# OPTIMIZATION OF INDIGENOUS MICROALGAE FOR HIGH BIOMASS AND LIPIDS PRODUCTIVITY AND UTILIZATION OF ALGAL BIOMASS IN BIOREFINERY APPROACH


**By**

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**2021**

# OPTIMIZATION OF INDIGENOUS MICROALGAE FOR HIGH BIOMASS AND LIPIDS PRODUCTIVITY AND UTILIZATION OF ALGAL BIOMASS IN BIOREFINERY APPROACH


Thesis Submitted by

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to

**Department of Microbiology,**

In the partial fulfillment of the requirement for the degree of

**Doctor of Philosophy**

in

Microbiology

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad, Pakistan
2021

*This thesis is dedicated to;*

*The sake of Allah, my Creator and my Master,*

*Great teacher and His Messenger, the Prophet Muhammad (PBUH), who taught us the purpose of life,*

*My great parents, who never stop giving of themselves in countless ways,*

*My beloved brothers and sisters,*

*To all my family, the symbol of love and giving,*

*My friends who encourage and support me,*

*All the people in my life who touch my heart,*

*I dedicate this research.*

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# ACKNOWLEDGEMENTS

In the Name of **Allah**, the Most Merciful, the Most Compassionate all praise be to Allah, the Lord of the worlds; and prayers and peace be upon **Muhammad** His servant and messenger. First and foremost, I must acknowledge my limitless thanks to Allah, the Ever-Magnificent; the Ever-Thankful, for His help and bless. I am totally sure that this work would have never become truth, without His guidance.

I owe a deep debt of gratitude to our university for giving us an opportunity to complete this work. I am grateful to some people, who worked hard with me from the beginning till the completion of the present research particularly my supervisor **Prof. Dr. M. Ishtiaq Ali** and Co-supervisor **Dr. Asif Jamal**, who have been always generous during all phases of the research, and thanks to **Prof. Dr. Amir Ali Shah**, Chairman, Department of Microbiology, and all my respected Teachers for their moral and academic support. I am very grateful to **Department of Chemistry, QAU Islamabad, Pakistan State Oil, National University of Science and Technology and National Centre of Physics** for their consistent help and facilitation throughout the research work. I would like to take this opportunity to say warm thanks to all my beloved **friends**, who have been so supportive along the way of doing my thesis.

I also would like to express my wholehearted thanks to **my family** for their generous support they provided me throughout my entire life and particularly during PhD. Because of their unconditional love and prayers, I have the chance to complete this thesis.

Last but not least, deepest thanks go to all people who took part in making this thesis real.

**Farhana Bibi**

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# List of Abbreviations

| | |
|---|---|
| ACCase | Acetyl-coA carboxylase |
| ATP | Adenosine triphosphate |
| BBD | Box-Behnken design |
| BBM | Bold basal medium |
| CDW | Cellular dry weight |
| CCD | Central composite design |
| CPAM | Cationic polyacrylamide |
| CFPP | Cold filter plugging point |
| DGAT | Diacyl glycerol acyl transferase |
| DGTS | Diacyglyceryl-N, N, N-trimethyl homoserine |
| DME | Dimethyl ether |
| ENR | Enoyl-ACP reductase |
| EPA | Eicosapentaenoic acid |
| FAMEs | Fatty acid methyl esters |
| FFA | Free fatty acids |
| GA | Gibberellic acid |
| GPAT | Glycerol 3-Phosphate dehydrogenase |
| HAD | 3-hyroxy acyl-ACP dehydratase |
| IAA | 3-Indoleacetic acid |
| JA | Jasmoic acid |
| KAR | 3-Ketoacyl-ACP reductase |
| KAS-3 | 3-Ketoacyl-ACP synthase |
| KIN | Kinetin |
| LPAAT | Lysophosphatidic acid acyl transferase |
| MAT | Malonyl-coA-ACP transacylase |
| MDA | Malondialdehyde |
| PAP | Phosphatidic acid phosphatase |

| | |
|---|---|
| PBRs | Photobioreactors |
| PCA | Principal Component Analysis |
| PBR | Photobioreactor |
| PCI | Phenol Chloroform Isoamyl Alcohol |
| PDH | Pyruvate dehydrogenase |
| PK | Pyruvate Kinase |
| ROS | Reactive oxygen species |
| TAGs | Triacylglycerol's |
| TN | Tetradesmus nygaardii |
| TE | Fatty acyl –ACP thioesterase |

# Abstract

Microalgae have been determined as alternative and potential feedstock for carbon neutral biofuels production over other sources due to its ability to grow at higher rates with less generation time, high-energy rich molecules formation, ability to sequester $CO_2$ form environment and ability to treat wastewater. The abiotic stresses have been appeared as promising strategy for induction of desired metabolite in this respect. The main objective of this study was to delineate the effect of various abiotic stresses on microalgal biofuels production. Several abiotic growth factors such as temperature, pH, nitrogen and phosphorus concentration were focused in this study to facilitate the cultivation of microalgae for higher lipids production and biomass utilization in energy efficient and cost-effective way. The present study was resulted in successful isolation of four indigenous algal strains. Based on initial screening of growth characteristics and biochemical composition, two isolates FSL and F2 were selected for Identification, cultivation, optimization and biofuels production. This initial screening provided bases for further study design for strains optimization and biofuels production. The aim of this study was to enhance the specific rate of growth of *Closteriopsis acicularis*, (green microalgae, freshwater, family *Chlorellaceae)*, with the impact of concentration of phosphate and pH to get optimum productivity of biomass. Present research investigated the independent and coupled impact of pH and concentration of phosphate on characteristics of photoautotrophic growth for *Closteriopsis acicularis* to produce bioethanol. Statistical experimental design (CCD) coupled with Response Surface Method (RSM) was utilized to optimize the growth characteristics and production of bioethanol in lab facility. The experimental outcomes revealed the high specific rate of growth and productivity of biomass as 0.342 $day^{-1}$ and 0.497 g $L^{-1}$ $day^{-1}$ respectively, attained at high pH (9) and high concentration of phosphate (0.115 g $L^{-1}$) at late exponential phase of growth. The composition of elements in optimized biomass revealed increased accumulation carbon, oxygen, phosphorus as macronutrients and sodium, magnesium, aluminum, potassium, calcium and iron other than sulfur and nitrogen as

micronutrients. It was observed 58% carbohydrate content in biomass after optimization and acid catalyzed saccharification resulted in 29.3 g $L^{-1}$ of monosaccharides. The yield of bioethanol was calculated as 51% g ethanol/g glucose and a maximum of 14.9 g/L of ethanol, which infers the successful optimization strategy for growth of *Closteriopsis acicularis* to get enhanced algal yield, biomass and bioethanol production. In this study also, the Box-Behnken statistical design (BBD) of experiment was used to optimize growth parameters (N-concentration, Temperature and pH) for high biomass and lipids accumulation of indigenous microalgae *Tetradesmus nygaardii*. The results show that high level of parameters favors high biomass production (543 mg/L) while lipids accumulation was found maximum (272 mg/L). The Response surface methodology (RSM) assessed interaction AC (N-concentration and pH) as the strongly affecting interaction for biomass and lipids production. C16 and C18 chain length fatty acids were found dominant contributor of *Tetradesmus nygaardii* oil and the biodiesel properties determined were in accordance with ASTM D6751 and EN14214 standards of biodiesel specification. These results demonstrate the potential of *Tetradesmus nygaardii* to produce high lipids as a promising feedstock for biodiesel production. The bio-methane potential of two defatted algal biomass co-digested with eucalyptus leaves waste was assessed and the results illustrated that maximum biogas yield and methane content was obtained highest for *Closteriopsis acicularis* + eucalyptus leaves as 1182 mL/g VS and 74.8%, respectively, which concluded that co-digestion of microalgae with carbon rich substrate is more feasible option for cost-effective and enhanced biogas production from microalgae.

# Chapter 1

# Introduction

# 1 Introduction

The world energy demand is exponentially growing due to ongoing increase in population and industrialization across the globe. The energy consumption and demand are expected to double by 2050, which will consequently elevate the burden on oil and gas sector. Currently the world energy consumption of 80% is obtained by fossil fuels, which are depleting gradually from this planet (Salam *et al.*, 2020). Also, the use of fossil derived fuels is polluting our land, air and water bodies at an alarming pace. The dire need of alternative energy supplies is one of the most intimidating challenges in today's society. Now days, renewable energy have been considered an attractive alternative of fossil fuels. Renewable energy is a form of energy derived from natural resources such as sunlight, rain, biomass and thermal energy because these natural resources are environment friendly and sustainable. Also, these renewable resources do not release loads of $CO_2$ in environment, which is a leading cause of climate change worldwide. Besides $CO_2$, use of fossil fuels is also leaving environment with tons of harmful gases and mercury. For instance, sulfur dioxide ($SO_2$) and nitrogen oxides ($NO_x$) released upon burning of fossil fuels are causing acid rain and smog, respectively (Kim *et al.*, 2020). Mercury causes soil and water contaminations, which in turn is accumulating in bodies of marine life and drastically affecting food chain (KS, Ramya and Varjani, 2019).

Pakistan being an underdeveloped country with more the 190 million population, which is expected to increase three-fold by 2050, is experiencing a serious energy crisis and our fossil fuels import burden accounts for 60% of Pakistan's total foreign exchange. The alarming increase in GHGs and associated climate change is one of the major issues in Pakistan. There is dire need of sustainable alternative renewable energy resource capable of removing loads of GHGs and production of bioenergy simultaneously to fill the gap between national energy demand and supply for development and establishment of sustainable energy resources (Kalair *et al*., 2019).

Biomass derived biofuels such as bioethanol, biodiesel, biogas and biobutanol are considered important renewable and alternative source of energy. This is because biomass derived biofuels offer carbon neutral energy production and do not contribute towards greenhouse gas (GHG) emissions. The $CO_2$ they release in environment upon burning gets utilized again for their growth and $O_2$ production via process of photosynthesis (Chen *et al.*, 2015). Generally, Biofuels are categorized into four generations based upon type of raw material used for their production. First generation biofuels are made from edible sources such as vegetable oils, animal fats, cereals such as; corn, sugarcane etc. One of the major drawbacks associated with first generation biofuels is their competition in human and animal food chain. Second generation biofuels are made from non-edible cellulosic resources such as oil crops (Jatropha, Miscanthus) and agricultural wastes. Second generation biofuels are mainly suitable for bioethanol production and not for biodiesel production. As the biodiesel production from these crops requires extensive processing and cost competitiveness over fossil derived fuels. Third generation biofuels also known as algal fuels are made up from macro and microalgae. Being photosynthetic organism, algae require simple nutrients to grow in an energy rich biomass. One of the major drawbacks with this type of fuels is expensive production of algae at large scale to achieve a threshold for production and commercialization of algal-based fuels in comparison to fossil fuels. Fourth generation biofuels are newly introduced field and scientist are hunting ways to secure a source of energy that increases at double rates with increase in sequestration of CO2 from environment via biotechnology (Jambo *et al.*, 2016)(Dahman *et al.*, 2019).

Microalgae, being third generation biofuel resource offers various advantages over another feedstock. By simply growing, it benefits environment and energy generation systems simultaneously. By capturing sunlight, sequestering loads of CO2 from environment, removing inorganic chemicals from wastewater, salt water or brackish water, it converts all these into energy rich molecules such as lipids, carbohydrates, proteins, vitamins, carotenoids and pigments (Hiibel *et al.*, 2015)(Baldisserotto *et al.*, 2021). These vast bio products derived from algae are useful in wide range of application

such as biofuels (biodiesel, bioethanol, biogas, biobutanol, bio hydrogen), pharmaceuticals, therapeutics, fertilizers, health supplements and cosmetics (Rasala and Mayfield, 2011)(Ameen, AlNadhari and Al-Homaidan, 2021)(DOE, 2016)(Yan *et al.*, 2016)(Khan *et al.*, 2019).

With this diverse and tremendous ability of bimolecular formation, a new concept named as biorefinery is introduced recently, which designs processing routes for complete utilization of algal biomass into multiple products in more environment friendly and cost-effective way. Recently, a lot of studies are reported to present the algae biorefinery for complete utilization of algal biomass for production of biofuels and high value added products simultaneously (di Visconte *et al.*, 2019)(Khoo et al., 2019)(Figueroa-Torres *et al.*, 2020). However, still there are certain challenges persisting to unveil the potential of algae-based biofuel and biorefinery. For instance, the challenges for large-scale production of algal biomass in upstream processing are; selection of key algal strain with ability to grow fast, higher productivity of biomass, maximum neutral lipids accumulation in the form of TAGs and high total carbohydrate content. The downstream processing includes; cost effective and more efficient harvesting and oil extraction techniques, environment friendly bioprocessing and conversion routes of biomass to multiple end products via biological or chemical reactions.

Microalgae based biofuels are advantageous over other biofuels feedstock as being nontoxic, do not require arable land for their source cultivation, do not compete with food chain and are carbon neutral entities. Moreover, microalgae possess less generation time and high rate of growth in comparison to other plant based biofuels (Hossain, Mahlia and Saidur, 2019). Biodiesel is one of the major types of biofuels in limelight by researchers and is made up from neutral lipids content called triacylglycerol's (TAGs) in microalgae. Extraction of lipids is followed by their conversion into fatty acid methyl esters (FAMEs) in a process called transesterification, for biodiesel synthesis. There are mainly two methods of transesterification reactions; (1) two stage transesterification which is more common and easily scalable approach, involves solvent based extraction of lipids in the first step and conversion of oils to FAMEs in the presence of methanol and catalyst (acid

or base) in the second step, (2) in-situ direct transesterification of algal lipids in a single step which utilizes algal biomass directly when mixed with ethanol and catalyst for a certain period of time at specific reaction temperature to synthesize biodiesel (Ghosh, Banerjee and Das, 2017)(de Jesus *et al.*, 2020) .Microalgae is considered preferable due to its tremendous potential to accumulate high amounts of neutral lipids (TAGs) upon un-favorable growth condition or upon abiotic stresses during cultivation with in their cells. After prolonged exposure to abiotic stresses, microalgae are able to synthesize and store TAGs ranges from 20%-60% of CDW, in their specialized organelles known as lipids bodies (Klok *et al*., 2014). The current focus is on enhancement of these neutral lipids content via various techniques such as optimization of growth conditions, nutrient starvations, metabolic or genetic engineering (Chiranjeevi and Mohan, 2016)(Arguelles and Martinez-Goss, 2021)(Huang and Daboussi, 2017).

Optimization of growth conditions is first line of tool to enhance lipids accumulation within algal cells. It is also a necessary tool to understand the behavior of microalgae towards utilization and requirement of nutrients as well as production of biomass rich in lipids or another targeted biomolecule. With the aid of this technique, researchers can better understand the mechanism of lipids synthesis in particular algal strain, which provides insight in further engineering of strain. The ability to accumulate targeted biomolecules in microalgae varies from specie to specie and even the same specie with different geographical origin. For instance, the lipids content of commercially important microalgae species *Chlamydomonas reinhardtii*, is reported 80% (Li *et al.,* 2021) and 35.4% (Meng *et al.,* 2020) depending upon different to geographical origin and cultivation conditions in recent studies.

The isolation and characterization of dominant indigenous microalgae is important, as it provides better adaptability to environment for growth factors and also resists contamination by overproduction of itself over other microorganisms. For successful large-scale production and commercialization of algal-based biofuels, algal strain with rapid rate of growth and high productivity of biomass is one of the key challenges to overcome till now. There are very few algal strains (*Chlorella vulgaris,*

*Botryococcus Braunii, Scenedesmus sp. Rhizocolonium hieroglyphicum, Nannochloropsis oculata, Euglena viridis and Chlorella pyrenoidosa,*) isolated and explored for characterization and biofuels production potential in Pakistan (Manzoor *et al.*, 2015), with very little optimization studies of indigenous microalgae for biomass and lipids production (Alam *et al.*, 2019).

It is well known fact that microalgae is able to synthesize and store neutral lipids (TAGs) upon optimization of various abiotic factors of growth and cultivation (Santhakumaran *et al.*, 2020)(Kim *et al.*, 2019). The isolation and screening of dominant indigenous microalgae and optimization of its biomass and lipids production is prerequisite for large-scale production of algal-based biofuels at national level. Statistical design of experiment for optimization studies facilitates the better understanding of behavioral and growth characteristics of microalgae. Statistical designs are helpful in understanding the independent as well as cumulative effect of various growth conditions in an extensive and precise way (Fozer *et al.*, 2019). Additionally, it is important to develop a bioprocessing route for conversion of whole algal biomass into energy using biorefinery approach. Generally, Biodiesel, bioethanol and biogas are common type of biofuels used in transportation and energy generation systems (Chen *et al.*, 2015). For biodiesel production, it is important to enhance lipids content and more polyunsaturated fatty acids (PUFAs) content is suitable for high quality biodiesel synthesis (Santhakumaran *et al.*, 2020). Bioethanol is fermented product of carbohydrates, mainly in the presence of *Saccharomyces cerevisiae* (Faizal, Sembada and Priharto, 2021). The anaerobic digestion of algal biomass to biogas is common techniques for biomethane generation (Ras *et al.*, 2011). However, there are few studies mentioned for defatted algal biomass conversion to biogas. The low carbon to nitrogen ration of defatted algal biomass requires suitable substrate with high C/N ration for co-digestion purposes, which is reported to enhance biogas production of algal biomass significantly (Solé-Bundó *et al.*, 2017).

## 1.1 Aim and objectives of research

The aim and over all focus of this research was the optimization of biomass and lipids productivity of indigenous microalgae for biofuels production in a cost effective biorefinery approach. The specific objectives of this study were as follows:

I. Isolation, screening and identification of dominant indigenous microalgae
II. Optimization of physicochemical parameters with the help of statistical design of experiment and response surface method (RSM), for high biomass & lipid productivity in selected algal strain
III. Characterization of synthesized biofuel (bioethanol & biodiesel)
IV. Anaerobic co-digestion of defatted algal biomass for enhanced biogas production

# Chapter 2

# Literature review

# 2 Literature review

Energy is the lifeblood of modern societies. In the past decades, the world's energy consumption and associated CO2 emissions increased rapidly due to the increases in population and energy demand (Jeffry *et al*., 2021). World primary energy consumption grew by 45% over the past 20 years, and is likely to grow by 39% over the next 20 years. Global energy consumption growth averages 1.7% p.a. from 2010 to 2030, with growth decelerating gently beyond 2020. The demand of energy increases by 1.3% each year till 2040, with rising demand for energy uncontrolled by more struggles to increase efficiency (World Energy Outlook, 2019). While this is well below the remarkable 2.3% growth seen in 2018, it would result in a relentless upward march in energy-related emissions, as well as growing strains on almost all aspects of energy security. The past decade has seen strong growth in the deployment of renewable energy technologies (Global and Russian Energy Outlook, 2019). Recently more efforts are made to date in curbing greenhouse gases from the transportation sector have focused on the simultaneous production of biofuels.

Biofuels are promising eco-friendly alternative source of renewable energy due to its ability to adjust with gasoline for as high as 85% blends without any modifications in engines. For this purpose, scientist from last few decades is researching various sources suitable for biofuels production. For instance, microalgae based biofuels give prominence to researches due to several advantages over other plants based biofuel resources such as; (1) being loaded resource of carbohydrates, proteins lipids, and various valued products (2) short generation time (3) do not compete with human and animal food chain (4) easy to cultivate in in-door and out-door facilities (5) no arable land requirement (6) ability to thrive in waste, brackish and saline water (7) ability to sequester loads of CO2 from

environment (8) significant contributor towards $O_2$ generation system (9) ability to grow whole year by harvesting sunlight (Hossain, Mahlia and Saidur, 2019).

Yet there are some bottlenecks of algae-based biofuels towards commercialization, which includes high cost in upstream and downstream processes of microalgae. To date a lot of efforts are made to combat these challenges and to reduce cost of algal based products such as metabolic and genetic engineering, optimization of cultivation conditions, technologies development for efficient harvesting and oil extraction processes and selection of high lipids producing strains (Yu, Chen and Zhang, 2015)(Shah *et al.*, 2018). However, optimization of cultivation condition has been used as first line of tool for high biomass and lipids production in microalgae.

Microalgae accumulates large quantities of neutral lipids mainly in the form of TAGs, which are most suitable candidates for biodiesel synthesis. Under abiotic stresses, the enhanced accumulation of these fatty acids serves as energy reserves for cell survival and maintenance. Increased synthesis of polyunsaturated fatty acids (PUFAs) also serves to maintain fluidity of biological membranes during stressful growth conditions. To date, there are several studies reported for increase in mass fraction of lipids under various abiotic stress conditions for *Chlamydomonas reinhardtii* (Li *et al.*, 2021), *Monoraphidium sp.* (Bohnenberger and Crossetti, 2014) *Chlorella sp.* (Moreno-Garcia *et al.*, 2019) and *Chloroidium sp.* (Santhakumaran *et al.*, 2020). However, the response to various abiotic stresses varies from species to species and even for same specie with different geographical origin. Therefore, there is dire need to better understand the cellular responses associated with abiotic stresses to unveil the regulatory elements responsible for enhanced lipids synthesis and to identify challenges in economical production of microalgae derived biofuels. This understanding will help researchers to engineer microalgae to develop more adaptable nature and desired properties and to build metabolic model of algae with worthwhile characteristics. Moreover, by understanding and optimizing the role of abiotic factors in algal growth, we can reduce the chances of

contamination associated with growth of unwanted microorganism and poses serious challenges towards large scale cultivation of microalgae in out-door facilities.

The current review contextualized the (1) Recent advances to utilize whole algal biomass in biorefinery approach to reduce cost of biodiesel synthesis and to make process more economically viable (2) role of abiotic stresses towards enhanced lipids accumulation in microalgae (3) the biosynthetic gene responses of algal cells up on abiotic stressed conditions.

## 2.1 Algal Biorefineries as a promising route

Microalgae are photosynthetic unicellular prokaryotic (Cyanophyceae) and eukaryotic (Chlorophyta) organisms with ability to rapidly grow in diverse aquatic environments such as fresh water, wastewater, and marine waters. Biochemically, Microalgal cells vary according to species and mainly depends on their geographical and cultivation conditions. Bio molecular accumulation within cells such as carbohydrates, proteins, lipids, enzymes, pigments are strongly affected by abiotic factors of growth and cultivation of algae (Klin, Pniewski and Latała, 2020). It was observed a vast variation in biochemical nature of species among each other and even the same specie with different geographical and growth conditions are accumulating these biomolecules differently (Ferreira *et al.*, 2019). In recent years, frequently researched concept of algae biorefinery describes the bioprocessing of algae biomass to biofuels coupled with production of high value added products, in efforts to utilize whole biomass resource and to make bioprocessing more sustainable and cost effective (Khoo *et al.*, 2019). Figure 2.1 demonstrates a general framework of major algae biorefinery routes for utilization of biomass chemical components for energy production. Whole biomass of microalgae could be utilized via four different conversion processes such as catalytic conversion, thermochemical conversion, biochemical conversion and photosynthetic conversion to formulate microbial fuel cell. Such framework can help building energy efficient and profitable algal circular economy by integrating energy efficient bioprocessing routes with waste reduction and high value-added products generation strategies.

Although, microalgae portray competitive potential as a biomass resource over other oil-bearing crops, still various challenges persist in algal biorefinery. The expensive techniques used for upstream processes such as cultivation, and downstream process such as harvesting and oil extraction are limiting the rate of algal production by increasing the cost of overall process. Currently, the microalgae derived biomass production at industrial scale is about 15000 tons/year, which is considerable low in comparison to demand. Therefore, the extraction and commercialization of algae derived high value-added products are gaining attention recently to balance the production cost of microalgae and biofuels. Biofuel's production should be performed at low energy expenditure to counter the price of fossil fuels in market. Unfortunately, this challenge has still not been overcome (Koyande *et al.*, 2019).

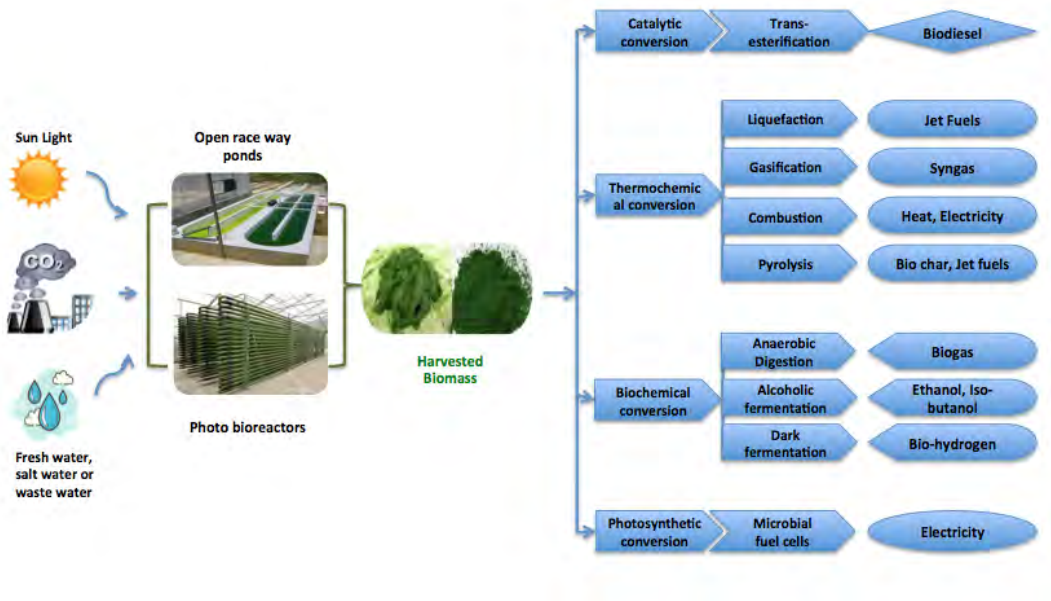


**Figure 2.1**: General framework and biomass conversion techniques for algal biorefinery.

Various strategies for optimization of raw material such as nutrients, light, temperature and CO2 level have been derived and proved effective for enhanced biomass accumulation of microalgae recently (Santhakumaran *et al.*, 2020). Certain general considerations to unravel the potential of algae-based biofuel biorefinery includes; upstream processing such as selection of key algal strain capable of rapid rate of growth, higher productivity of biomass, maximum neutral lipids accumulation in the form of

TAGs and high total carbohydrate content, whereas downstream processing involves cost effective and ecofriendly bioprocessing and conversion routes of biomass to multiple end products via biological or chemical reactions.

### 2.1.1 Algal Carbohydrates

In algal cells, the carbohydrates are synthesized inside chloroplast (eukaryotes) and cytosol (prokaryotes), mainly consisting of monosaccharaides and their associated polymers for structural and metabolic functions (Özçimen, Gülyurt and İnan, 2012). The carbohydrates content of algal cells varies according to species and cultivation conditions. The species with high carbohydrate content can be potential source of biofuels production via various biomass conversion processes to end product such as bioethanol, bio butanol, biogas and bio hydrogen. The major pathways of biofuels production form carbohydrates are fermentation and anaerobic digestion. Fermentation is generally carried out in the presence of *Saccharomyces cerevisiae* to convert fermentable sugars into bioethanol (Phwan *et al.*, 2018). Anaerobic digestion is used to convert carbohydrates and proteins into biogas and biomethane with the aid of various methanogen bacteria (Fatima *et al.*, 2021).

There are several studies reported in efforts to optimize carbohydrates production in algal cells to get higher yield of these biofuels. Recently, optimization studies on growth conditions of *Anabaena variabilis* demonstrated the effect of pH, $MgSO_4$ and $NaHCO_3$ on total carbohydrates yield for bioethanol production, which was consequently increased carbohydrates up to 2.4 folds upon optimization of cultivation parameters (Deb, Mallick and Bhadoria, 2021). Another study reported biorefinery approach of biodiesel and bioethanol production from *Chlamydomonas sp.* KNM 0029C, upon optimization of light intensity which yielded 50.5% of total carbohydrates for downstream processing to bioethanol and 19% lipids for biodiesel production (Kim *et al.*, 2020). Microalgae based biorefinery has also been studied for integrated production of biodiesel and biobutanol. The process resulted in conversion of carbohydrates content into 10.31% (g $g^{-1}$ CDW) and 10.07% (g $g^{-1}$ glucose) butanol after optimization of Acetone-Butanol-Ethanol fermentation process via Response surface method (Figueroa-Torres *et al.*, 2020). The

co-production of microalgae derived biodiesel and biogas has been studied extensively recently which demonstrated this biorefinery approach as a promising route for integrated biofuel production and usage of whole biomass resource for energy generation (González-González *et al.*, 2018; Mendoza *et al.*, 2020; Zewdie and Ali, 2020).

### 2.1.2 Algal Proteins

Microalgae possess significantly high level of proteins that plays important role in cellular functions. Algal species with high proteins content containing all essential amino acids can act as desirable source of nutrients and feed for humans and animals' consumption. Algae are a rich source of proteins similar to those of traditional sources of protein such as soybean, meat, milk and egg. Algae are more viable source of protein production in terms of productivity and nutritional value over terrestrial plants. Microalgae yields up to 4-15 tons/Ha/year of proteins over terrestrial high protein crops i.e., soybean with 0.6-1.2 tons/Ha/year proteins (Bleakley and Hayes, 2017).

### 2.1.3 Algal Lipids

Algal lipids accounts for (20%-70%) of total biomass, which makes it suitable and attractive renewable source of oil production in comparison to other, oil crops. Algal cells are comprised of polar and non-polar lipids, where non-polar lipids are major center of focus in algal biorefineries for biodiesel production. Non-polar lipids are stored form of fats, also known as neutral lipids comprising mainly of triacylglycerol's (TAGs) (DOE, 2016). TAGs are mainly produced and enhanced under abiotic stresses such as nutrient deprivation, light, temperature, pH and salt stresses (Peng *et al.*, 2020a; Sulochana and Arumugam, 2020; Li *et al.*, 2021; Zhao *et al.*, 2021). After prolonged exposure to abiotic stresses, microalgae are able to synthesize and store TAGs ranges from 20%-60% of dry cellular weight, in their specialized organelles known as lipids bodies (Klok *et al.*, 2014).
Algal lipids are low value-added products and this is one of the important criteria for development of processes and technologies for cost effective production of algae derived biodiesel. Microalgae strain characteristics that play decisive role in selection of desirable feedstock for biodiesel production generally applied for factors such as high lipids content and productivity, high productivity of biomass, faster rate of growth and suitable

fatty acid profile. Biomass production and neutral lipids content in terms of TAGs, together are key characteristics for techno-economic viability of algae derived fuels. Several studies have been reported that the optimization of cultivation conditions can possibly alter lipids contents, profile and productivity, where as there are still efforts required to attain maximum lipids and productivity of biomass simultaneously to achieve profitable and cost-effective biodiesel production form algae (Akubude, Nwaigwe and Dintwa, 2019).

