using the pH meter. pH meter is first calibrated with known solution of pH 4 and 7.

3.2.3. Conductivity

Digital conductivity meter is used for measuring the conductivity of the sample. Conductivity meter consists of an electrode which when immersed in the sample gives reading in μS/cm. The conductivity meter works at a temperature of $27^{0}C$. The readings are taken at room temperature.

3.2.4. TDS

TDS= 0.65*Conductivity (μS/cm)

3.2.5. Phosphorous (Jal TARA water testing kit)

12 ml of sample was transferred in the test tube. Then, 0.4 ml of Ammonium molybdate reagent by the help of 2 ml syringe was taken and transferred into the test tube. Shake the test tube and then add 2 drops of stannous chloride and again shake the test tube thoroughly. Wait for 10 minutes for color development and then compare the color with standard color chart.

3.2.6. Nitrate (Jal TARA water testing kit)

Transfer 5 ml of water sample to the cylinder. Then, heat the sample on a spirit lamp till the liquid evaporates to dryness. Let it cool to room temperature and after the test tube has been cooled, 8-9 drops of Phenol Disulphonic Acid (PDA). Rotate the cylinder slowly so that the PDA dissolves the entire residue at the bottom of the tube. Then, take 2 ml of distilled water in 2 ml syringe and dilute the solution by adding 2 ml of distilled water. Add about 23 drops of ammonia solution. Shake the cylinder and add distilled water till 5 ml mark with 2 ml syringe. Now compare the color with standard color chart.

3.2.7. Chemical Oxygen Demand (COD)

COD test is commonly used to measure the amount of organic and inorganic compound in water. It is expressed in gm/l, which indicates the mass of oxygen consumed per litre of solution. COD determines the quantity of oxygen required to oxidize the organic and inorganic matter in water under specific conditions of oxidizing agent, temperature and time. Organic and inorganic compounds present in sample get oxidized completely by potassium dichromate in presence of sulphuric acid, silver sulphate and mercuric sulphate to produce CO_2 and H_2O. The dichromate consumed by sample is equivalent to the amount of O_2 required to oxidize organic and inorganic matter.

Preparation of reagents

Standard potassium dichromate reagent- digestion: Weigh accurately 4.913 gm of potassium dichromate which is previously dried at 103^0C for 2-4 hrs and transfer it to a beaker. Weigh accurately 33.3 gm of mercuric sulfate and add to the same beaker. Measure 167 ml conc. Sulfuric acid using measuring cylinder and transfer it into the beaker. Dissolve the contents and cool down to room temperature. Take 1000ml standard flask and transfer the contents to the 1000 ml standard flask and make up the volume up to 1000 ml using distilled water.

Sulfuric acid reagent- catalyst solution: Weigh accurately 5.5gm silver sulfate crystal to dry clean 1000 ml beaker. Now add 500 ml conc. Sulfuric acid and allow standing for 24 hrs so that the silver sulfate crystal gets completely dissolved.

Procedure: 2 COD vials are taken with stopper (one for sample and one for blank). Now, 2.5 ml of the sample was added to one COD vial and 2.5 ml of distilled water to another COD vial. Then, add 1.5 ml of potassium dichromate reagent to each COD vials. Now, add 3.5 ml of sulfuric acid reagent to both the vials and tightly close the vials. Keep the vials in COD digester for 2 hrs at 150^0C. Now, cool at room temperature after digestion. Then measure the value of COD through COD meter. (COD METER HANNA INSTRUMENTS).

3.2.8. Biochemical Oxygen Demand (BOD)

Biochemical Oxygen Demand or BOD is a chemical procedure for determining the amount of dissolved oxygen needed by aerobic biological organism in a body of water to break down organic material present in a given water sample at certain temperature over a specific period of time. BOD is measured as per APHA, Standard Methods for the Examination of Water and Wastewater 1999. 5-days BOD is found for all the samples.

BOD_5 = {(initial D.O. – final D.O.)$_{Sample}$ – (initial D.O. – final D.O.)$_{Blank}$}* Dilution factor

Dilution factor = Total volume of BOD bottle / Volume of sample taken

Initial dissolved oxygen of both blank and diluted sample is determined by Winkler's Method. The BOD bottles were then incubated at 20^0C for 5 days. Final D.O. of diluted sample and blank is determined after incubation for 5 days.

3.2.9. Iron (Jal TARA water testing kit)

Transfer 5 ml of sample in cylinder. Now, take 0.4 ml of HCL with 2 ml syringe and transfer it to the cylinder. Then, add 4 drops of hydroxyl amine hydrochloride and heat the sample on spirit lamp till the liquid reduces to 1/3 of its initial volume. Now add few drops of ammonium acetate buffer and mix it thoroughly. Then after 5 mins add 2-4 drops of phenanthroline and mix the components thoroughly. Now, make the sample up to 4ml by adding distilled water and then observe the change in color.

3.3. SCREENING, ISOLATION & IDENTIFICATION OF MICROALGAL STRAIN

3.3.1. Screening & Isolation

Algal samples were collected from 4 different areas of Delhi from the river Yamuna and these samples were inoculated in the N-11 medium to check the presence and growth of the unknown microalgal strains. Plates were prepared by spreading the inoculated samples to obtain colonies of microalgae. After that, to obtain the pure colonies streaking was done and pure colonies were obtained after several repetition. Pure colonies obtained from streaking plates were then inoculated in the N-11 medium and their growth was observed.

Screening of microalgal samples were done by observing their growth and biomass yield. Among 7 Yamuna river samples, the best 3 are selected for further process.

3.3.2. DNA Extraction

HiMedia Marine Algal DNA Purification kit has been used to carry out the extraction process.

Sample Preparation (Manual Disruption with mortar and pestle)

Weigh up to 250 mg of the finely cut plant material and grind properly using a mortar and Pestle in liquid nitrogen to a fine powder. Allow the liquid nitrogen to evaporate. DO NOT ALLOW THE SAMPLE TO THAW (keep samples on ice if needed). Proceed immediately to the DNA isolation protocol.

NOTE: Delay in DNA isolation after sample preparation will result in DNA degradation and reduced yield.

Protocol:

1. To the ground material, immediately add 3 ml of STE Buffer (with b – mercapthoethanol and Bovine Serum Albumin) and mix thoroughly. [Do not grind the plant material after the addition of STE Buffer, as it may cause shearing of DNA]. Proceed immediately to step 2 without delay.

NOTE: For the preparation of STE Buffer, refer General Preparation Instructions.

2. Load sample in HiShredder (DSCA02) Add the sample to the HiShredder placed in a 2.0 ml collection tube and centrifuge for 2 minutes at a maximum speed (≈13,000 rpm). Discard the flow-through fraction without disturbing the cell pellet.

NOTE: The above step should be performed in two different HiShredders (provided) as the entire sample cannot be processed in the same HiShredder.

3. To each pellet, add 200 µl of CTAB Extraction Buffer, mix thoroughly and pool the contents of both the tubes into a single tube (not provided).

4. Optional RNase A treatment: If RNA-free genomic DNA is required, add 20 µl of RNase A Solution (DS0003), mix and incubate for 10 minutes at room temperature (15-25°C), then continue with step 5.

5. Incubate the samples at 65°C for 30 minutes. 6. Add 400 µl of Chloroform: Isoamyl alcohol (24:1) (Product Code: MB115). Mix the sample gently by inversion for 10 minutes. Centrifuge for 10 minutes at a maximum speed (≈13,000 rpm).

7. Following centrifugation, mixture separates into three phases: lower organic phase, an interphase containing debris and an upper aqueous phase containing DNA.

8. Transfer the top aqueous layer into a fresh tube. Add 2 volumes of ethanol (96-100%) and 0.1 volumes of 3M Sodium acetate, pH 5.2; mix gently.

9. Incubate the samples at -20°C for 10 minutes. Centrifuge for 10 minutes at a maximum speed (≈13,000 rpm). Discard the supernatant.

10. Resuspend the pellet in 500 µl of 70% ethanol and centrifuge for 10 minutes at a maximum speed (≈13,000 rpm). Discard the supernatant.

11. Air dry the pellet to remove traces of ethanol.

NOTE: Do not over-dry the pellet.

12. Dissolve the pellet gently in 200 µl of Elution Buffer (ET) (DS0040) by pipetting. Storage of the eluate with purified DNA: The eluate contains pure genomic DNA. For short term storage (24-48 hours) of the DNA, 2-8°C is recommended. For long-term storage, -20°C or lower temperature (-80°C) is recommended. Avoid repeated freezing and thawing of the sample which

may cause denaturing of DNA. The Elution Buffer (ET) will help to stabilize the DNA at these temperatures.

General Preparation Instructions

1. Preheat a water bath or heating block to 65°C.
2. **Preparation of STE Buffer**

Add 2 µl of b - mercapthoethanol and 2 mg Bovine Serum Albumin (BSA) per ml of STE Buffer prior to use.

NOTE: STE buffer should be freshly prepared before the experiment.

3. **Thoroughly mix reagents**

Examine the reagents for precipitation. If any kit reagent forms a precipitate (other than enzymes), warm at 55-65°C until the precipitate dissolves and allow cooling to room temperature (15-25°C) before use.

4. Ensure that clean & dry tubes and tips are used for the procedure.
5. Pre-chill the mortar and pestle at -20°C.

Concentration, yield and purity of DNA

Spectrophotometric analysis and agarose gel electrophoresis will reveal the concentration and the purity of the genomic DNA. Use Elution Buffer (ET) to dilute samples and to calibrate the spectrophotometer; measure the absorbance at 260 nm, 280 nm and 320 nm in DNA nanodrop. Absorbance readings at 260 nm should fall between 0.1 and 1.0. The 320 nm absorbance is used to correct for background absorbance. An absorbance of 1.0 at 260 nm corresponds to approximately 50 µg/ml of DNA. The $A_{260}–A_{320}$ /$A_{280}–A_{320}$ ratio should be 1.6–1.9. Purity is determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. DNA purified by HiPurA Algal DNA Purification Kit is free of protein and other contaminants that can inhibit PCR or other enzymatic reactions.

Concentration of DNA sample (µg/ml) = 50 x A260 x dilution factor.

3.3.3. Polymerase Chain Reaction (PCR)

A region of approx. 700 bp of 18S rRNA gene was amplified by PCR using eukaryotic primers
Fw: 5'-GTCAGAGGTGAAATTCTTGGATTTA-3'

Rev: 5'- AGGGCAGGGACGTAATCAACG-3'.

PCR reactions were performed in 50 µl reaction mixture containing $MgCl_2$ (2mM), Template (2.5 µl), Forward Primer (2.5 µl), Reverse Primer (2.5 µl), 2X GoTaq Mixture (25 µl), and

Nuclease free water (12.5 µl). The following PCR program was conducted to carry out the reactions: 1 cycle at 95°C for 3 mins followed by 35 cycles at 94°C for 1 min, 48°C for 1:30 mins, 72°C for 2 mins and a final cycle at 72°C for 3 mins. The amplified PCR products were then purified.

3.3.4. PCR product Purification

Take 50µl PCR product and in that add 3M sodium acetate and 100µl chilled absolute ethanol and mix them thoroughly. Now, store the mixture at -20°C for 30-50 mins. Then, centrifuge at 14000 rpm for 30 mins. Discard the supernatant and wash the pellet with 70% ethanol and again spin at 14000 rpm for 5 mins. Now discard the supernatant and dry the pellet. Dissolve the pellet in milliq or TE.

3.3.5. Agarose Gel Electrophoresis

1. Prepare a 50x stock solution of TAE buffer in 1000m of distilled H_2O:
   For this weigh 242 g of Tris base in a chemical balance. Transfer this to a 1000ml beaker. Prepare EDTA solution (pH 8.0, 0.5M) by weighing 9.31g of EDTA and dissolve it in 40ml distilled water. EDTA is insoluble and it can be made soluble by adding sodium hydroxide pellets. Check the pH using pH meter. Make the solution 100ml by adding distilled water. Pipette out 57.1 ml of glacial acetic acid.
   Mix the Tris base, EDTA solution and glacial acetic acid and add distilled water to make the volume to 1000ml

2. Prepare sufficient electrophoresis buffer (usually 1x TAE) to fill the electrophoresis tank and to cast the gel:
   For this we take 2ml of TAE stock solution in an Erlenmeyer flask and make the volume to 100ml by adding 98ml of distilled water. The 1x working solution is 40 mM Tris-acetate/1 mM EDTA. It is important to use the same batch of electrophoresis buffer in both the electrophoresis tank and the gel preparation.
3. Prepare a solution of agarose in electrophoresis buffer at an appropriate concentration:
   For this usually 2 grams of agarose is added to 100ml of electrophoresis buffer.
4. Loosely plug the neck of the Erlenmeyer flask. Heat the slurry in a boiling-water bath or a microwave oven until the agarose dissolves. The agarose solution can boil over very easily so keep checking it. It is good to stop it after 45 seconds and give it a swirl. It can become superheated and NOT boil until you take it out whereupon it boils out all over you hands. So

wear gloves and hold it at arm's length. You can use a Bunsen burner instead of a microwave - just remember to keep watching it.

5. Use insulated gloves or tongs to transfer the flask/bottle into a water bath at 55°C. When the molten gel has cooled, add 0.5µg/ml of ethidium bromide. Mix the gel solution thoroughly by gentle swirling.

   (For the preparation of ethidium bromide adds 1 g of ethidium bromide to 100 ml of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the 10 mg/ml solution to a dark bottle and store at room temperature.)

6. While the agarose solution is cooling, choose an appropriate comb for forming the sample slots in the gel.

7. Pour the warm agarose solution into the mold.

   (The gel should be between 3 - 5 mm thick. Check that no air bubbles are under or between the teeth of the comb.)

8. Allow the gel to set completely (30-45 minutes at room temperature), then pour a small amount of electrophoresis buffer on the top of the gel, and carefully remove the comb. Pour off the electrophoresis buffer. Mount the gel in the electrophoresis tank.

9. Add just enough electrophoresis buffers to cover the gel to a depth of approx. 1mm.

10. Mix the samples of DNA with 0.20 volumes of the desired 6x gel-loading buffer.

11. Slowly load the sample mixture into the slots of the submerged gel using a disposable micropipette or an automatic micropipettor or a drawn-out Pasteur pipette or a glass capillary tube. Load size standards into slots on both the right and left sides of the gel.

12. Close the lid of the gel tank and attach the electrical leads so that the DNA will migrate toward the positive anode (red lead). Apply a voltage of 1-5 V/cm (measured as the distance between the positive and negative electrodes). If the electrodes are 10cm apart then run the gel at 50V. It is fine to run the gel slower than this but do not run it any faster. Above 5V/cm the agarose may

heat up and begin to melt with disastrous effects on your gel's resolution. If the leads have been attached correctly, bubbles should be generated at the anode and cathode.

13. Run the gel until the bromophenol blue and xylenecyanol FF have migrated an appropriate distance through the gel.
    (The presence of ethidium bromide allows the gel to be examined by UV illumination at any stage during electrophoresis).

14. The gel tray may be removed and placed directly on a transilluminator. When the UV is switched on we can see orange bands of DNA.

3.3.6. Gel Extraction

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow. The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix. Do not centrifuge the sample at this stage.

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

8. Discard flow-through and place QIAquick column back in the same collection tube. Collection tubes are re-used to reduce plastic waste.

9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min. This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription or microinjection.

10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

Note: If the DNA will be used for salt sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at ≥10,000 x *g* (~13,000 rpm).

12. Place QIAquick column into a clean 1.5 ml micro centrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

3.3.7. DNA Sequencing

For DNA sequencing samples were sent to KPCL Life Sciences Pvt. Ltd. (Delhi).

3.3.8. BLAST

The Basic Local Alignment Search Tool (BLAST) discovers areas of local closeness between groupings. The program thinks about nucleotide or protein successions to grouping databases and figures the measurable hugeness of matches. Impact can be utilized to derive utilitarian and transformative connections between arrangements and in addition help distinguish individuals from quality families. Impact discovers districts of closeness between organic groupings. The program thinks about nucleotide or protein arrangements to succession databases and computes the measurable importance.

CHAPTER 4

RESULTS & DISCUSSION

4.1. Biodiesel Production

4.1.1. Growth curve of microalgae *Scenedesmus obliquus*

Scenedesmus obliquus was grown in N11 medium for 35 days of incubation period to observe its growth pattern in the medium. The growth was monitored at each 5 days interval by measuring the optical density of culture at wavelength of 540 nm against the blank and it was observed that *Scenedesmus obliquus* showed maximum growth about 25 days of incubation period. So 25 days incubation period is best to harvest the test microalgae to obtain maximum biomass yield as shown in Figure 3.