Besides lipids content, the length and degree of unsaturation of neutral lipids are also key characteristics to consider for selection of suitable microalgal specie as a feedstock for biodiesel production. According to literature reported, Palmitic acid (16: 0), Myristic acid (14:0), Stearic acid (18:0), Palmitoleic acid (16:1), Linoleic acid (18: 2), Oleic acid (18:1), and Linolenic acids (18:3) are most common FA's in most algal species (Hoekman *et al.*, 2012; Song *et al.*, 2013). Generally, algal oils contain considerably high amounts of poly unsaturated fatty acids (PUFAs), that are desirable for certain ideal biodiesel standards and specification such as Iodine value, cetane number, cold filter plugging point and ignition value (Schlagermann *et al.*, 2012). Iodine value is measure of degree of unsaturation in oils. Cetane number affects the characteristics of fuels auto-ignition quality (Hoekman *et al.*, 2012). Increase in degree of unsaturation in FAMEs content results in decreasing Cetane number (Knothe, 2011). CFPP refers to the lowest temperature at which volume of pure biodiesel flows through filters with in time limit of 1 min (Knothe and Steidley, 2005). Cold flow properties depend on saturated fatty acids content in biodiesel, higher saturated fatty acids results in poor cold flow properties.

The techno-economic analysis of microalgae-based biodiesel biorefineries, coupled and integrated with high value-added chemicals and energy generation systems are more promising and cost-effective way of bioenergy production. However, still the microalgae based large scale and cost-effective biodiesel production faces challenges from mass cultivation, upstream processes and downstream processing of algal products.

## 2.2 Advancements in techniques for mass cultivation of microalgae

There are several techniques for cultivation of microalgae such as open systems (open raceway ponds), closed systems (fermenters, photo bioreactors), hybrid and attached

growth systems (DOE, 2016). Commercialization of algae derived low and high value-added products requires economic viability of mass cultivation systems to produce tons of algal biomass.

### 2.2.1 Open raceway ponds

Open ponds are currently most used systems for large-scale production of microalgae biomass. Open ponds such as natural waters, lakes, lagoons, artificial ponds or containers are easy to construct and operate under minimal cost. Raceway ponds of varied lengths and diameters are usually built in compacted earth, lined with plastics and cultures are fed continually during day time with the aid of paddle wheel which sustains the flow of cultures (Karthikeyan, Muthukumaran and Balakumar, 2016). However, there are still major challenges in this cultivation system such as uncontrolled environmental factors necessary for algal growth, poor light penetration and $CO_2$ sequestration ability in densely populated ponds, losses due to evaporation and microorganisms' contamination. Therefore, large-scale cultivation of microalgae is restricted to strains that are mainly genetically modified or have faster rate of growth and better stability in certain environmental conditions that are optimum for their robust growth over other contaminants (Menetrez, 2012; L. Zhang *et al.*, 2020).

### 2.2.2 Photobioreactors

Photobioreactors (PBRs) are closed vessels for photoautotrophic production of microalgal biomass whereas fermenters are closed vessels provided with carbon source for heterotrophic production of biomass. Generally, closed systems are more efficient systems and mitigate most of problems associated with cultivation in open pond systems. Closed systems offer better control over environmental factors such as light, pH, temperature, $CO_2$ supply, density and continuous mixing. However, closed systems are expensive in terms of operational and construction cost (Powell and Hill, 2009; KS, Ramya and Varjani, 2019).

Various types of PBRs designed for mass cultivation of microalgae are; vertical tubular PBR, Bubble column PBR, Airlift PBR, Flat panel PBR, Horizontal tubular PBR, Helical type PBR, Stirred tank PBR and Hybrid type PBR. For general considerations to

construct Photobioreactors, it was suggested by researchers that design of PBR should support the cultivation of different algal species, design must distribute fast and uniform light and CO2 to cultures, design must resist the fouling of reactor at its light transmitting surfaces (fouling usually caused by adhesive nature of algae), design should support high rates of mass transfer without suppressing growth of cultures and design should contain maximum or complete illuminated part (Singh and Sharma, 2012).

### 2.2.3 Hybrid cultivation systems

Hybrid cultivation systems are developed to separate biomass growth from the lipids accumulation phase. These systems are advantageous and efficient in biodiesel biorefineries where oil separation is an expensive step in processing till product. However, there are very few studies carried out on hybrid cultivation systems; still some studies demonstrated considerably controlled environmental impact on algal growth in hybrid cultivation systems in comparison to open and closed cultivation systems (Su *et al.*, 2011; Adesanya *et al.*, 2014; Narala *et al.*, 2016). The biochemical composition of microalgae biomass strongly depends upon types of cultivation system.

An ideal cultivation strategy enables higher rate of growth and lipids productivity simultaneously. In previous studies, higher productivity of biomass was observed with lower lipids content for most of algal strains. It is also observed that due to stress or unfavorable conditions, higher lipids accumulation within cells result in loss of biomass concentration. A hybrid cultivation system was studied with different nitrogen sources used in first and second stage of cultivation, suggested simultaneous increase in lipids and productivity of biomass with maximum productivity of biomass of 0.427 g $L^{-1}$ $day^{-1}$ and higher lipids content of 499-698 g $kg^{-1}$ CDW in microalgae (Dahmen-Ben Moussa *et al.*, 2019). Recently, a two-phase system of algal cultivation has been proposed that gives optimal and synchronized lipid and biomass production. This system utilizes nutrient rich growth media in first stage of cultivation to achieve maximum productivity of biomass whereas the second phase of strategy introduces stress to induce lipids with in cells. There are number of studies reported in favor of efficiency of two phase cultivation system for higher lipids accumulation and improved biomass production, which includes

switching one or more mode of growth (phototrophic, heterotrophic, mixotrophic), mode of operation (batch cultures, semi or fed batch cultures, continuous) and abiotic factors (nutrients, temperature, pH, $CO_2$, light intensity, salinity) (Su *et al.*, 2011; Adesanya *et al.*, 2014; Wirth *et al.*, 2015; Narala *et al.*, 2016; Minyuk, Sidorov and Solovchenko, 2020; Liyanaarachchi *et al.*, 2021).

### 2.2.4 Harvesting strategies

Harvesting is the process of separating and dewatering algal biomass from growth medium. The economy of algal biodiesel biorefineries are significantly influenced by processing cost of harvesting biomass that accounts for up to 30% of total processing cost (Roy and Mohanty, 2019). Fig. 2 shows various harvesting technologies to yield concentrated algal biomass with minimum moisture content. Generally, concentration techniques are based on physical, chemical and biological processes. Physical harvesting such as ultrasonic waves and electrolysis destabilizes algal cells with aid of electrical or mechanical forces. Chemical harvesting involves use of inorganic or organic additives to increase coagulation whereas biological harvesting processes makes use of natural or spontaneous flocculation with aid of bacteria, fungi or another algal specie and do not requires chemical for coagulation (Deconinck *et al.*, 2018).

There are mainly two steps involved in harvesting technologies, primary harvesting is generally carried out by techniques known as floatation, sedimentation, flocculation or combination of these processes where, the secondary harvesting is carried out by centrifugation and filtration to remove maximum of moisture content left over by primary harvesting. Some studies have reported maximum harvesting capacity of microalgal biomass by having combined one or more of harvesting technologies.

Recently, a high performance and low-cost strategy has been reported with 94% harvesting efficiency when saltwater chlorella vulgaris biomass was concentrated using cationic polyacrylamide (CPAM) combined with clay particles. The combination strategy showed best synergistic effect due to its excellent charge patch and bridging adsorption (You *et al.*, 2019). Another study reported technologies based on organic, electrolytic and magnetic flocculation as successful harvesting strategy for algal biomass (Deconinck *et*

*al.*, 2018). However, there is less data reported about extent of efficiency of these novel-harvesting technologies. Microalgae cell walls are negatively charges entities and could be attracted to cationic polymers for adhesion to surfaces. Microalgae adheres to solid surfaces to minimize free interfacial energy via non-covalent van der walls forces, electrostatic and acid-base interactive forces. Modification of these solid surfaces can improve adhesion of algal cell significantly. Magnetic nanoparticles are recently studies by many researchers with aim to improve harvesting technologies of algal biomass and resulted in considerably high and efficient harvesting (Kucmanová and Gerulová, 2019).

The combination of microalgae cultivation, harvesting, oil extraction and transesterification technologies helps to identify the potent strategies for large scale production and commercialization of algae derived biofuels as well as to overcome bottlenecks in processing technologies (Shi, Handler and Shonnard, 2019).

### 2.2.5 Oil extraction techniques

Oil extraction is one of the major and energy intensive step in microalgae-based biodiesel production. There are various techniques to extract lipids from algal biomass (Figure 2.2), still there are limitation necessary to address and resolve for further cost reduction in processes for biodiesel production. Solvent based oil extraction techniques are most commonly used for algal biomass, recently a study reported >96% of total lipids extracted using 33ml of optimal solvent mixture; chloroform, methanol and water (5.7:3:1) in 2h extraction process at 25 $^{o}$C (Chatsungnoen and Chisti, 2016). Another study reported in-situ, nondestructive, repetitive extraction of lipids from Botryococcus braunii as a cost effective (up to 50%) oil production process in microalgal biorefineries (Jackson, Bahri and Moheimani, 2020). Subcritical dimethyl ether (DME) has been reported as green solvent for economical extraction and recovery of total fatty acids (54.4%) from flocculation thickened microalgae (Wang, Oshita and Takaoka, 2021). Super critical fluids are more recently studied alternative methods of lipids extraction reported for as high as 40% more lipids extraction in comparison to traditional solvents that are able to extract only 10-15% of fatty acids (Xue *et al.*, 2020).

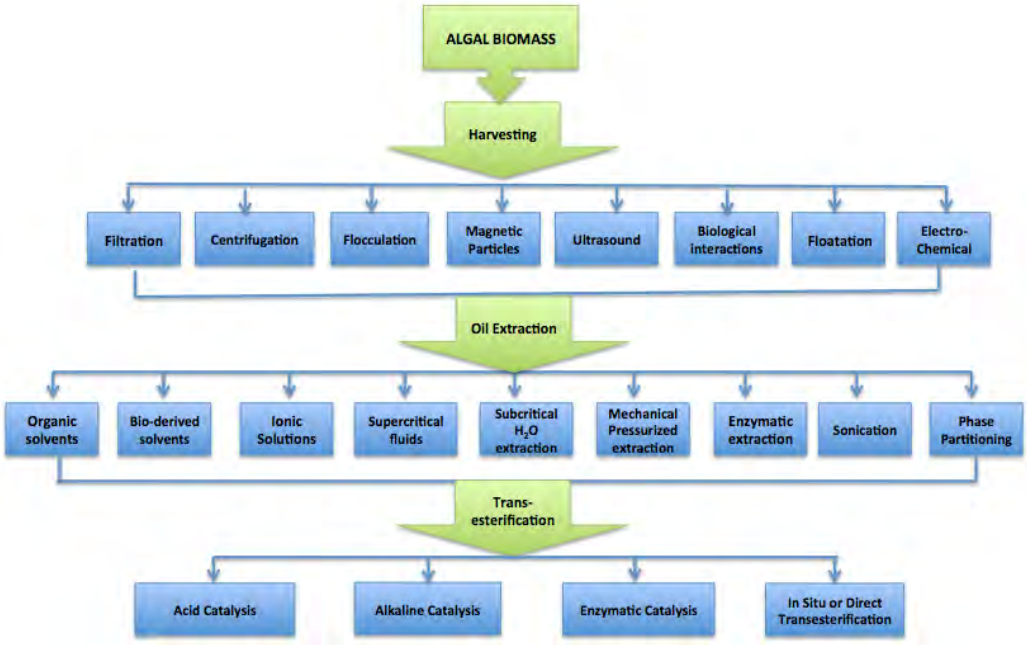


**Figure 2.2:** Advenced strategies in down stream processing of algal biomass.

### 2.2.6 Transesterification of oils to biodiesel

Oil extraction in algal biorefineries is followed by conversion of lipids to fatty acid methyl esters (FAMEs) in a process called transesterification. There are various methods of transesterification (Fig.3), which involves two stage, single stage or direct transesterification reactions. Two stage transesterification is commonly used and easily scalable approach that involves solvent based extraction of lipids in first step where the conversion of extracted lipids to methyl esters using methanol and catalyst (acid or base) in second step. This process is tedious and leaves environmental and health hazards in form of storage and handling of large volumes of solvents.

More recently, an integrated concept of direct use of microalgae has been introduced to avoid drying of biomass and oil extraction in biodiesel synthesis via in-situ direct transesterification of algal lipids. Single stage or direct transesterification is emerging concept of transesterification which utilizes algal biomass directly when mixed with ethanol and catalyst for certain period of time at specific reaction temperature to attain trans-esterified products. Several studies have been reported for optimization of reaction conditions for biodiesel synthesis and maximum yield (Ghosh, Banerjee and Das, 2017;

Torres *et al.*, 2017; Kim *et al.*, 2019).

Catalyst based in-situ transesterification is another alternative and less energy expensive method for biodiesel production. A recently reported solid acid catalyst prepared from sulfonated rice husk was employed for in-situ transesterification of Parachlorella kesslari biomass and process yielded highest FAMEs within 30 mins of reaction time at room temperature suggesting more cost effective and convenient transesterification of lipids to biodiesel (Wadood *et al.*, 2020).

## 2.3 Optimization Strategies to induce Lipids via Abiotic Stresses

Different abiotic stresses are first line of tool for induction of microalgal growth and lipids production. There are various factors that affect the biomass and lipids accumulation in algal cells. However, this review paper will discuss effects of stresses related to nutrients starvation, temperature, pH, salinity, light intensity, and organic chemicals and their impact on synthesis and enhancement of lipids in microalgae.

### 2.3.1 Nutrient stresses

#### *2.3.1.1 Nitrogen*

Nitrogen is an essential macronutrient that plays direct role in synthesis of lipids, proteins and carbohydrates (Yaakob *et al.*, 2021). Microalgae sequesters nitrogen in the form of nitrates, nitrites, urea and ammonium (Flynn and Butler, 1986). Nitrogen is considered vital nutrient for growth of microalgae as it constitutes all structural and functional entities of algal cells such as proteins, enzymes, pigments, energy transfer molecules and genetic makeup (Kim, Mujtaba and Lee, 2016). Optimization of Nitrogen source and concentration for particular algal strain could be effective strategy to enhance lipids induction in much cheaper and ecofriendly way. Recently a study reported 2.27 folds increase in lipids production during nitrogen stress in a fresh water microalgae *Scenedesmus quadricauda*. Several biochemical changes have been observed that were associated with nitrogen depletion such as generation of reactive oxygen species $H_2O_2$, OH- and $O_2$- along with several energy equivalents that eventually lead to the increase in lipids accumulation in algal cell (Sulochana and Arumugam, 2020).

It was recently reported a nitrogen depletion related 38.14% ± 0.82% increase in lipids content in *Scenedesmus rubescens* cells (Jo *et al.*, 2020). Role of nitrogen has also been

investigated for production and release of extracellular matter in *Nannochloropsis salina, Chlorella sp*. and *Haematococcus pluvialis*, which showed decrease in proteins content on a per cell basis and increase in total biomass upon nitrogen starvation during algal cultivation (Baroni *et al.*, 2020). NaNO3 with effective concentration of 0.375 g L-1 was found optimized nitrogen concentration in cultivation of Chlorobion sp. and Chlorella sp., which resulted in considerably high lipids content of 31.61% and 28.77%, respectively (Arguelles and Martinez-Goss, 2021). Nitrogen limitation at a minimum concentration of 3.5mM $NaNO_3$ in *Eustigmatos vischen Jhsu-01* strain of microalgae has achieved 49% increase in total lipids content (Xu *et al.*, 2020). Similarly, nitrogen concentration was optimized for *Chloroidium saccharophillum* and observed 2.9mM nitrates concentration optimized for higher lipids productivity of 40.37% $L^{-1}$ $day^{-1}$ (Santhakumaran *et al.*, 2020). Generally, Nitrogen depletion causes lipids enhancement in algal cells. However, there are several studies that reported loss of productivity of biomass in efforts to induce lipids synthesis via nitrogen starvation (Huesemann *et al.*, 2016; Qi *et al.*, 2019; An *et al.*, 2020). This inverse relation between nitrogen concentration in growth medium and lipids production might be explained on the basis of facts that low levels of nitrogen results in reduction of Proteins and carotenoids synthesis, hence triggers the lipids accumulation by reducing competition of carbon sources. Also the depletion of nitrogen in results in degradation of nitrogenous compounds within cells to provide energy storage and carbon source for accumulation of neutral lipids in response to stress (Wan *et al.*, 2014; Li-Beisson, Beisson and Riekhof, 2015; T.-Q. Shi *et al.*, 2020).

#### *2.3.1.2 Phosphorus*

Phosphorus is another essential nutrient that plays important role in microalgal growth, lipids productivity and vital cellular processes such as photosynthesis, synthesis of nucleic acids, phospholipids and ATP. Microalgae sequesters phosphorus in the form of polyphosphate or orthophosphate to build cellular components and nutritional content. Phosphorus is also an important regulator of several metabolic pathways. (Meng *et al.*, 2019) proposed that phosphate starvation triggers increase in biosynthesis of DGTS (diacyglyceryl-N, N, N-trimethyl homoserine), which is central intermediate in

glycolipids synthesis. Phosphorus depletion resulted in enhanced biosynthesis of EPA (Eicosapentaenoic acid) and other glycolipids in *Nannochloropsis oceanica*. Another study reported significantly higher biosynthesis of arachidonic acid and Eicosapentaenoic acid in phosphorus deficient cultures of *Nannochloropsis oculata* but with reduced chlorophyll a content, photosynthesis activity and rate of growth (Matsui *et al.*, 2020).

Phosphorus starvation in *Chlorella sp.* has been resulted in 16% increase in total lipids content (Liang *et al.*, 2013). Similarly, the optimization study of phosphorus for Chloroidium saccharophillum has been resulted in 33.57 % increase in lipids yield $L^{-1}$ $day^{-1}$ with 0.5mM concentration of phosphate (Santhakumaran *et al.*, 2020).

Even though phosphorus and nitrogen deficiencies alone improve lipids production in microalgal cells, it is also observed decreased total biomass content of microalgae associated with nutrients starvation, which in turns in failure of algal oil and biodiesel production. Recent efforts are made to couple nitrogen starvation with phosphorus supplementation. Phosphorus supplementation in the presence of nitrogen starvation offers an attractive strategy for simultaneous high biomass and lipids productivity (Chu *et al.*, 2013; Fu *et al.*, 2017, 2019). The hormesis effect of phosphorus on microalgae has achieved 10.2% increase in biomass along with 39.3% increase in total lipids content of *Chlorella vulgaris* cultures grown under nitrogen starved conditions (Fu *et al.*, 2019). Similarly, enhanced biomass production of 4.53 g/L has been reported for chlorella vulgaris grown under nitrogen depletion and phosphorus supplementation, which also enhanced lipids content to 42.3% than those in control (Fu *et al.*, 2017). *Chaetoceros muelleri*, a common microalgae in water bodies of Mexico has been examined for effect of different phosphorus concentrations on lipids synthesis and observed highest total lipids accumulation at lowest phosphorus concentration (7μMP) suggesting phosphorus limitation significantly triggers lipids production (Lovio-Fragoso, Hayano-Kanashiro and López-Elías, 2019).

#### *2.3.1.3 Iron*

Iron is an essential micronutrient that plays important role in several biochemical pathways and acts as cofactor in all electron transfer reactions during oxygenic

photosynthesis (Devadasu *et al.*, 2016). Depletion of iron has been reported to results in conversion of membrane lipids into free fatty acids in the form of lipids droplet in *D. tertiolecta* (Chen *et al.*, 2011)(Rizwan, Mujtaba and Lee, 2017). A significantly increase in lipids, specifically triacylglycerol's (TAGs) have been observed in *C. reinhardtii* upon iron stress. TAGs accumulation was accompanied by overexpression of diacyl glycerol acyl transferase protein. Iron stress significantly increased the level of C16, C18: 2 and C18: 3 fatty acids (Devadasu and Subramanyam, 2021). Another study reported iron limitation associated enhanced rate of growth of five microalgal strains *Chlorella sp, Chlorococcum sp., Phormidium sp., Chlorella sp., and Cosmarium sp.* (Aslam *et al.*, 2021).

### 2.3.2 PH

PH is one of the important key factors that plays important role in growth metabolism of microalgae and controls the solubility and accessibility of various nutrients and $CO_2$ (Qiu, Gao, Paola A Lopez, *et al.*, 2017). pH associated growth and biomass production of algae varies in many ways. It generally affects the distribution of carbon, bioavailability and improves the availability of various trace metals and inorganic nutrients by posing linear impact physiologically (Chen and Durbin, 1994; Suthar and Verma, 2018). Temporary changes in pH can decrease the growth of unwanted microorganisms and favors growth of axenic algal cell densities. PH also plays vital role in building environmental societies and neighborhoods as increase or decrease in pH selects the cultivation conditions of cultures. It is also observed that microalgae thrives in environments that ranges in pH 7-7.5 (Jabir *et al.*, 2021).

There are several studies that reported pH 7.5-9 as optimum culture condition for high lipids and biomass accumulation (Bartley *et al.*, 2014)(Sakarika and Kornaros, 2016). In one the recent study, pH 9 was reported as optimum pH level for cultivation of *Chloroidium saccharophillum* which resulted in 70.32% increase in lipids productivity recorded $L^{-1}$ $day^{-1}$ (Santhakumaran *et al.*, 2020). Another study reported pH 7 as best cultivation pH for growth and lipids production of *Chlorella sorokiniana* DOE 1412 strain with 25.7% increase in total lipids production (Qiu, Gao, Paola A. Lopez, *et al.*,

2017). However, there are few studies that show higher biomass formation and lipids production in low pH cultivation such as one of the studies reported optimum pH for algal growth as 2.5 for *Chlorella sp.* Another study documented pH 5 as most suitable pH for optimum growth of *Chlorella* (Sakarika and Kornaros, 2016). Peng *et al.* found pH 6 as most suitable culture condition of *Nannochloropsis sp.* MASCC11 strain that resulted in maximum lipids production of 108.2 mg L-1 (Peng *et al.*, 2020b).

### 2.3.3 Salinity stress

Salinity is another important factor that controls the growth and biological activities of microalgae. High salinity is challenging and unavoidable environmental truth for photosynthetic organisms to fight with. Especially, fresh water microalgal strains shows reluctance to survive in high saline environment where as algal strains inhabitant of brackish water can easily survive and thrive in high salinity environment. It is critical to decide any optimum saline condition for microalgae as a group of organism as because salt tolerance of microalgae species depends upon their genetic makeup, morphology and evolutionary selection process (Shetty, Gitau and Maróti, 2019).

The synthetic mechanism of microalgae for lipids accumulation can be optimized under salt stresses. Salt related stress shifts the biochemical pathways of starch towards lipids accumulation to reserve energy and survival. A halo tolerant microalga *Scenedesmus sp.* IITRIND2 was investigated for its physiological and metabolic adaptabilities under saline conditions. It was observed salinity derived metabolic adjustments that include accumulation of negative charged lipids, high proline and sugars synthesis and direction of metabolic flux towards synthesis of TAGs (Arora *et al.*, 2019). Two species of fresh water microalgae *Chlorella Sorokiniana* CG12 and *Desmodesmus* GS12 were studied for effect of various salt concentrations. $CaCl_2$ was found the most affecting salt for enhancement of lipids accumulation up to 40.02% and 44.97% in these strains, respectively (Srivastava, Nishchal and Goud, 2017).

The interactive effect of high light and salinity stress was examined for microalgae *Chromochloris zofingiensis*. The coupled effect of 0.25M NaCl and 400$\mu Em^{-2}s^{-1}$ was found effective for as high as 15-folds increase in TAGs content (Kou *et al.*, 2020). The

effect of melatonin and salinity stress was assessed for Monoraphidium sp. QLY-1, which resulted in 51.74% increase in total lipids content with 0.34M NaCl and 10μM MT concentration (Zhao *et al.*, 2021). Another study was designed to examine the mutual effect of H2O2 and salinity stress on growth and lipids synthesis of *Monoraphidium sp.* QLY-1 strain. The effective H2O2 and saline concentration was suggested as 2mM and 171.12mM respectively, which yielded 24.09% increase in total lipids content (Qiao *et al.*, 2021). A marine microalgae *Tetraselmis suecica* was examined for effect of various salinity concentrations on growth, biomass, biochemical composition and lipids productivity. It was observed an osmotic and ionic imbalance on very low salinity (10ppt) and increase in salinity from 30 to 60ppt significantly improved the total lipids accumulation by 22% (Pugkaew *et al.*, 2019).

### 2.3.4 Temperature

Temperature is one of the important cultivation factors that affect the growth and lipids production in microalgae. All metabolic reactions and enzymatic activities are greatly affected by temperature fluctuations (Zhao, Han and Cao, 2020). These temperature fluctuations are observed more prominent in temperate regions where greenhouse effect also contributes to temperature fluctuations between 10-45 $^{o}$C in outdoor microalgae cultivation facilities. Increase in temperature till range of optimum can have positive effect on photosynthetic and metabolic activities of microalgae mainly due to increase in Calvin cycle related enzymatic activities. This relation between microalgal rate of growth and temperature was fairly modeled by using Arrhenius equation suggesting the rate of growth and biomass accumulation doubles by increase in each 10$^{o}$C rise in temperature till optimum level. Above optimum temperature, the enzymatic activities halt due to proteins denaturation consequently resulting in algal growth inhibition (Ras, Steyer and Bernard, 2013).

There are several studies documenting temperature dependent increase in biomass and lipids accumulation in microalgae. For instance, *Chaetoceros sp.* was observed most suitable specie for outdoor cultivation in Thailand tropical climate due to its ability to grow and thrive in wide range of temperature, up to 40 $^{o}$C (Chaisutyakorn, Praiboon and

Kaewsuralikhit, 2018). A study examined heat stress for enhancement of lipids in *Glacilariopsis lemaneiformis* at high temperature as 33 ºC. It was observed an increase in PUFA content of total lipids yield along with 88% increase in C20: 5 fatty acids (X. Zhang *et al.*, 2020). The effect of temperature was studied for *Chlamydomonas reinhardtii*, a commercially important microalgae strain. It was resulted in 80% increase in mass fraction of lipids with growth temperature in range of 30-32 ºC (Li *et al.*, 2021). The effect of temperature for optimum lipids productivity in microalgae *Nannochloropsis sp.* MASCC11 strain was studied. It was suggested 35 ºC as optimum temperature for maximum lipids productivity of 134.6 mg $L^{-1}$ (Peng *et al.*, 2020b). Similarly another study reported maximum rate of growth, biomass and lipids accumulation at 25-30 ºC for *Nannochloropsis sp* (Zhao, Han and Cao, 2020). However, the growth and lipids accumulation of microalgae depends strongly on environmental and climate condition for where it was isolated.

The microalgal strains that are isolated from cold regions can only survive in lower temperature ranges and higher temperature halts their enzymatic activities. For instance, a cold stress was given to microalgae *Porosira glacialis* strain. It was observed a 33.4 % total lipids accumulation at lower temperature of 2 ºC (Svenning *et al.*, 2019). Another study examined the effect of cold temperature on lipids accumulation of *Chlamydomonas malina* RCC2488 and determined 32% accumulation of total lipids content at 8 ºC (Morales-Sánchez *et al.*, 2020).

### 2.3.5 Light Intensity

Light is an important and indispensible environmental factor that greatly affects the photosynthetic and metabolic pathways of microalgae. The accumulation of neutral lipids suitable for biodiesel production is mainly synthesized under stressful cultivation conditions of microalgae (He *et al.*, 2015). These neutral lipids act as energy reserves for cell survival and consequently results in continuous harvesting of more light. There are several studies reported for high lipids accumulation under high light intensities. For instance, increase in light intensity during cultivation of *Desmodesmus sp*. and *Scenedesmus obliquus* positively affected the synthesis of fatty acids and biomass

simultaneously (Nzayisenga *et al.*, 2020). Similarly, a higher rate of growth and increased total lipids content was observed with high light intensity using red and white LED lamps for cultivation of *Chlorella vulgaris* in green house facility (Metsoviti *et al.*, 2020).

Another study demonstrated the effect of photoperiods on lipids accumulation of *Verrucodesmus verrucosus*, resulting 50.42% higher lipids accumulation in 12hrs light: 12hrs dark photoperiod under higher light intensity of 2000 lux (Vélez-Landa *et al.*, 2021). Recently there are various studies reported for coupled effect of light intensities and other abiotic stresses such as temperature, salinity and chemicals related stress that impacts positively towards accumulation of higher lipids content. For instance, the coupled effect of light intensity and glycerol was examined for *Chlorella sp*. It was observed a 17.2 % higher lipids content with 3 g $L^{-1}$ glycerol and 40µmol.$m^{-2}$.$s^{-1}$ light (Moreno-Garcia *et al.*, 2019). However, there are no clear data documented for high light intensity related lipids induction mechanism for microalgae, unraveling of theses mechanisms can further help to elucidate the light stress related biosynthetic pathways of lipids in algal cells.