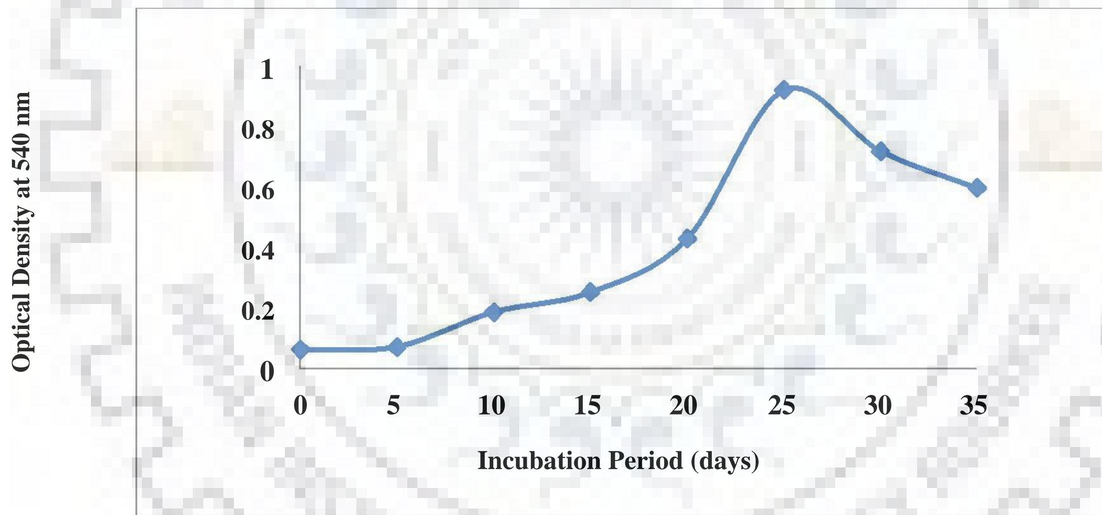

Figure 3: Showing optical density of *Scenedesmus obliquus* in nutrient rich media.

Figure 3 show the growth curve of *Scenedesmus obliquus* in the nutrient rich N-11 media. The optical density at 540 nm is maximum at 25th day i.e. 0.9213. *Scenedesmus obliquus* shows an exponential growth in the nutrient rich N-11 media.

4.1.2. Biomass yield

- **Biomass yield in Control Media**

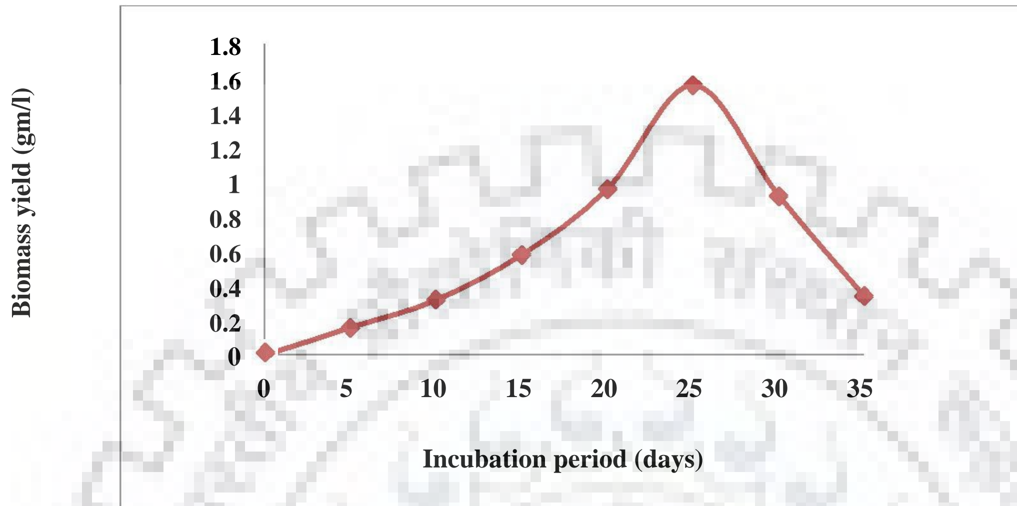

Figure 4: Showing biomass yield of *Scenedesmus obliquus* in nutrient rich media.

From the above Figure 4, we can find that the Biomass yield of the microalgal species *Scenedesmus obliquus* was observed maximum at 25th day i.e. 1.56 gm/l in nutrient enriched N-11 media.

- **Biomass yield in Phosphorus deficient media**

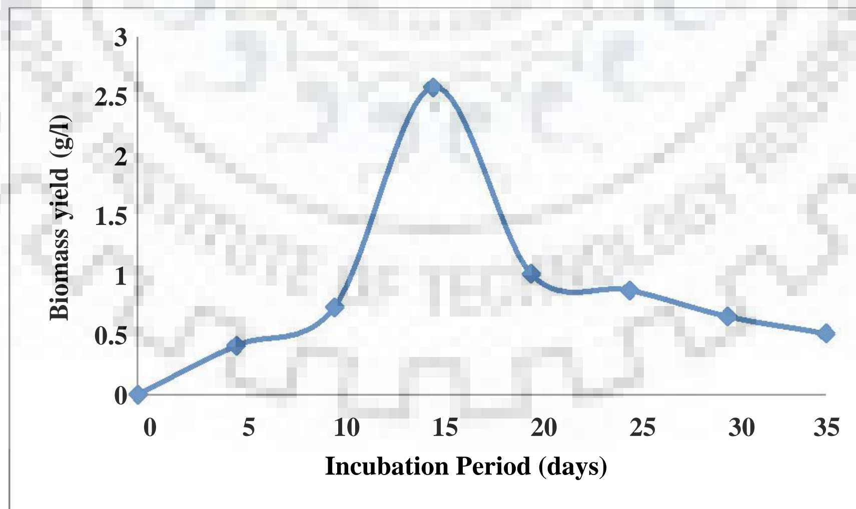

Figure 5: Showing biomass yield of *Scenedesmus obliquus* in P deficient media.

Biomass yield of the microalgal species *Scenedesmus obliquus* was observed maximum at 15th day i.e. 2.58 gm/l in phosphorous deficient N-11 media.

- **Biomass yield in Nitrogen deficient media**

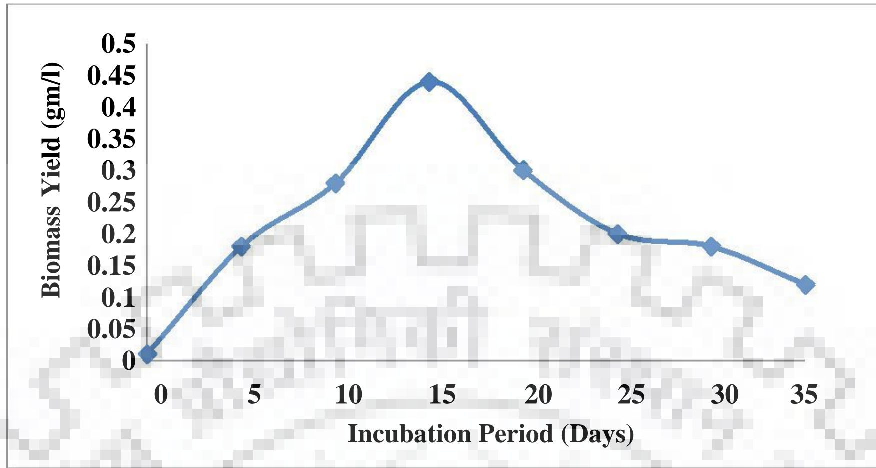

Figure 6: Showing biomass yield of *Scenedesmus obliquus* in P deficient media.

From the above Figure 6, we can find that the Biomass yield of the microalgal species *Scenedesmus obliquus* was observed maximum at 15th day i.e. 0.45 gm/l in Nitrogen deficient N-11 media.

- **Biomass yield in Potassium deficient media**

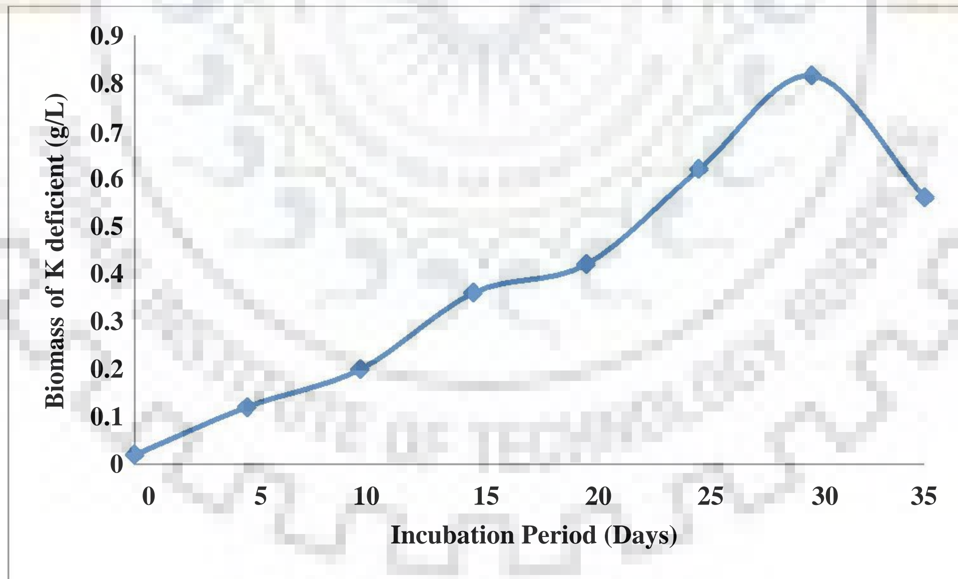

Figure 7: Showing biomass yield of *Scenedesmus obliquus* in K deficient media.

From the above Figure 7, we observed that the Biomass yield of the microalgal species *Scenedesmus obliquus* was observed maximum at 30th day i.e. 0.816 gm/l in Potassium deficient N-11 media.

- **Biomass yield in Calcium deficient media**

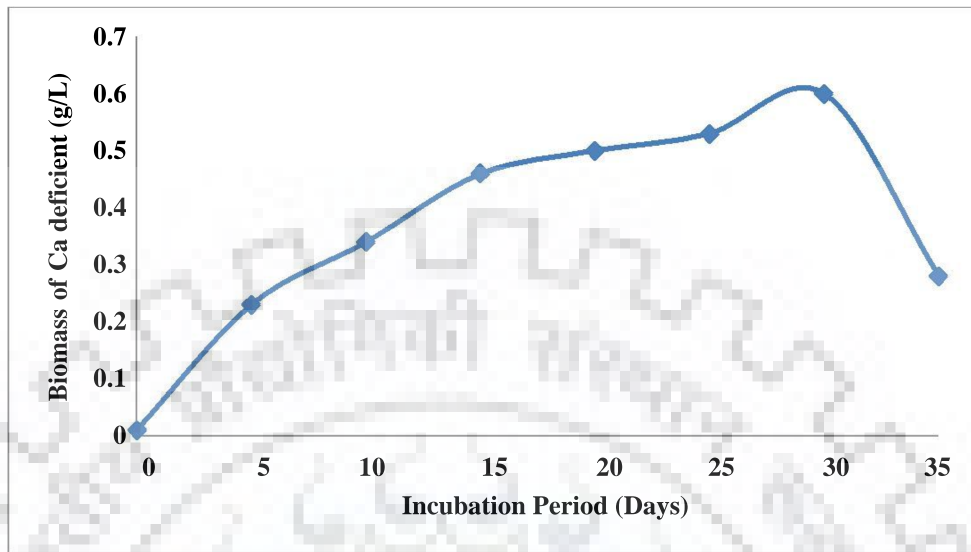

Figure 8: Showing biomass yield of *Scenedesmus obliquus* in Ca deficient media.

From Figure 8, we found that the Biomass yield of the microalgal species *Scenedesmus obliquus* was maximum at 30th day i.e. 0.60 gm/l in Calcium deficient N-11 media.

- **Biomass yield in NP deficient media**

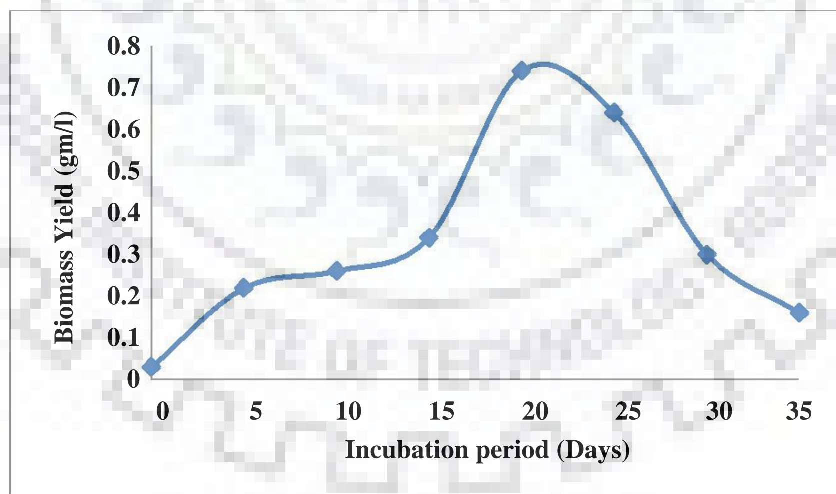

Figure 9: Showing biomass yield of *Scenedesmus obliquus* in PN deficient media.

From the above Figure 9, we can observe that the Biomass yield of the microalgal species *Scenedesmus obliquus* was maximum at 20th day i.e. 0.74 gm/l in Nitrogen-Phosphorous deficient N-11 media.

- **Biomass yield in PK deficient media**

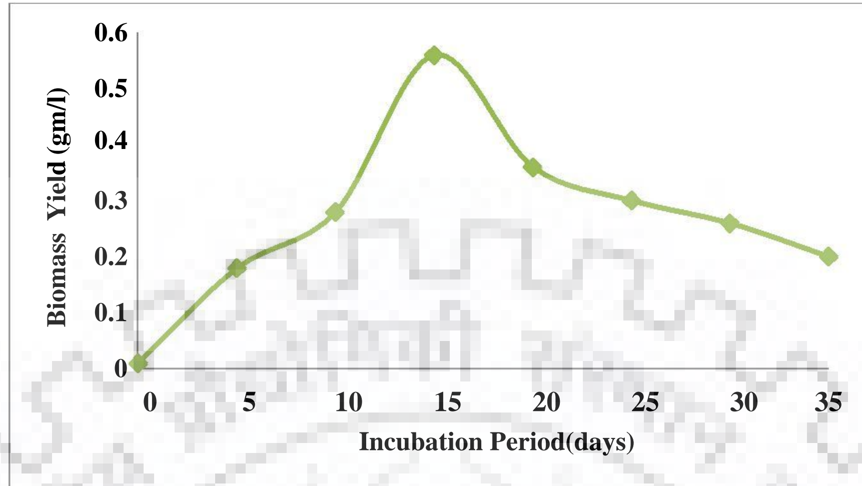

Figure 10: Showing biomass yield of *Scenedesmus obliquus* in PN deficient media.

From the above Figure 10, we can observe that the Biomass yield of the microalgal species *Scenedesmus obliquus* was maximum at 15th day i.e. 0.56 gm/l in Phosphorous-Potassium deficient N-11 media.

- **Biomass yield in NK deficient media**

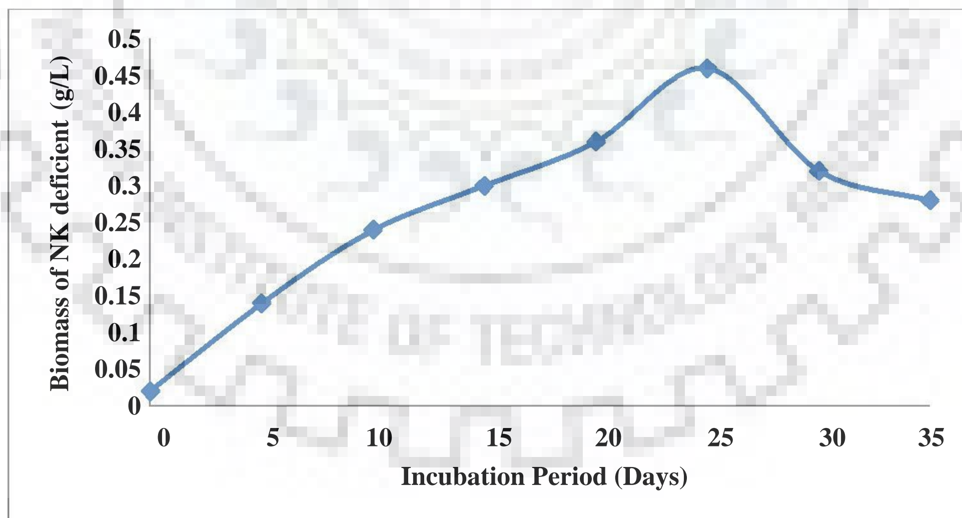

Figure 11: Showing biomass yield of *Scenedesmus obliquus* in NK deficient media.

From the Figure 11, we can observe that the Biomass yield of the microalgal species *Scenedesmus obliquus* was maximum at 20th day i.e. 0.46 gm/l in Nitrogen-Potassium deficient N-11 media.

- **Biomass yield in Ca/Fe deficient media**

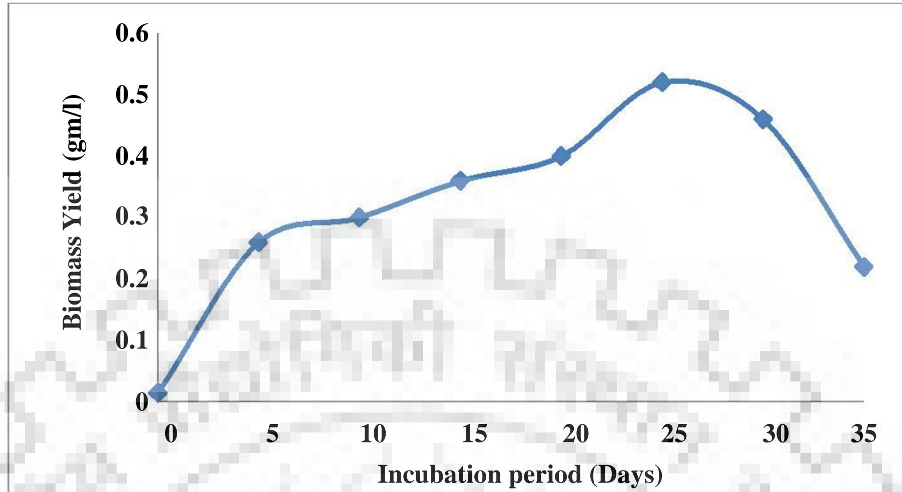

Figure 12: Showing biomass yield of *Scenedesmus obliquus* in Ca/Fe deficient media.

From the above Figure 12, we can say that the Biomass yield of the microalgal species *Scenedesmus obliquus* was observed maximum at 25th day i.e. 0.52 gm/l in Calcium-Iron deficient N-11 media.

- **Biomass yield in NPK deficient media**

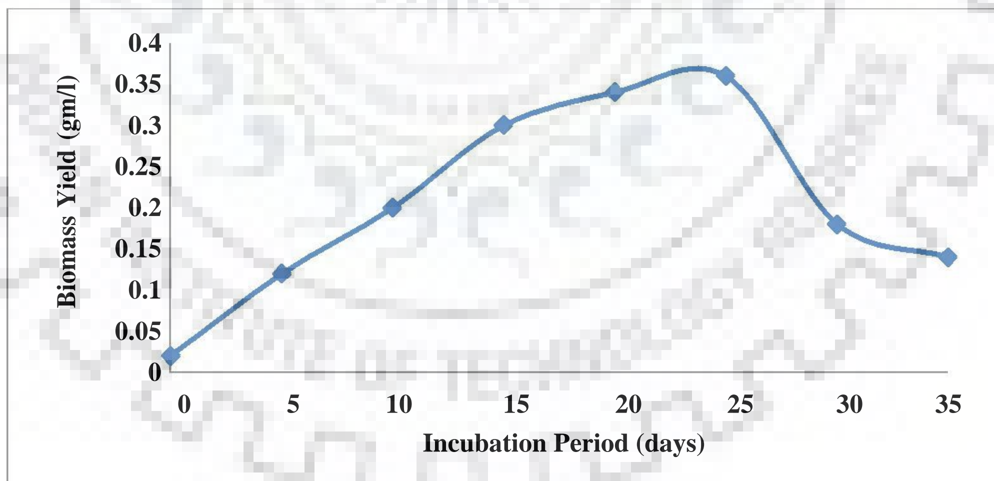

Figure 13: Showing biomass yield of *Scenedesmus obliquus* in NPK deficient media

From the above Figure 13, we can observe that the Biomass yield of the microalgal species *Scenedesmus obliquus* was maximum at 25th day i.e. 0.36 gm/l in Nitrogen-Phosphorous-Potassium deficient N-11 media.

- **Comparison of Biomass yield with nutrient enriched and nutrient starved media**

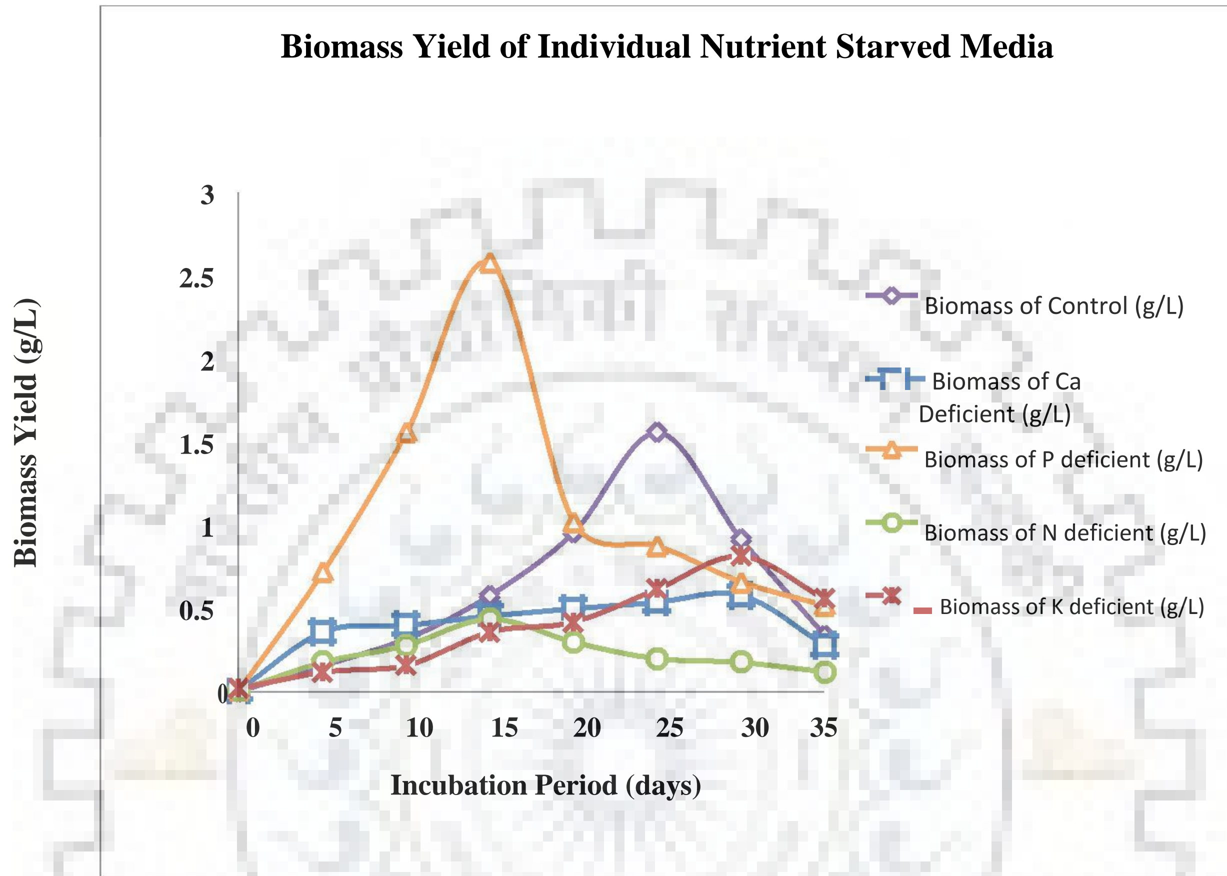

Figure 14: Showing Biomass yield of individual nutrient starved media.

From the above Figure 14, we can find that the Biomass yield from *Scenedesmus obliquus* was observed maximum in Phosphorous deficient N-11 media on 15th day i.e. 2.58 g/L. As compared with the nutrient rich N-11 media to the other deficient stress conditions we can see that Nitrogen deficient media is producing maximum biomass yield at 15th day i.e. 0.44 g/L; Potassium at 30th day i.e. 0.816 g/L; and Calcium at 30th day i.e. 0.58 g/L.

- **Effect of combine starved media on biomass yield**

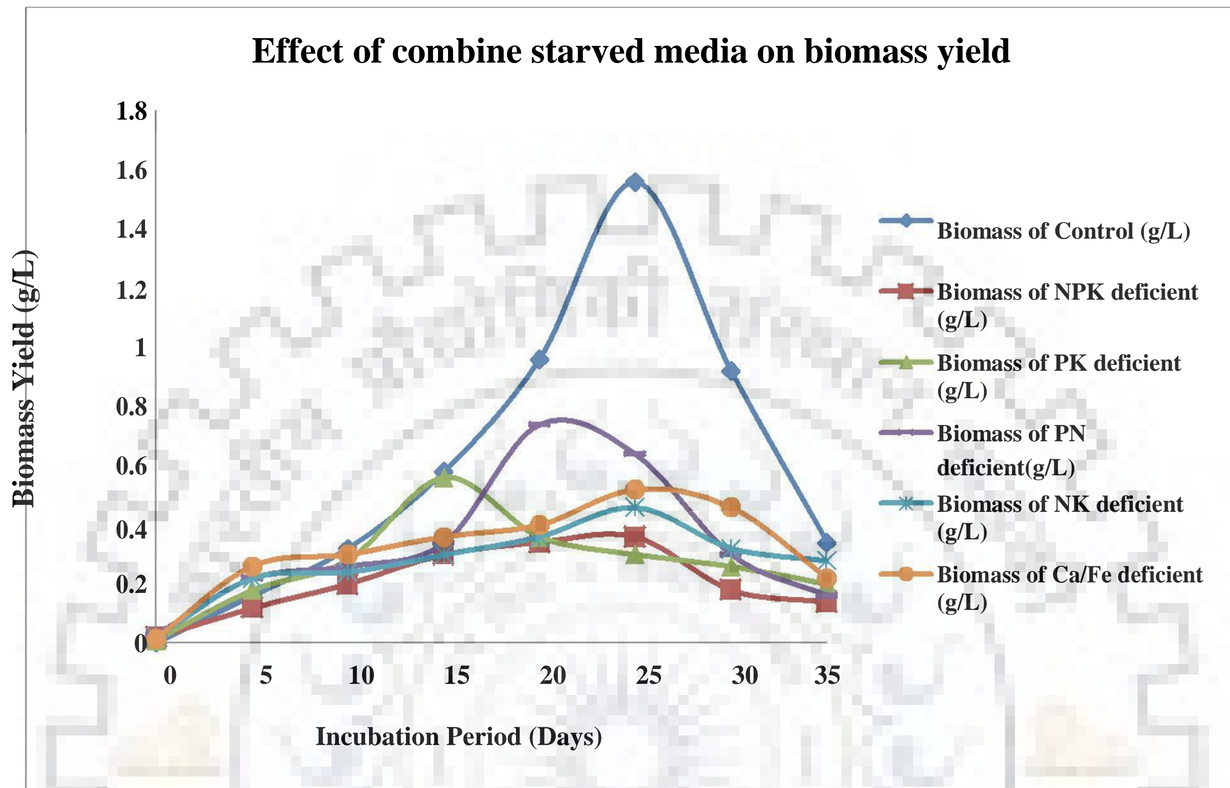

Figure 15: Showing Biomass yield of combined nutrient starved media.

From the above Figure 15, we can find that the Biomass yield from *Scenedesmus obliquus* was observed maximum in Phosphorous-Nitrogen deficient N-11 media on 20th day i.e. 0.74 g/L. As compared with the nutrient rich N-11 media to the other combined nutrient deficient stress conditions we can see that Nitrogen-Potassium deficient media is producing maximum biomass yield at 25th day i.e. 0.46 g/L; Potassium-Phosphorous at 15th day i.e. 0.56 g/L; Nitrogen-Phosphorous-Potassium deficient at 25th day i.e. 0.36 g/L and Calcium-Iron at 25th day i.e. 0.52 g/L.

4.1.3. Lipid Content (%) (w/w)

- **Lipid content in nutrient enriched media**

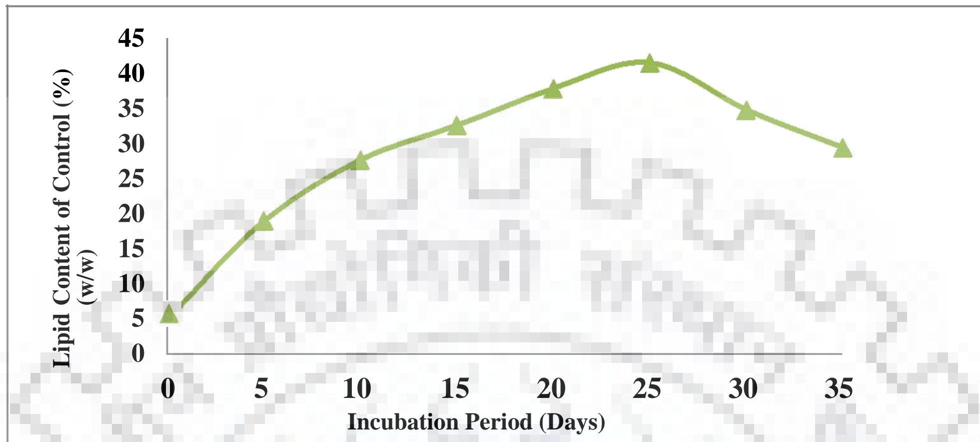

Figure 16: Showing Lipid Content (%) (w/w) in nutrient enriched N-11 media.

Above mentioned Figure 16 shows the Lipid Content (%) (w/w) present in *Scenedesmus obliquus* in nutrient rich N-11 media. Lipid content was observed maximum at 25th day i.e.41.46% in nutrient enriched N-11 media.

- **Lipid content in Nitrogen deficient media**

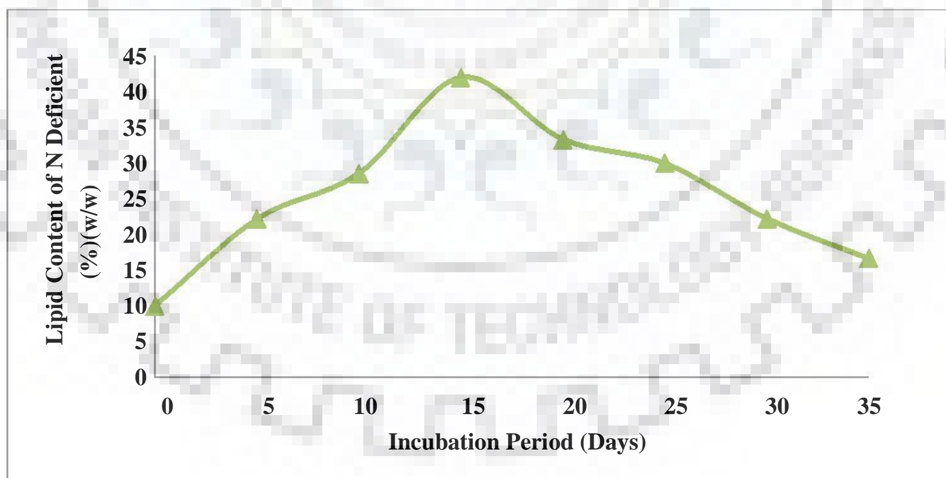

Figure 17: Showing Lipid Content (%) (w/w) in N deficient N-11 media.

Figure 17 showing Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed at 15th day i.e.42.045% in Nitrogen deficient N-11 media.

- **Lipid content in Phosphorous deficient media**

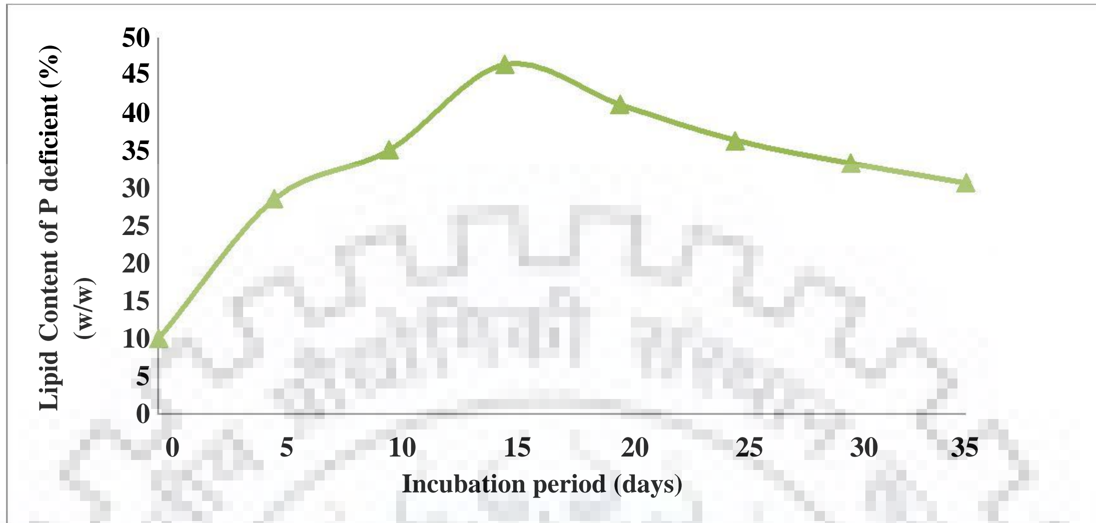

Figure 18: Showing Lipid Content (%) (w/w) in P deficient N-11 media.