### 2.3.6 Effect of different chemicals on lipids production

Various chemicals act as metabolic activators or triggers that are capable to directly regulate the metabolic reactions in algal cells towards enhanced lipids accumulation. This alternative method of lipids induction relies on phenotypic screening of microalgae and does not require knowledge related to biosynthetic pathways of lipids synthesis (Yu, Chen and Zhang, 2015). A recent study examined the effect of antioxidants on lipids synthesis of *Heveochlorella sp.* YU strain. The maximum of 31.83% increase in lipids content was recorded with 40µmol$L^{-1}$ melatonin (Cui *et al.*, 2021). Another study determined the effect of glyphosate herbicide for lipids induction in *Chlorella sorokiniana*. There was observed 17% increase in lipids synthesis with 30.10ppm ($IC_{50}$) supplementation (Jaiswal *et al.*, 2020). The effect of walnut shell extract (WSE) was studied on growth and lipids accumulation of *Haematococcus pluvialis*. With supplementation of 15% WSE, it was observed a 23.39% increase in lipids yield (Yu *et al.*, 2021). Effect of acidification was determined on lipids synthesis of *Scenedesmus*

*obtusiusculus* AT-UAM strain. With 1N HCl and CO2 addition, it was observed a 32% increase in lipids with 60% of total lipids obtained (Sánchez-García *et al.*, 2020). Another study examined the effect of phytohormones Kinetin (KIN), Jasmoic acid (JA) and Gibberellic acid (GA) on fatty acid synthesis of *Aurantinochytrium sp.,* documented the increase in growth and fatty acid by 16%-28% and 22%-36%, respectively. The combinatorial effect of these phytohormones resulted in decreased levels of reactive oxygen species (ROS) and malondialdehyde (MDA) and augmented the certain antioxidants and enzymes involved in lipogenesis (Nazir *et al.*, 2020).

There are various studies reported recently to study the interactive effect of different chemicals and other abiotic growth factors on various commercially viable microalgae, for increased lipids synthesis. For intense, the coupled effect of melatonin and nitrogen stress was determined for lipids accumulation of Chlamydomonas reinhardtii. It was observed 35.4% increase in total lipids content with 5μM MT and nitrogen starvation (Meng *et al.*, 2020). Another study examined the interactive effect of phytohormones for lipids synthesis in *Chlorella sorokiniana.* The combined effect of 10 mg $L^{-1}$ cytokinin and kinetic resulted in 35.85% increase in lipids content (Guldhe *et al.*, 2019a). A recent study showed that N-depletion coupled with 3-Indoleacetic acid (IAA) supplementation significantly increased the lipids yield in *Nannochloropsis oceanica*. The IAA supplementation enhanced the growth and lipids productivity by stimulating the biosynthesis of pigments. It was observed an increase in omega-6 and omega-3 fatty acids due to combinatorial effect of N-depletion and IAA supplementation on algal cells (Guldhe *et al.*, 2019b).

## 2.4 Biosynthetic gene response of algae towards abiotic stresses for lipids induction

For technological advancements in algal biorefinary, it is important to get deep insight in regulatory mechanism of lipids synthesis in microalgae. There are several studies reported for reprogramming of biosynthetic pathways of various biomolecules such as carbohydrates and proteins towards lipids accumulation (Lu *et al.*, 2019a)(L. Zhang *et al.*, 2020). Stressed cultivation conditions stimulate or triggers algal regulatory

mechanisms towards more neutral lipids (TAGs) synthesis as a mechanism of defense and cell survival (Soós *et al.*, 2021).

There are various studies reported recently for study of the regulation of genes and their impact on lipids synthesis under various stresses in microalgae. For instance, the effect of high light and salinity stress was examined for regulation of lipogenic genes in *Chromochloris zofingiensis.* It was observed a 49.76% increase in total lipids content and Up-regulation of lipogenic genes involved in the expression of; 3-ketoacyl-ACP synthase (*kas*), stearoyl-ACP desaturase (*sad*), ω-3 fatty acid desaturase (*fad*) by 5.81-, 1.22-, 3.75 fold increase, respectively (Kou *et al.*, 2020). Another study was reported for determination of nitrogen limitations effect on TAGs synthesis. For *Eustigmatos vischeri* JHsu-01 strain, it was observed a significantly increase in biosynthesis of TAGs with 50.9% increase in total lipids content. It was also observed the up regulation of genes involved in de-novo fatty acid synthesis (K09458, K00059, K00208 and K01964), in Kennedy pathway (K11155, K00128, K00002 and K00011) and in Acetyl transfer pathway from membrane lipids (K00679 and K00993) (Xu *et al.*, 2020).

Various studies documented the abiotic stress related gene regulation and the key enzymes associated with increased synthesis of TAGs. Pyruvate dehydrogenase (PDH) is found an important gene in biosynthetic pathways of lipids that enhances lipids accumulation in microalgae (Lu *et al.*, 2019b). One of the recent studies examined the effect of phytohormones on the growth and productivity of biomass in Chlorella sorokiniana. It was observed 7.83-fold increase in expression of rbcL gene and 4.15-fold increase in accD gene with increase in productivity of biomass by 45.55% (Guldhe *et al.*, 2019c). Similarly increasing activity of ACCase has been observed to utilize substrate Malonyl-CoA for excess lipids formation. Fatty acid synthase is another rate limiting enzyme in lipids synthesis. Overexpression of genes related to FAS complex such as KAS-3 has been reported to increase levels of Palmitic acid in total lipids content whereas up regulation of KAS-1 has been reported to have significantly increased lipids synthesis. It was also suggested that the multiple genes regulations in biosynthetic pathways of microalgae could have more prominent effect on lipids synthesis (Yu *et al.*,

2011). The phosphorus depletion was studied for its effect on lipids synthesis and associated genes regulation in *Nannochloropsis oceanica.* Experimentation resulted with maximum lipids productivity of 141.4 mg $L^{-1}$ $day^{-1}$. It was observed an Up-regulation of genes involved in expression of aconitrate hydratase (ACH), 2-oxoglutarate dehydrogenase (OGDH), succinyl-coA synthetase (SCA), succinate dehydrogenase (SDH) and malate dehydrogenase of TCA cycle. Down regulation of four genes encoding lipases (Y. Shi *et al.*, 2020). The interactive effect of high light and nutrient limitation in *Chlorella sorokiniana* SLM2 strain resulted in up-regulation of genes involved in metabolic flux of oxidative pentose phosphate pathway and glycolysis pathway, Down regulation of TCA cycle and Triggering shunt of gamma-amino butyric acid (GABA). It was observed a significant increase in NADPH and lipids accumulation upon stress (Mingcan *et al.*, 2019).

# Chapter 3

# Material and methods

# 3 Sampling

Samples were collected from local region of Islamabad (Rawal lake). The climate of region at time of sampling was humid subtropical with temperature 30ºC and pH 8.1. Samples were stored in 100 ml of sterile falcons tubes. Physicochemical properties like Salinity (electrical conductivity), nitrogen (colorimetric method- Kjeldahl nitrogen), Phosphate (Orthophosphate-pretreatment followed by colorimetric analysis), sulphate (Turbidimetric analysis) and nitrates (colorimetric analysis-NEDA method) (CPCB, 2006) content of samples were measured.

## 3.1 Isolation and growth conditions

The isolation of major algal strains was carried out using Standard plating method in modified Bold Basal Medium of growth (1.5% agar). Prior to samples transfer, the fungal and bacterial contamination was avoided by adding antibacterial (Ampicillin) and antifungal (Carbendazim) in enrichment media with mild doses (0.1µg/ml) (Mustapa *et al.*, 2016). The chemical composition of modified BBM media in g/L dist. $H_2O$ as; $NaNO_3$ (0.25), $CaCl_2$ (0.025), $MgSO_4$ (0.075), $K_2HPO_4$ (0.075), $KH_2PO_4$ (0.175), NaCl (0.025), EDTA (0.05), KOH (0.31), $FeSO_4.7H_2O$ (0.049), $H_3BO_3$ (0.114), $ZnSO_4$ (0.0882), $MnCl_2.4H_2O$ (0.014), $MoO_3$ (0.007), $CuSO_4.5H_2O$ (0.015), Co ($NO_3$). 6 $H_2O$ (0.0048). 0.5 mL of original sample was transferred to media plate and spread uniformly on agar plates with the help of sterilized loop. The plates were re-streaked for few cycles by carefully picking fresh cells every time until successful isolation of axenic cultures. The colonies were picked from dilution plate on the bases of morphology and microscopic examination and by avoiding contaminated areas. Plates were provided constant growth conditions of temperature 27°C ± 1°C and light intensity of 50 µmol photon $s^{-1}m^{-2}$ using fluorescent lamps along with photoperiod of 12 h light: 12 h dark cycle (18 days). The algal isolates were labeled and stored on cool shelf with low light. The cultivation of algal strains was carried out in Erlenmeyer glass flask with capacity of

1L enrichment media. The continuous aeration and bubbling sterile air were provided to cultures to maintain carbon source.

## 3.2 Morphological and molecular identification of Microalgal Strains

The light microscope (MCX100-micros Austria) was used to assess the morphological characteristics of algal strains. For molecular identification, the genomic DNA was extracted from algal cells by using Modified Cetyl trimethylammonium bromide (CTAB) method. For that, 0.25 g of harvested and grounded algal cells was solubilized in 1.5ml of CTAB buffer (pre-heated, 65 ºC). 3mm sterile glass beads were used to mechanically digest the cells using vortex. Samples were then degraded enzymatically by using Proteinase K (5µl), 10 % SDS (30µl) & lysozyme (10µl) and provided incubation for 1h at 37 ºC. CTAB (100ml) and 5M NaCl (80µl) were added in digested algal cells solution for DNA segregation and purification following incubation in water bath at 65ºC for 15 mints. In order to separate proteins from genomic DNA, Phenol: Chloroform: Isoamyl alcohol (PCI)(500µl) was used and solution was centrifuged (10,000rpm) for 20mints. After adding PCI, the solution was separated in two different phases (organic & liquid phase). The phase of liquid was carefully picked and transferred to new Eppendorf tube following repeat washing with PCI. The chilled isopropanol (500µl) and 3M sodium acetate (300µl) solutions were added to separated liquid phase and provided incubation at 4 ºC (overnight), to get precipitation. The next day, centrifugation at 10000rpm and -4 ºC temperature was carried out to pellet out precipitates. The precipitates were then washed with 70% ethanol solution (200µl) twice to remove any impurity. At the end, the palettes were dissolved in TE buffer (50µl) for Standard Gel electrophoresis as well as DNA sequencing. Standard Gel Electrophoresis was used to find out integrity of DNA fragments. For this purpose, 1% agarose gel has been prepared and DNA samples of 5µl has been resolved elecrophorically on gel for 30mins. Gel was stained with 5µl of ethidium bromide to visualize DNA on UV transilluminator (Jagielski *et al.*, 2017). DNA sequencing was obtained from MACROGEN public biotechnology company of South Korea. The sequences obtained were BLASTn in NCBI genbank database by using alignment tool (MUSCLE) to search homology of algal DNA sequences in database (Rismani-Yazdi *et al.*, 2011). Genbank ID/accession number for DNA sequence of

respective algae strains was obtained from NCBI. In order to estimate maximum likelihood and evolutionary distance between algal species, MEGA version 7 was utilized to construct phylogeny linkage trees (Chaidir *et al.*, 2016).

## 3.3 Growth characterization

Specific rate of growth and biomass production were evaluated by estimating microalgal cellular dry weight and optical density (750nm). Cellular dry weight was measured by using 100ml of microalgal culture centrifuged at 4000rpm for 15 min following washing of cellular pallets twice with distilled water and drying in oven. The biomass Production was estimated by measuring cellular dry weight (mg/L) at late exponential phase of cultivation. The specific rate of growth (g $L^{-1}$ $d^{-1}$) was calculated using following formula;

Specific rate of growth = $\mu = \ln N_2 - \ln N_1 . (t_2 - t_1)^{-1}$ (1)

Where $N_1$ and $N_2$ is the cellular dry weight of biomass taken at initial ($t_1$) and late exponential phase ($t_2$) respectively (Krzemińska *et al.*, 2014).

The productivity of biomass (g $d^{-1}$ $L^{-1}$) was calculated at exponential phase of growth with the help of equation, where $Z_2$ and $Z_1$ were cellular dry biomass (g $L^{-1}$) obtained at initial and final time by using equation (2).

Productivity of biomass = $(Z_2-Z_1)/(t_2-t_1)$ (2)

Using estimated specific rate of growth, the generation time (T) was calculated with the help of equation (3);

$T = \ln 2 / \mu$ (3)

## 3.4 Biochemical composition

Bligh and dyer method has been used to extract total lipids. Briefly, Dried algal pallets were homogenized for 15mins in (2:1 v/v) chloroform / methanol solution. Mixtures were centrifuged at 5000rpm for 12mins and 12ºC, twice to separate supernatants. 0.9% NaCl solution was added to extract lipids at (1:5 v/v). After gentle shaking for 2mins, the

solutions were allowed to undergo phase separation for next 30 mins. Rotary evaporator was used to recover chloroform. Further quantification of total lipids was done gravimetrically (BLIGH and DYER, 1959). Lipids content was measured using equation;

Lipids (w/w %) = (Total lipids obtained mg) / (Total dry Biomass used mg) x 100 (4)

The modified phenol-sulfuric acid method was used for the estimation of total carbohydrates content (21 days aged cellular cultures) after acid catalyzed pretreatment and fixing standard glucose (Dubois *et al.*, 1956).

## 3.5 Optimization of biomass and lipids production in strain 1

### 3.5.1 Experimental Design and response surface Methodology

In order to optimize biomass and lipids production, Box-Benhken Design (BBD) was utilized for Nitrogen concentration, Temperature and pH as independent variables. Based on preliminary studies, the three coded levels corresponding to low, high and center of independent variables has been chosen (Table 3.1). Total 15 treatments including three control levels were obtained from the design of experiment. The linear, square as well as 2-way interaction of variable and their associated significance were estimated by Analysis of variance (ANOVA) and Pareto charts at confidence level of 95% ($p<0.05$). The response surface & regression analysis, ANOVA, factorial and Surface plots (2D Counter and 3D) were obtained for assessment of interactions of variable and values of response by using Minitab (Software, 18).

**Table 3.1: Optimization of growth parameters via BBD for biomass and Lipids Production**

| | | Range and Levels | | |
|---|---|---|---|---|
| **Experimental parameters** | **Symbols** | **-1** | **0** | **+1** |
| **N-Concentration g/L** | A | 0.1 | 0.3 | 0.5 |
| **Temperature $^{o}$C** | B | 25 | 30 | 35 |
| **PH** | C | 6 | 7 | 8 |

## 3.6 Synthesis and characterization of biodiesel Fatty acid methyl esters (FAMEs) using advanced analytical techniques

Transesterification of fatty acids were performed using modified protocol of (Zhao *et al.*, 2016). The extracted lipids were added with 2ml of 3% $H_2SO_4$ in methanolic solution to

prepare methyl esters of fatty acids. The reaction time and temperature were maintained as 4h and 70ºC in water bath. After esterification, 2ml of n-hexane was added in reaction mixture and left standing for 4h. The top n-hexane layer was then removed and was projected to **FT-IR** (Bruker, Perkin Elmer Tensor 27 with ATR detector) analysis in range of 4000-600 $Cm^{-1}$ for identification of functional groups and structural arrangements FAMEs.

The **$^{1}$H NMR** and **$^{13}$CNMR** of TN oil and Biodiesel were carried out at 300MHz by using CDCl3 solvent in NMR spectrometer (Bruker). **GC-MS analysis** (GC-6890N coupled with MS-5973 Mass Selective Detector) was carried out for identification and characterization of methyl esters. The dimensions of capillary column used for separation were 30m x 0.32mm with 0.25µm film thickness. Initial temperature of oven was set as 120ºC and gradually increased at the rate of 10ºC/min to 300ºC. Helium was used as carrier gas at the rate of 1.5ml/min. Relative proportion of fatty acids was calculated via area normalization method.

The biodiesel specifications, i.e., Density at 15ºC (ASTM D1298-Hydrometer method), Flash point (ASTM D93-Pensky Martens closed cup method), Cetane number (ASTM D613-standerd method), Saponification value (ASTM D1962-titration method|), Acid value (ASTM D664- standard method), Heating value (Bomb Calorimeter), Solidifying point and Cold filter plug point (CFPP) (ASTM D5551-standered method), were investigated and compared with ASTM 6571 and EN 14214 standards value.

## 3.7 Optimization of specific rate of growth and productivity of biomass of strain 2

### 3.7.1 Design of experiment and response surface Methodology

In order to optimize productivity of biomass and specific algal rate of growth, central composite Design (CCD) was utilized as design of experiment by taking independent variables (pH and concentration of phosphate), which resulted in 2 levels of factor along with 5 repetitions of central points. The lowest and highest parametric values were assigned using factor levels (-1) and (+1), respectively, by maintaining the value of alpha (α), 1 (Table 3.2). There were 13 treatments that includes 5 levels of control resulted from

the design of experiment. The linear, square and 2-way variable interaction and their associated significance were estimated by Analysis of variance (ANOVA) and Pareto charts at confidence level of 95% ($p<0.05$). The response surface & regression analysis, ANOVA, factorial and Surface plots (2D Counter and 3D) were obtained for assessment of interactions of variable and values of response by using Minitab (Software, 18).

**Table 3.2: Optimization of growth parameters via CCD for high specific rate of growth and productivity of biomass**

| | | Range | |
|---|---|---|---|
| Experimental parameters | Symbols | -1 | +1 |
| Concentration of phosphate (g/L) | A | 0.023 | 0.115 |
| PH | B | 6 | 9 |

## 3.8 Biomass Analysis

The optimized and un-optimized biomass was analyzed for biomolecules accumulation differences using Fourier transform Infrared spectroscopy (**FT-IR)**. For that, 10ml of cellular suspension were harvested by centrifugation at 5000rpm for 10 mins. Cellular pallets were then washed twice with distilled water and dried in electric desiccator (35 mins). The FTIR spectrometer (Perkin-Elmer Tensor 27 (Bruker)) equipped with ZnSe ATR detector (resolution of 1 cm & 15 scans) was used for analysis in range of 4000-600$cm^{-1}$. The carbohydrates, lipids and proteins quantities were evaluated and compared by using the intensity of absorbance at respective spectral peaks allotted to biomolecules. The elemental and surface analysis of optimized biomass was performed by scanning electron microscopy (SEM, Vega3 TESCAN) equipped with energy dispersive X-ray spectroscopy system (EDS, Bruker).

## 3.9 Saccharification and submerged Fermentation (SmF)

The acid catalyzed hydrolysis of biomass for saccharification was carried out using 500mg dried and grounded biomass subjected to 2% $H_2SO_4$ solution following autoclave (121$^oC$, 15mins) (Hossain, Basu and Mamun, 2015). The carbohydrates catalysis to fermentable sugars, the hydrolysate was subjected to continuous agitation at 200rpm and 45$^oC$ for 48hours. The bioethanol production was carried out using *Saccharomyces*

*cerevisiae* in submerged fermentation. Yeast cells were cultured in yeast extract peptone dextrose (YDP) medium. Cultures from active (logarithmic) phase were taken following centrifugation at 9000rpm (10mins). Pelleted active yeast cells were then used ferment hydrolyzed sugars solution by providing continuous agitation at 800rpm (30$^{o}$C, 5 days) (Selvan *et al.*, 2019).

### 3.9.1 Estimation and characterization of bioethanol

The concentration of bioethanol was assessed by method of solvent extraction and dichromate oxidation. For that, 15g of $K_2Cr_2O_7$ were dissolved in 100ml of 5M $H_2SO_4$ solution. The bioethanol extractant used to recover bioethanol from aqueous solution was Tri-n-butyl phosphate (TBP). For that, solutions of ethanol (0%-30% standard) combined with TBP (1:1) eppendorf tubes (2ml) following vigorously vortex until separation of two different phases. The upper phase solution (500µl) was then shifted to a clean Eppendorf tube and assorted with equal quantity reagent of dichromate. Solution was then vortexed till separation of phase. After it, the lower phase was diluted 10X using standard solutions as well as sample (Miah *et al.*, 2017). The optical densities were calculated (595nm) using UV-spectrophotometer (analytikjena Germany, SPECORD 200 plus). Ethanol stander curve was used to estimate the unknown bioethanol concentration in sample.

## 3.10 Anaerobic Co-digestion of defatted biomass

### 3.10.1 Substrate for co-digestion

For enhancement of biogas and methane synthesis, the defatted microalgae biomass was co-digested with carbon rich substrate that is dried eucalyptus leaves are selected because of high C/N ratio (Table 3.3). The leaves samples collected from garden were washed twice and dried, to remove the soil and dust. The dried leaves were then grinded and further proceeded for biogas production experimentation.

### 3.10.2 Inoculum preparation

Inoculum used in this study was obtained from the anaerobic digester of Biorefinery and Sustainable Energy Lab at Quaid-I-Azam University. Incubation time was given to the inoculums so that microbes can degrade the organic matter present in it. It was incubated

at 37 ºC for one week. When the inoculum was fully developed, it was stored at room temperature to be used in anaerobic digestion. Total solids (TS), Volatile solids (VS) and the Moisture content of inoculums were found out by standard protocols (Table 3.3) (Filer, Ding and Chang, 2019).

**Table 3.3: Organic and inorganic content of substrates**

| Substrate | TS% | VS of TS (%) | C/N | Moisture (%) | VS of sample g VS |
|---|---|---|---|---|---|
| Inoculum | 1.67 | 94.59 | 1.1 | 98.33 | 1.58 |
| *Closteriopsis* sp. biomass | 51.5 | 73.42 | 5.84 | 50 | 36.71 |
| *Tetradesmus* sp. biomass | 50 | 67.86 | 4.79 | 49.85 | 34.03 |
| Dried eucalyptus leaves | 93.55 | 93.1 | 36.5 | 6.45 | 87.1 |

### 3.10.3 Biomethane potential test (BMP) and experimental procedure

This study was designed to evaluate the effect of co-digestion of eucalyptus leaves with defatted microalgae for enhanced biogas and methane generation. The reactor setup is based on comparative study of biogas production from two different strains of defatted microalgae samples alone and with co-digestion using carbon rich substrate. The batch experiment was done in 500 ml reactors containing working volume of 250 ml. The temperature of the incubator was set at 39 ºC and the pH of reactors was set to 7-7.5. The substrate to inoculum ratio was 4. Each experiment was performed in duplicates. In order to determine the amount of biogas produced in the background the negative control was run which contain only inoculums. In order to determine the net biogas production by the substrate in the test reactors the amount of biogas produced by the negative control was subtracted from the test reactors. For anaerobic digestion of microalgae biomass and its co-digestion with dried eucalyptus leaves, ten reactors are used; two for microalgal strain *Closteriopsis* sp., two for microalgal strain *Tetradesmus* sp., two for co-digestion of *Closteriopsis* sp. with dried eucalyptus leaves (*Closteriopsis* sp.+ Eucalyptus leaves waste), two for co-digestion of *Tetradesmus* sp. with dried eucalyptus leaves (*Tetradesmus* sp. + Eucalyptus leaves waste) and two for negative control. For the

working volume of 250 mL the amount of substrate and inoculums was determined by following formula.

$$\text{Amount of substrate added} = \frac{\text{Working volume (ml)} \times \text{VS of inoculum (mg)}}{\text{VS of inoculum (mg)} + 4 + \text{VS of substrate (mg)}}$$

Amount of inoculum added = working volume – amount of substrate added

After adding the calculated amounts of the substrate and inoculum to each reactor they were flushed with nitrogen gas to remove the oxygen from the reactors. Shaking was given to reactor on daily basis to mix the inoculum and substrate. The daily production of biogas and purified methane volume was determined using NaOH scrubbing method (Fatima *et al.*, 2021). Briefly, a thick layer of steel wool was used to eliminate moisture and excess gasses such as hydrogen sulfide and $CO_2$ using 40% NaOH solution, upon subsequently passing biogas through it.

### 3.10.4 Analytical methods

Fourier transform infrared spectroscopy **(FTIR)** was used to better identify structural changes and functional groups in anaerobically digested and degraded samples of microalgal biomass alone and in co-digestion with carbon rich substrate using PerkinElmer 56 FTIR in the wavelength range of 3600-1600 cm-1. The C/N ratio of substrates and inoculum was determined by **Elemental analysis** (NC Technologies, Innovative Elemental µ-Analysis). The effect of co-digestion on methanogen bacterial colonization in samples was determined using the **Scanning Electron Microscopy (SEM)** to better understand the effect of co-digestion in different microalgae strains for methane generation (SEM, Vega3 TESCAN).

# Chapter 4

# Results and discussion

# 4 Results and discussion

## 4.1 Isolation, screening and identification of dominant indigenous microalgae

### 4.1.1 Physicochemical properties of sample water

The temperature of area of Rawal Lake was measured as 30ºC at the time of sampling and sampling was done during daytime. All physiochemical characteristics (table 4.1.1) of sampling water were measured in order to assess the natural growth condition of microalgae in water medium, which proved helpful in lab scale cultivation and growth parameters.

**Table 1.1.1: Physiochemical properties of sampled water to assess natural growth conditions of Indigenous microalgal strains**

| Sr. No. | Physicochemical characters | Concentration |
|---|---|---|
| 1 | Nitrates | 4.8 mg/L |
| 2 | pH | 8 |
| 3 | Salinity | 78 g/kg |
| 4 | Total Nitrogen | 51 mg/L |
| 5 | Phosphates | 47 mg/L |
| 6 | Sulfates | 22 mg/L |

### 4.1.2 Isolation and screening

For isolation, culture medium and all equipment's were sterilized to avoid any contamination. Careful isolation has been carried out in order to obtain axenic microalgae strains from sample by using standard-plating method. Initially, there were four different strains (MSB, FSL, MRA, F2) isolated from culture based upon colony morphology (Figure 4.1.1). Isolates color varied from dark green to light green. The isolates were cultivated in flask with capacity of 1 liter (Erlenmeyer glass) with modified media (BBM) for initial screening of growth characteristics and biochemical composition such as lipids and carbohydrates content (Table 4.1.2). The growth patterns and biomass

accumulation were assessed by monitoring optical density of biomass at 750nm using spectrophotometer (SPECORD 200 plus, analytikjena Germany).

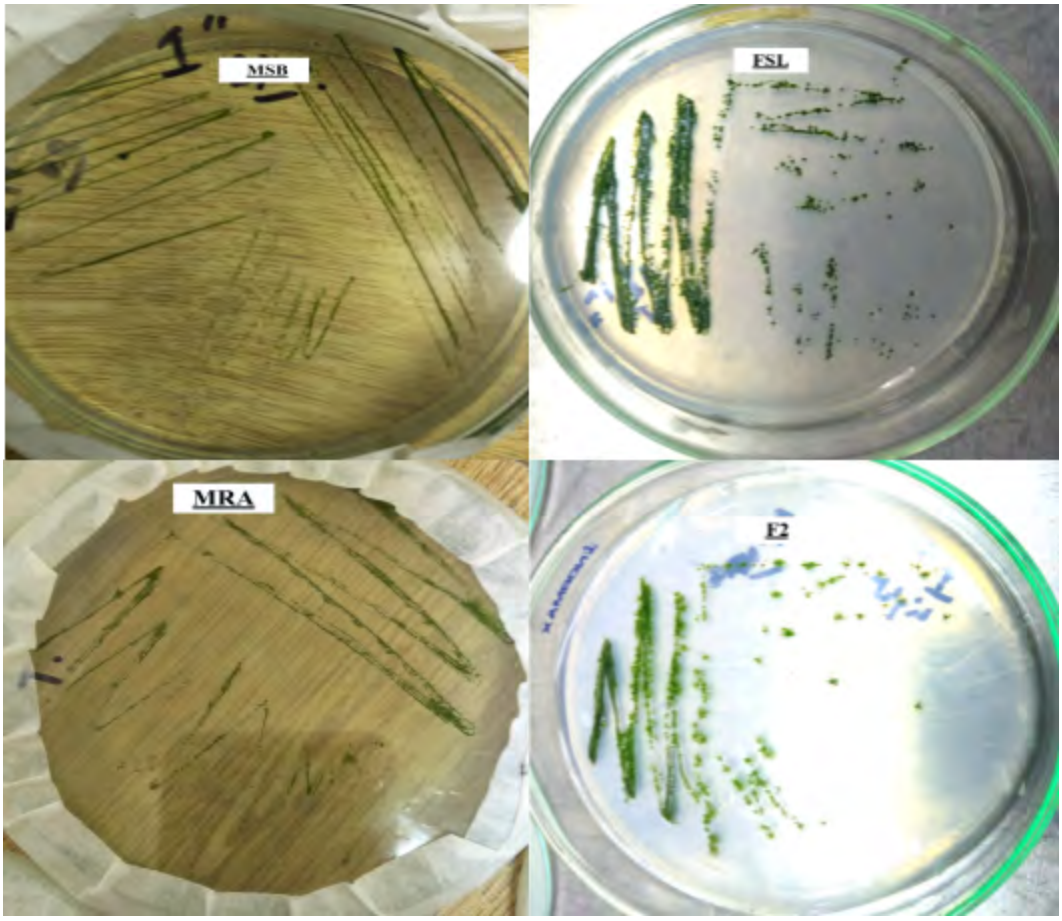


**Figure 4.1.1: Isolated sample of microalgae grown on BBM nutrient media**

Figure 4.1.2 shows the growth pattern of each isolate during 21 days of cultivation. Isolates FSL and F2 have shown high density of biomass with prolonged exponential phase till day 11 of cultivation following short stationary phase of 2 days. It was observed comparatively less dense biomass formation in case of isolate MSB and MRA with slightly prolonged exponential phase till day 13$^{th}$ of inoculation following 4 days long stationary phase. These results depict the faster biomass accumulation of isolate FSL and F2 with high biomass formation capability. Further, the rate of growth, generation time and productivity of biomass of isolates were calculated by measuring cellular dry weight obtained at the initial exponential phase (day 3 of inoculation) and end of stationary phase (day 15th and 17th of inoculation of isolate FSL & F2 and MSB & MRA,

respectively). The isolate FSL and F2 has shown higher rate of growth and productivity of biomass with less generation time as compared to other strains (Table 4.1.2). The order of productivity of biomass (mg $d^{-1}$ $L^{-1}$) and rate of growth (g $d^{-1}$ $L^{-1}$) was found in manner; FSL>F2>MRA>MSB. The biochemical composition analysis has revealed higher lipids accumulation in case of isolate FSL with maximum of 30%, whereas higher carbohydrates content was observed in case of F2 with maximum of 38%. The results of initial screening revealed the potential and suitability of isolate FSL and F2 with comparatively high growth as well as productivity of biomass, for further optimization and biofuels production studies.

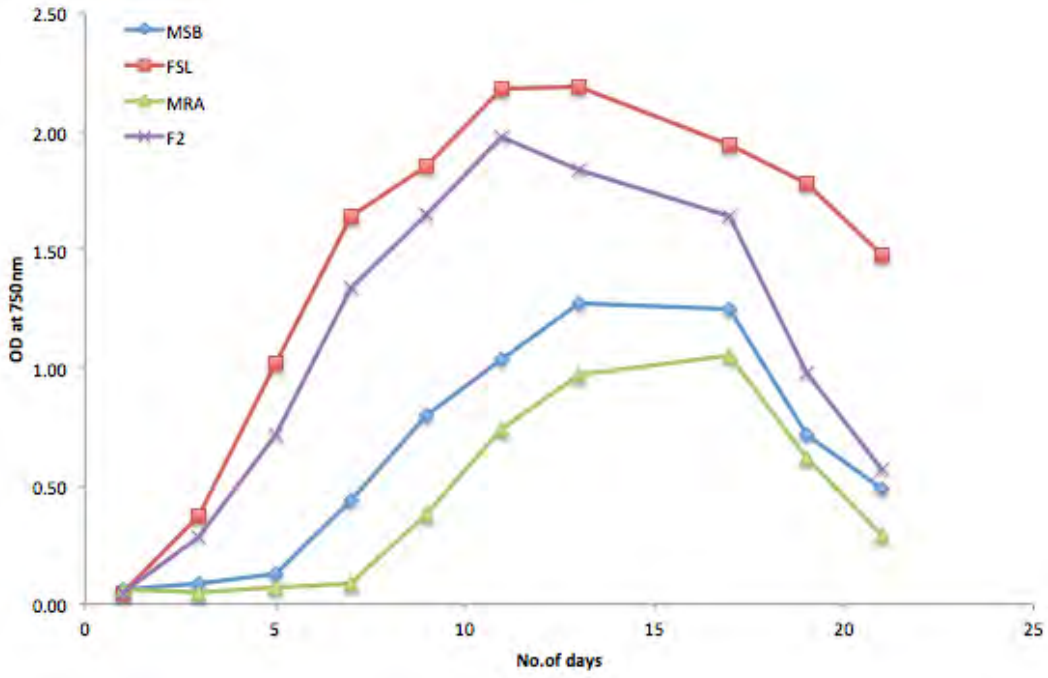


**Figure 4.1.2: Growth pattern and biomass accumulation of algal isolates by spectrophotometric analysis**

**Table 4.1.2: Growth characteristics and biochemical composition of algal isolates**

| | F2 | FSL | MSB | MRA |
|---|---|---|---|---|
| **Rate of growth (g $d^{-1}$ $L^{-1}$)** | 0.169 | 0.175 | 0.132 | 0.141 |
| **Generation time (day)** | 4.1 | 3.96 | 5.25 | 4.9 |
| **Productivity of biomass (mg $d^{-1}$ $L^{-1}$)** | 121 | 187 | 97 | 102 |
| **Lipids Content %** | 17 | 30 | 11 | 14 |
| **Carbohydrates Content %** | 38 | 27 | 21 | 19 |

### 4.1.3 Morphological identification of selected strains

The present study conducted to identify the potential dominant algal strain in fresh water of Islamabad region. Based on initial screening, two isolates FSL and F2 were selected for further optimization and biofuel production. Light microscopy has been used to preliminary identify the isolated strains (100X lens with oil emulsion). Two microalgae strains with comparatively faster rate of growth have been successfully isolated and selected from culture as shown in Fig (4.1.3 & 4.1.4). The algal isolate morphologically examination showed unicellular, and narrow pointed needle like configuration of F2 isolate (Figure 4.1.3), resembling fresh water algal member of family: *Chlorellaceae*, genus: *Closteriopsis* and specie: *acicularis* (John, Whitton and Brook, no date).

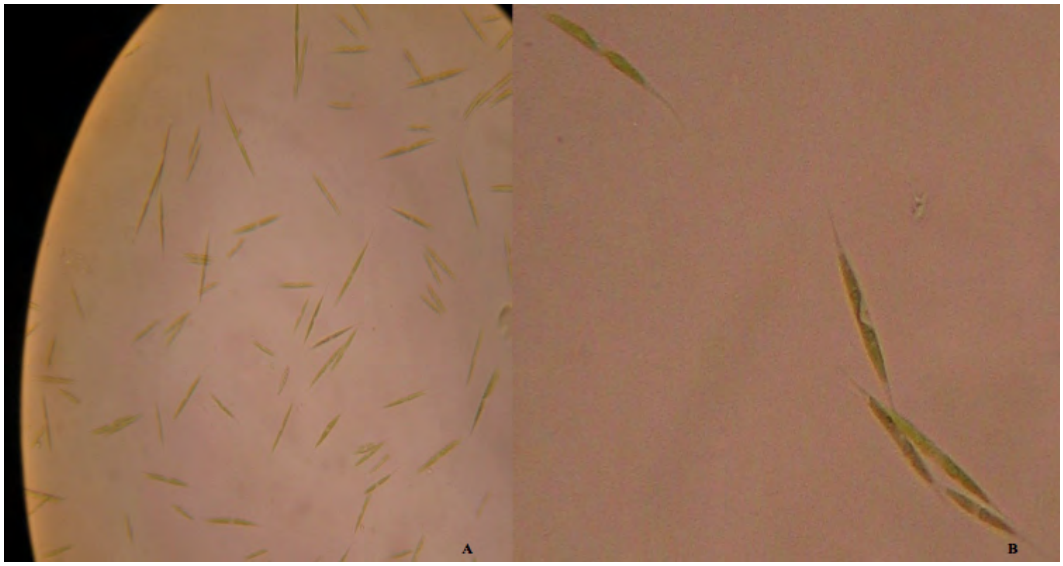


**Figure 4.1.3: Morphological examination of algal isolate F2 with the aid of Light Microscopy**

Isolate FSL shows a colonial organization of group of cells in row (Figure 4.1.4). Colonial organization of cells is usually a characteristic of members of family *Scenedesmaceae*; *Scenedesmus sp*., *Acutodesmus sp*. or *Tetradesmus sp*. Containing 2, 4, 8, 16 or 32 cells grouped together in a row. Shape of cells varies from oval and round to cylindrical (Hegewald and Hangata, 2000; Chaidir *et al.*, 2016). Due to morphological plasticity of members of family *Scenedesmaceae*, it is considerably difficult to determine taxonomic position of each genus and specie morphologically (Kravtsova *et al.*, 2013).

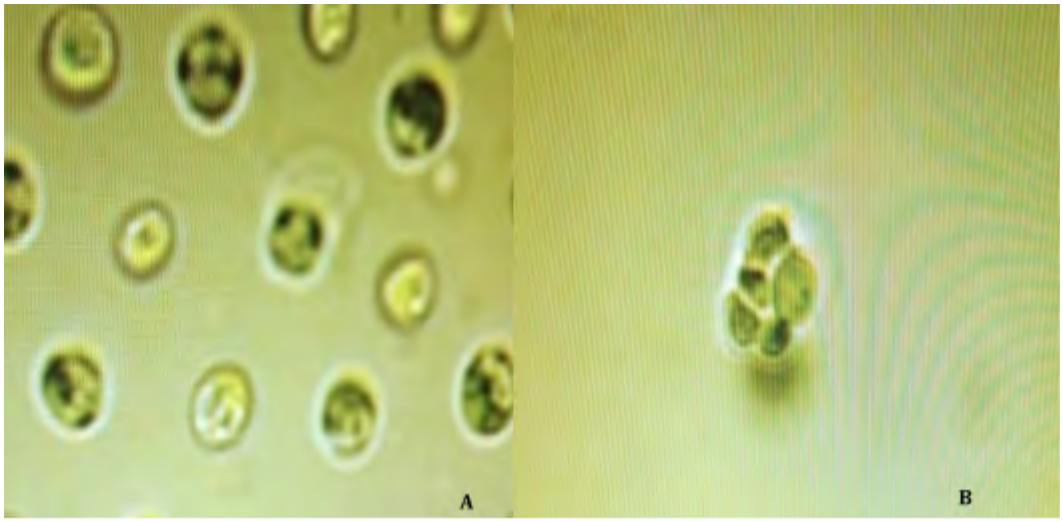


**Figure 4.1.4: Morphological examination of algal isolate FSL with the aid of Light Microscopy**

### 4.1.4 Molecular identification

Standard Gel Electrophoresis of DNA extracts showed considerably narrow, thick and large fragments of microalgae isolates. DNA extraction methods that generate high molecular weight DNA fragments have been considered more suitable for second and third generation sequencing technologies and other downstream processes. Moreover, thick and high molecular weight fragments are crucial for high quality libraries preparation and further efficient genomic assembly using long reads. In agarose gel (Figure 4.1.5), along with large fragments, a high DNA concentration with broader length distribution has been seen. Taken together, higher the average molecular weight of DNA fragment depicts the better the quality of Genomic DNA (Chakraborty *et al.*, 2016; Jagielski *et al.*, 2017).

Genomic DNA sequencing was carried out by Macrogen South Korea, which involves amplicon sequencing of whole DNA. Extracted Genomic DNA samples were amplified by Macrogen using PCR to assess 18S rRNA regions for identification of microorganism prior to DNA sequencing. The sequenced Data of isolates was then converted to FASTA files using NCBI GenBank database. All raw amplicon homology were identified using Basic Local Alignment Search Tool for Nucleotide sequences (BLASTn) (Yun, Kim and Yoon, 2019). The phylogenetic tree describes the evolutionary lineage of species, organisms from one distinct ancestor (Yanuhar, Caesar and Musa, 2019). Thus, the

phylogenetic relationship of strains were inferred based on similarity of sequenced data with GenBank database and by Aligning sequences in MUSCLE Alignment tool and constructing maximum likelihood consensus tree to infer the phylogenetic kinship of isolates with class and species, the genetic distance of strains have been estimated using the Tamura-Nei method (Alonso *et al.*, 2012) in MEGA software (version 7). The bootstrap values were calculated on the basis of 550 bootstrap replicates to assign degree of confidence to the nodes in phylogenetic tree.

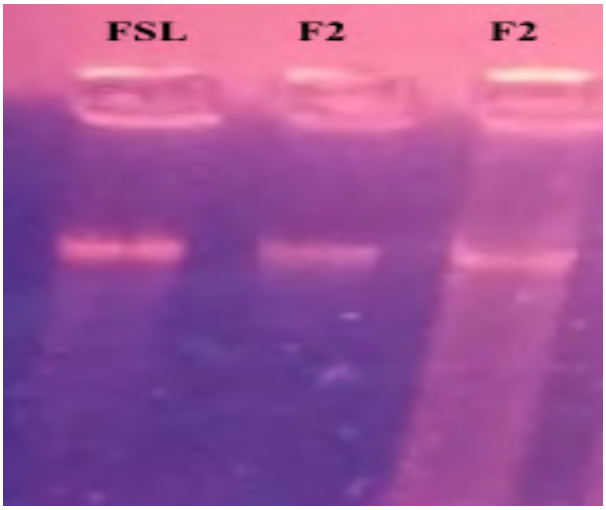


**Figure 4.1.5: Agarose gel showing Genomic DNA extracted for algal isolate FSL and F2**

In case of F2 isolate, the complete raw genomic sequence obtained was BLAST in NCBI GenBank to compare the sequence homology of isolate for identification purpose, which resulted in the sequence homology of 96.28% out of 672 bases, with member of family *Chlorellaceae* (green Algae), genus *Closteriopsis* and specie *acicularis* with the E-value 0.0 which shows significant homology of sequence data F2 isolate (Figure 4.1.6). The phylogenetic tree obtained by maximum likelihood method has shown close kinship of F2 isolate with the member of family *Chlorellaceae* (green Algae); specie *Closteriopsis acicularis.* The bootstrap value of the tree based on the analysis of 550 bootstrap replicates has resulted in 100% confidence support assigned to the node, which is strongly supporting that the isolated and sequenced strain of microalgae is *Closteriopsis acicularis*. **(Accession ID =*Closteriopsis acicularis,* MT 858355.1)**

The BLASTn of FSL isolate sequence has resulted in 98% sequence identity out of 667 bases, with members of family *Scenedesmaceae* (green algae), Genus Tetradesmus, specie *nygaardii* with significant homology of sequence resulted in E-value of 0.0 (Figure 4.1.7). The phylogenetic tree based on maximum likelihood inferred FSL is a novel strain having kinship with *Tetradesmus nygardii strain* with relatively moderate bootstrap value of 65%, which indicates that the organisms of this monophyletic group have a close sequence similarity. **(Accession ID= *Tetradesmus nygaardii,* MT858750)**

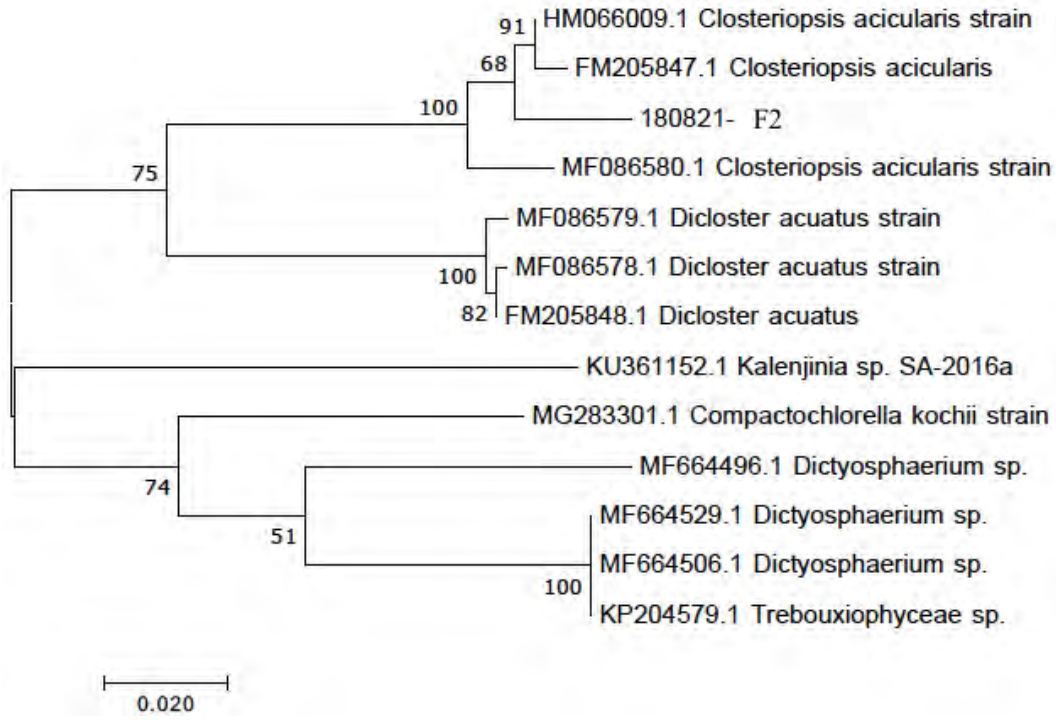


**Figure 4.1.6:** The phylogeny of microalgae *Closteriopsis acicularis* (strain F2)

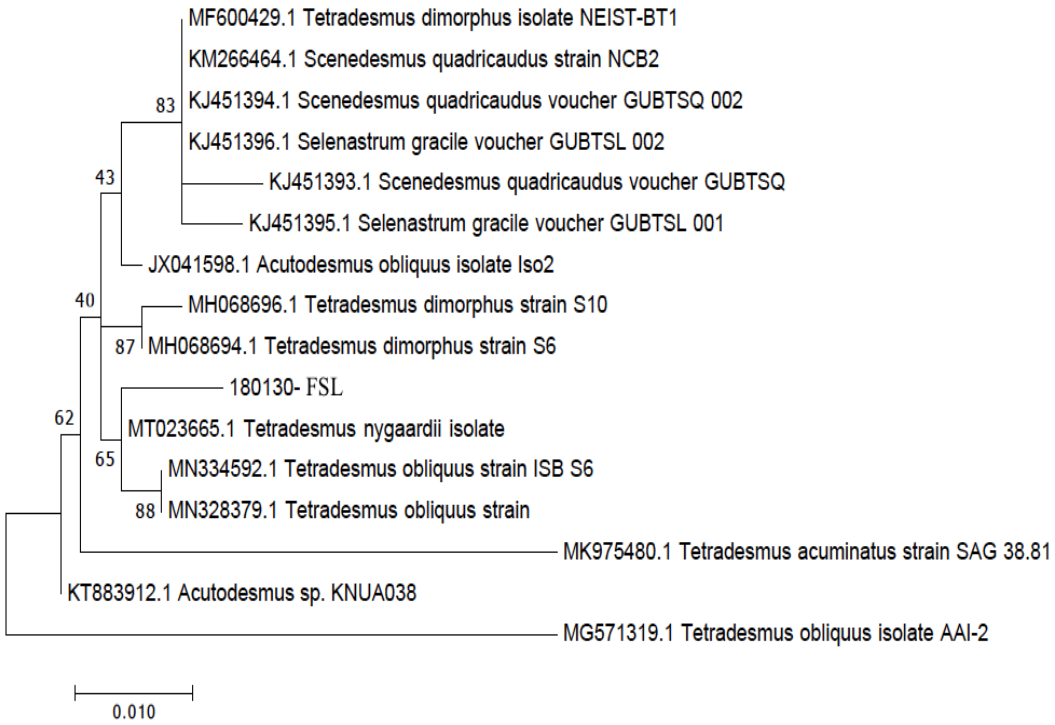


**Figure 4.1.7:** The phylogeny of Tetradesmus nygaardii (strain FSL)

## 4.2 Optimization of physicochemical parameters using Central Composite design (CCD) of experiment and response surface method (RSM) for high specific rate of growth and productivity of biomass of *Closteriopsis acicularis*

### 4.2.1 Statistical analysis and optimization of growth factors (phosphate-concentration and pH)

The metabolism for synthesis of biomolecules such as high biomass and lipids production could be advanced by varying cultivation parameters such as temperature, salinity, nutrient concentration, light, pH and $CO_2$ level. So far various studies have been carried out to demonstrate the individual and combinatorial effects of various parameters for biomass and lipids production in microalgae (Yang *et al.*, 2014; Miranda *et al.*, 2016; Peng *et al.*, 2020)(Yaakob *et al.*, 2021).

This study demonstrates the important growth parameters effect (concentration of phosphate and pH) for specific rate of growth and productivity of biomass for *Closteriopsis acicularis*. The values of response obtained during experiment (linear, square and interaction terms), were assessed by formulating the second order polynomial model following analysis of multiple regression (Minitab software, version 18). The composition of treatments was carried out using Central composite Design (CCD) of experiment (Table 3.2).

It was noticed a constant growth pattern for all 13 treatments of *Closteriopsis acicularis,* with considerably 2 days lag phase and prolong exponential phase of growth till day 21$^{th}$ of cultivation. Generally, an inoculum from healthy exponential phase culture shows very short lag phase (Talling and Fogg, 1966). The values of response associated with specific rate of growth (day$^{-1}$) as well as productivity of biomass (g L$^{-1}$ day$^{-1}$) for cultures harvested from exponential phase (late) of growth, was well correlated with the variables with the help of model equation of predictive regression in coded units as equations (4) and (5) respectively.

**Specific rate of growth** = 0.3301 + 1.058A – 0.01674 B – 7.708 A.A + 0.000973 B.B + 0.0616 A.B (4)

**Productivity of biomass** = -0.148 + 3.663 A + 0.0340 B – 21.48 A.A – 0.00042 B.B + 0.2246 A.B (5)

The results obtained from model of predictive regression revealed the two variables (concentration of phosphate and pH) affected strongly the output responses (Table 4.2.1). For commercialization and large scale production of algal biomass, rapid and high productivity of biomass are key factors that favors the over production and growth of microalgae over other microorganisms that contaminates and potentially kills algae in outdoor facilities of cultivation for bulk production (Tan and Lee, 2016). The highest specific rate of growth ($day^{-1}$) observed in present study was 0.342 $day^{-1}$ with generation time of 48.72 hrs., under growth conditions of concentration of phosphate 0.115 g $L^{-1}$ and pH 9 (Table 4.2.1), which is commensurate with specific rate of growth of microalgae; *Monoraphidium sp.* (0.35 ± 0.01) $day^{-1,}$ *Chlorella sp.* (0.39 ± 0.01), (Klin, Pniewski and Latała, 2018) and *Chlorococcum littorale* (0.134 $h^{-1}$) (Kurano and Miyachi, 2005). The maximum productivity of biomass observed was 0.497 g $L^{-1}$ $day^{-1}$ under growth conditions of concentration of phosphate (0.115 g/L) and pH 9, which is comparable with biomass productivities of *C. minutissima* 494 (0.396 g $L^{-1}$ $day^{-1}$), *Chlorella sp*. 800 (0.495 g $L^{-1}$ $day^{-1}$), *Chlorella sp*. 313 (0.451 g $L^{-1}$ $day^{-1}$) and higher than that of *Chlorella vulgaris* (75.57±1.93 mg $L^{-1}$ $day^{-1}$) reported in previous studies (Hempel, Petrick and Behrendt, 2012)(Fozer *et al.*, 2019), respectively.

The alkaline pH and high concentration of phosphate have resulted in considerably faster rate of growth and high productivity of biomass. The robust growth associated with alkaline pH (pH 8 to 9) has been reported in previous studies. pH > 9 could potentially restrain sequestration of $CO_2$ and $HCO_3^{-1}$ necessary for growth and productivity of biomass of algae (Difusa *et al.*, 2015)(Gardner *et al.*, 2011)(Bartley *et al.*, 2016). It was also observed robust growth of microalgae with slight rise of pH, whereas pH 8.2 is reported favorable for high nitrogen sequestration and formation of ammonia and nitrates. This also explains the slightly high alkalinity at later stage of exponential phase in algal cultures (Difusa *et al.*, 2015)(Eisele and Ullrich, 1977). The rate of growth and

productivity of biomass in microalgae are linearly proportional concentration of phosphate in medium (Yaakob *et al.*, 2021).

Our findings are compatible to findings in literature reported earlier, which associates robust growth and biomass production with high concentration of phosphate and concludes that the optimum concentration of phosphate of microalgae ranges in 0.001 g $L^{-1}$ to 0.179 g $L^{-1}$ (Roopnarain, Gray and Sym, 2014).

**Table 4.2.1: CCD and Values of response**

| Run Order | Phosphate. Conc. (g/L) | PH | Specific rate of growth ($day^{-1}$) | | Productivity of biomass (g $L^{-1}$ $day^{-1}$) | |
|---|---|---|---|---|---|---|
| | | | Experimental | Predicted | Experimental | Predicted |
| 1 | +1 | -1 | 0.33 | 0.33 | 0.32 | 0.33 |
| 2 | +1 | +1 | 0.34 | 0.34 | 0.5 | 0.49 |
| 3 | +1 | 0 | 0.33 | 0.33 | 0.42 | 0.42 |
| 4 | 0 | 0 | 0.33 | 0.33 | 0.36 | 0.35 |
| 5 | -1 | +1 | 0.29 | 0.23 | 0.26 | 0.24 |
| 6 | 0 | -1 | 0.33 | 0.33 | 0.23 | 0.26 |
| 7 | 0 | 0 | 0.33 | 0.33 | 0.34 | 0.35 |
| 8 | 0 | 0 | 0.33 | 0.33 | 0.36 | 0.35 |
| 9 | -1 | -1 | 0.23 | 0.23 | 0.15 | 0.15 |
| 10 | 0 | +1 | 0.33 | 0.33 | 0.4 | 0.41 |
| 11 | 0 | 0 | 0.33 | 0.33 | 0.34 | 0.35 |
| 12 | -1 | 0 | 0.23 | 0.29 | 0.18 | 0.2 |
| 13 | 0 | 0 | 0.33 | 0.33 | 0.35 | 0.35 |

The coefficients of determination, $R^2$ were calculated to evaluate the prediction capability of model, which reveals the correlation between predicted and values of experimental response (specific rate of growth & productivity of biomass). The prediction of accurate statistical model was assessed by observing high $R^2$ and near adj. $R^2$ values for responses (Table 4.2.2 and 4.2.3).

The effect of main parameters (concentration of phosphate & pH) upon specific rate of growth and productivity of biomass was observed using factorial plots, which revealed significantly higher influence on phosphate intake on both responses. The pH revealed more prominent effect on productivity of biomass only (Fig.4.2.1a and 4.2.1c). Fig. 4.2.1b shows the interaction effect of phosphate (0.1 g $L^{-}$) and pH (9) was optimum for

specific rate of growth, whereas the coupled effect on productivity of biomass was observed optimum at pH 9 and concentration of phosphate > 0.1 g $L^{-1}$.

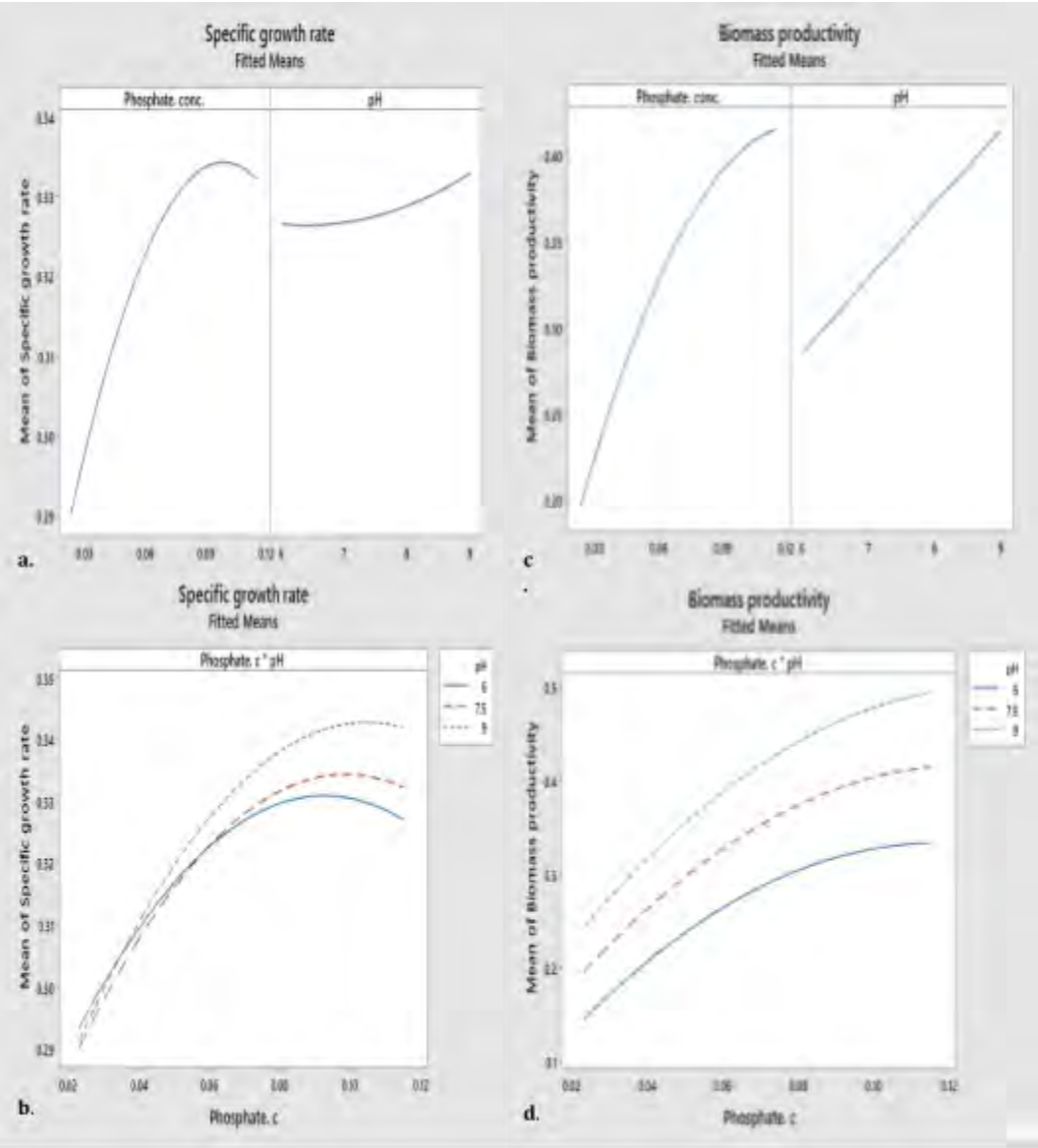


**Figure 4.2.1**: ***Closteriopsis acicularis;*** **Factorial plots of individual effect (a, c) & interactive effect (b, d) for specific rate of growth and productivity of biomass**

The Pareto Charts (Standardized) at confidence level of 95% were plotted to evaluate the effect of variables significance on linear (A, concentration of phosphate & B, pH), square and interaction terms for responses (specific rate of growth & productivity of biomass). In Fig. 4.2.2 the prominent line drawn vertically specifies the threshold with average value of 2.36 statistically significant for both responses. In Fig. 4.2.2a and 4.2.2b Pareto

chart depicts model terms associated significantly or insignificantly with responses other than square pH, which showed insignificant association among responses.

To evaluate further, for estimation of F-statistics and Probability, the ANOVA was performed. The significance or insignificance of modal variables and their interactions could be indicated by high F-value and low P-value ($P<0.05$) (Naghipour *et al.*, 2016; Sultana *et al.*, 2020). The ANOVA results for specific rate of growth and productivity of biomass has been shown in table 4.4 and 4.5, respectively. It can be observed extremely low P-values of the model for both responses as ($P=0.000$), indicating significance of respective predictive model associated with the response. It was also observed high significance at confidence level of 95% ($P<0.05$, $\alpha=0.05$) for the linear terms of both variables. However, the square terms for pH in case of both responses revealed insignificant. The interaction term (AB) of both responses was found significant. The overall ANOVA results were according to results obtained from conclusions of data from Pareto charts.