Figure 18 showing Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed at 15th day i.e. 46.511% in Phosphorous deficient N-11 media.

- **Lipid content in Calcium deficient media**

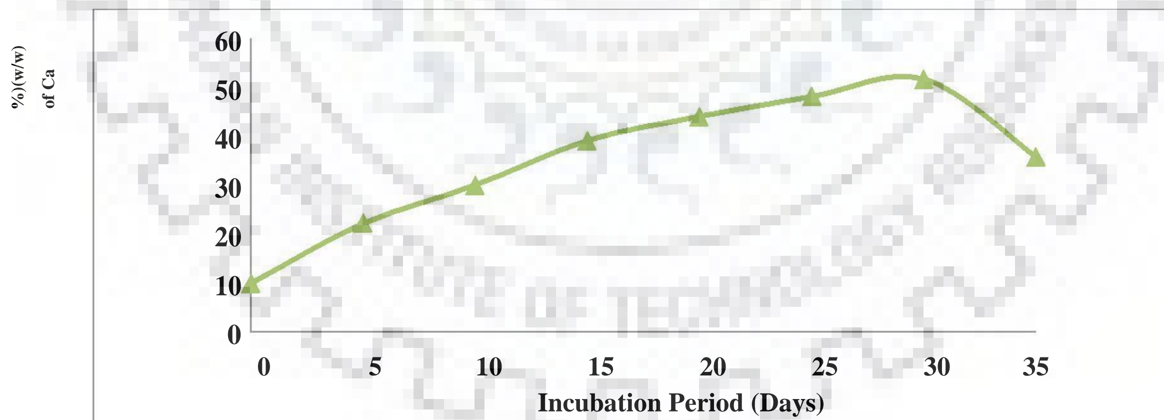

Figure 19: Showing Lipid Content (%) (w/w) in Ca deficient N-11 media.

Figure 19 shows Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed maximum at 30th day i.e.51.721% in Calcium deficient N-11 media.

- **Lipid content in Potassium deficient media**

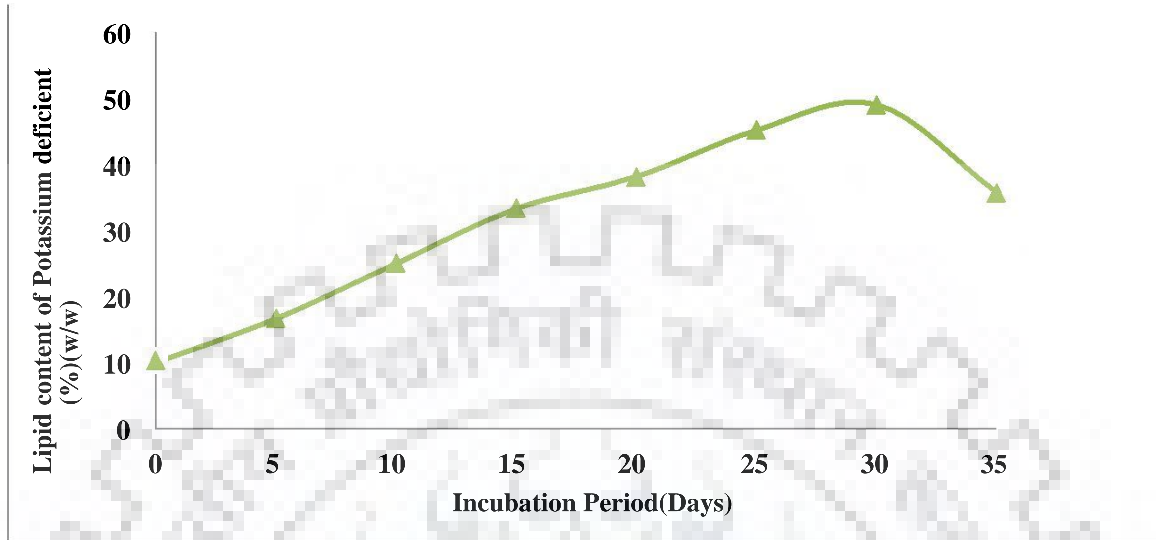

Figure 20: Showing Lipid Content (%) (w/w) in K deficient N-11 media.

Figure 20 depicts Lipid Content (%) (w/w) and in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed maximum at 30th day i.e.49.012% in Potassium deficient N-11 media.

- **Lipid content in Nitrogen-Potassium deficient media**

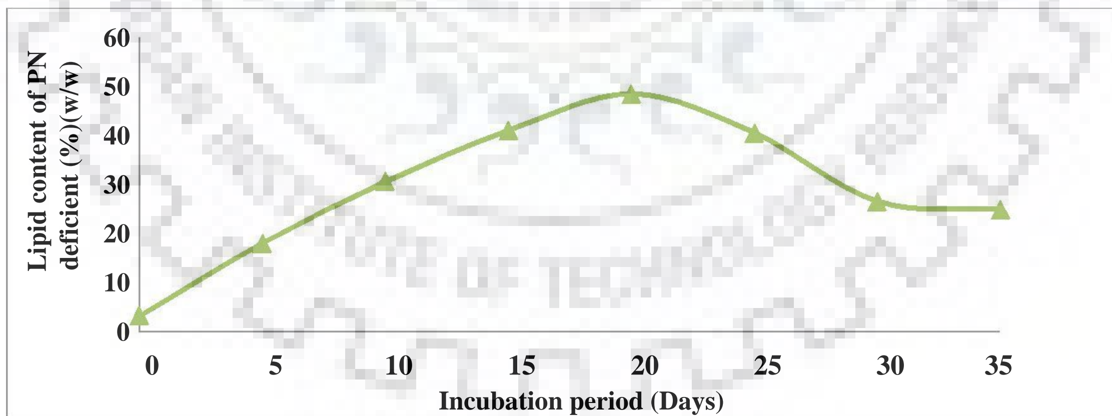

Figure 21: Showing Lipid Content (%) (w/w) in NK deficient N-11 media.

Figure 21 shows Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed maximum at 20th day i.e.48.648% in Nitrogen-Potassium deficient N-11 media.

- **Lipid content in Potassium-Phosphorous deficient media**

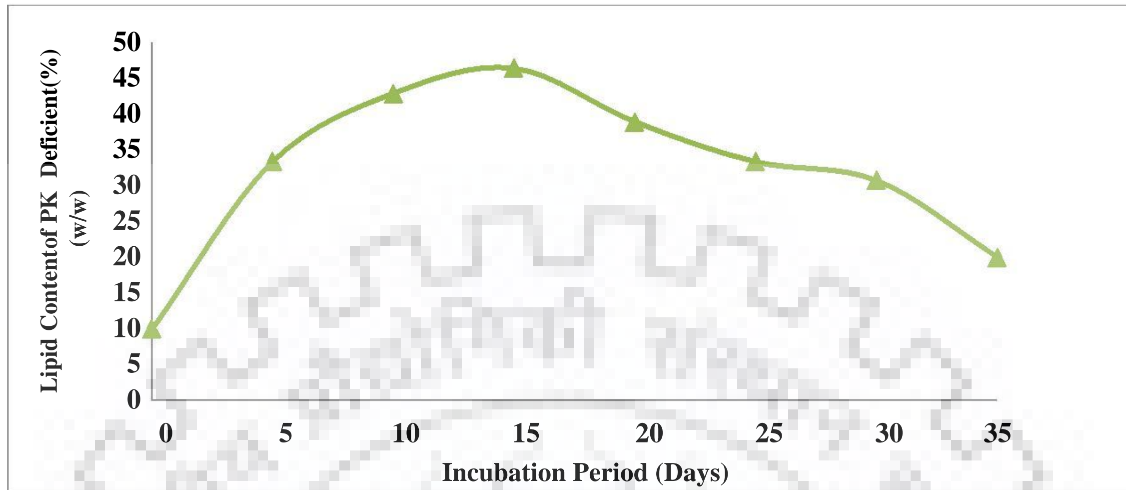

Figure 22: Showing Lipid Content (%) (w/w) in PK deficient N-11 media.

Figure 22 shows Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed maximum at 15th day i.e.46.428% in Phosphorous-Potassium deficient N-11 media.

- **Lipid content in Nitrogen-Phosphorous deficient media**

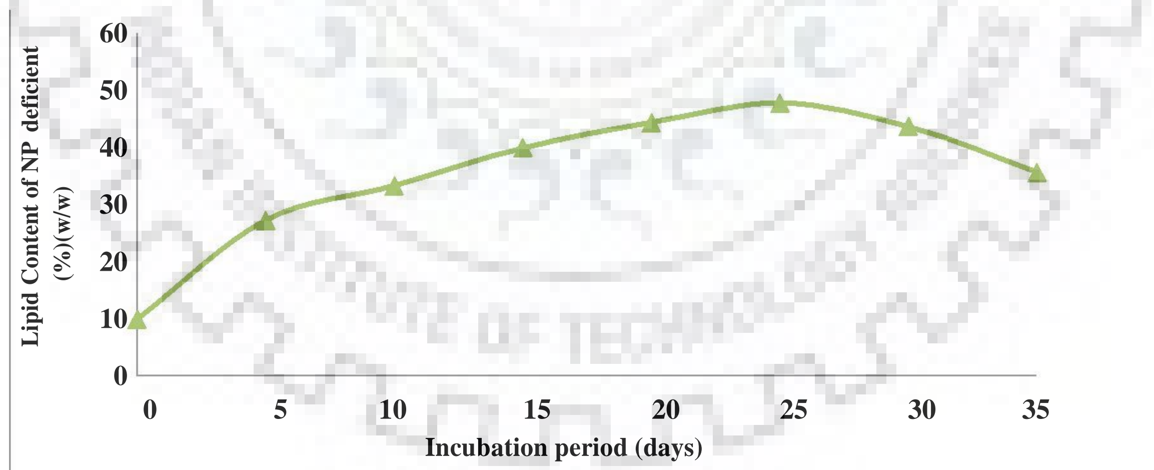

Figure 23: Showing Lipid Content (%) (w/w) in NP deficient N-11 media.

Figure 23 shows Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed maximum at 25th day i.e.47.826% in Nitrogen-Phosphorous deficient N-11 media.

- **Lipid content in Calcium-Iron deficient media**

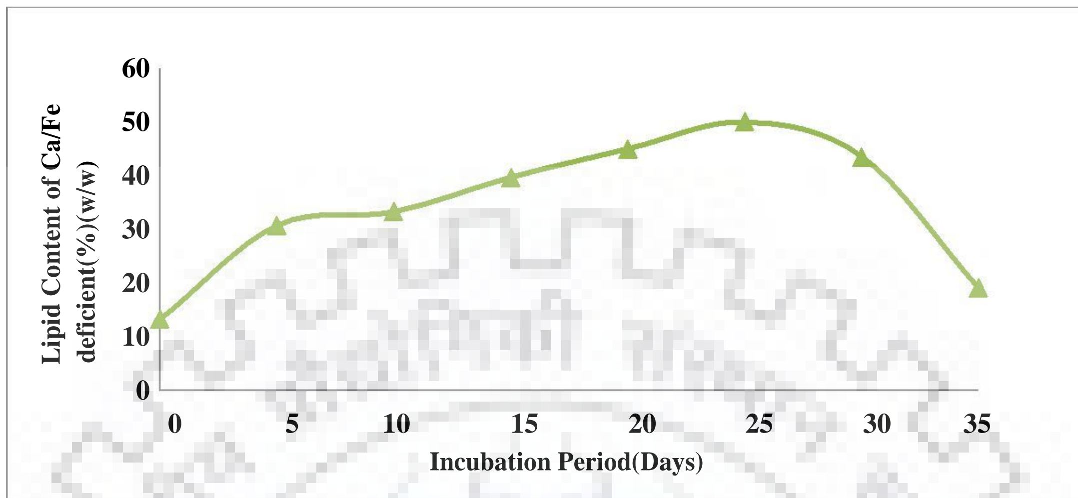

Figure 24: Showing Lipid Content (%) (w/w) in Ca-Fe deficient N-11 media.

Figure 24 depicts the Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed maximum at 25th day i.e.50% in Calcium-Iron deficient N-11 media.

- **Lipid content in Nitrogen-Phosphorous-Potassium deficient media**

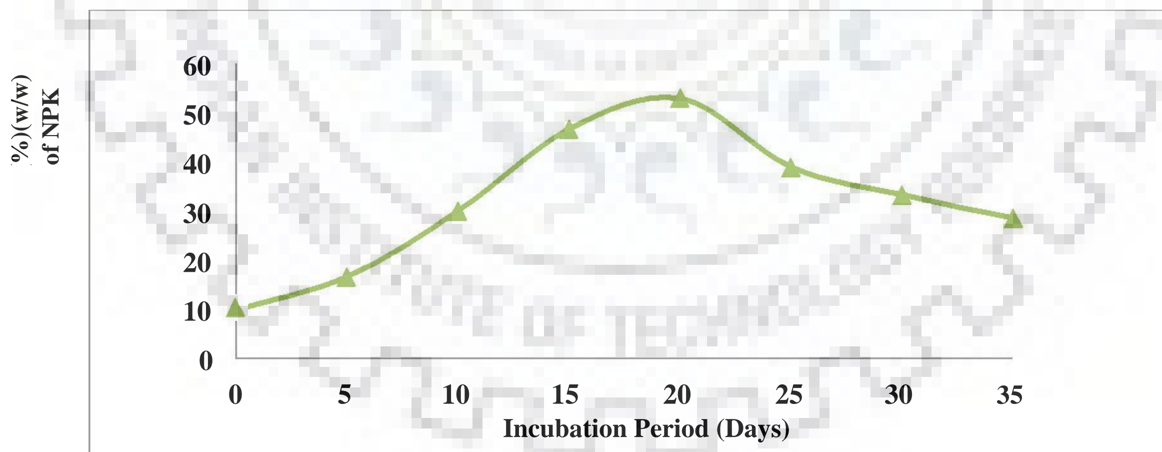

Figure 25: Showing Lipid Content (%) (w/w) in NPK deficient N-11 media.

Figure 25 shows the Lipid Content (%) (w/w) in microalgal species *Scenedesmus obliquus*. Maximum Lipid Content (%) (w/w) was observed maximum at 20th day i.e.52.94% in NPK deficient N-11 media.

- **Comparison of Lipid Content with nutrient enriched and nutrient starved media**

Table 2: Showing Lipid Content in with nutrient enriched and nutrient starved media.

Days	Lipid Content in Control (%)(w/w)	Lipid Content in P deficient (%) (w/w)	Lipid Content in N Deficient (%) (w/w)	Lipid Content in Ca Deficient (%) (w/w)	Lipid content in Potassium deficient (%)(w/w)
0	5	10	10	10	10
5	19	28.57142857	22.22222222	22.22222222	16.66666667
10	27.66	35.13513514	28.57142857	30	25
15	32.58	46.51162791	40.90909091	39.13043478	33.33333333
20	37.82	41.17647059	33.33333333	44	38.0952381
25	41.46	36.36363636	30	48.14814815	45.16129032
30	34.7826087	33.33333333	22.22222222	51.72413793	49.01960784
35	29.4117647	30.76923077	16.66666667	35.71428571	35.71428571

From the above Table 2, we can find that the Lipid Content in *Scenedesmus obliquus* was observed maximum in Calcium deficient N-11 media on 30th day i.e. 51.72%. As compared with the nutrient rich N-11 media to the other deficient stress conditions we can see that Nitrogen deficient media is producing maximum Lipid Content at 15th day i.e.40.90%; Potassium deficient at 15th day i.e.46.51%; and Phosphorous at 15th day i.e.46.51%.

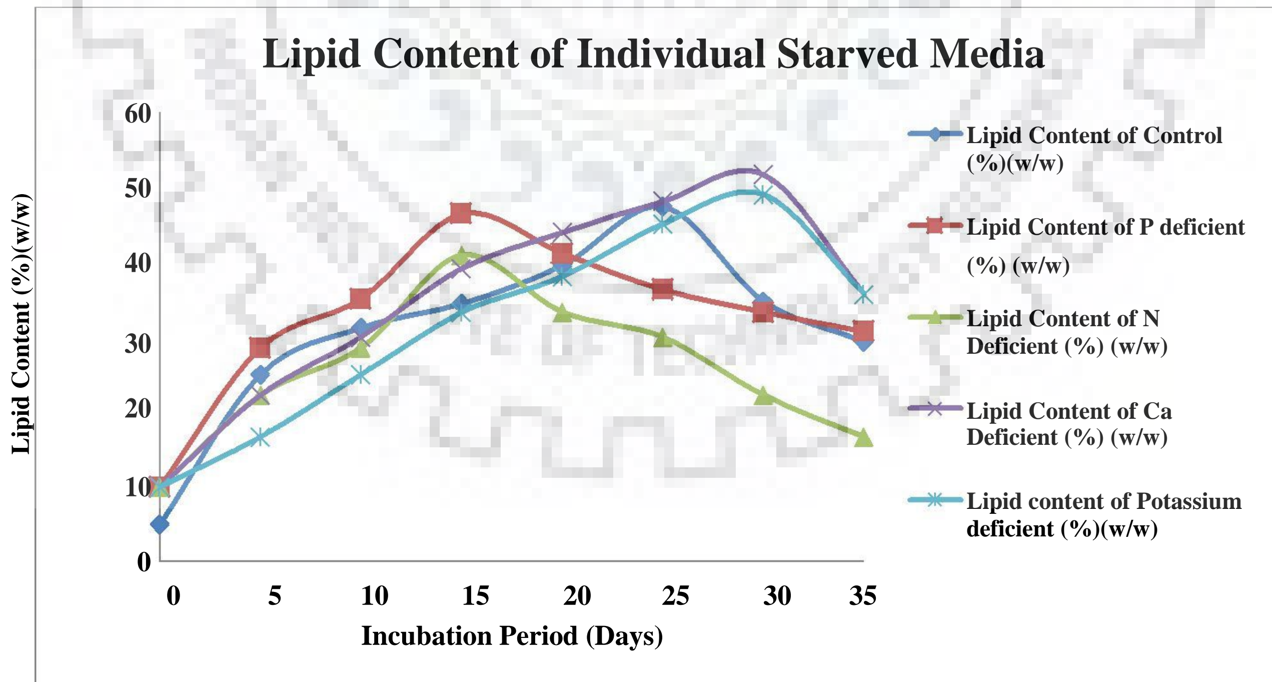

Figure 26: Showing Lipid Content (%) (w/w) in individual starved N-11 media.