### 4.2.2 Response Surface Analysis

The response Surface method was utilized to assess the optimum responses after interaction of experimental variable (concentration of phosphate & pH) fixed at two levels. 2 dimensional counter plots and 3 dimensional surface plots for response are shown in figure 4.2.3 and 4.2.4. The variable range optimum for high specific rate of growth of *Closteriopsis acicularis* is shown in figure 4.2.3, which depicts that the specific rate of growth increased with high concentration of phosphate and alkaline pH. The concentration of phosphate and pH (+1 level) has resulted in maximum rate of growth. The maximum specific rate of growth (>0.336 g $day^{-1}$) was obtained with coupled effect of alkaline pH and high concentration of phosphate (Fig. 4.2.3). The productivity of biomass was obtained maximum (>0.45 g $L^{-1}$ $day^{-1}$) with alkaline pH and high concentration of phosphate at +1 levels (Fig. 4.2.4), which infers that the algae (*Closteriopsis acicularis*) has resulted in higher specific rate of growth and productivity of biomass with the high pH and concentration of phosphate and our findings are in

accordance with the reports of literature earlier (Roopnarain, Gray and Sym, 2014)(Difusa *et al.*, 2015)

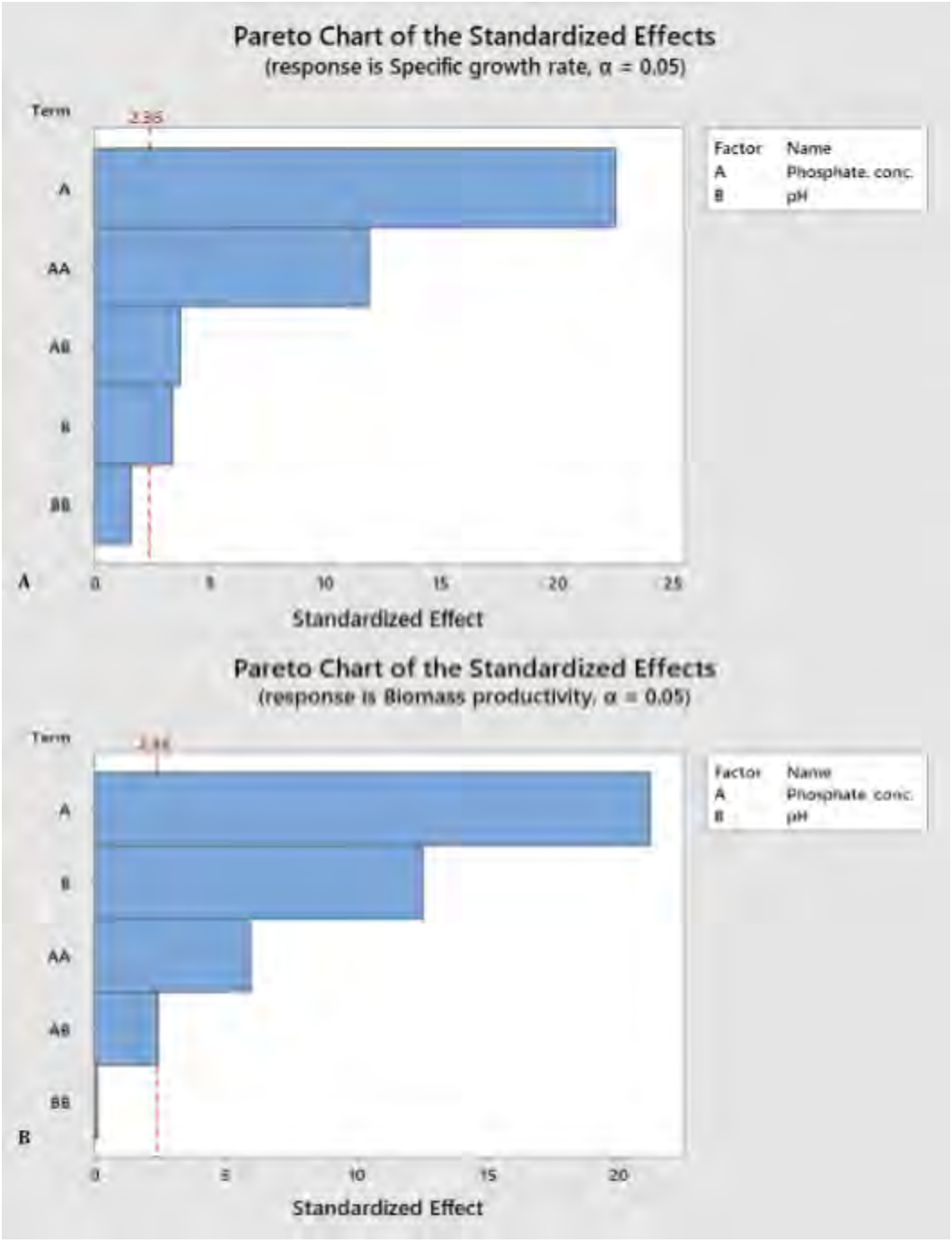


**Figure 4.2.2: Pareto chart (Standardized) for *Closteriopsis acicularis* variables screening included in optimization of ; (a) Specific rate of growth, (b) Productivity of biomass.**

**Table 4.2.2: CCD statistical analysis ANOVA for Specific rate of growth ($day^{-1}$)**

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Model | 5 | 0.0036 | 0.0007 | 136.6 | 0 |
| Linear | 2 | 0.0028 | 0.0014 | 259.3 | 0 |
| Phosphate. Conc. | 1 | 0.0027 | 0.0027 | 507 | 0 |
| PH | 1 | 0.0000 | 0.0000 | 11.5 | 0.012 |
| Square | 2 | 0.0008 | 0.0004 | 75.4 | 0 |
| Phosphate. conc.*Phosphate. Conc. | 1 | 0.0007 | 0.0001 | 140.8 | 0 |
| PH*pH | 1 | 0.0000 | 0.0000 | 2.5 | 0.155 |
| 2-Way Interaction | 1 | 0.0001 | 0.0001 | 13.8 | 0.007 |
| Phosphate. Conc.*pH | 1 | 0.0001 | 0.0001 | 13.8 | 0.007 |
| Error | 7 | 0.0000 | 0.0000 | | |
| Lack-of-Fit | 3 | 0.0000 | 0.0000 | 2.5 | 0.201 |
| Pure Error | 4 | 0.0000 | 0.0000 | | |
| Total | 12 | 0.0036 | | | |

$R^2$ = 98.99%, $R^2$ (adj.) = 98.26%, $R^2$ (pred.) = 94.35%

**Table 4.2.3**: **CCD statistical analysis ANOVA for Productivity of biomass ($g\ L^{-1}\ day^{-1}$)**

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Model | 5 | 0.1051 | 0.0211 | 130.5 | 0.0 |
| Linear | 2 | 0.0974 | 0.0487 | 302.2 | 0.0 |
| Phosphate. conc. | 1 | 0.0722 | 0.0722 | 447.9 | 0.0 |
| pH | 1 | 0.0252 | 0.0252 | 156.5 | 0.0 |
| Square | 2 | 0.0068 | 0.0034 | 21.1 | 0.001 |
| Phosphate. conc.*Phosphate. conc. | 1 | 0.0057 | 0.0057 | 35.4 | 0.001 |
| pH*pH | 1 | 0.0000 | 0.0000 | 0.02 | 0.905 |
| 2-Way Interaction | 1 | 0.0010 | 0.0010 | 6 | 0.045 |
| Phosphate. conc.*pH | 1 | 0.0010 | 0.0010 | 6 | 0.045 |
| Error | 7 | 0.0011 | 0.0002 | | |
| Lack-of-Fit | 3 | 0.0009 | 0.0003 | 4.5 | 0.092 |
| Pure Error | 4 | 0.0003 | 0.0001 | | |
| Total | 12 | 0.1063 | | | |

$R^2$ = 98.94%, $R^2$ (adj.) = 98.18%, $R^2$ (pred.) = 91.62%

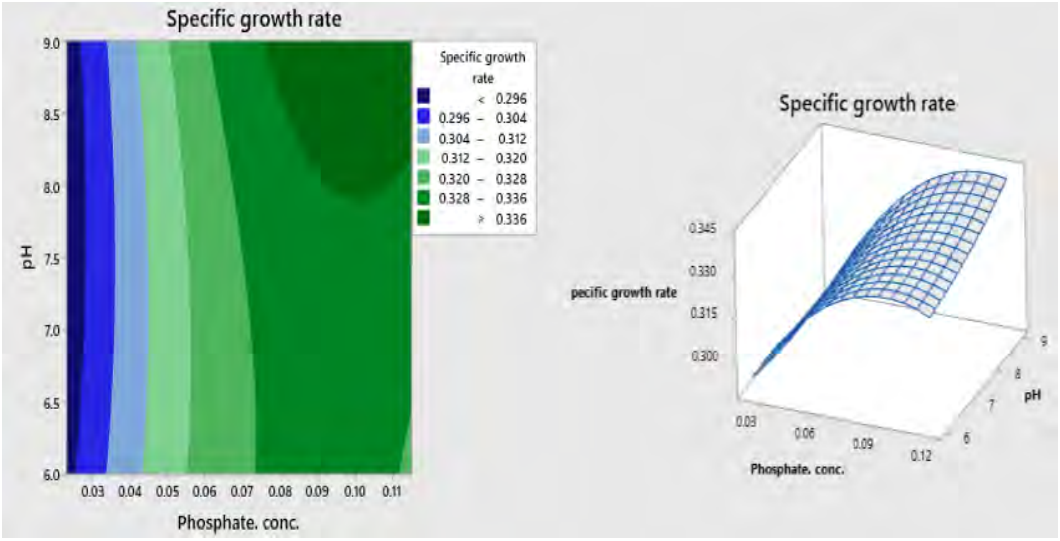


**Figure 4.2.3: 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for specific rate of growth as a function of concentration of phosphate and pH in *Closteriopsis acicularis***

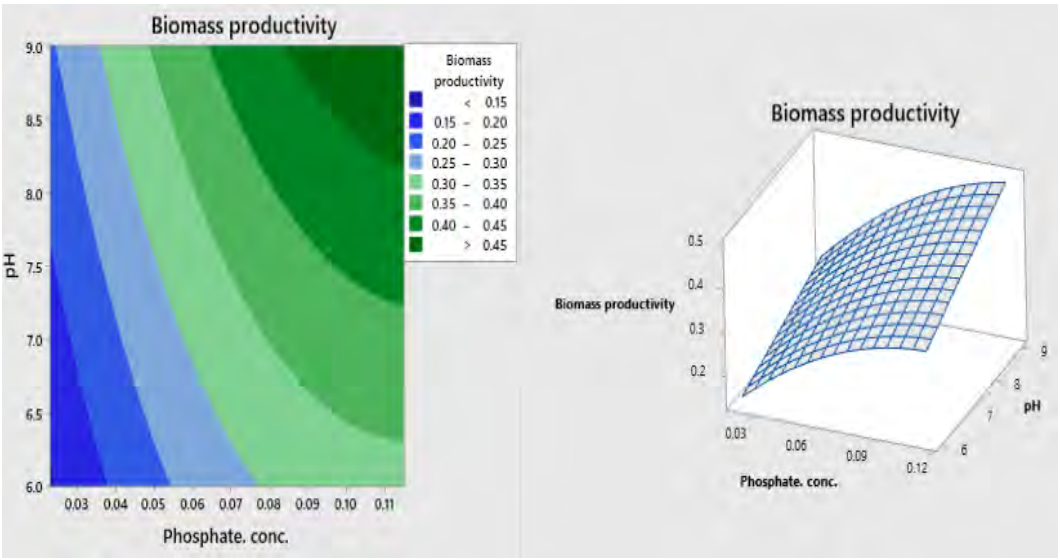


**Figure 4.2.4: 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for productivity of biomass as a function of concentration of phosphate and pH in *Closteriopsis acicularis***

## Characterization of biomass by FT-IR and SEM-EDS

In order to visualize the optimum cultivation conditions effect on *Closteriopsis acicularis*, the biomass harvested from four different treatments (2, 3, 4 & 10) with considerably high productivity of biomass was characterized with the aid of FT-IR analysis.

The biochemical composition of these biomasses was visualized by the FTI-R spectra. Generally, the spectral peaks for characterization of proteins, lipids and carbohydrates biochemically disseminates the helpful information in range of 600-4000 $cm^{-1}$. Generally, the spectral bands in the spectral range of 900-1200$cm^{-1}$, 1540-1640$cm^{-1}$ and 1735-2917$cm^{-1}$ specifies the carbohydrates, proteins and lipids functional groups in algal biomass (Piasecka *et al.*, 2020).

Figure 4.2.5d, 4.2.5a, 4.2.5b and 4.2.5c depicts the FTIR biomass spectra of treatment 2, 3, 4 and 10 (Table 4.2.1), respectively. According to Beer-lambert's law, the light absorbance is directly proportional to the compound concentration and their length of path (Lee, 1999)(Huesemann *et al.*, 2016). The biomolecules accumulation in biomass was compared based on values of absorbance and intensity of spectral peaks (Fig. 4.2.5e). The absorption maximum and spectral peaks intensity was observed in biomass derived from treatment with high concentration of phosphate and alkaline pH, for stretching of -C-O-C- group of carbohydrates (1059.67$cm^{-1}$) (Giordano *et al.*, 2001), the functional groups for amide I & amide II of proteins (1627.64 &1537.58$cm^{-1}$) (Movasaghi, Rehman and Rehman, 2008)(Naumann, Fabian and Lasch, 2009)(Coates, 2006)(Stehfest, Toepel and Wilhelm, 2005), whereas symmetric and asymmetric stretching of saturated fatty acids methylene group (2920.16 &2851.44$cm^{-1}$) (Naumann, Fabian and Lasch, 2009)(Giordano *et al.*, 2001).

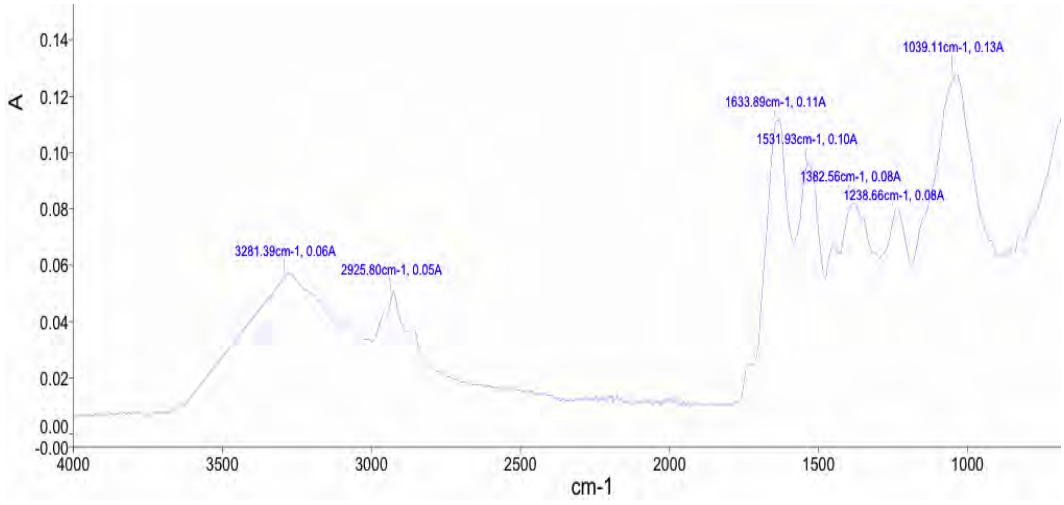


**Figure 4.2.5a**: FT-IR analysis of biomass grown in treatment 3 for *Closteriopsis acicularis*

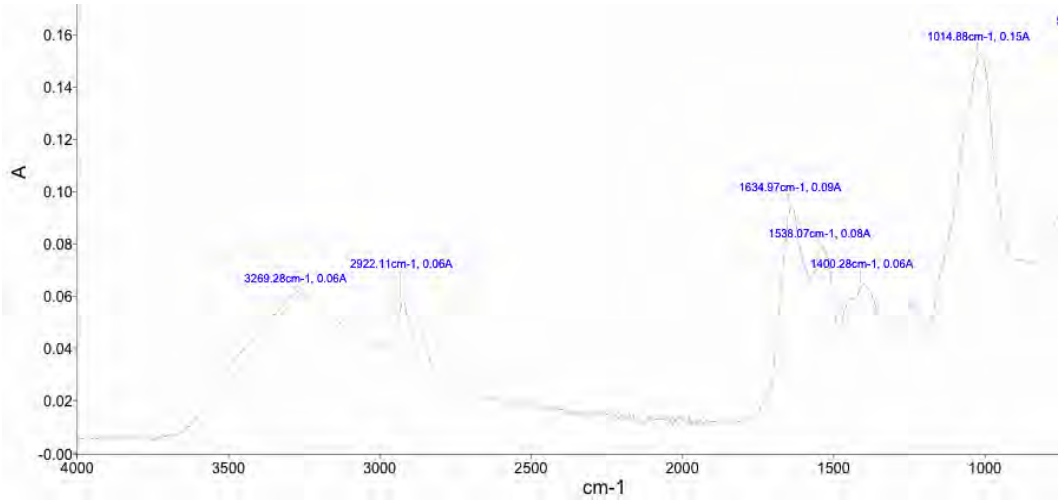


**Figure 4.2.5b**: FT-IR analysis of biomass grown in treatment 4 for *Closteriopsis acicularis*

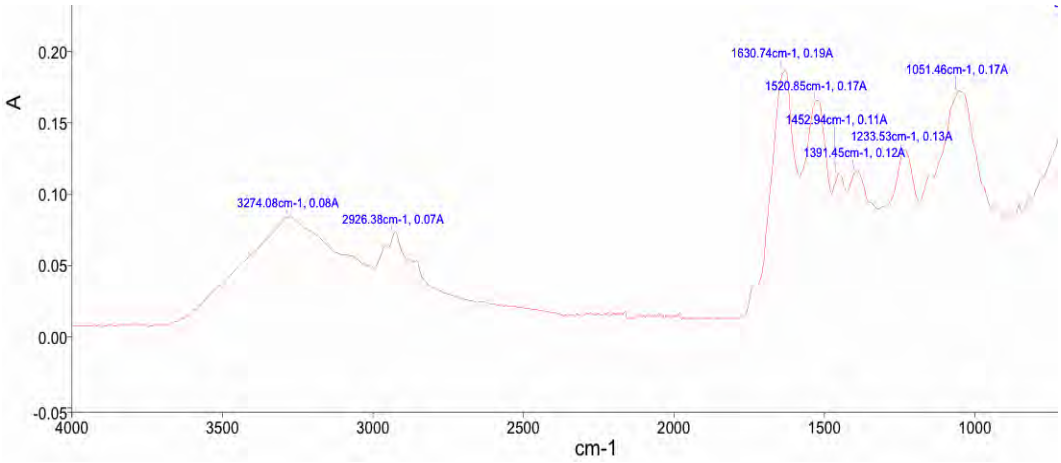


**Figure 4.2.5c**: FT-IR analysis of biomass grown in treatment 10 for *Closteriopsis acicularis*

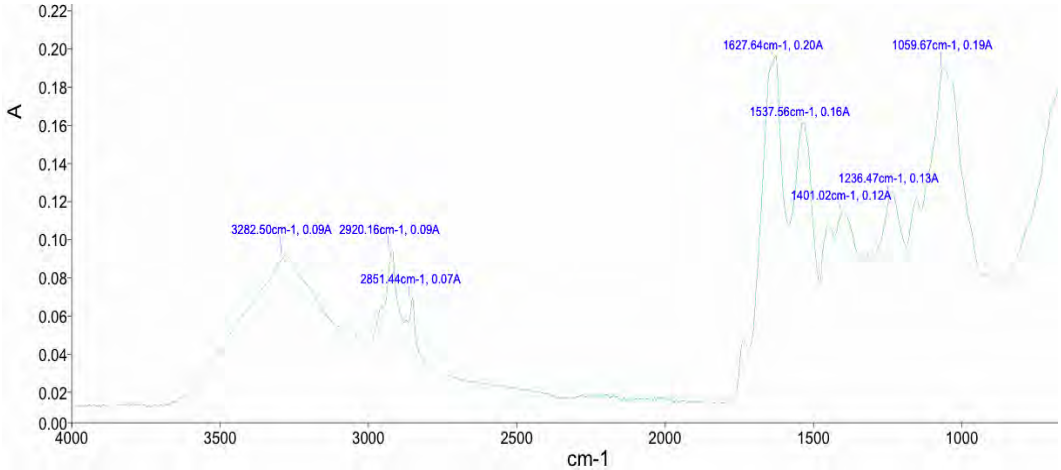


**Figure 4.2.5d**: FT-IR analysis of biomass grown in treatment 2 for *Closteriopsis acicularis*

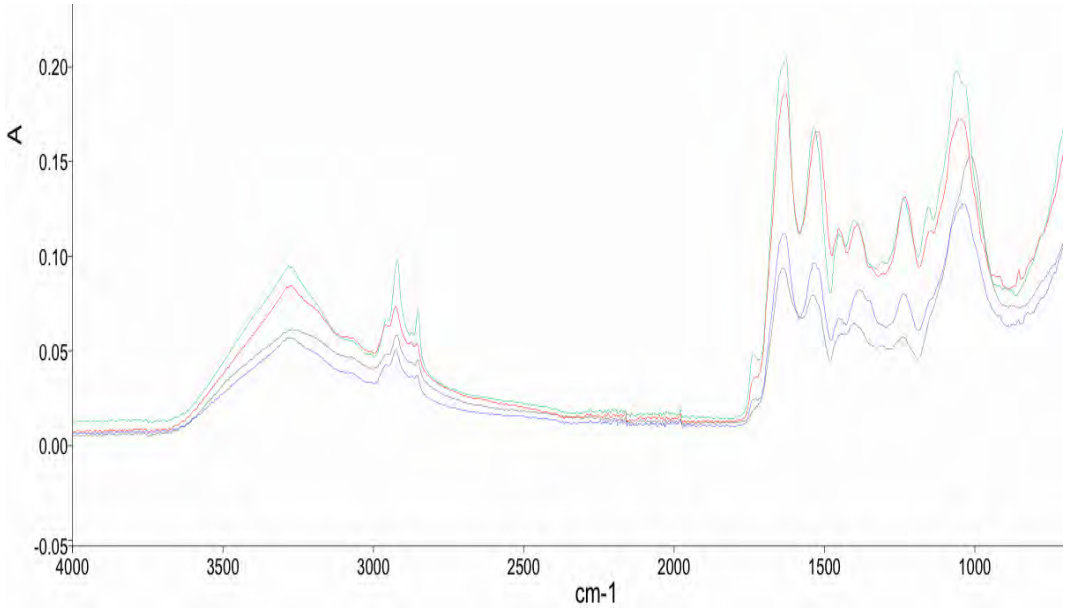


**Figure 4.2.5e**: FT-IR spectra for comparative analysis of *Closteriopsis acicularis* biomass under different treatments, depicting biomass under optimum conditions in treatment 2

It is also observed clearly from figure 4.2.5e, that the biochemical composition in all biomasses have appeared to show almost same assignments of spectral peaks in specified functional group regions allotted to lipids, carbohydrates and proteins but with varying values of absorption and spectral peaks intensity.

Phosphorus is an essential nutrient for microalgae growth and reproduction. It has tremendous ability to sequester phosphorus from nutrient medium and extent of biomass production is directly proportional to phosphorus uptake (Chu *et al.*, 2013)(Yaakob *et al.*, 2021). The concentration of phosphate and alkaline pH effect was assessed on sequestration of organic and inorganic elements by finding out the elemental composition biomass taken from treatments (2 and 4, Table 4.2.1) with optimized and un optimized biomass.

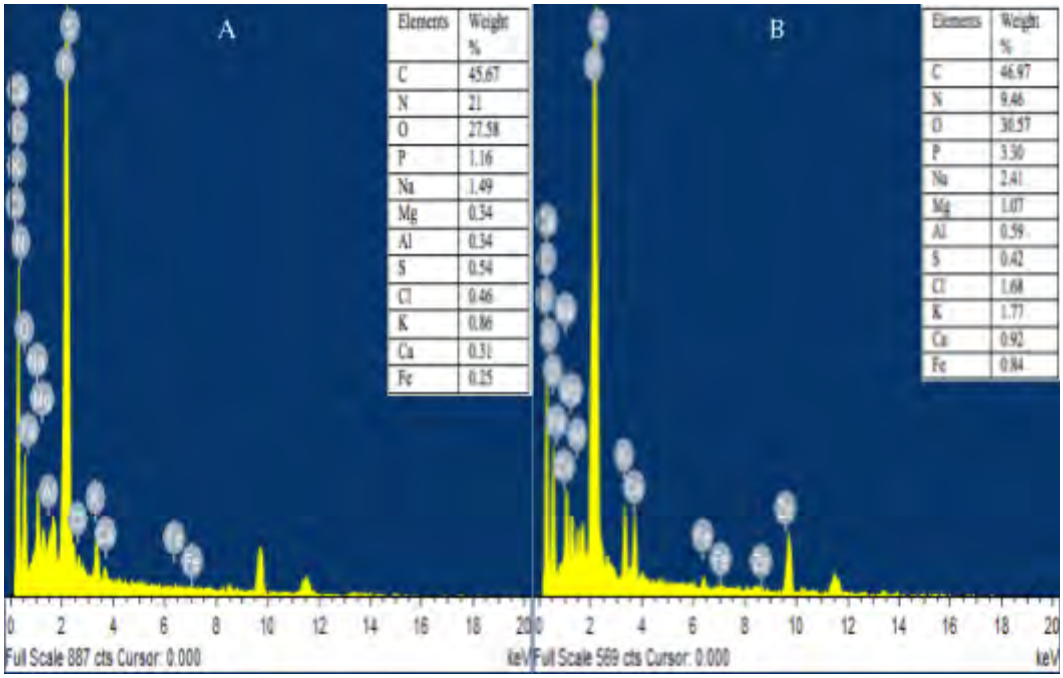


**Figure 4.2.6**: **SEM-EDS analysis of biomass grown under low concentration of phosphate and pH (A) & high concentration of phosphate and pH (B), for *Closteriopsis acicularis***

It was observed high carbon, oxygen, phosphorus and inorganic elements accumulation such as sodium, magnesium, potassium except Sulphur and nitrogen, which are necessary for proteins formation, biomass harvested from culture conditions with high concentration of phosphate and alkaline pH (Fig. 4.2.6b). The elemental makeup and composition was found higher in optimized biomass in present study as compared to reported in earlier studies for microalgae (Rendón-Castrillón *et al.*, 2021)(Rendón-Castrillón *et al.*, 2021). It is also reported that the high elemental concentration tends to enlarge the size of cells and surface to volume ratio that increase the amount of chloroplast and photosynthesis (Acevedo and Ramírez, 2003). Thus, according to results attained by characterization of microalgae biomass in this study, the concentration of phosphate and pH optimization has resulted in considerably nutrient rich and high biomass production.

### 4.2.3 Production and estimation of Bioethanol

The total carbohydrates content of biomass derived from *Closteriopsis acicularis* was calculated as 58% (w/w) of CDW. For ethanol production, the high carbohydrate content rich in fermentable sugars (monosaccharide) are suitable. In previous studies, the

maximum carbohydrate content of 55% and 60% has been reported for microalgae *C. reinhardtii* (Kim *et al.*, 2006) and *C. vulgaris* (Brennan and Owende, 2010), respectively. The amount of glucose was estimated after the acid catalyzed saccharification of biomass as 29.3 g/L. It was observed that approximately whole amount of the monosaccharide was utilized by *Saccharomyces cerevisiae* after about 12hrs of incubation and yielded maximum ethanol as 14.9 g/L. In the fermentation process, the yield of ethanol was estimated as 51% g ethanol/g glucose, which is comparable with ethanol yield obtained as 0.51 and 0.48 g ethanol/g glucose in earlier studies (Kim *et al.*, 2020)(Adela, B. N. and Loh, S. K., 2015), respectively.

## 4.3 Optimization of physicochemical parameters using Box-Behnken design (BBD) of experiment and response surface method (RSM) for high biomass and lipids production in *Tetradesmus nygaardii*

### 4.3.1 Statistical analysis optimization of growth factors (N-concentration, Temperature and pH)

The metabolism for synthesis of biomolecules such as high biomass and lipids production could be advanced by varying cultivation parameters such as temperature, salinity, nutrient concentration, light, pH and $CO_2$ level. So far various studies have been carried out to demonstrate the individual and combinatorial effects of various parameters for biomass and lipids production in microalgae (Yang *et al.*, 2014; Miranda *et al.*, 2016; Peng *et al.*, 2020).

This study was conducted to demonstrate the effect of pH, nitrogen concentration and temperature on biomass production, total lipids Production and lipids content (% of CDW) of microalgae *Tetradesmus nygaardii* (TN). The experimental values of response for linear, square and interaction terms were assessed by formulating the second order polynomial model and conducting the multiple regression analysis (Minitab, 18). The composition of treatments at level of factors (low, -1, Center, 0 & high, +1) was carried out using Box-Behnken Design (BBD) of experiment (Table 3.1).

A constant growth pattern was observed for *Tetradesmus nygaardii* in all 15 treatments. Generally, an inoculum from healthy exponential phase culture shows very short lag phase(Talling and Fogg, 1966). By using predictive model of regression equation in coded units, the values of response of biomass production (mg/L), lipids production (mg/L) and lipids content (% of CDW) of cultures harvested were correlated with variables (pH, N-concentration, Temperature), as equations (6), (7) and (8) respectively.