- **Effect of combine starved media on Lipid Content**

Table 3: Showing Comparison of Lipid Content with nutrient enriched and combined nutrient starved media.

Days	Lipid Content in NK deficient (%)(w/w)	Lipid content in PN deficient (%) (w/w)	Lipid Content in PK Deficient (%) (w/w)	Lipid Content in NPK Deficient (%)(w/w)	Lipid Content in Ca/Fe deficient (%)(w/w)
0	10	3.333333	10	10	13.3333
5	27.2727	18.18181	33.3333	16.666	30.7692
10	33.3333	30.76923	42.8571	30	33.3333
15	40	41.17647	46.4285	40	39.7222
20	44.444444	48.64864	38.8888	52.941	45
25	47.826086	40.625	33.3333	38.888	50
30	43.75	26.66666	30.7692	33.333	43.4782
35	35.714285	25	20	28.571	19.0909

From the above Table 3, we can find that the Lipid Content in *Scenedesmus obliquus* was observed maximum in Phosphorous-Nitrogen-Potassium deficient N-11 media on 20th day i.e. 52.941 %. As compared with the nutrient rich N-11 media to the other combined nutrient deficient stress conditions we can see that Nitrogen-Potassium deficient media is producing maximum Lipid Content at 20th day i.e. 48.64 %; Potassium-Phosphorous at 15th day i.e. 46.42 %; and Calcium-Iron at 25th day i.e. 50 %.

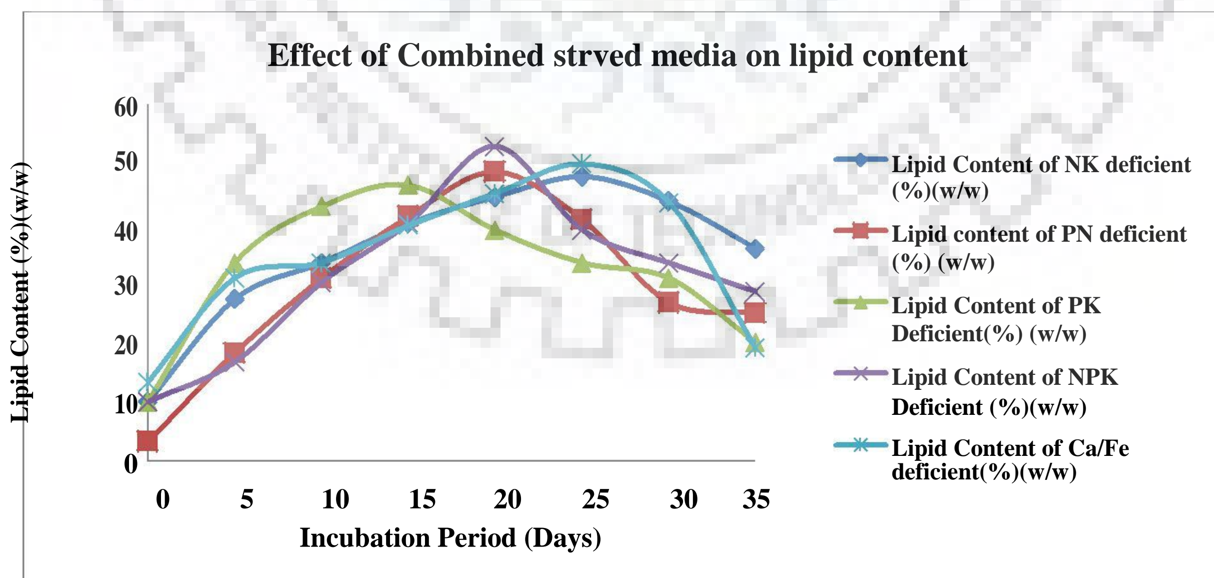

Figure 27: Showing Lipid Content (%) (w/w) in combined starved N-11 media.

4.1.4. FAME Analysis

Table 4: FAME composition of *Scenedesmus obliquus* by transesterification.

Compounds	P deficient	N deficient	K deficient	NPK deficient	PN deficient	Nutrient rich
C 12:1	0.86%	1.31%				1.12%
C 13:0	0.74%					
C 14:1		3.65%				
C 16:0	20.84%	24.35%	1.25%	32.67%	31.25%	35.66%
C 16:1	4.39%	10.19%			5.24%	3.41%
C 17:1	23.84%		25.39%			
C 17:2	1.09%					
C 18:0					3.76%	2.49%
C 18:1	28.53%	29.59%	18.89%	8.67%	12.19%	28.29%
C 18:2	2.11%	13.37%	15.83%		41.23%	7.78%
C 18:3			17.87%	29.14%		
C 19:1	12.46%					
C 20:0	2.34%					
C 20:1	0.59%	2.56%	5.65%	13.94%	2.98%	18.44%
C 21:1	1.01%	1.36%	7.69%			
C 22:1	2.62%	2.11%	6.67%	15.57%		
C 23:1		13.38%			3.15%	
C 27:1	0.46%					
C 36:0		0.70%				
C 8: 1						2.88%

Table 4, shows the composition of various fatty acids i.e. Saturated Fatty Acids (SFA), Unsaturated Fatty Acids i.e. Monounsaturated Fatty Acids (MUFA) and Polyunsaturated Fatty Acids (PUFA) in 6 samples of *Scenedesmus obliquus.*

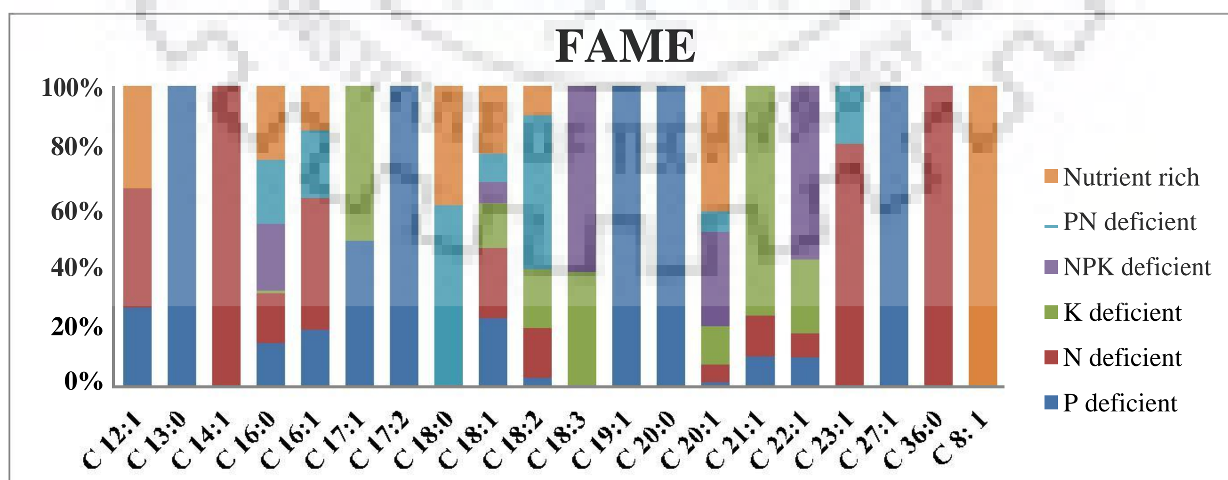

Figure 28: FAME composition of *Scenedesmus obliquus*.

Table 5: Total % of Fatty Acid present in the samples.

Fatty Acids	P deficient	N deficient	K deficient	NPK deficient	PN deficient	Nutrient rich
MUFA	74.76%	60.50%	64.29%	38.1800%	23.56%	54.14%
PUFA	3.20%	13.37%	33.70%	29.14%	41.23%	7.78%
SFA	23.92%	25.05%	1.25%	32.67%	35.01%	38.15%

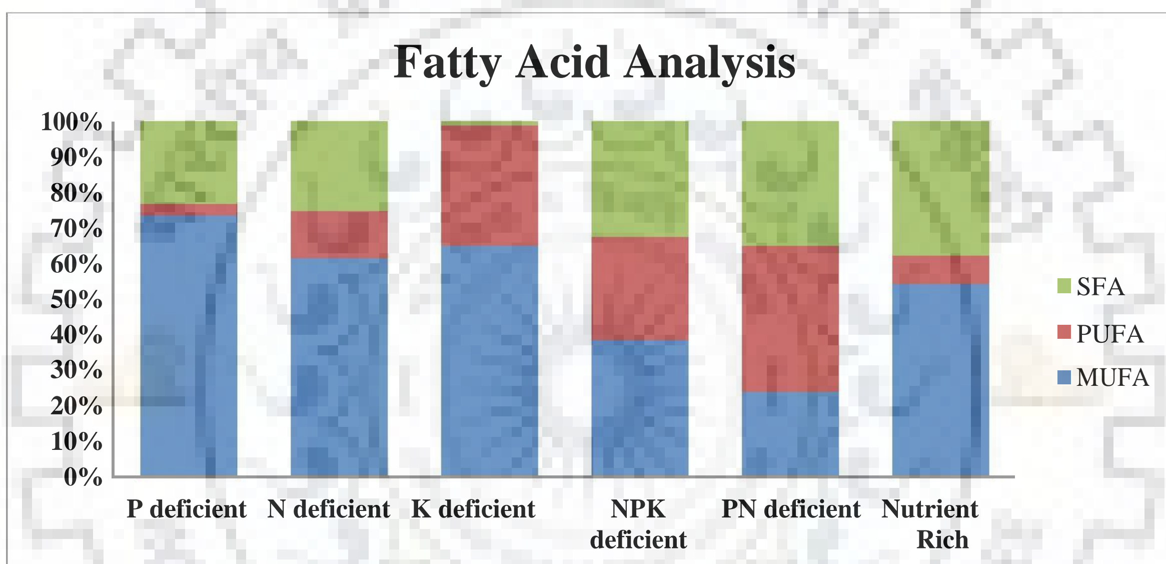

Figure 29: Total Fatty Acid Analysis of 6 samples.

From the above Figure 29 and Table 5 we can say that the maximum amount of Monounsaturated Fatty Acids (MUFA) is present in P deficient i.e. 74.76% followed by Sample 3 i.e. 64.29%, N deficient i.e. 60.40%, and Nutrient rich i.e. 54.14% . The maximum amount of Saturated Fatty Acids (SFA) is present in P deficient i.e. 38.16% followed by PN deficient i.e. 35.01%, NPK deficient i.e. 32.67% and N deficient i.e. 25.05%. The maximum amount of Polyunsaturated Fatty Acids (PUFA) is present in PN deficient i.e. 41.25%. From this, we can say that among all the samples P deficient can be considered the best as it produce adequate amount of MUFA and SFA which is good for the biodiesel followed by Nutrient rich and N deficient, respectively.

4.1.5. Results of Experimental Design

Table 6: Shows the Modeling factors and their response.

Std	Run	Block	Factor1 A:N deficient(g/L)	Factor 2 B: P deficient (g/L)	Factor 3 C: K deficient (g/L)	Response 1 Biomass Yield (g/L)	Response 2 Lipid Production (g/L)
7	1	Block 1	0	10	10	2.53	1.3
8	2	Block 1	10	10	10	2.54	1.06
14	3	Block 1	5	5	13.41	2.28	1.19
19	4	Block 1	5	5	5	2.21	1.22
9	5	Block 1	3.41	5	5	2.51	1.26
12	6	Block 1	5	13.41	5	2.89	1.24
18	7	Block 1	5	5	5	2.52	1.25
3	8	Block 1	0	10	0	2.7	1.24
20	9	Block 1	5	5	5	2.21	1.21
17	10	Block 1	5	5	5	2.21	1.22
1	11	Block 1	0	0	0	2.25	1.11
10	12	Block 1	13.41	5	5	2.64	1.19
13	13	Block 1	5	5	3.41	2.26	1.24
4	14	Block 1	10	10	0	2.54	1.28
11	15	Block 1	5	3.41	5	2.62	1.21
6	16	Block 1	10	0	10	2.63	1.21
5	17	Block 1	0	0	10	2.44	1.27
15	18	Block 1	5	5	5	2.21	1.22
16	19	Block 1	5	5	5	2.26	1.21
2	20	Block 1	10	0	0	2.52	1.25

All the runs were put according to the values showed in all the 3 Factors (N deficient, P deficient and K deficient) as mentioned in the above table at the incubator shaker at $27^{0}C$ for 15 days. Responses (Biomass Yield in g/L and Lipid Production in g/L) were calculated to get the optimum condition for maximum Biomass Yield and Lipid production. From the responses the best condition to get the maximum biomass yield and Lipid Production was Run 6 (5 g N deficient, 13.41 g P deficient and 5 g K deficient) giving 2.52 g/L Biomass Yield and 1.25 g/L Lipid Production.

Response: Biomass Productivity

ANOVA for Response Surface Quadratic Model

Analysis of Variance table (Partial sum of squares)

Table 7: Showing the results of ANOVA

Source	Sum of Squares	DF	Mean Square	F value	Prob >F	
Model	0.67	1	0.075	8.32	0.0014	Significant
A	0.020	1	0.020	2.28	0.1618	
B	0.063	1	0.063	6.97	0.0247	
C	7.961E-003	1	7.961E-003	0.22	0.6501	
A^2	0.15	1	0.15	16.52	0.0023	
B^2	0.39	1	0.39	43.76	<0.0001	
C^2	5.992E-004	1	5.992E-004	0.067	0.8013	
AB	0.047	1	0.047	5.19	0.0460	
AC	1.012E-003	1	1.012E-003	0.11	0.7438	
BC	0.028	1	0.028	3.08	0.1098	
Residual	0.090	10	8.968E-003			
Lack of Fit	0.013	5	2.536E-003	0.16	0.9652	Not Significant
Pure Error	0.077	5	0.015			
Cor Total	0.76	19				

The Model F-value of 8.32 implies the model is significant. There is only a 0.14% chance that a "Model F-value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

In this case, B, $A^{2,}$ $B^{2,}$ AB are significant model terms.

The "Lack of fit F-value" of 0.013 implies that lack of fit is not significant relative to pure error. There is 96.52% chance that a "Lack of fit F-value" this large could occur due to noise. Non-significant lack of fit is good.

Values greater than 0 .1000 indicates that the terms are not significant.

Table 8: Showing the various mathematical results of ANOVA

Std. Dev.	0.095	**R-squared**	0.8822
Mean	2.45	**Adj R-squared**	0.7761
C.V	3.87	**Pred R-squared**	0.7119
PRESS	0.22	**Adeq Precision**	9.243

The “Pred R-squared” of 0.7119 is in reasonable agreement with the “Adj R-squared” of 0.7761.

“Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 9.243 indicates an adequate signal. This model can be used to navigate the design space.

Table 9: Showing the coefficient and standard error.