**Biomass production (Y1)** = 745 - 833.1 A + 10.83 B – 112.4 C – 187.5 A.A -0.4500 B.B + 3.00 C.C + 11.25 A.B + 102.5 A.C + 1.950 B.C. (6)

**Lipids Production (Y2)** = -452 + 446.9 A + 26.03 B + 122.3 C – 108.3 A.A –0.3133 B.B -5.58 C.C – 4.50 A.B – 56.25 A.C – 1.750 B.C. (7)

**Lipids Content (Y3)** = -80.7 +143.1 A + 3.73 B + 28.63 C + 3.1 A.A – 0.0250 B.B – 1.125 C.C – 1.500 A.B – 20.00 A.C – 0.450 B.C. (8)

The predictive regression model show that pH, Nitrogen concentration, and temperature influenced the output responses strongly (Table 4.3.1). The highest biomass concentration in this study was observed as 543 mg/L with N-Concentration 0.5 g/L, pH 8 and temperature 30ºC which is comparable with highest biomass production of *Chlorella sp.* and *Tetradesmus sp.* reported in recent studies (Bunkaew and Kongruang, 2020; Piasecka *et al.*, 2020). The maximum lipids production and lipids content was found as 272mg/L and 60% of CDW, respectively, with 0.1 g/L N-concentration, pH 7 and temperature 25ºC which is comparable with lipids production and % lipids content of *Chlorella sp*. and *Scenedesmus sp.* reported in previous studies (Yang *et al.*, 2014; Bunkaew and Kongruang, 2020). Lower levels of nitrogen concentration coupled with low pH or temperature and low temperature coupled with acidic pH resulted in considerably high lipids accumulation in certain treatments. Significantly high lipids Content (56-60% of CDW) was observed in treatment 3, 9, 12, which had low nitrogen concentration, acidic pH and temperature ranging between 25-30ºC, However, high lipids accumulation in these treatments resulted in slightly low biomass concentration (Table 4.3.1). Overall, the gain of lipids resulted in loss of biomass in present study. Our experimental findings are in accordance with results reported by earlier researchers(Rodolfi *et al.*, 2009; Mujtaba *et al.*, 2012; Ma *et al.*, 2014; Chaisutyakorn, Praiboon and Kaewsuralikhit, 2018; Peng *et al.*, 2020).

The coefficients of determination, $R^2$ were calculated to evaluate the prediction capability of model, which reveals the correlated predicted and experimental values of response (Y1, Y2 and Y3). The prediction of model as statistically accurate, was assessed by observing high $R^2$ and close adjacent $R^2$ values for responses (Table 4.3.2,4,5). The Pareto Charts of Standardized effect with confidence level of 95% was plotted for values of response (Y1=Biomass production (mg/L), Y2=lipids production (mg/L) and Y3=lipids content (% of CDW)) to find the significant variables effect on their linear (A=N- concentration, B=Temperature and C=pH), square ($A^2$, $B^2$ and $C^2$) and interaction

terms (AB, AC and BC) for biomass and lipids production, From Figure 4.3.1, 4.3.2 & 4.3.3, the dominant line drawn vertically specifies the threshold statistically significant with average value of 2.57 for all responses. The Pareto chart depicts the individual, linear, squares and their interactions of variables as associated significantly with output responses other than square terms of lipids content (Fig. 4.3.3), $C^2$ for biomass production (Fig. 4.3.1) and $A^2$ and AB for lipids production (Fig. 4.3.2), which shows response associated insignificance of variables.

Table 4.3.1**:** BBD and values of response (Y)

| **Run order** | **N-Conc. A** | **Temperature B** | **PH** | **Biomass production Y1 (mg / L)** | | **Lipids production Y2 (mg / L)** | | **Lipids content Y3 (% Of CDW)** | |
|---|---|---|---|---|---|---|---|---|---|
| | **(g/L)** | **($^o$C)** | | Exp. | Pred. | Exp. | Pred. | Exp. | Pred. |
| 1 | 0.1 | 35 | 7 | 436 | 436.4 | 221 | 217.5 | 50 | 49.1 |
| 2 | 0.5 | 30 | 6 | 460 | 462 | 226 | 224.9 | 49 | 48.6 |
| 3 | **0.1** | **25** | **7** | 451 | 450.4 | **272** | **272.3** | **60** | **59.9** |
| 4 | 0.3 | 35 | 6 | 454 | 451.4 | 215 | 216.4 | 47 | 47.3 |
| 5 | 0.3 | 30 | 7 | 479 | 484 | 227 | 227.7 | 47 | 47 |
| 6 | 0.3 | 30 | 7 | 485 | 484 | 231 | 227.7 | 48 | 47 |
| 7 | 0.5 | 35 | 7 | 502 | 502.6 | 150 | 149.8 | 30 | 30.1 |
| 8 | 0.5 | 25 | 7 | 472 | 471.6 | 219 | 222.5 | 46 | 46.9 |
| 9 | 0.1 | 30 | 6 | 457 | 459.3 | 259 | 261.1 | 56 | 56.6 |
| 10 | 0.3 | 25 | 8 | 478 | 480.63 | 231 | 229.6 | 48 | 47.8 |
| 11 | 0.3 | 30 | 7 | 488 | 484 | 225 | 227.7 | 46 | 47 |
| 12 | 0.3 | 25 | 6 | 464 | 462.4 | 265 | 262.6 | 57 | 56.5 |
| 13 | **0.5** | **30** | **8** | **543** | **540.8** | 154 | 151.9 | 28 | 27.4 |
| 14 | 0.1 | 30 | 8 | 458 | 456 | 232 | 233.1 | 51 | 51.4 |
| 15 | 0.3 | 35 | 8 | 507 | 508.6 | 146 | 148.4 | 29 | 29.5 |

To evaluate further, for estimation of F-statistics and Probability , the ANOVA was performed. The significance or insignificance of modal variables and their interactions could be indicated by high F-value and low P-value ($P<0.05$) (Naghipour *et al.*, 2016; Sultana *et al.*, 2020). Table 4.3.2, 4.3.3 and 4.3.4 show Analysis of variance for response Y1, Y2 and Y3 respectively. It can be observed easily that, P-values of the model for both responses are significantly low ($P=0.000$) indicating significance of respective predictive

model associated with the response. The square terms $A^2$ and $B^2$ for biomass production, $B^2$ and $C^2$ for lipids production were significant, whereas all interaction terms for three responses except AB of lipids production were significant.

**Table 4.3.2**: Statistical analysis (ANOVA) of BBD for Biomass Production (mg/L), response Y1

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|
| Model | 9 | 10091.4 | 1121.26 | 69.86 | 0 |
| Linear | 3 | 6822.8 | 2274.25 | 141.7 | 0 |
| N-Conc. | 1 | 3828.1 | 3828.12 | 238.51 | 0 |
| Temperature | 1 | 144.5 | 144.5 | 9 | 0.03 |
| PH | 1 | 2850.1 | 2850.13 | 177.58 | 0 |
| Square | 3 | 701.1 | 233.7 | 14.56 | 0.007 |
| N-Conc.*N-Conc. | 1 | 207.7 | 207.69 | 12.94 | 0.016 |
| Temperature*Temperature | 1 | 467.3 | 467.31 | 29.12 | 0.003 |
| PH*PH | 1 | 33.2 | 33.23 | 2.07 | 0.21 |
| 2-Way Interaction | 3 | 2567.5 | 855.83 | 53.32 | 0 |
| N-Conc.*Temperature | 1 | 506.2 | 506.25 | 31.54 | 0.002 |
| N-Conc.*PH | 1 | 1681 | 1681 | 104.74 | 0 |
| Temperature*PH | 1 | 380.2 | 380.25 | 23.69 | 0.005 |
| Error | 5 | 80.3 | 16.05 | | |
| Lack-of-Fit | 3 | 38.3 | 12.75 | 0.61 | 0.671 |
| Pure Error | 2 | 42 | 21 | | |
| Total | 14 | 10171.6 | | | |

$R^2$ = 99.21%, $R^2$ (adj.) = 97.7%, $R^2$ (pred.) = 93.05%

**Table 4.3.3**: Statistical analysis (ANOVA) of BBD for Lipids Production, Response Y2

| Source | DF | Adj SS | Adj MS | F-Value | P-Value |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| Model | 9 | 21386.5 | 2376.28 | 169.94 | 0 |
| Linear | 3 | 20131.8 | 6710.58 | 479.9 | 0 |
| N-Conc. | 1 | 6903.1 | 6903.13 | 493.67 | 0 |
| Temperature | 1 | 8128.1 | 8128.13 | 581.27 | 0 |
| PH | 1 | 5100.5 | 5100.5 | 364.76 | 0 |
| Square | 3 | 361.2 | 120.41 | 8.61 | 0.02 |
| N-Conc.*N-Conc. | 1 | 69.3 | 69.33 | 4.96 | 0.076 |
| Temperature*Temperature | 1 | 226.6 | 226.56 | 16.2 | 0.01 |
| PH*PH | 1 | 115.1 | 115.1 | 8.23 | 0.035 |
| 2-Way Interaction | 3 | 893.5 | 297.83 | 21.3 | 0.003 |
| N-Conc.*Temperature | 1 | 81 | 81 | 5.79 | 0.061 |
| N-Conc.*PH | 1 | 506.3 | 506.25 | 36.2 | 0.002 |
| Temperature*PH | 1 | 306.3 | 306.25 | 21.9 | 0.005 |
| Error | 5 | 69.9 | 13.98 | | |
| Lack-of-Fit | 3 | 51.2 | 17.08 | 1.83 | 0.372 |
| Pure Error | 2 | 18.7 | 9.33 | | |
| Total | 14 | 21456.4 | | | |

$R^2$ = 99.67%, $R^2$ (adj.) = 99.09%, $R^2$ (pred.) = 95.98%

**Table 4.3.4**: Statistical analysis (ANOVA) of BBD for Lipids content, Response Y3

| **Source** | **DF** | **Adj SS** | **Adj MS** | **F-Value** | **P-Value** |
|---|---|---|---|---|---|
| Model | 9 | 1340.48 | 148.943 | 141.85 | 0 |
| Linear | 3 | 1241.25 | 413.75 | 394.05 | 0 |
| N-Conc. | 1 | 512 | 512 | 487.62 | 0 |
| Temperature | 1 | 378.13 | 378.125 | 360.12 | 0 |
| PH | 1 | 351.13 | 351.125 | 334.4 | 0 |
| Square | 3 | 5.98 | 1.994 | 1.9 | 0.248 |
| N-Conc.*N-Conc. | 1 | 0.06 | 0.058 | 0.05 | 0.824 |
| Temperature*Temperature | 1 | 1.44 | 1.442 | 1.37 | 0.294 |
| PH*PH | 1 | 4.67 | 4.673 | 4.45 | 0.089 |
| 2-Way Interaction | 3 | 93.25 | 31.083 | 29.6 | 0.001 |
| N-Conc.*Temperature | 1 | 9 | 9 | 8.57 | 0.033 |
| N-Conc.*PH | 1 | 64 | 64 | 60.95 | 0.001 |
| Temperature*PH | 1 | 20.25 | 20.25 | 19.29 | 0.007 |
| Error | 5 | 5.25 | 1.05 | | |
| Lack-of-Fit | 3 | 3.25 | 1.083 | 1.08 | 0.513 |
| Pure Error | 2 | 2 | 1 | | |
| Total | 14 | 1345.73 | | | |

$R^2$ = 99.61%, $R^2$ (adj.) = 98.91%, $R^2$ (pred.) = 95.80%

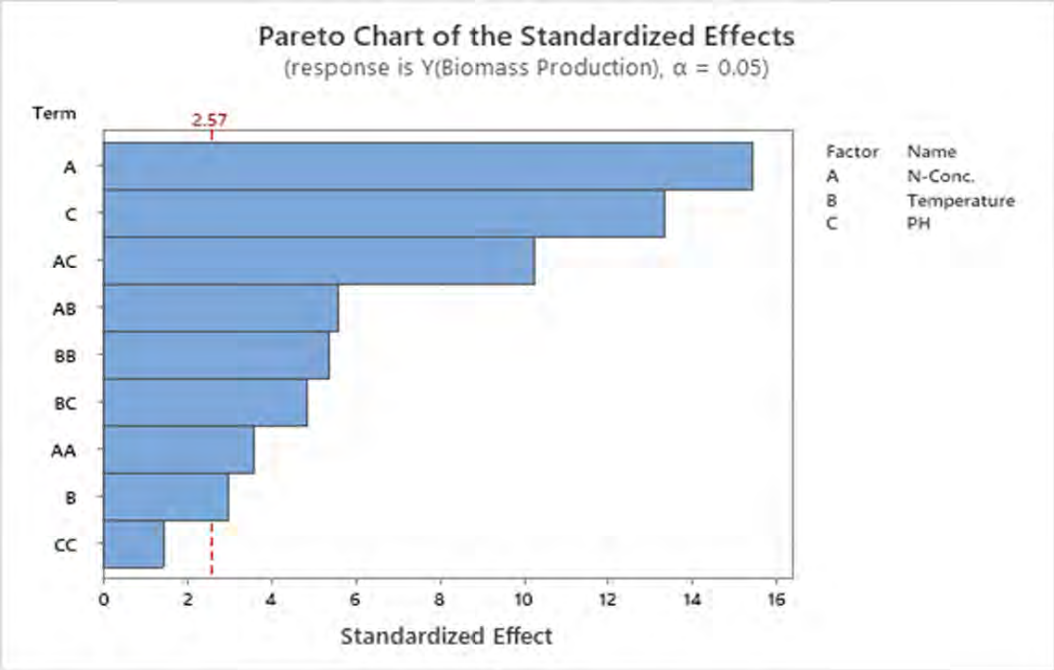


**Figure 4.3.1:** Pareto chart (Standardized) after screening of parameters upon optimization of Tetradesmus nygaardii for Biomass production mg/L.

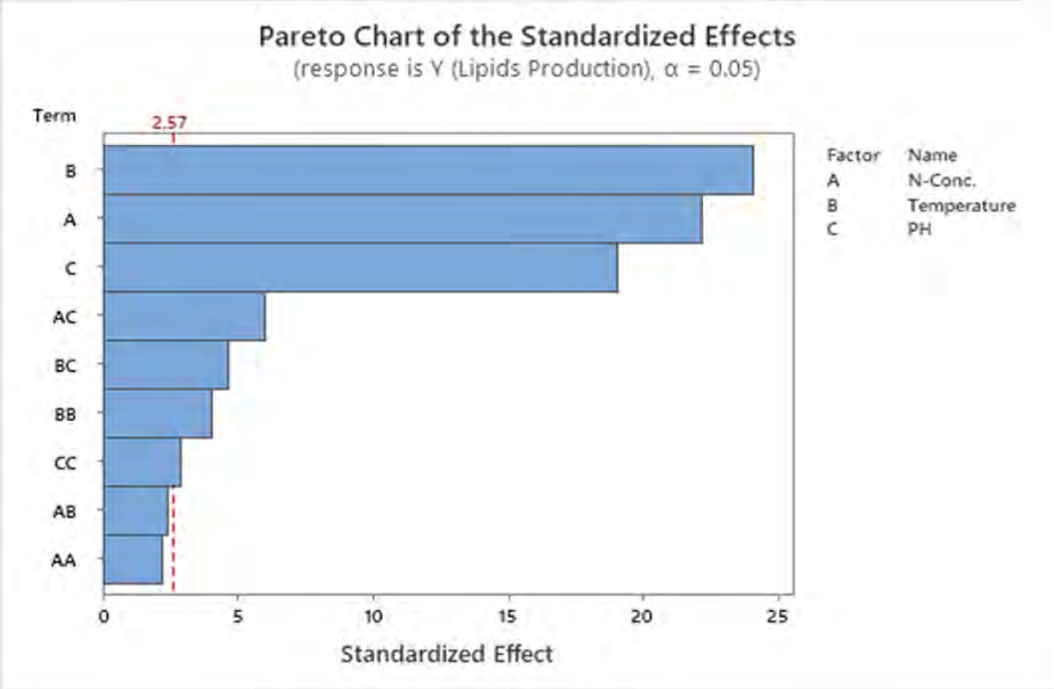


**Figure 4.3.2:** Pareto chart (Standardized) after screening of parameters upon optimization of *Tetradesmus nygaardii* for lipids production mg/L.

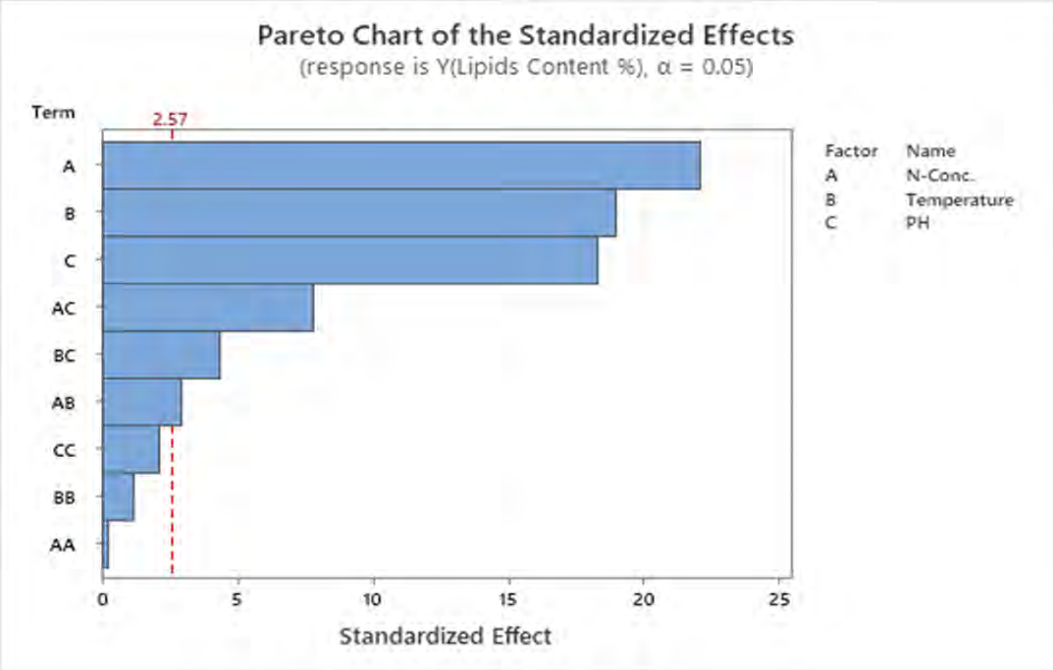


**Figure 4.3.3:** Pareto charts (Standardized) after screening of parameters involved in optimization of *Tetradesmus nygaardii* for lipids content % CDW.

### 4.3.2 Response Surface Analysis

The response Surface method (RSM) was utilized to assess the interaction of pH, N-concentration and temperature for values of optimized responses by maintaining one variable at 0 level while other two variables between higher and lower (-1 & +1) levels, respectively. The plots (2 dimensional counter & 3 dimensional surface) for responses (mg/L) are depicted in figure 4.3.4 to 4.3.9. Figure 4.3.4, 4.3.5 and 4.3.6 shows the optimum variable range for high biomass production. The interactive effect of growth parameters strongly affected the output values of response. It was observed increase in biomass production with increase in N-concentration, temperature and pH. The maximum biomass was obtained with N-concentration at higher parametric level (0.55 g/L), temperature between 0 and +1 level (30-35$^{o}$C) and pH at +1 level (8), which suggests the aided higher uptake of nitrogen at alkaline pH, which consequently resulted in higher biomass formation. The highest response value for biomass production (>540 mg/L) was obtained with interactive effect of alkaline pH and high N-concentration as depicted in surface and counter plots (interaction AC) (Fig. 4.3.5).

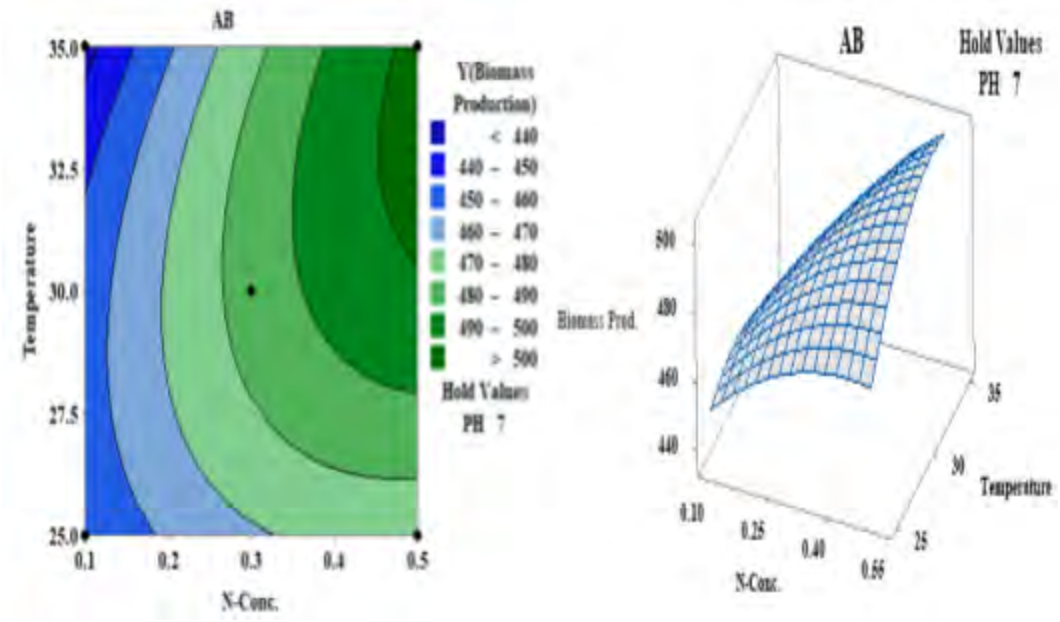


**Figure 4.3.4:** 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for biomass production of Tetradesmus nygaardii as a function of N-concentration and Temperature

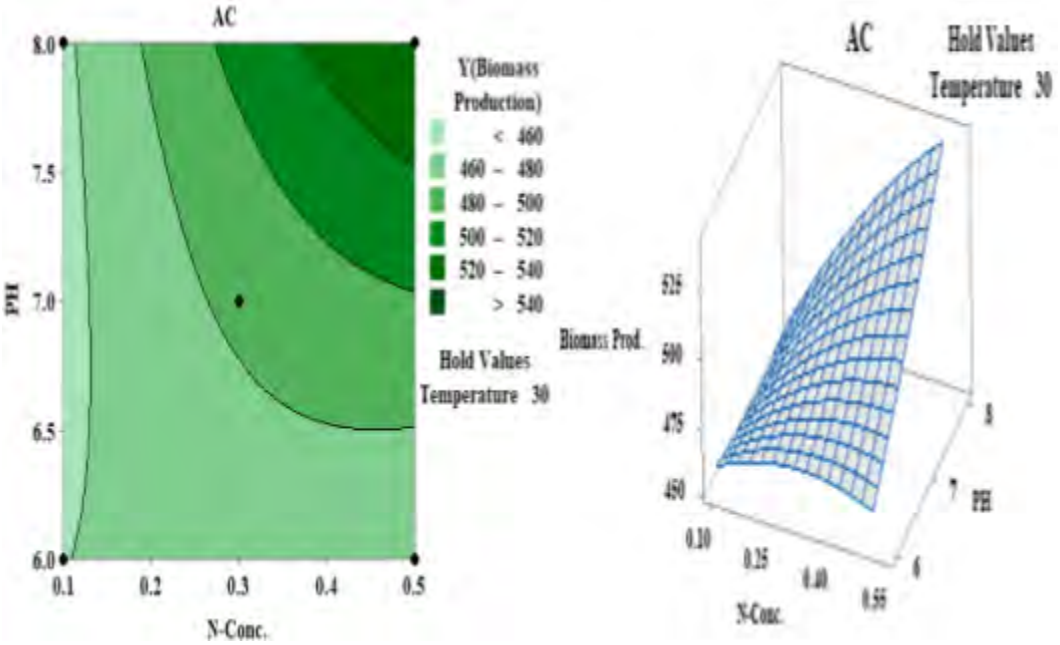


**Figure 4.3.5**: 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for biomass production of *Tetradesmus nygaardii* as a function of N-concentration and pH

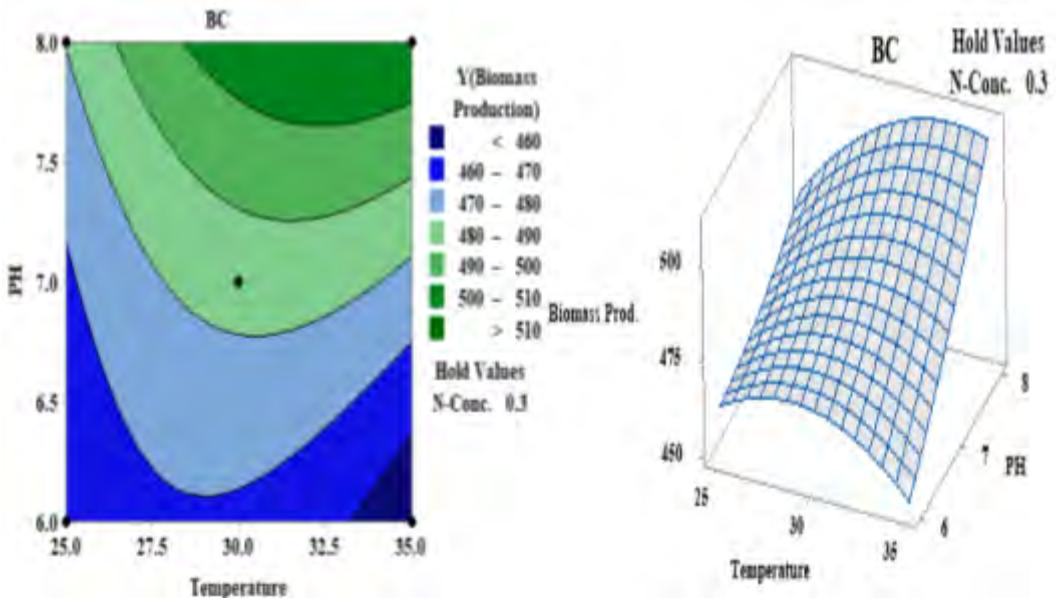


**Figure 4.3.6**: 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for biomass production of *Tetradesmus nygaardii* as a function of Temperature and pH

The lipids Production was observed maximum for interaction AC (Fig. 4.3.8). The pH and N-concentration strongly affected the lipids accumulation in present study, by showing maximum lipids accumulation of (>260 mg/L) with pH and N-concentration at -1 levels (Fig 4.3.8). The acidic pH, low temperature and low N-concentration (Treatment 3) have resulted in 43% increase in lipids content as compared to (Treatment 9). It is observed 17% increase in biomass production by increasing the concentration of nitrogen and pH in growth media while in case of lipid production, an inverse relation has been found (Treatment 9, Table 4.3.1). However, the gain of lipids was observed more prominent as compared to loss of biomass. The loss of biomass could be due to limited availability of nitrogen in growth medium.

According to results attained, the microalgae used in this study (*Tetradesmus nygaardii*) has shown high lipids accumulation in limited nitrogen concentration and low pH. The highest lipids production of strain in present study was comparable to the lipids production (299.48 ± 21.47 mg/L) reported earlier by *Tetradesmus obliquus* grown autotrophically in BBM media (Piasecka *et al.*, 2020).