Factor	Coefficient Estimate	DF	Standard Error	95% Cl Low	95% Cl High	VIF
Intercept	2.27	1	0.039	2.18	2.36	
A-N deficient	0.039	1	0.026	-0.018	0.096	1
B-P deficient	0.068	1	0.026	0.011	0.12	1
C-K deficient	0.012	1	0.026	-0.045	0.069	1

Final Equation in terms of Coded factor:

Biomass Productivity " = $+2.27+0.039*A+0.068*B+0.012*C+0.10*A^2 + 0.17*B^2 - 6.448E\text{-}003*C^2 - (0.076*A*B) + (0.011*A*C) - (0.059*B*C)$

Final equation in terms of Actual Factors:

Biomass Productivity = +2.28884 – 0.019813*N deficient – 0.025477*P deficient +0.014476*K deficient + 4.05542E-003* N $deficient^2$ + 6.60100E-003 * P $deficient^2$ -2.57932E-4 *K $deficient^2$ -3.0500E-003* N deficient*P deficient+4.5000E-004*N deficient*K deficient-2.35000E-003*P deficient*K deficient

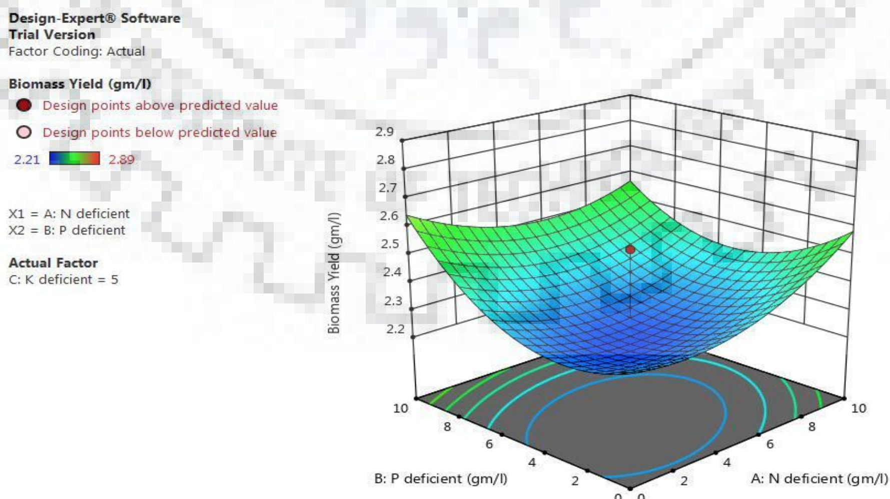

Fig 30: Shows the graph of quadratic model of Biomass Yield at optimum conditions.

Response: Lipid Content

ANOVA for Response Surface 2FI Model

Analysis of Variance table (Partial sum of squares)

Table 10: Showing the results of ANOVA

Source	Sum of Squares	DF	Mean Square	F value	Prob >F	
Model	0.054	6	9.044E-003	43.30	<0.0001	Significant
A	4.138E-0.003	1	4.138E-003	19.81	0.0007	
B	5.991E-004	1	5.991E-004	2.87	0.1141	
C	1.128E-003	1	1.128E-003	5.40	0.0370	
AB	9.800E-003	1	9.800E-003	46.92	<0.0001	
AC	0.029	1	0.029	137.89	<0.0001	
BC	9.800E-003	1	9.800E-003	46.92	<0.0001	
Residual	2.715E-003	13	2.089E-004			
Lack of Fit	1.623E-003	8	2.040E-004	0.94	0.5538	Not Significant
Pure Error	1.083E-003	5	2.167E-004			
Cor Total	0.057	19				

The Model F-value of 43.30 implies the model is significant. There is only a 0.01% chance that a "Model F-value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

In this case, A, C, AB, AC, BC are significant model terms.

Values greater than 0 .1000 indicates that the terms are not significant.

If there are many insignificant model terms (not counting those required to support hierarchy) , model reduction may improve the model.

The "Lack of fit F-value" of 0.94 implies that lack of fit is not significant relative to pure error. There is 55.38% chance that a "Lack of fit F-value" this large could occur due to noise. Non-significant lack of fit is good.

Table 11: Showing the various mathematical results of ANOVA

Std. Dev.	0.014	R-squared	0.9523
Mean	1.22	Adj R-squared	0.9304
C.V	1.19	Pred R-squared	0.8065
PRESS	0.011	Adeq Precision	26.294

The “Pred R-squared” of 0.8065 is in reasonable agreement with the “Adj R-squared” of 0.9304.

“Adeq Precision” measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 26.294 indicates an adequate signal. This model can be used to navigate the design space.

Table 12: Showing the coefficient and standard error.

Factor	Coefficient Estimate	DF	Standard Error	95% Cl Low	95% Cl High	VIF
Intercept	1.22	1	3.232E-003	1.21	1.23	
A-N deficient	-0.017	1	3.911E-003	-0.026	-8.958E-003	1
B-P deficient	6.623E-003	1	3.911E-003	-1.825E-003	0.015	1
C-K deficient	-9.086E-003	1	5.110E-003	-0.018	-6.376E-004	1
AB	-0.035	1	5.110E-003	-0.046	-0.024	1
AC	-0.060	1	5.110E-003	-0.071	-0.049	1
BC	-0.035	1	5.110E-003	-0.046	-0.024	1

Final Equation in terms of Coded factor:

Lipid Content, = +1.22 -0.017*A + 6.623E-003*B – 9.086E-003*C – 0.035*A*B - 0.060*A*C – 0.035*B*C

Final equation in terms of Actual Factors:

Lipid Content, = +1.10887 + 0.015519*N deficient + 0.015325*P deficient + 0.017183*K deficient – 1.40000E-003* N deficient * P deficient^2 -2.40000E-003* N deficient*P deficient - 1.40000E-003 *P deficient *K deficient

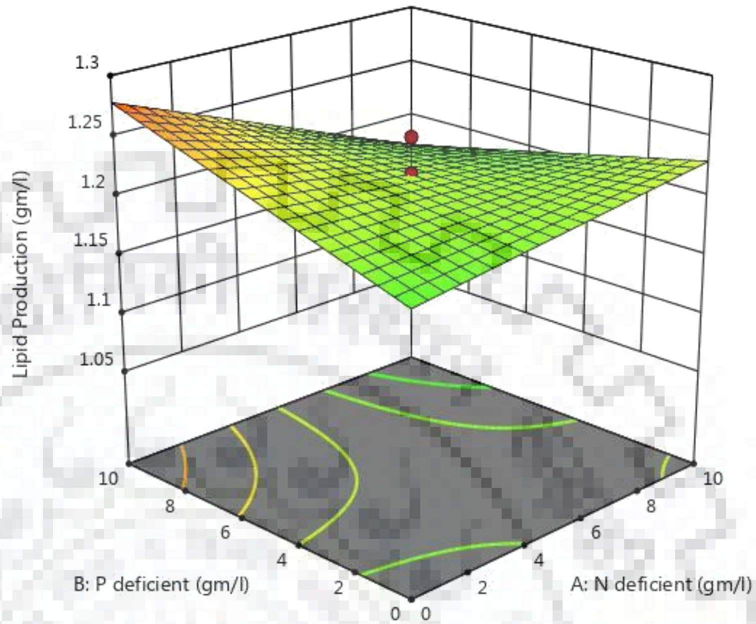

Fig 31: Shows the graph of 2FI model of Lipid Production at optimum conditions.

From the above Response results and graphs, we can conclude that the best fit model for Response 1: Biomass Production (g/L) was Quadratic model. The F value for this model was found to be 8.32 which was significant for this model. The best fit model for Response 2: Lipid Production (g/L) was 2FI mode. The F value for this model was 43.30 which was significant to this model.

4.2. Waste Water Treatment

4.2.1. Characterization of grey water

Table 13: Showing the characterization of grey water sample collected from Cautley Bhawan.

Parameters	Average Value
pH	9.7
TDS (mg/l)	1711
Nitrate (mg/l)	110
Phosphorous (mg/l)	2
Iron (mg/l)	1.5
Chemical Oxygen Demand (COD)(mg/l)	1350
Biochemical Oxygen Demand (BOD)(mg/l)	145

4.2.2. Characterization of grey water after *S. obliquus* inoculation (Secondary culture)

Table 14: Showing the characterization of grey water sample after the secondary inoculation of *S. obliquus*

Parameters	Average Value
pH	8.3
TDS (mg/l)	893
Nitrate (mg/l)	50
Phosphorous (mg/l)	0.7
Iron (mg/l)	0.5
Chemical Oxygen Demand (COD)(mg/l)	275
Biochemical Oxygen Demand (BOD)(mg/l)	40

4.2.3. Characterization of grey water after *S. obliquus* inoculation (Primary culture)

Table 15: Showing the characterization of grey water sample after the direct inoculation of *S. obliquus*

Parameters	Average Value
pH	8.2
TDS (mg/l)	897
Nitrate (mg/l)	45
Phosphorous (mg/l)	0.8
Iron (mg/l)	0.7
Chemical Oxygen Demand (COD)(mg/l)	289
Biochemical Oxygen Demand (BOD)(mg/l)	45

From the above mentioned data we can say that after the secondary inoculation of ***S. obliquus*** as well as after the primary inoculation of ***S. obliquus***, all the parameters of grey water decreased and reached to the range which is close to the drinking water or we can say clean water.

From the above results we can observe that the *Scenedesmus obliquus* microalgal strain can treat the grey water in an efficient and effective manner in both the primary as well as the secondary inoculation. Only small difference among the various parameters was observed when we compare the inoculation process. From the above results, we found that the grey water pH was reduced from 9.7 to 8.3 in secondary inoculation and 8.2 in primary inoculation. The TDS reduced from 1711 mg/L to 893 mg/L and 897 mg/L in secondary and primary inoculation, respectively. The Nitrate value decreased to 50 mg/L and 45 mg/L from 110 mg/L. The phosphorous reduced from 2 mg/L to 0.7 mg/L and 0.8 mg/L. The Iron content reduced from 1.5 mg/L to 0.5 mg/L and 0.8 mg/L. The Chemical Oxygen Demand (COD) reduced from 1350 mg/L to 275 mg/L in secondary inoculation and 289 mg/L in primary inoculation. The Biochemical Oxygen Demand (BOD) was reduced to 40 mg/ L in secondary inoculation and 45 mg/L in primary inoculation from 145 mg/L in grey water sample.

So, we can say that the microalgal strain *Scenedesmus obliquus* has the great potential to treat the grey water to make it cleaner so that it can be reused to fulfill the water requirements.

4.3. Isolation, Screening and Identification of microalgal species collected from Yamuna water

4.3.1. Plating

Fig 32: Showing the images of streaking of various samples collected from Yamuna water sample for isolation of microalgal strain.

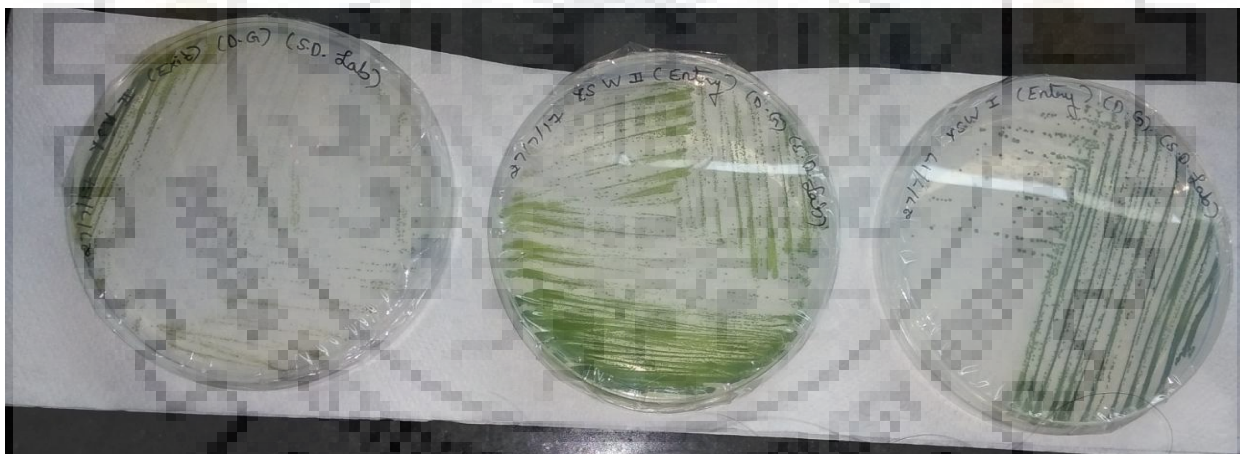

Fig 33: Showing the images of streaking of various samples collected from Yamuna water sample for isolation of microalgal strain.

Figure 32 and Figure 33 shows the streak plates from which the pure colony of algal strain was collected and again inoculated in N-11 agar plates to get the pure colonies.

4.3.2. Screening of best samples

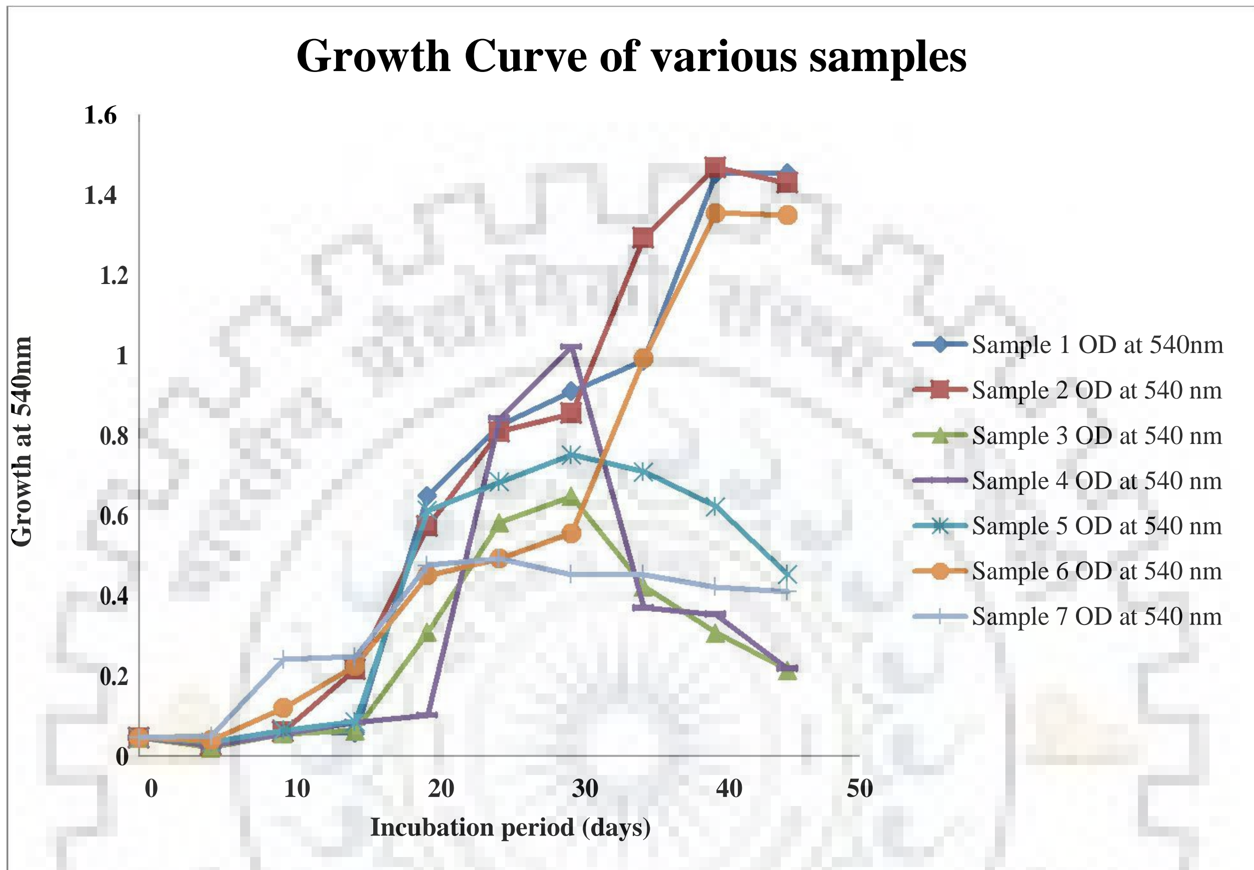

Figure 34: Growth curve of various samples collected from Yamuna River

Figure 34 shows the growth curve of various water samples which were collected to check their growth in N-11 media. We can see that these microalgal strain samples have shown a longer growth period of 50 days. Sample 1, Sample 2, Sample 4, Sample 5 & Sample 6 have shown better growth in the media for longer period of time as compared to Sample 3 and Sample 7. Among them Sample 2 and Sample 4 were considered for DNA sequencing for further process.

4.3.3. Gel electrophoresis

Figure 35: Gel run image of YRS 2.