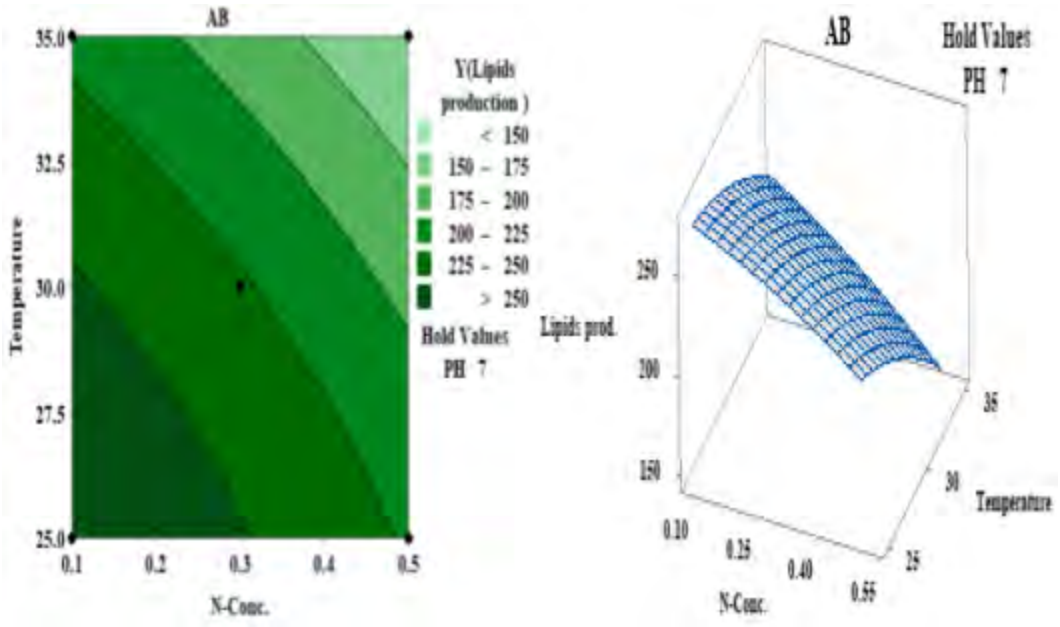


**Figure 4.3.7: 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for lipids production of *Tetradesmus nygaardii* as a function of N-concentration and Temperature**

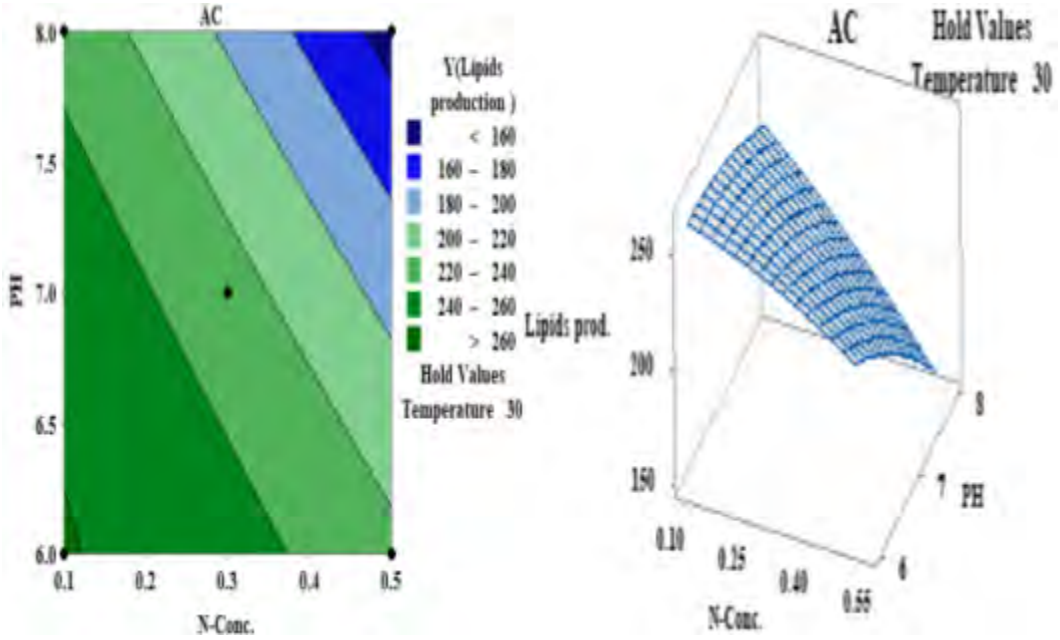


**Figure 4.3.8: 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for lipids production of *Tetradesmus nygaardii* as a function of N-concentration and pH**

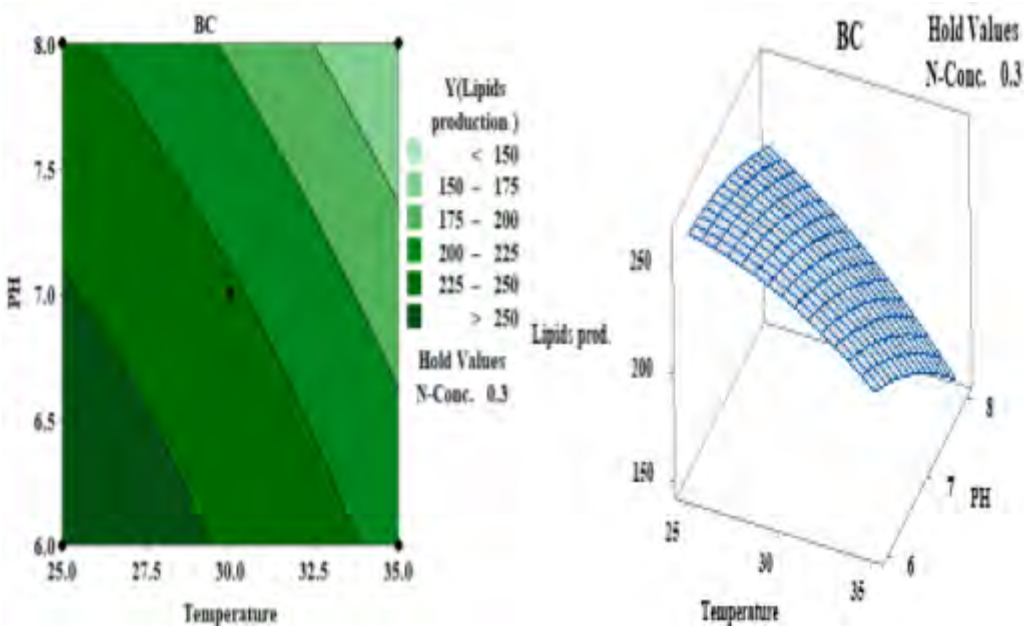


**Figure 4.3.9: 2 dimensional counter plots & 3 dimensional surface plots of 2-way variables interaction) for lipids production of *Tetradesmus nygaardii* as a function of temperature and pH**

### 4.3.3 Biomass Characterization by FTIR

Biomass and lipids production of microalgae are affected directly by changing concentration of temperature, pH and nitrogen (Chaisutyakorn, Praiboon and Kaewsuralikhit, 2018; Qi *et al.*, 2019; Minyuk, Sidorov and Solovchenko, 2020). The impact of optimized cultivation conditions was analyzed for high biomass and lipids production of *Tetradesmus nygaardii*, the biomass of treatment 3 which yielded highest lipids (272 mg/L) and treatment 13 which resulted in highest biomass production (543 mg/L) was characterized by FT-IR analysis.

The biochemical composition of these biomasses was visualized by the FTI-R spectra. Generally, the spectral peaks for characterization of lipids, proteins and carbohydrates disseminates the helpful assignments in range of 600-4000 $cm^{-1}$. Generally, the spectral assiognments in the range of 900-1200$cm^{-1}$, 1540-1640$cm^{-1}$ and 1735-2917$cm^{-1}$ specifies the carbohydrates, proteins and lipids functional groups, respectively in algal biomass (Piasecka *et al.*, 2020). The spectral assignments for distinctive functional groups of biomolecules have been shown in Table 4.3.5.

The FTIR spectra of biomass derived from optimum conditions (treatment 3) and control (treatment 13) have been shown in figure 4.3.10 and 4.3.11 respectively. From Fig. 4.3.10, the FTIR of algal biomass grown at low pH, low N- concentration and low temperature conditions have shown strong bands in range of 1540-1640$cm^{-1}$ with maximum absorption at 1627$cm^{-1}$ and 1532$cm^{-1}$, which is specified region op protein's Amide I and Amide II functional groups. Furthermore, spectral band appearing with maximum absorption at 1402$cm^{-1}$ and 3274$cm^{-1}$ are assigned to Symmetrical stretching of -COO and N-H functional group of proteins.

The spectral bands appearing in range of 2918-2927$cm^{-1}$ and 2850-2854$cm^{-1}$ with maximum absorption at 2920$cm^{-1}$ and 2851$cm^{-1}$ are assigned to asymmetric and symmetric stretching of methylene groups of fatty acids. Additionally, the band residing in range of 1052-1061$cm^{-1}$ with the maximum absorption at 1058 has been assigned to C-O-H functional group of carbohydrates.

Fig 4.3.11 represents the FTIR spectra of algal biomass cultivated in optimized conditions. The spectral bands appearing in range of 2918-2927$cm^{-1}$ with maximum of absorption peaks at 2922$cm^{-1}$ and 2853$cm^{-1}$ are assigned to asymmetric and symmetric stretching of methylene and methyl group of fatty acids. Additionally, the band appearing in range of 1740-1743$cm^{-1}$ with maximum absorption peak at 1743$cm^{-1}$ shows stretching of C=O functional group of fatty acids. The two bands appearing at 1463$cm^{-1}$ and 1377$cm^{-1}$ are also assigned to bending of methylene and methyl group of fatty acids.

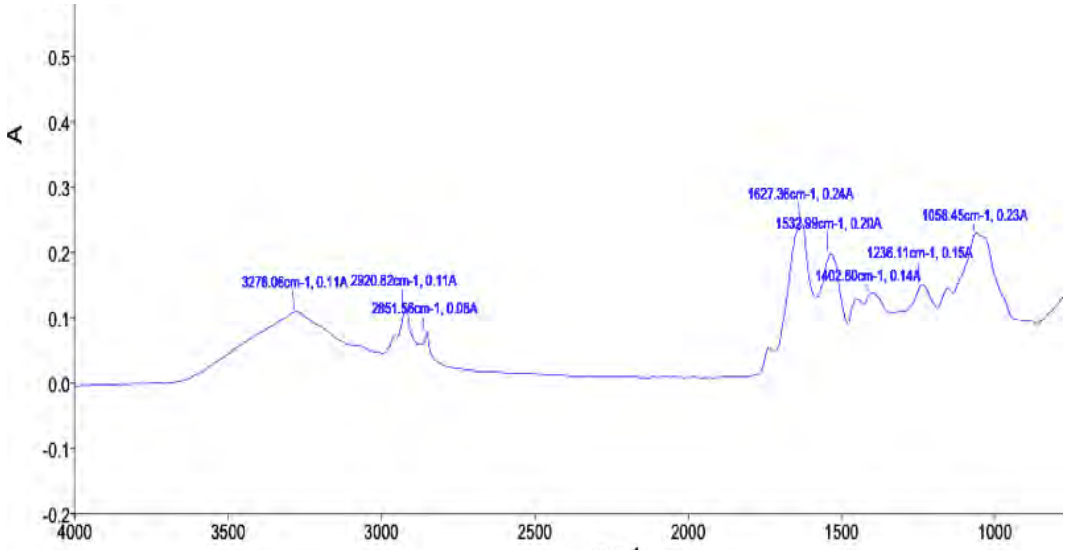


**Figure 4.3.10:** FT-IR spectrum of un-optimized biomass of *Tetradesmus nygaardii*

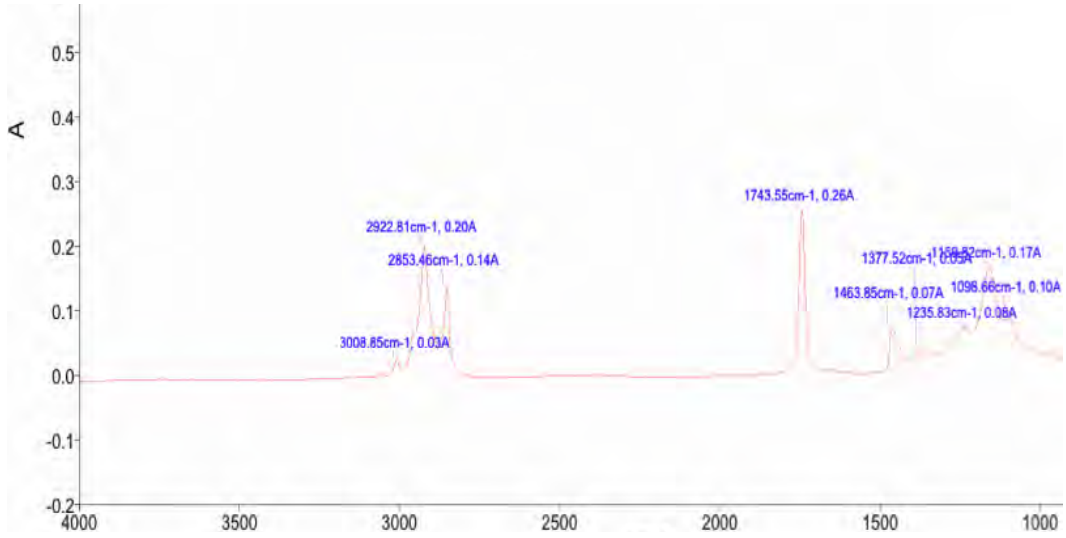


**Figure 4.3.11:** FT-IR spectrum of optimized biomass of *Tetradesmus nygaardii*

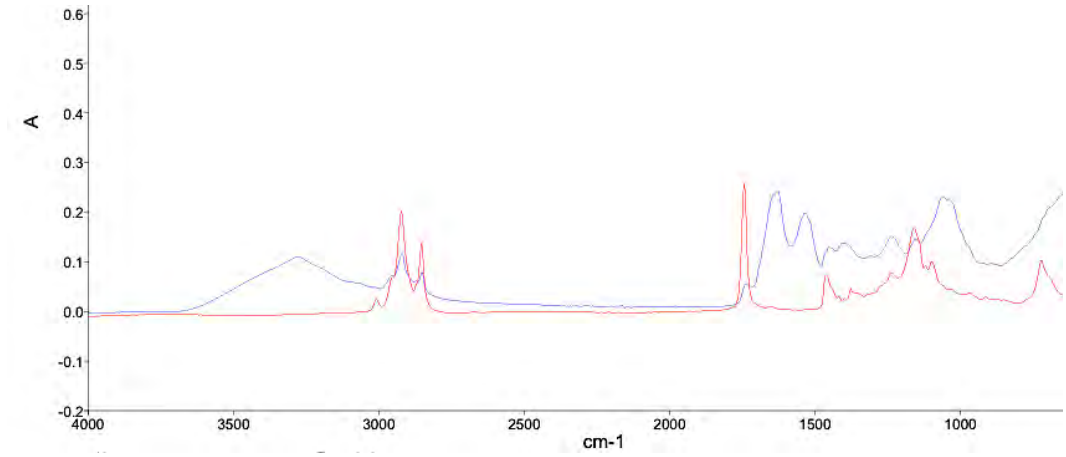

**Figure 4.3.12:** FT-IR spectrum of comparative analysis of optimized and un-optimized biomass of *Tetradesmus nygaardii*

Fig. 4.3.12 shows the comparative analysis of two independent spectra (fig. 4.3.10 & 4.3.11), where peak shifts has been observed in functional group region. The optimized biomass has appeared to show more spectral bands in region of fatty acids functional groups while control has depicted frequent bands in spectral region characterized for proteins. The bands assigned to carbohydrates region are more intense in control biomass. Our findings are in accordance with some previous studies, which reports microalgae metabolic behavior shifts preferentially toward synthesis, and storage of fatty acids in starvation conditions (Pancha *et al.*, 2014; Yu *et al.*, 2018) while other reports metabolic shifts towards protein synthesis on high nitrogen concentration (Long, Jones and

Orr, 2001). These findings could be explained by the fact that transformation mechanism of organic matter to biomolecules such as Lipids, proteins and carbohydrates in microalgae depends on cultivation parameters such as nutrients concentration, temperature, pH, sunlight and $CO_2$ levels.

**Table 4.3.5**: Band assignments of FT-IR spectrum for biochemical composition of *Tetradesmus nygaardii* biomass.

| Wave Number $cm^{-1}$ in literature | Wave number $cm^{-1}$ OB | Wave number $cm^{-1}$ UOB | Spectral Assignments | References |
|---|---|---|---|---|
| 3200-3400 | 3008 | 3276 | O-H or N-H of $H_2O$/Proteins | (Piasecka *et al.*, 2020)<br>(Arrondo and Goñi, 1998) |
| 2918-2927 | 2922 | 2920 | $CH_2$ str.asym. Saturated Fatty acids | (Naumann, Fabian and Lasch, 2009)<br>(Lazar *et al.*, 2012)(Arrondo and Goñi, 1998)(Naumann, Fabian and Lasch, 2009) |
| 2850-2854 | 2853 | 2851 | $CH_2$ str. Sym.Fatty acids chain | (Giordano *et al.*, 2001)(Piasecka *et al.*, 2020) |
| 1740-1743 | 1743 | | C=O str. From fatty acids | (Movasaghi, Rehman and Rehman, 2008)(Naumann, Fabian and Lasch, 2009) |
| 1618-1690 | | 1627 | Amide I of proteins | (Coates, 2006)(Stehfest, Toepel and Wilhelm, 2005) |
| 1532-1540 | | 1532 | Amide II of proteins | (Liang *et al.*, 2013) |
| 1463 | 1463 | | $CH_2$ bend. Of fatty acids | (Barth, 2007) |
| 1402 | | 1402 | COO- str. Sym. Of proteins | (Barth, 2007) |
| 1377 | 1377 | | $CH_3$ bend. of fatty acids | (Coates, 2006)(Stehfest, Toepel and Wilhelm, 2005) |
| 1230-1250 | 1235 | 1236 | P=O of nucleic acids or phospholipids | (Yoshida *et al.*, 1997)(Lazar *et al.*, 2012) |
| 1052-1061 | 1159 | 1058 | C-OH, str.of carbohydrates | |
| 900-1200 | 1098 | | C-O-C, str. Of Carbohydrates | (Giordano *et al.*, 2001) |

## 4.4 Characterization of Biodiesel

### 4.4.1 Fatty acid methyl ester (FAME) Analysis

#### *4.4.1.1 FTIR Analysis*

The FTIR spectra of TN (*Tetradesmus nygaardii*) oil and biodiesel has shown minor differences in terms of spectral bands intensity and absorbance frequencies due to small variations in nature of oil and biodiesel (Guille, 1997).

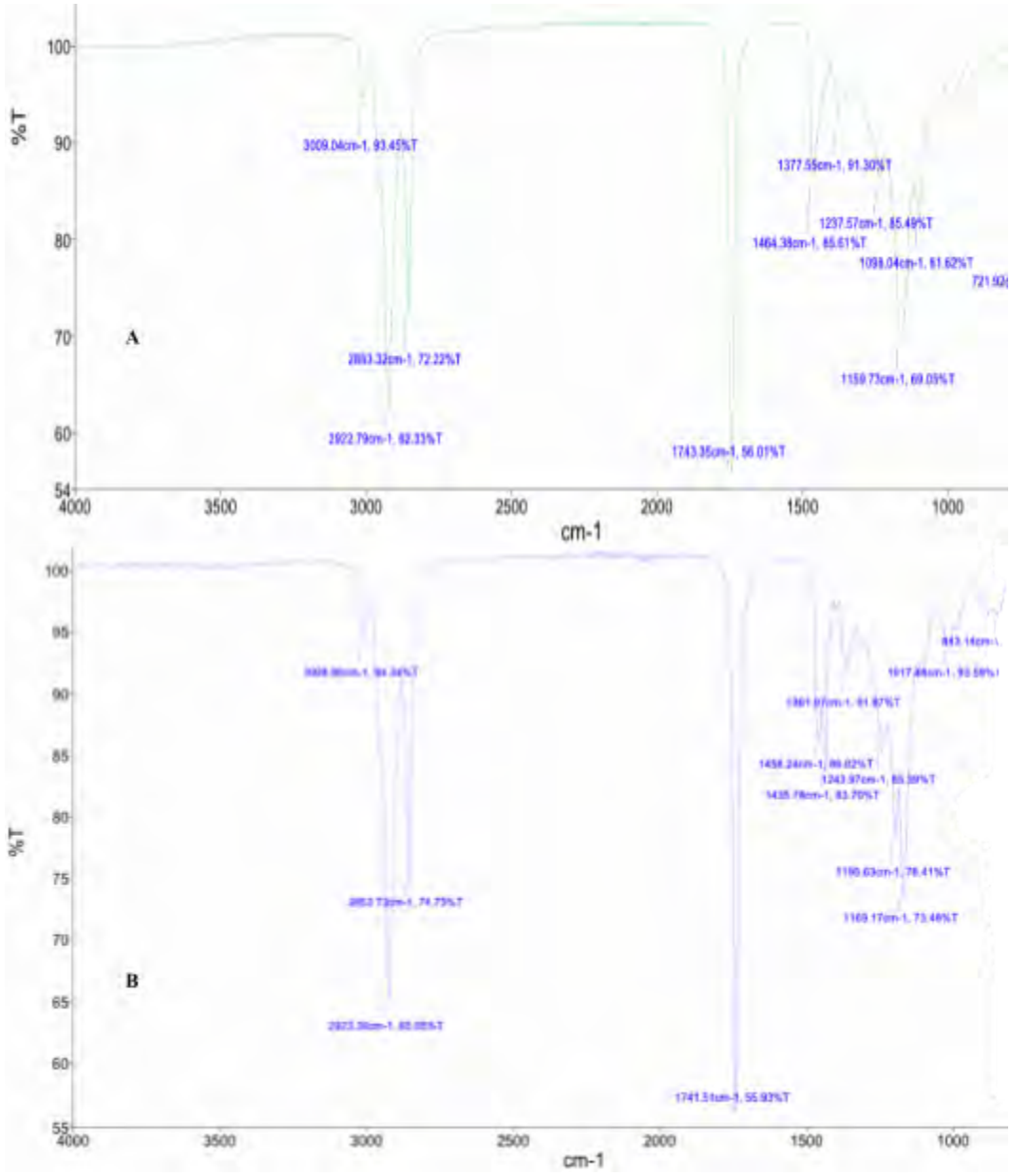


**Figure 4.4.1**: FT-IR spectrum of *Tetradesmus nygaardii* oil (A) & biodiesel (B).

In TN oil, the strong spectral peaks of triglycerides functional group appeared at 2922.79cm-1 ($SP^2$ C-H str.), 2853.32cm-1 ($SP^3$ C-H str.), 1743.35cm-1 (C=O str.) and 1464.38cm-1 (C-H bend.) (Fig.4.4.1a). However, the methyl esters in TN biodiesel have shown two distinctive spectral peaks of methoxy carbonyl and carbonyl stretching. The spectral peak of C=O stretch in ester functional group appeared at 1741.51cm-1. The peaks appeared at 2853.73cm-1 and 2923.30cm-1 shows the $SP^3$ C-H and $SP^2$ C-H stretching respectively. The methyl and methylene bending vibrations appeared at 1361.97cm-1 and 1458.24cm-1 (Fig 4.4.1b).

The IR spectra in range of 1500-500cm-1 have strong impact on change of ester functional group towards methyl esters. The spectral peaks appearing at 1435.78cm-1 displays the most important difference between TN oil and biodiesel. Another difference observed for spectral peak at 1159.73cm-1 in TN oil spectra, split in two peaks 1169.17cm-1 and 1195.63cm-1 in TN biodiesel. These Characteristic peaks in TN biodiesel, therefore, confirms the fatty acid methyl esters formation and structure and are in accordance with previous studies(Munir *et al.*, 2019).

#### *4.4.1.2 NMR Analysis*

$^1$H NMR and $^{13}$C NMR are efficient spectroscopic techniques for evaluation of transesterification process and biodiesel quality on the basis of characteristics signals appearance in NMR spectra. The characteristics chemical shifts of $^1$H NMR spectrum of TN biodiesel are shown in Fig 4.4.2b; δ (ppm): 0.872 (t, -$CH_3$), 1.295 (m, -$CH_3$), 1.609 (m, -$CH_2$-$CH_2$-), 2.292(t, -$CH_2$-COO-), 5.33 (t, -CH=CH-), and the appearance of new strong signal peak of methoxy group at 3.652ppm that is absent in TN oil spectrum, is the most significant change in TN biodiesel spectra. The peak positions are in line with the previous work (Munir *et al.*, 2019). The characteristics signals and chemical shifts of $^{13}$CNMR are (Fig 4.4.3b): δ (ppm); 29.15 (-$CH_2$-) n, 77.49 (-C=O), 129.69 (-CH=CH-), 174.23 (-COO-). The total percentage conversion of TN oil to biodiesel was calculated using following equation (Tariq *et al.*, 2011);

$$C = 2A(Me) / 3A(CH_2) \quad (9)$$

Where C is percentage conversion of oil to biodiesel, A (Me) and A ($CH_2$) is integration values of protons for methoxy and α-methylene. The total conversion of TN oil to biodiesel was 82%.

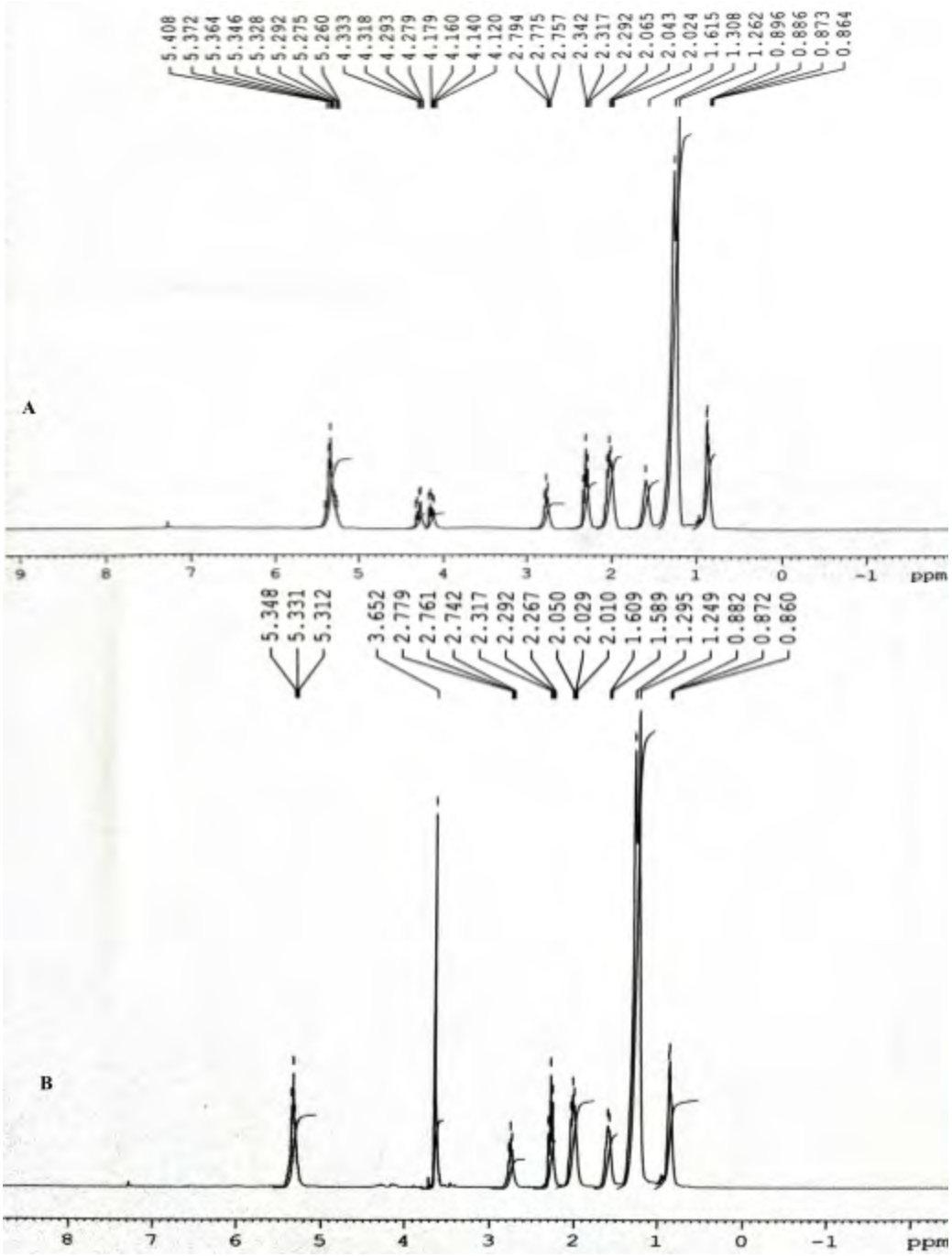


**Figure 4.4.2**: $^{1}H$ NMR spectra of (a) *Tetradesmus nygaardii* oil, (b) *Tetradesmus nygaardii* Biodiesel

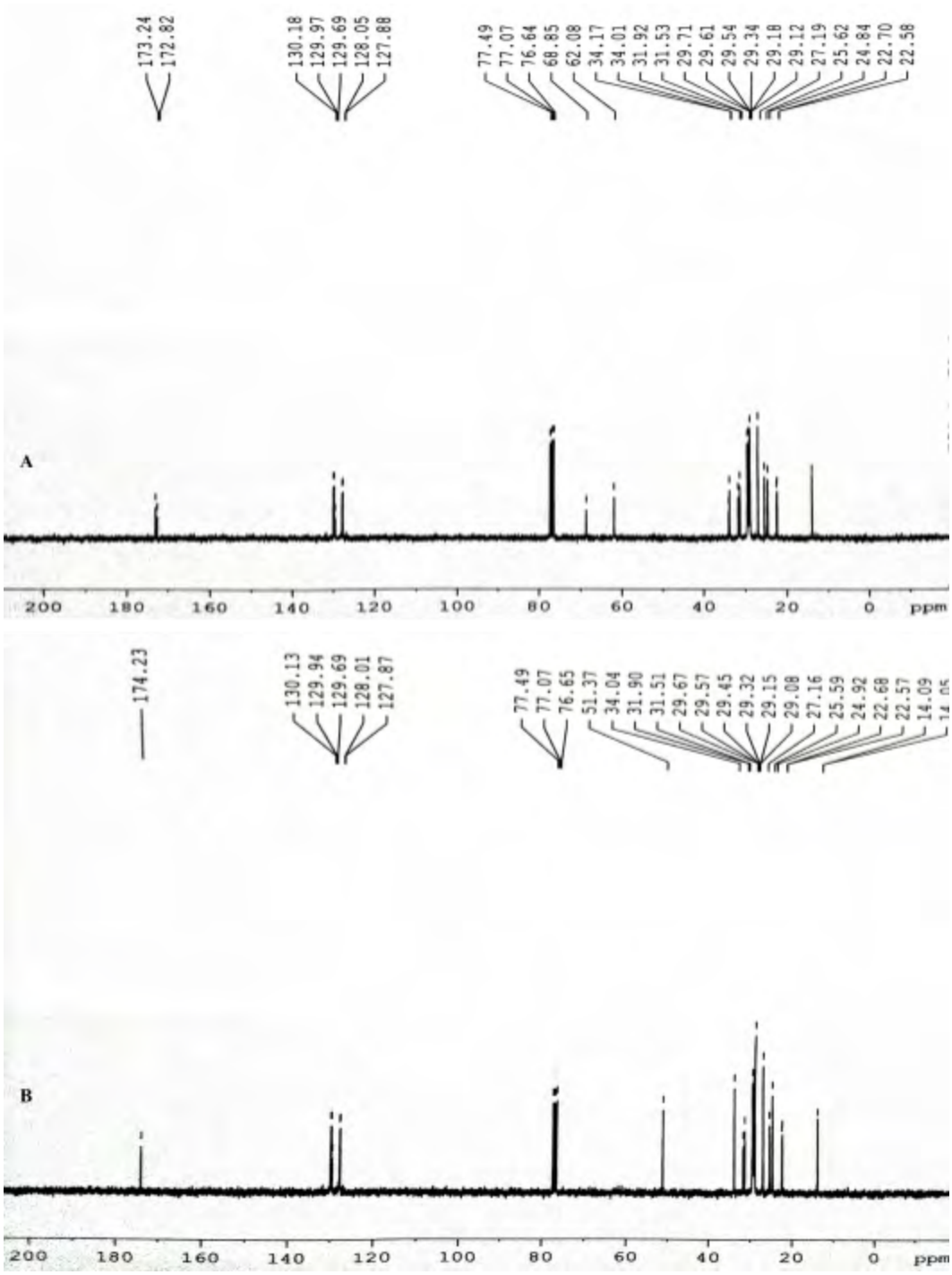


**Figure 4.4.3**: 13C NMR spectra of; (a) *Tetradesmus nygaardii* oil, (b) *Tetradesmus nygaardii* Biodiesel

#### *4.4.1.3 GC-MS Spectroscopic Analysis*

The composition of fatty acids methyl esters of biodiesel sample prepared from lipids extraction was calculated by using Gas chromatography-Mass Spectrometry (GC/MS).