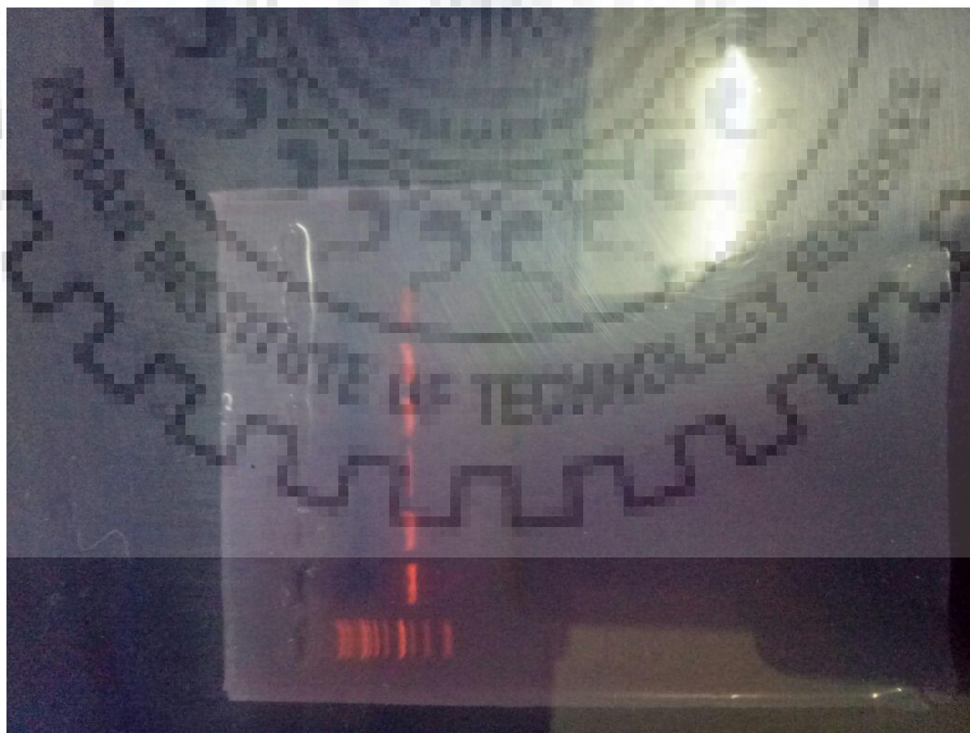

Figure 36: Gel run image of YRS 4.

4.3.4. DNA Sequencing

- **Forward FASTA Sequence of sample 5**

>SA7 ARKRMCTYYCTTCSRAGRCWTTKGCCAAGGATGTTTTCATTARTCAAGAA

CGAAAGTTGGGGGCTCGAAGACRATYAKWTACCGTCCTAGTCTCAACCAT

AAACGATGCCGACTAGGGATCGGCGGATGTTTCTTCGATGACTCCGCCGG

CACCTTATGAGAAATCAAAGTTTTTGGGTTCCGGGGGGAGTATGGTCGCA

AGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGKGGAGCC

TGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGACATAG

TGAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGTGGTGGTGC

ATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTCAGGTTGATTCCGGTAACG

AACGAGACCTCAGCCTGCTAAATAGTCACGGTTGGTTCGCCAGCCGGCGG

ACTTCTTAGAGGGACTATTGGCGACTAGCCAATGGAAGCATGAGGSAATR

ASRGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTG

ATGCATTCAACGAGCCTAGCCTTGGCCGAGAGGCCCGGGTAATCTTTGAA

ACTGCATCGTGATGGGGATAGATTATTGCAATTATTAATCTTCAACGAGG

AATGCCTAGTAAGCGCAAGTCATCAGCTTGCGTKGATTACGTCCCCTGCC MMT

- **Reverse FASTA sequence of sample 5**

>SA8 CGKTCAGCGCTMTAGGCATTCCTCGTTGAGATTAATAATTGCAATAATCT

ATCCCCATCACGATGCAGTTTCAAAGATTACCCGGGCCTCTCGGCCAAGG

CTAGGCTCGTTGAATGCATCAGTGTAGCGCGCGTGCGGCCCAGAACATCT

AAGGGCATCACAGACCTGTTATTGCCTCATGCTTCCATTGGCTAGTCGCC

AATAGTCCCTCTAAGAAGTCCGCCGGCTGGCGAACCAACCGTGACTATTT

AGCAGGCTGAGGTCTCGTTCGTTACCGGAATCAACCTGACAAGGCAACCC

ACCAACTAAGAACGGCCATGCACCACCACCCATAGAATCAAGAAAGAGCT

CTCAATCTGTCAATCCTCACTATGTCTGGACCTGGTAAGTTTTCCCGTGT

TGAGTCAAATTAAGCCGCAGGCTCCACGCCTGGTGGTGCCCTTCCGTCAA

TTCCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCGGAACCCAAAAAC

TTTGATTTCTCATAAGGTGCCGGCGGAGTCATCGAAGAAACATCCGCCGA

TCCCTAGTCGGCATCGTTTATGGTTGAGACTAGGACGGTATCTAATCGTC

**TTCGAGCCCCCAACTTTCGTTCTTGATTAATGAAAACATCCTTGGCAAATGCTTTCGCAGTAGTTCGTCTTTCATAAATCCA
AGAATTTACCCCTCTGACA**

4.3.5. BLAST

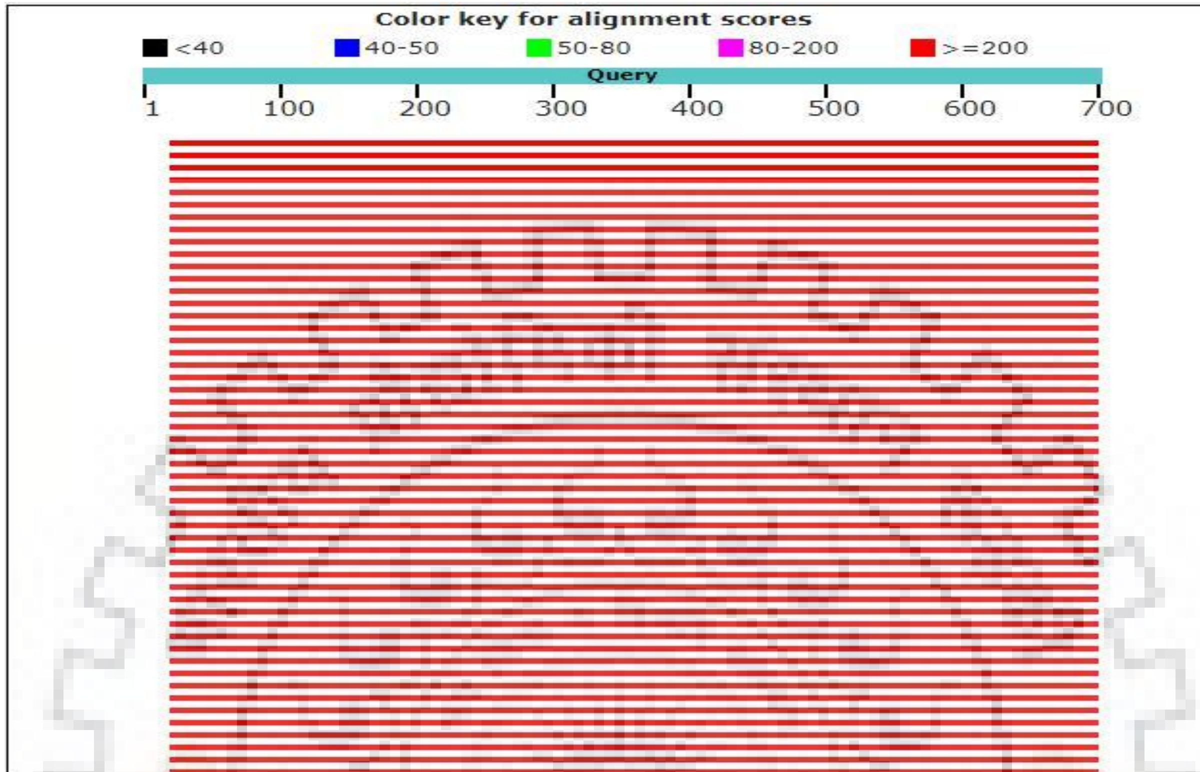

Fig 37: Shows the BLAST results from NCBI.

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Chlorella sorokiniana strain NZmm3W1 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KY054944.1
Chlorella sorokiniana voucher BR001 small subunit ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KY303731.1
Chlorella sorokiniana isolate 19-4 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KU948990.1
Chlorella sorokiniana strain KAS908 isolate 2 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KT886083.1
Chlorella sorokiniana strain KLL-G018 clone c 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spa	1205	1205	96%	0.0	98%	KP726220.1
Chlorella sorokiniana 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KP771817.1
Micractinium reisseri clone EdL_Cl1_MAF 18S ribosomal RNA gene and internal transcribed spacer 1, partial sequence	1205	1205	96%	0.0	98%	KF887344.1
Chlorella sp. GC 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KF773743.1
Chlorella sorokiniana strain SAG 211-31 18S ribosomal RNA gene, complete sequence	1205	1205	96%	0.0	98%	KF673387.1
Chlorella sorokiniana strain B4Riv 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KJ173792.1
Chlorella sorokiniana strain A102 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KF981996.1
Chlorella sorokiniana strain LB75 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KJ616757.1
Chlorella sorokiniana strain LB65 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KJ616755.1
Chlorella sorokiniana 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KJ149805.1
Chlorella sp. T302 18S ribosomal RNA gene, partial sequence	1205	1205	96%	0.0	98%	KF879597.1

Fig 38: Shows the BLAST results having Identity % from NCBI.

From the DNA sequencing results, we have found that the microalgal sample which we have isolated shows 98% identity with ***Chlorella sorokiniana***. ***Chlorella sorokiniana*** is a freshwater green microalga with a characteristic emerald-green color and pleasant grass odor. Its cell division rate is quite fast and divides into four new cells every 17 to 24 hours. This microalga strain also has the potential to produce biodiesel.

CHAPTER 5

CONCLUSION

Scenedesmus obliquus strain was grown in the N-11 media and its growth has been observed in various nutrient deficient conditions. The amount of biomass yield was found 1.56 g/L and lipid content was 41.46 % on 25th day in nutrient rich N-11 media. Different patterns of growth curve, Biomass yield and Lipid content was calculated under different deficient conditions. Biomass yield from *Scenedesmus obliquus* was observed maximum in Phosphorous deficient N-11 media on 15th day i.e. 2.58 g/L. Biomass yield from *Scenedesmus obliquus* was observed maximum in Phosphorous-Nitrogen deficient N-11 media on 20th day i.e. 0.74 g/L. Lipid Content in *Scenedesmus obliquus* was observed maximum in Phosphorous-Nitrogen-Potassium deficient N-11 media on 20th day i.e. 52.941 %. Lipid Content in *Scenedesmus obliquus* was observed maximum in Calcium deficient N-11 media on 30th day i.e. 51.72%.

From the FAME analysis we found that the maximum amount of Monounsaturated Fatty Acids (MUFA) is present in P deficient (74.76%), followed by K deficient (64.29%), N deficient (60.40%), and Nutrient Rich (54.14 %.) The maximum amount of Saturated Fatty Acids (SFA) is present in Nutrient Rich (38.16%) followed by PN deficient (35.01%), NPK deficient (32.67%) and N deficient (25.05%). The maximum amount of Polyunsaturated Fatty Acids (PUFA) is present in PN deficient i.e. 41.25%. From this, we can say that among all the samples P deficient can be considered the best as it produce adequate amount of MUFA and SFA which is good for the biodiesel followed by Nutrient rich and N deficient, respectively.

By using Response Surface Method (RSM), we found the optimum condition to get the high yield of biomass and Lipid production at particular stress condition. The optimum condition (5 g N deficient, 13.41 g P deficient and 5 g K deficient) was giving 2.52 g/L Biomass Yield and 1.25 g/L Lipid Production. The ANOVA shows quadratic model for Biomass Yield and 2FI model for Lipid Production.

Various tests were performed to check the different parameters of grey water and effect on parameters when *Scenedesmus obliquus* was inoculated. After the secondary inoculation of ***S. obliquus*** as well as after the primary inoculation of ***S. obliquus***, all the parameters of grey water decreased and reached to the range which is close to the drinking water or we can say clean water.

The algal samples collected from Yamuna River were grown in N-11 media. 7 different strains were isolated by streaking and then these colonies were grown in N-11 media to observe their growth to select most suitable microalgae for biomass yield. The selected strain was identified as C*hlorella sorokininana* by 18 s RNA sequencing. Further 18 s RNA sequence was deposited to NCBI under the accession no- …………

CHAPTER 6

REFERENCES

[1]. Blight, E.G. and Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian Journal of Biotechnology and Physiology 37, 911-917.

[2]. Brune, D.E., Lundquist, T.J., Benemann, J.R., 2009. Microalgal biomass for greenhouse gas reductions: Potential for replacement of fossil fuels and animal feeds. J. Environ. Eng. 135, 1136–1144.

[3]. Darley, W.M., 1982. Algal Biology; A physiological Approach. Basic Microbiology, vol. 9. Blackwell Scientific Publications, Oxford

[4]. Oswald, W.J., 1988a. Large-scale algal culture systems (engineering aspects). In: orowitzka, M.A., Borowitzka, L.J. (Eds), Micro-Algal Biotechnology, Cambridge Univ. Press, Cambridge, pp. 357– 394.

[5]. Oswald, W.J., 1988b. Micro-algae and wastewater treatment. In: Borowitzka, M.A., Borowitzka, L.J. (Eds.), Micro-algal Biotechnology, Cambridge Univ. Press, pp. 305–328.

[6]. Tam, N.F.Y., Wong, Y.S., 1989. Wastewater nutrient removal by Chlorella pyrenoidosa and Scenedesmus sp. Environ. Pollut. 58, 19– 34.

[7]. Sialve B, Bernet N, Bernard O. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol Adv 2009;27:409–16.

[8]. Chisti Y. Response to Reijnders: do biofuels from microalgae beat biofuels from terrestrial plants? Trends Biotechnol 2008;26:351–2.

[9]. Tredici MR. Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. Biofuels 2010;1:143–62.

[10]. Norsker NH, Barbosa MJ, Vermuë MH, Wijffels RH. Microalgal production – a close look at the economics. Biotechnol Adv 2011;29:24–7.

[11]. Chisti Y. Constraints to commercialization of algal fuels. J Biotechnol 2013;167:201–14.

[12]. Williams PJLB, Laurens LML. Microalgae as biodiesel & biomass feedstocks:review & analysis of the biochemistry, energetics & economics. Energy Environ Sci 2010;3:554–90.

[13]. Chen CY, Yeh KL, Aisyah R, Lee DJ, Chang JS. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: a critical review. Bioresour Technol 2011; 102:71–81.

[14]. Talebian-Kiakalaieh A, Amin NAS, Mazaheri H. A review on novel processes of biodiesel production from waste cooking oil. Appl Energy 2013; 104:683–710.

[15]. Liu CZ, Wang F, Stiles AR, Guo C. Ionic liquids for biofuel production:opportunities and challenges. Appl Energy 2012; 92:406–14.

[16]. Balat M, Balat H. Recent trends in global production and utilization of bioethanol fuel. Appl Energy 2009; 86:2273–82.

[17]. Tian ZY, Chafik T, Assebban M, Harti S, Bahlawane N, Mountapmbeme Kouotou P, et al. Towards biofuel combustion with an easily extruded clay as a natural catalyst. Appl Energy 2013; 107:149–56.

[18]. Rawat I, Ranjith Kumar R, Mutanda T, Bux F. Dual role of microalgae:phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Appl Energy 2011; 88:3411–24.

[19]. McGinn PJ, Dickinson KE, Bhatti S, Frigon JC, Guiot SR, O'Leary SJB. Integration of microalgae cultivation with industrial waste remediation for biofuel and bioenergy production: opportunities and limitations. Photosynthetic Resource 2011; 109:231–47.

[20] Takagi M, Karseno, Yoshida T. Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae Dunaliella cells. J Biosci Bioeng 2006; 101:223–6.

[21] Daroch M, Geng S, Wang G. Recent advances in liquid biofuel production from algal feedstocks. Appl Energy 2013; 102:1371–81.

[22] Darzins A, Pienkos P, Edye L. Current status and potential for algal biofuels production. BioIndustry Partnes & NREL: Bioenergy Task; 2010. 131.

[23] Mulbry W, Kondrad S, Pizarro C, Kebede-Westhead E. Treatment of dairy manure effluent using freshwater algae: algal productivity and recovery of manure nutrients using pilot-scale algal turf scrubbers. Bioresour Technology.2008; 99:8137–42.

[24] Olguín EJ. Phycoremediation: key issues for cost-effective nutrient removal processes. Biotechnol Adv 2003; 22:81–91.

[25] Lam MK, Lee KT. Microalgae biofuels: a critical review of issues, problems and the way forward. Biotechnol Adv 2012; 30:673–90.

[26] Yanagi M, Watanabe Y, Saiki H. CO2 fixation by Chlorella sp. HA-1 and its utilization. Energy Convers Manage 1995;36:713–6.

[27] Kishimoto M, Okakura T, Nagashima H, Minowa T, Yokoyama SY, Yamaberi K.

CO2 fixation and oil production using micro-algae. J Ferment Bioeng 1994;78:479–82.

[28] Dalrymple OK, Halfhide T, Udom I, Gilles B, Wolan J, Zhang Q, et al.Wastewater use in algae production for generation of renewable resources: a review and preliminary results. Aquat Biosyst 2013;9.

[29] Peccia J, Haznedaroglu B, Gutierrez J, Zimmerman JB. Nitrogen supply is an important driver of sustainable microalgae biofuel production. Trends Biotechnol 2013; 31:134–8.

[30] Yang J, Xu M, Zhang X, Hu Q, Sommerfeld M, Chen Y. Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. Bioresour Technol 2011; 102:159–65.

[31] Leite GB, Abdelaziz AEM, Hallenbeck PC. Algal biofuels: challenges and opportunities. Bioresour Technol 2013; 145:134–41.

[32] Bilanovic D, Holland M, Armon R. Microalgal CO2 sequestering – modeling microalgae production costs. Energy Convers Manage 2012; 58:104–9.

[33] Christenson L, Sims R. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. Biotechnol Adv 2011; 29:686–702.

[34] Sialve B, Bernet N, Bernard O. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol Adv 2009; 27:409–16.

[35] Chisti Y. Response to Reijnders: do biofuels from microalgae beat biofuels from terrestrial plants? Trends Biotechnol 2008; 26:351–2.

[36] Tredici MR. Photobiology of microalgae mass cultures: understanding the tools for the next green revolution. Biofuels 2010; 1:143–62.

[37] Norsker NH, Barbosa MJ, Vermuë MH, Wijffels RH. Microalgal production – a close look at the economics. Biotechnol Adv 2011; 29:24–7.

[38] Chisti Y. Constraints to commercialization of algal fuels. J Biotechnol 2013;167:201–14.

[39] Lam MK, Lee KT. Potential of using organic fertilizer to cultivate Chlorella vulgaris for biodiesel production. Appl Energy 2012; 94:303–8.

[40] Mallick N. Biotechnological potential of immobilized algae for wastewater N, P and metal removal: a review. Biometals 2002; 15:377–90.

[41] Bank W. World Development Indicators 2012. World Bank Publications; 2012.

[42] de la Noüe J, Laliberté G, Proulx D. Algae and waste water. J Appl Phycol 1992;4:247–54.

[43] Martijn EJ, Redwood M. Wastewater irrigation in developing countries – limitations for farmers to adopt appropriate practices. Irrig Drain 2005;54:S63–70.

[44] Correll DL. The role of phosphorus in the eutrophication of receiving waters: a review. J Environ Qual 1998;27:261–6.

[45] Anderson DM, Glibert PM, Burkholder JM. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 2002;25:704–26.

[46] Diaz RJ, Rosenberg R. Spreading dead zones and consequences for marine ecosystems. Science 2008;321:926–9.

[47] Cai T, Park SY, Li Y. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew Sust Energy Rev 2012;19:360–9.

[48] Flotats X, Bonmatí A, Fernández B, Magrí A. Manure treatment technologies: on-farm versus centralized strategies. NE Spain as case study. Bioresource Technol 2009; 100:5519–26.

[49] Lam MK, Lee KT. Immobilization as a feasible method to simplify the separation of microalgae from water for biodiesel production. Chem Eng J 2012;191:263–8.

[50] Zhou W, Min M, Li Y, Hu B, Ma X, Cheng Y, et al. A hetero-photoautotrophic two-stage cultivation process to improve wastewater nutrient removal and enhance algal lipid accumulation. Bioresour Technol 2012;110:448–55.

[51] Wang L, Min M, Li Y, Chen P, Chen Y, Liu Y, et al. Cultivation of green algae Chlorella sp. in different wastewaters from municipal wastewater treatment plant. Appl Biochem Biotechnol 2010;162:1174–86.

[52] Samorì G, Samorì C, Guerrini F, Pistocchi R. Growth and nitrogen removal capacity of Desmodesmus communis and of a natural microalgae consortium in a batch culture system in view of urban wastewater treatment: Part I. Water Res 2013;47:791–801.

[53] Salama ES, Kim HC, Abou-Shanab RAI, Ji MK, Oh YK, Kim SH, et al. Biomass, lipid content, and fatty acid composition of freshwater Chlamydomonas mexicana and Scenedesmus obliquus grown under salt stress. Bioprocess Biosyst Eng 2013;36:827–33.

[54] Singh A, Olsen SI. A critical review of biochemical conversion, sustainability and life cycle assessment of algal biofuels. Appl Energy 2011;88:3548–55.

[55] Obaja D, MacÉ S, Mata-Alvarez J. Biological nutrient removal by a sequencing batch reactor (SBR) using an internal organic carbon source in digested piggery wastewater. Bioresour Technol 2005;96:7–14.

[56] Bich NN, Yaziz MI, Bakti NAK. Combination of Chlorella vulgaris and Eichhornia crassipes for wastewater nitrogen removal. Water Res 1999;33:2357–62.

[57] Lopez, C.V.G., Fernandez, F.G.A., Sevilla, J.M.F., Fernandez, J.F.S., Garcia, M.C.C., Grima, E.M., 2009. Utilization of the cyanobacteria Anabaena sp ATCC 33047 in CO2 removal processes. Biores. Technol. 100 (23), 5904–5910.

[58] Mandal, S., Mallick, N., 2009. Microalga Scenedesmus obliquus as a potential source for biodiesel production. Appl. Microbiol. Biotechnol. 84 (2), 281–291.

[59] Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renew. Sust. Energ. Rev. 14 (1), 217–232.

[60] Matsunaga, T., Matsumoto, M., Maeda, Y., Sugiyama, H., Sato, R., Tanaka, T., 2009. Characterization of marine microalga, Scenedesmus sp strain JPCC GA0024 toward biofuel production. Biotechnol. Lett. 31 (9), 1367–1372.

[61] Guo, F.J., Wang, H., Wang, J.F., Zhou, W.J., Gao, L.L., Chen, L., Dong, Q.Z., Zhang, W., Liu, T.Z., 2014. Special biochemical responses to nitrogen deprivation of filamentous oleaginous microalgae Tribonema sp. Bioresour. Technol. 158, 19–24.

[62] Illman, A.M., Scragg, A.H., Shales, S.W., 2000. Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme Microb. Technol. 27, 631–635.

[63] Knothe, G., 2009. Improving biodiesel fuel properties by modifying fatty eater composition. Energy Environ. Sci. 2, 759–766.

[64] Knothe, G., 2005. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process. Technol. 86, 1059–1070.

[65] Li, Y., Fei, X., Deng, X., 2012. Novel molecular insights into nitrogen starvation induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae Micractinium pusillum. Biomass Bioenergy 42, 199–211.

[66] Lin, Q., Lin, J., 2011. Effect of nitrogen source and concentration on biomass and oil production of a Scenedesmus rubescens like microalga. Bioresour. Technol. 102, 1615–1621.

[67] Park, J.B.K., Craggs, R.J., Shilton, A.N., 2011. Wastewater treatment high rate algal ponds for biofuel production. Bioresour. Technol. 102, 35–42.

[68] Prathima Devi, M., Venkata Subhash, G., Venkata Mohan, S., 2012. Heterotrophic cultivation of mixed microalgae for lipid accumulation and wastewater treatment during

sequential growth and starvation phases: effect of nutrient supplementation. Renew. Energy 43, 276–283.

[69] Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L., Pérez, A., 2009. Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour.Technol. 100, 261–268.

[70] Ruangsomboon, S., Ganmanee, M., Choochote, S., 2013. Effects of different nitrogen, phosphorus, and iron concentrations and salinity on lipid production in newly isolated strain of the tropical green microalga, Scenedesmus dimorphus KMITL. J.Appl. Phycol. 25, 867–874.

[71] Ruiz, J., Arbib, Z., Álvarez-Díaz, P.D., Garrido-Pérez, C., Barragán, J., Perales, J.A., 2014. Influence of light presence and biomass concentration on nutrient kinetic removal from urban wastewater by Scenedesmus obliquus. J. Biotechnol. 178, 32–37.

[72] Hili AM, Feinberg DA. Fuel from microalgae: lipid products, energy from biomass: building on a generic technology base. In: Second technical review meeting; 1984.

[73] Kulay LA, Silva GA. Comparative screening LCA of agricultural stages of soy and castor beans. In: 2nd international conference on life cycle management – LCM2005; 2005. pp. 5–7.

[74] Mobius BioFuels. What is biodiesel? Mobius Biofuels, LLC; 2008. Accessed in December at: http://www.mobiusbiofuels.com/biodiesel.htm.

[75] Nielsen DC. Oilseed productivity under varying water availability. In: Proceedings of 20th annual central plains irrigation conference and exposition; 2008. pp. 30–33.

[76] Zappi M, Hernandez R, Sparks D, Horne J, Brough M, Swalm DC, et al. A review of the engineering aspects of the biodiesel industry. MSU E-TECH Laboratory Report ET-03-003; 2003.

[77] Bahadur NP, Boocock DGB, Konar SK. Liquid hydrocarbons from catalytic pyrolysis of sewage sludge lipid and canola oil: evaluation of fuel properties. Energy Fuels 1995;9:248–56.

[78] Babu BV. Biomass pyrolysis: a state-of-the-art review. Biofuels Bioproducts Biorefinin 2008;2(5):393–414.

[79] Boateng AA,Mullen CA, Goldberg N, Hicks KB, Jung HJG, Lamb JFS. Production of bio-oil from alfalfa stems by fluidized-bed fast pyrolysis. Industrial and Engineering Chemistry Research 2008;47:4115–22.

[80] Maxwell EL, Folger AG, Hogg SE. Resource evaluation and site selection for microalgae production systems. SERI/TR-215-2484; May 1985.

[81] Gressel J. Transgenics are imperative for biofuel crops. Plant Science 2008;174(3):246–63.

[82] De Pauw N, Morales J, Persoone G. Mass culture of microalgae in aquaculture systems: progress and constraints. Hydrobiologia 1984;116/117: 121–34.

[83] Grima EM, Belarbi EH, Fernandez FGA, Medina AR, Chisti Y. Recovery of microalgal biomass and metabolites: process options and economics. Biotechnol Adv 2003;20:491–515.

[84] Janaun J, Ellis N. Perspectives on biodiesel as a sustainable fuel. Renew Sust Energy Rev 2010;14:1312–20.

[85] Patil V, Tran K-Q, Giselrod HR. Towards sustainable production of biofuels from microalgae. J Mol Sci 2008;9:1188–95.

[86] Balat M, Balat H. Progress in biodiesel processing. Appl Energy 2010;87: 1815–35.

[87] Minowa T, Yokoyama A-Y, Kishimoto M, Okakurat T. Oil production from algal cells of Dunaliella tertiolecta by direct thermochemical liquefaction. Fuel 1995;74(12):1735–8.

[88] Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: a review. Renew Sust Energy Rev 2010;14:217–32.

[89] Huang G, Chen F, Wei D, Zhang X, Chen G. Biodiesel production by microalgal biotechnology. Appl Energy 2010;87:38–46.

[90] Miao X, Wu Q. Biodiesel production from heterotrophic microalgal oil. Bioresour Biotechnol 2006;97:841–6.

[91] Converti A, Casazza AA, Ortiz EY, perego P, Borghi MD. Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production. Chem Eng Process Process Intens 2009;48:1146–51.

[92] Lee J-Y, Yoo C, Jun S-Y, Ahn C-Y, Oh H-M. Comparison of several methods for effective lipid extraction from microalgae. Bioresour Technol 2010;101:575–7.

[93] Griffiths MJ, Harrison STL. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. In: 3rd Congress of the International Society for Appl Phycol; 2009.

[94] Mandal S, Mallick N. Microalgae Scenedesmus obliquus as a potential source for biodiesel production. Appl Microbiol Biotechnol 2009.

[95] Gouveia L, Oliveira AC. Microalgae as a raw material for biofuels production. J Ind Microbiol Biotechnol 2009;36:269–74.

[96] Demirbas A. Production of biodiesel from algae oils. Energy Sources Part A 2009;31:163–8.

[97] Liu Z-Y, Wang G-C, Zhou B-C (2008) Effect of iron on growth and lipid accumulation in Chlorella vulgaris. Bioresour Technol 99:4717–4722

[98] Miao X, Wu Q (2004) High yield bio-oil production from fast pyrolysis by metabolic controlling of Chlorella protothecoides. J Biotechnol 110:85–93

[99] Vílchez C, Garbayo I, Lobato MV, Vega JM. Microalgae-mediated chemicals production and wastes removal. Enzyme Microb Technol 1997;20:562–72.

[100] Lam MK, Lee KT. Renewable and sustainable bioenergies production from palm oil mill effluent (POME): win–win strategies toward better environmental protection. Biotechnol Adv 2011;29:124–41.

[101] Chinnasamy S, Bhatnagar A, Hunt RW, Das KC. Microalgae cultivation in a wastewater dominated by carpet mill effluents for biofuel applications. Bioresour Technol 2010;101:3097–105.

[102] Chinnasamy S, Bhatnagar A, Claxton R, Das KC. Biomass and bioenergy production potential of microalgae consortium in open and closed bioreactors using untreated carpet industry effluent as growth medium. Bioresour Technol 2010;101:6751–60.

[103] Bhatnagar A, Chinnasamy S, Singh M, Das KC. Renewable biomass production by mixotrophic algae in the presence of various carbon sources and wastewaters. Appl Energy 2011;88:3425–31.

[104] Hodaifa G, Martínez ME, Sánchez S. Daily doses of light in relation to the growth of Scenedesmus obliquus in diluted three-phase olive mill wastewater. J Chem Technol Biotechnol 2009;84:1550–8.

[105] Benitez J, Beltran-Heredia J, Torregrosa J, Acero JL, Cercas V. Aerobic degradation of olive mill wastewaters. Appl Microbiol Biotechnol 1997;47:185–8.

[106] Beccari M, Bonemazzi F, Majone M, Riccardi C. Interaction between acidogenesis and methanogenesis in the anaerobic treatment of olive oil mill effluents. Water Res 1996;30:183–9.

[107] Ammary BY. Nutrients requirements in biological industrial wastewater treatment. Afr J Biotechnol 2004;3:236–8.

[108] Jiang L, Luo S, Fan X, Yang Z, Guo R. Biomass and lipid production of marine microalgae using municipal wastewater and high concentration of CO2. Appl Energy 2011;88:3336–41.

[109] Lam MK, Lee KT, Mohamed AR. Current status and challenges on microalgae based carbon capture. Int J Greenh Gas Con 2012;10:456–69.

[110] Hempel N, Petrick I, Behrendt F. Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. J Appl Phycol 2012;24:1407–18.

[111] Pires JCM, Alvim-Ferraz MCM, Martins FG, Simões M. Carbon dioxide capture from flue gases using microalgae: engineering aspects and biorefinery concept. Renew Sust Energ Rev 2012;16:3043–53.

[112] Benemann JR, Koopman BL, Weissman JC, Oswald WJ. Solar energy conversion with microalgal sewage treatment ponds. Proc Annu Meet – Am Sect Int Sol Energy Soc 1977;1:25–6.

[113] Sydney EB, da Silva TE, Tokarski A, Novak AC, de Carvalho JC, Woiciecohwski AL, et al. Screening of microalgae with potential for biodiesel production and nutrient removal from treated domestic sewage. Appl Energy 2011;88:3291–4.

[114] Oswald WJ. Productivity of algae in sewage disposal. Solar Energy 1973;15:107–17.

[115] Yun YS, Lee SB, Park JM, Lee CI, Yang JW. Carbon dioxide fixation by algal cultivation using wastewater nutrients. J Chem Technol Biotechnol 1997;69:451–5.

[116] Lacerda LMCF, Queiroz MI, Furlan LT, Lauro MJ, Modenesi K, Jacob-Lopes E, et al. Improving refinery wastewater for microalgal biomass production and CO2 biofixation: predictive modeling and simulation. J Petrol Sci Eng 2011;78:679–86.

[117] Hu B, Min M, Zhou W, Li Y, Mohr M, Cheng Y, et al. Influence of exogenous CO2 on biomass and lipid accumulation of microalgae Auxenochlorella protothecoides cultivated in concentrated municipal wastewater. Appl Biochem Biotechnol 2012;166:1661–73.

[118] Park J, Seo J, Kwon EE. Microalgae production using wastewater: effect of light-emitting diode wavelength on microalgal growth. Environ Eng Sci 2012;29:995–1001.

[119] Huo S, Wang Z, Zhu S, Zhou W, Dong R, Yuan Z. Cultivation of Chlorella zofingiensis in bench-scale outdoor ponds by regulation of pH using dairy wastewater in winter, South China. Bioresour Technol 2012;121:76–82.

[120] Han F, Huang J, Li Y, Wang W, Wan M, Shen G, et al. Enhanced lipid productivity of Chlorella pyrenoidosa through the culture strategy of semicontinuous cultivation with nitrogen limitation and pH control by CO2. Bioresour Technol 2013;136:418–24.

[121] Riebesell U. Effects of CO2 enrichment on marine phytoplankton. J Oceanogr 2004;60:719–29.

[122] Sato N. Modulation of lipid and fatty acid content by carbon dioxide in Chlamydomonas reinhardtii. Plant Sci 1989;61:17–21.

[123] Ota M, Kato Y, Watanabe H, Watanabe M, Sato Y, Smith Jr RL, et al. Fatty acid production from a highly CO2 tolerant alga, Chlorocuccum littorale, in the presence of inorganic carbon and nitrate. Bioresour Technol 2009;100:5237–42.

[124] Prathima Devi M, Venkata Mohan S. CO2 supplementation to domestic wastewater enhances microalgae lipid accumulation under mixotrophic microenvironment: effect of sparging period and interval. Bioresour Technol 2012;112:116–23.