Table 4.4.1 shows the fatty acid profiling along with their relative proportion (%) and methyl esters retention time. It is found that the predominant methyl esters were palmitic acid (C16: 0) and linoleic acid (C18: 2). The fatty acid profiling revealed the relative proportion of monounsaturated fatty acids as 53.3%, polyunsaturated fatty acids as 35% and saturated fatty acids as 11.62% of total lipids content. Generally the high level of polyunsaturated fatty acids and low level of saturated fatty acids are favorable for good quality biodiesel and quality improvement, as it results in decreasing oxidative stability and cold flow problems (Knothe, 2009; Hoekman *et al.*, 2012).

Along with predominant palmitic acid (C16: 0), the other less dominant monounsaturated fatty acids were found as palmitoleic acid (16:1), oleic acid (18:1), eicosenoic acid (20:1) and erucic acid (22:1). Among monounsaturated fatty acids the palmitoleic acid (16:1) and oleic acid (18:1) methyl ester gives fine balancing of cold flow properties and oxidative stability (Hoekman *et al.*, 2012). Overall, in present study the TN biodiesel possessed predominant $C_{16}$-$C_{18}$ FAME's as 21.64% and 33.92% of total fatty acids, among which linoleic acid (18:2) methyl ester was dominant as 24.91%.

According to previous studies, palmitic acid (16: 0), myristic acid (14:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18: 2) and linolenic acids (18:3) are most common fatty acids in most algal species (Hoekman *et al.*, 2012; Song *et al.*, 2013). While fatty acids of high carbon chain length ($C_{20}$-$C_{22}$); eicosapentaenoic acid (20:5), eicosenoic acid (20:1), eicosanoic acid (20:0), erucic acid (22:1) and heneicosanoic acid (21:0) are less common fatty acids found in microalgal cell (Lang *et al.*, 2011; El-Kassas, 2013; Song *et al.*, 2013; Yun *et al.*, 2014; Rohit and Venkata Mohan, 2018; Tsarenko *et al.*, 2020). In present study, polyunsaturated fatty acids of $C_{18}$ chain length are less dominant (0.38%) (Table 4.4.1), which is in accordance with study reported earlier that $C_{18}$ polyunsaturated fatty acids are less prominent fatty acids in microalgal oils as compared to vegetable oils (Knothe, 2011).

**Table 4.4.1**: Fatty acid methyl ester profile of optimized *Tetradesmus nygaardii* biomass

| Peak | Retention Time | Chemical formula | Ester chemical structure | FA chain length | Relative prop. (%) |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| 2 | 17.212 | $C_{15}H_{30}O_2$ | Myristic acid, methyl ester | **C14: 0** | 0.64 |
| 3 | 19.801 | $C_{17}H_{32}O_2$ | Palmitoleic acid, methyl ester | **C16: 1** | 1.78 |
| 4 | 20.249 | $C_{17}H_{32}O_2$ | Palmitic acid, methyl ester | **C16: 0** | 19.86 |
| 6 | 22.865 | $C_{19}H_{34}O_2$ | Linoleic acid, methyl ester | **C18: 2** | 24.91 |
| 7 | 23.026 | $C_{18}H_{36}O_2$ | Stearic acid, methyl ester | **C18: 0** | 3.93 |
| 8 | 23.346 | $C_{57}H_{104}O_6$ | Oleic acid, 1,2,3- propanetriyl ester | **C18: 1** | 8.63 |
| 10 | 24.23 | $C_{19}H_{32}O_2$ | Linolenic acid, methyl ester | **C18: 3** | 0.38 |
| 11 | 24.407 | $C_{22}H_{34}O_2$ | Eicosapentaenoic acid, methyl ester | **C20: 5** | 0.95 |
| 12 | 24.793 | $C_{21}H_{40}O_2$ | Eicosenoic acid, methyl ester | **C20: 1** | 5.21 |
| 13 | 25.063 | $C_{21}H_{42}O_2$ | Eicosanoic acid, methyl ester | **C20 :0** | 2.11 |
| 14 | 27.01 | $C_{23}H_{44}O_2$ | Erucic acid, methyl ester | **C22: 1** | 4.43 |
| 15 | 27.243 | $C_{23}H_{46}O_2$ | Heneicosanoic acid, methyl ester | **C21 :0** | 2.01 |

### 4.4.2 Biodiesel Properties

Physicochemical properties of biodiesel were determined and compared with ASTM D6751 and EN14214 biodiesel standards (Table 4.4.2).

Fuel density is the key property that affects the air-fuel ratio and energy content in combustion chamber (Hoekman *et al.*, 2012). Degree of unsaturation in FAMEs composition directly affects the fuel density. Higher degree of unsaturation results in higher fuel density (Refaat, 2009). Density of TN biodiesel was found out to be 939Kg/$m^3$. Flash point refers to its inverse effect on fuel volatility (Hoekman *et al.*, 2012) and varies with variation in saturated fatty acids content in fuel. TN biodiesel has a flash point of 97$^o$C, which is comparable to ASTM and EN biodiesel standards.

Cetane number affects the characteristics of fuels auto-ignition quality (Hoekman *et al.*, 2012). Increase in degree of unsaturation in FAMEs content results in decreasing Cetane number (Knothe, 2011). Cetane number of TN biodiesel has found to be 54, which is in accordance with ASTM and EN standards. Saponification value refers to ester linkages content in biodiesel. Higher value of saponification relates to high percentage of ester bonds in Fuel. The saponification value of TN biodiesel was determined as 167 mgKOH/g. Cold flow properties refers to the fuel performance at low temperatures and generally, are indicated by cloud point and cold filter plugging point (CFPP). These properties are weather dependent and vary according to weather conditions of each country. Cloud point is the temperature at which the least soluble component of biodiesel

crystallizes from the solution (Hoekman *et al.*, 2012) and CFPP refers to the lowest temperature at which volume of pure biodiesel flows through filters with in time limit of 1 min (Knothe and Steidley, 2005). Cold flow properties depend on saturated fatty acids content in biodiesel, higher saturated fatty acids results in poor cold flow properties. The Cloud point and CFPP of TN biodiesel was determined as -15$^{o}$C and 13$^{o}$C respectively.

**Table 4.4.2:** Comparison of Biodiesel Properties of Tetradesmus nygaardii oil and ASTM, EN biodiesel standards

| | **ASTM biodiesel standards** | **EN biodiesel standards** | **TN biodiesel** |
|---|---|---|---|
| Density at 15$^{o}$C | - | 860-900 kg/m3 | 939 |
| Flash point | 93$^{o}$C | 101$^{o}$C | 97 |
| Cetane number | 47 min | 51 min | 54 |
| Saponification value | - | - | 167 |
| Acid value | 0.5mgKOH/g | 0.5mgKOH/g | 0.19 |
| Heating value | >35MJ/Kg | - | 45 |
| Cloud point ($^{o}$C) | - | - | -15 |
| CFPP ($^{o}$C) | - | - | 13 |

Acid value refers to corrosive property of fuel in internal combustion engine. Higher acid value indicates poor fuel quality and less engine efficiency. The acid value of TN biodiesel was found out to be 0.19 mg KOH/g, which is in accordance with ASTM and EN biodiesel standards. Heating Value refers to mass energy content of biodiesel. Fatty acid chain length has direct impact on heating values. Higher chain length in fatty acids results in higher heating value (Hoekman *et al.*, 2012). The heating value of TN biodiesel was determined as 45MJ/Kg, which relates well with ASTM biodiesel standards. This study, therefore, suggest TN oil as suitable feedstock of biofuel production.

## 4.5 Anaerobic Co-digestion of defatted microalgae biomass with eucalyptus leaves waste for enhanced biogas production

### 4.5.1 Assessment of Biomethane potential (BMP)

The biogas production in all four different treatments (including duplicates) started at day 2 and 3 of inoculation, which depicts healthy and active status of inoculum. The biogas production peaked between days 7 to 13 for treatments having algal biomass alone (*Closteriopsis acicularis*, *Tetradesmus nygaardii*). The biogas production in treatments of algal biomass co-digested with carbon rich substrate was found maximum between day 5 to 15 days inferring the prolonged availability of bioactive compounds for digestion and biogas production in samples *Closteriopsis acicularis* + eucalyptus leaves and *Tetradesmus nygaardii* + eucalyptus leaves. The highest cumulative yield of biogas was determined in *Closteriopsis acicularis* + eucalyptus leaves (Table 4.5.1, Fig. 4.5.1 & 4.5.2).

**Table 4.5.1**: Cumulative biogas yield, methane yield and methane percentage

| Batch | Biogas (mL) | Biogas yield (mL of CH4 / g of VS) | Methane content (mL) | Methane yield (mL of CH4 / g of VS) | Methane % |
|---|---|---|---|---|---|
| Control | 270 | 270.0 | 133 | 133.0 | 49.3 |
| *Closteriopsis acicularis* | 691 | 297.0 | 460 | 197.5 | 66.5 |
| *Tetradesmus nygaardii* | 666 | 280.0 | 466 | 196.0 | 69.9 |
| *Closteriopsis* sp. + Eucalyptus leaves | 1182 | 461.0 | 884 | 344.0 | 74.8 |
| *Tetradesmus* sp. + Eucalyptus leaves | 960 | 403.0 | 689 | 289.0 | 71.7 |

It was observed lower biogas yield in treatments with algal biomass alone. Co-digestion has showed advantage in biogas production over other treatments. The cumulative biogas produced by co-digestion was higher than cumulative biogas produced by microalgal biomass in case of both strains after 15 days of experiment. Methane content was found higher in co-digested samples treatment 884 mL/g VS for *Closteriopsis acicularis* + eucalyptus leaves and 689 mL/g VS for *Tetradesmus nygaardii* + eucalyptus leaves in comparison with treatments containing algal biomass only 691 mL/g VS for *Closteriopsis acicularis* and 537 mL/g VS for *Tetradesmus nygaardii* (Fig 4.5.3 & 4.5.4).

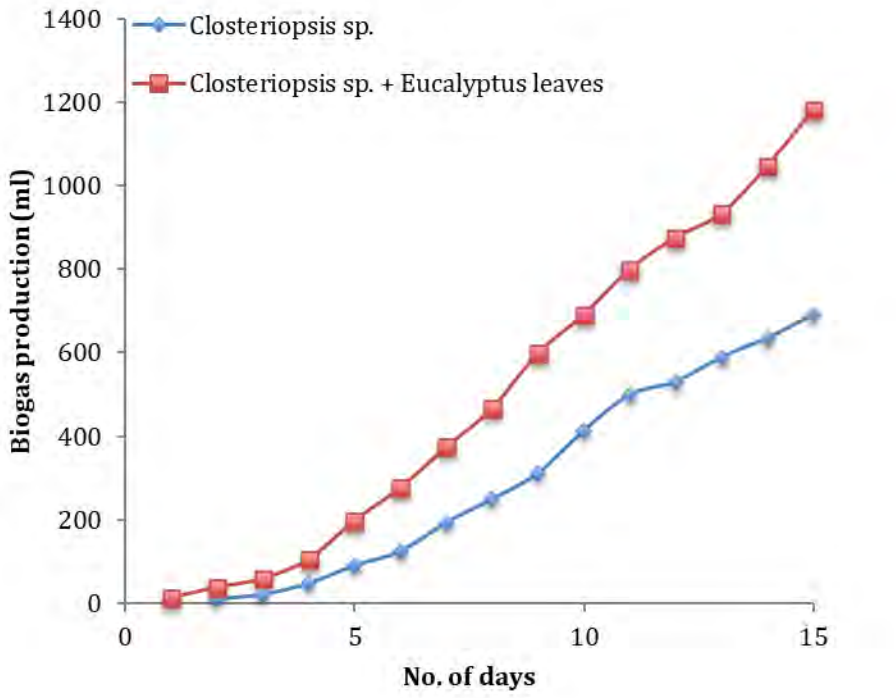


**Figure 4.5.1:** Cumulative biogas production of defatted algal biomass and co-digestion of strain *Closteriopsis acicularis & Closteriopsis acicularis* + eucalyptus leaves

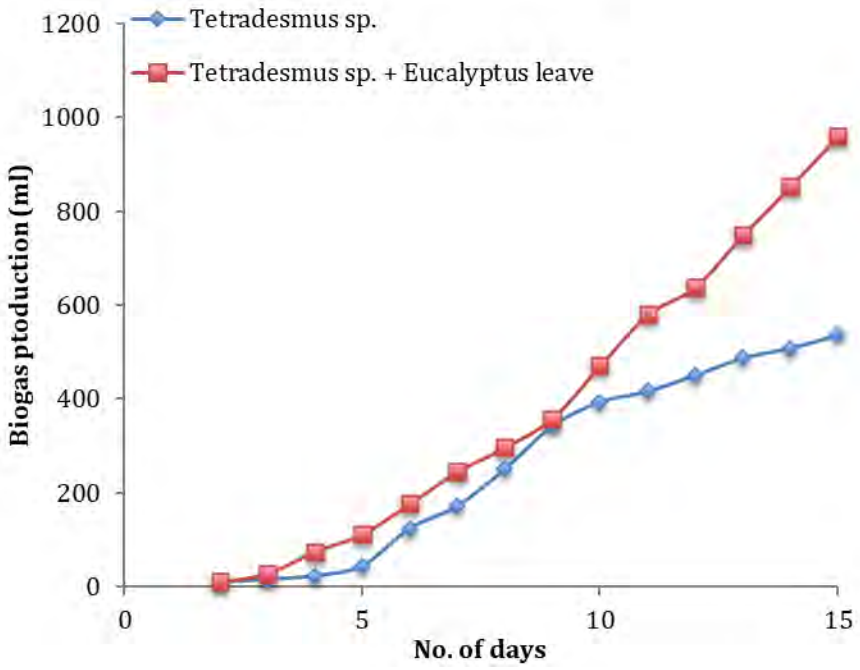


**Figure 4.5.2**: Cumulative biogas production of defatted algal biomass and co-digestion of strain *Tetradesmus* nygaardii & *Tetradesmus nygaardii* + eucalyptus leaves

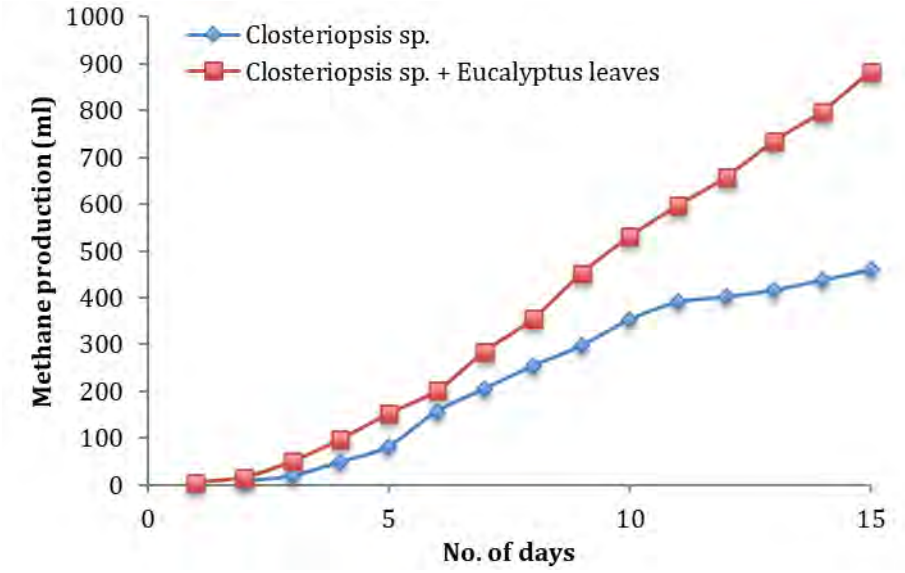


**Figure 4.5.3:** Cumulative methane generation of defatted algal biomass and co-digestion of strain *Closteriopsis acicularis* & *Closteriopsis acicularis* + eucalyptus leaves

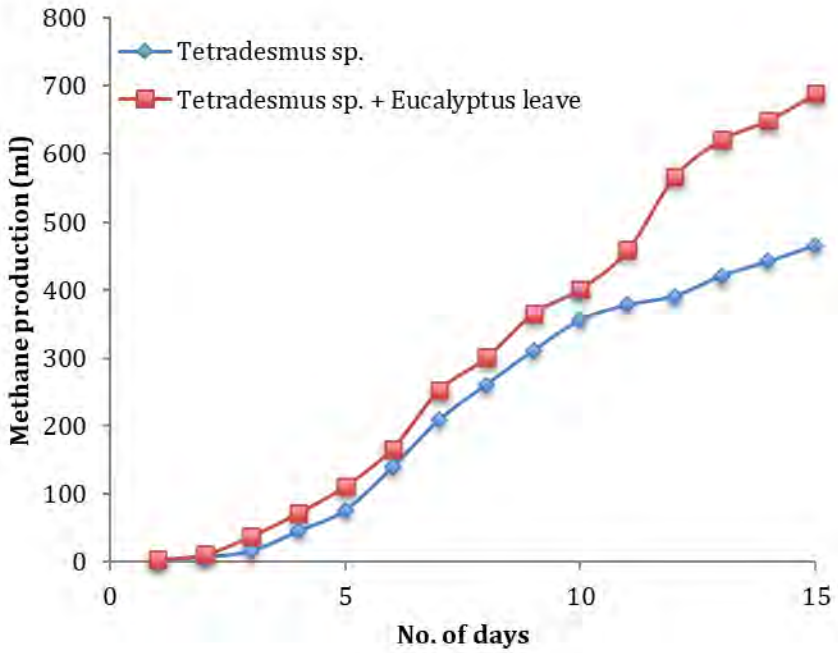


**Figure 4.5.4:** Cumulative methane generation of defatted algal biomass and co-digestion of strain *Tetradesmus nygaardii* & *Tetradesmus nygaardii* + eucalyptus leaves

Methane content was found higher in order of *Closteriopsis acicularis* + eucalyptus leaves > *Tetradesmus nygaardii* + eucalyptus leaves > *Tetradesmus nygaardii* > *Closteriopsis acicularis* > Control, with highest methane content of 74.8% in *Closteriopsis acicularis* + eucalyptus leaves (Table 4.5.1). Co-digestion significantly

increased the potential of biogas and methane generation in defatted algal biomass for both strains. The highest Biomethane yield (884 mL/g VS for *Closteriopsis acicularis* + eucalyptus leaves) observed in this study was found higher than previously reported data of anaerobic Co-digestion of defatted microalgae with rice straw as substrate (CG12, 573 mL/g VS) (Veerabadhran *et al.*, 2021). The variations and corresponding amount of bio methane determines the synthesis of biogas depends on type of substrate and rectors used during anaerobic digestion (Ishaq *et al.*, 2020). Co-digestion of algal biomass with carbon rich substrate has shown higher bio gasification potential through balancing lower C/N ratio of defatted algal biomass.

### 4.5.2 Effect of co-digestion on BMP of microalgae

The simultaneous digestion of two or more organic substrates increases the economic viability of anaerobic digestion of microalgae due to its potential of high methane yield as compared to the microalgae only. The enhanced methane production is mainly a result of improved organic loading rate and synergism (Mata, Martins and Caetano, 2010; Dębowski *et al.*, 2020; Digestion, 2020). Experimental results and cumulative biogas and methane production are shown in table 4.5.1.

During the initial days of experiment, the biogas yield was also higher in the reactors containing microalgae only. As microalgae was pretreated, it was easily available substrate for the anaerobic consortia to utilize and produce biogas at high rates during first week of experiment and it decreased gradually until the end of experiment. An increase in biogas production was observed in the co-digestion reactors during the second week of experiment. This is due to the slow degradation of co-digestion substrate. The methane yield of both microalgal strains increased by 5-8% upon co-digestion suggests the importance of co-digestion in the biogas production by microalgae.

The Scanning electron microscopy (SEM) was used to study the microbial colonization and surface morphology of *Closteriopsis acicularis* + eucalyptus leaves and *Tetradesmus nygaardii* + eucalyptus leaves substrates Fig 4.5.5 and 4.5.6.

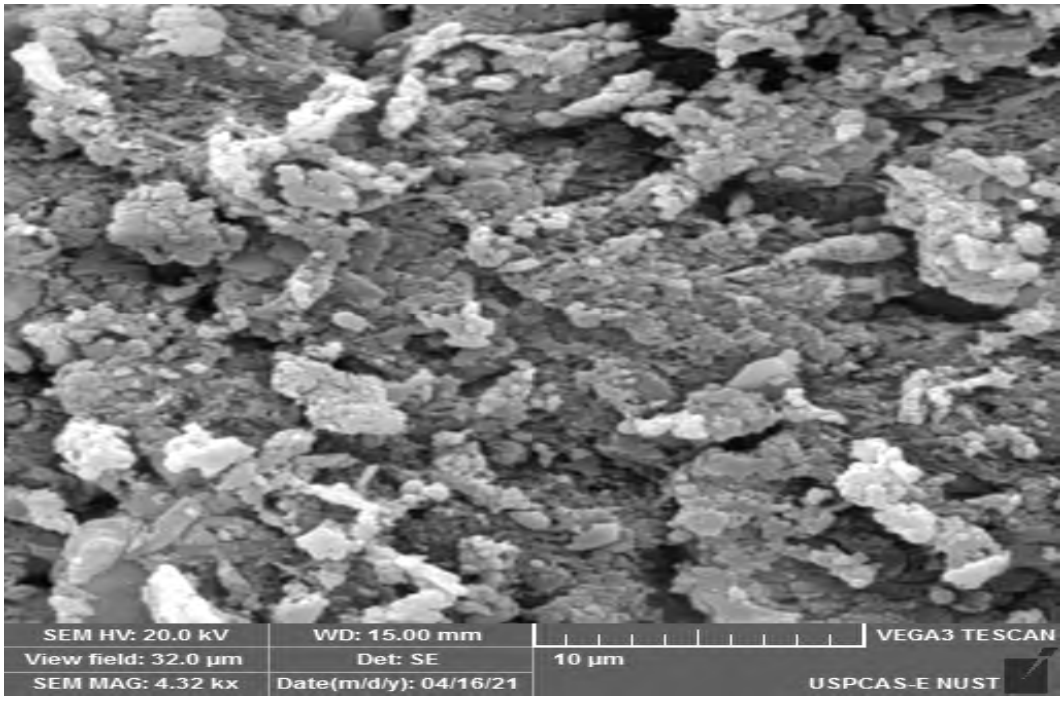


**Figure 4.5.5:** SEM analysis of anaerobically co-digested defatted microalgae biomass with Eucalyptus leaves waste showing surface morphology and microbial colonization of *Closteriopsis acicularis* + eucalyptus leaves

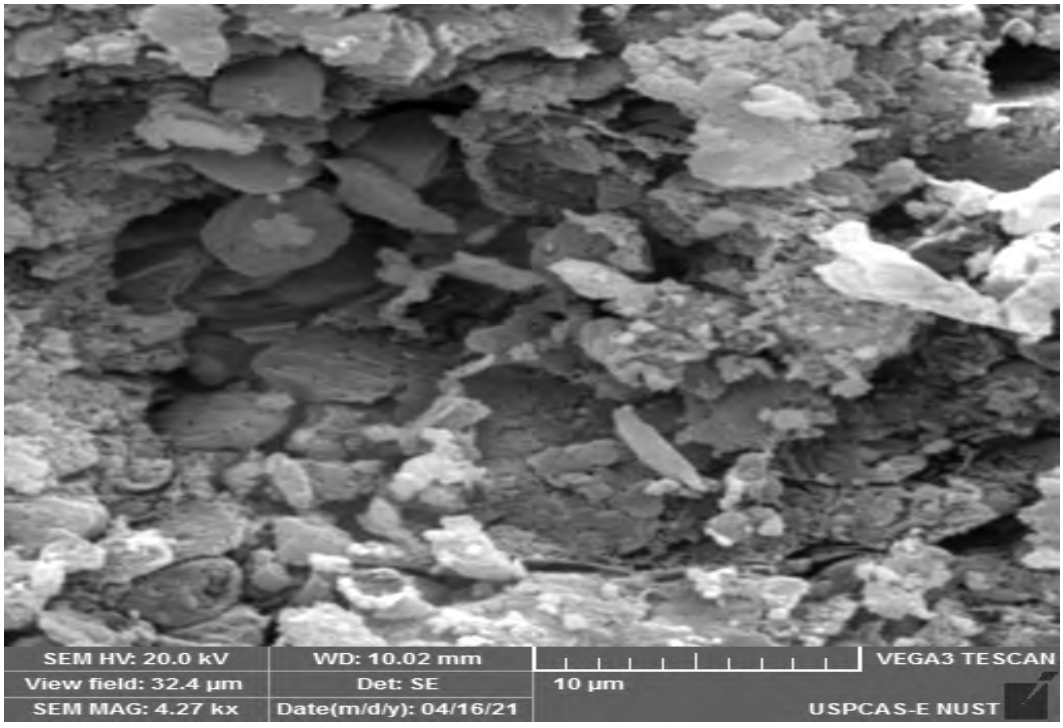


**Figure 4.5.6:** SEM analysis of anaerobically co-digested defatted microalgae biomass with Eucalyptus leaves waste showing surface morphology and microbial colonization of *Tetradesmus nygaardii* + eucalyptus leaves

The highest biogas and biomethane producing substrate (*Closteriopsis acicularis* + eucalyptus leaves) has shown high microbial attachment and degraded surface as compared to *Tetradesmus nygaardii* + eucalyptus leaves substrate, suggesting co-digestion has well supported the microbial colonization for degradation of complex macromolecules and synthesis of methane (González-González and De-Bashan,2021).

# CONCLUSION

# 5 Conclusion

The present study is an extensive investigation of optimization of indigenous microalgae for high biomass and lipids accumulation and its impact on biofuels production (biodiesel, bioethanol and biogas). It demonstrates the growth characterization and biochemical composition of algal strains as well as utilization of algal biomass in biorefinery approach. A comprehensive approach of study using statistical design of experiment (CCD & BBD) and response surface method accurately predicts the response (biomass production, lipids production, lipids content, specific rate of growth & productivity of biomass) upon optimization of abiotic growth factors (temperature, pH, phosphate & nitrate concentration). The author gives the extensive background of environmental factors that are significant for biomass and lipids accumulation, their key metabolic role as well as why they are important to be used as first line of tool in strain development and metabolic engineering for algae derived biofuels production. It provides a strong motivation and value for the current study that it could have potential in policy development and energy management in the region. The conclusions obtained in this study are vindicated by the experimental observations and statistical analysis and can greatly contribute to future policy decisions towards biofuels production and energy management in the region.

## 5.1 Key findings

- Based on the results, following key findings were drawn;

  Results of chapter 4 part (4.1) concluded that the samples from Rawal lake Islamabad are rich in green microalgae, which have tremendous ability of application in biofuel production. Moreover, the screening and identification revealed that *Closteriopsis acicularis* and *Tetradesmus nygaardii* were the potential algal strains to be considered for further downstream process and strain development for biofuels production.

- Based upon chapter 4 part (4.2), regarding the optimization study of *Closteriopsis acicularis* for high specific rate of growth and productivity of biomass, it is concluded that the alkaline pH resulted in high phosphate uptake by algal cells. The interactive effect of alkaline pH and high concentration of phosphate resulted in increase in specific rate of growth and productivity of biomass along with total carbohydrates content, which concludes the success of optimization strategy for strain development to be used as feedstock for bioethanol production.
- Chapter 4 part (4.3), stated that the coupled effect of low pH and nitrogen concentration in the nutrient media is effective strategy for enhancing lipids synthesis in *Tetradesmus nygaardii*. The combination of statistical analysis and experimental observations indicated that increase in lipids synthesis resulted in slightly decrease in biomass production perhaps; the gain of lipids was more prominent over loss of biomass. The characterization of lipids derived from optimized biomass revealed the suitable profile of fatty acid methyl esters for biodiesel synthesis. The synthesized biodiesel met most requirements of ASTM and EN standards of biodiesel specifications, which concludes the propriety of optimization strategy for metabolic engineering of strain and therefore reliability to be used as feedstock for biodiesel production.
- Chapter 4 (part 4.5) specified the biorefinery approach of study for integrated utilization of whole biomass in energy generation system. The defatted algal biomass co-digested with carbon rich waste has resulted in enhanced biogas and biomethane generation, which indicates the success of integrated waste reduction and energy generation plan of study

## 5.2 Limitations and future prospects

The body of present research work indicates several limitations and areas where further research would improve the bulk production of algal biomass and biofuels.

These limitations and future prospects are outlined below;

Firstly, in present study due to analytical and resources constraints, the sampling was restricted to single region. However, sampling, screening and identification of strains

from multiple regions could give more plausible results for hunting potential algal strain with robust growth and lipids productivity.

Secondly, integration of wastewater treatment with bulk production of algal biomass will give a more cost effective and ecofriendly route of biofuels production.

Thirdly, high value-added products extraction could considerably reduce the downstream processing cost of algal biomass to biofuels biorefinery.

Finally, Permanent strain development via genetic engineering, based on results obtained from present study could effectively increase the process efficiency for large-scale production of algal biomass.

## 5.3 Concluding Remarks

This thesis is an abstract of art explaining the comprehensive insight of abiotic growth factors impact on indigenous microalgae, as well as the strategic production of biofuels. This study apart from highlighting the key, alternative energy generation strategy, would also provide a base line for local and administrative authorities to formulate sustainable, cost effective and environment friendly way of energy production i.e., a step towards green energy.

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# Appendix I

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Original article

# Deciphering role of technical bioprocess parameters for bioethanol production using microalgae

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ARTICLE INFO

*Article history:*
Received 26 August 2021
Revised 3 October 2021
Accepted 4 October 2021
Available online xxxx

*Keywords:*
Microalgae
Biomass productivity
Specific growth rate
Optimization
RSM
Bioethanol

ABSTRACT

Microalgae biomass is considered an important feedstock for biofuels and other bioactive compounds due to its faster growth rate, high biomass production and high biomolecules accumulation over first and second-generation feedstock. This research aimed to maximize the specific growth rate of fresh water green microalgae *Closteriopsis acicularis*, a member of family *Chlorellaceae* under the effect of pH and phosphate concentration to attain enhanced biomass productivity. This study investigates the individual and cumulative effect of phosphate concentration and pH on specific growth characteristics of *Closteriopsis acicularis* in autotrophic mode of cultivation for bioethanol production. Central-Composite Design (CCD) strategy and Response Surface Methodology (RSM) was used for the optimization of microalga growth and ethanol production under laboratory conditions. The results showed that high specific growth rate and biomass productivity of 0.342 $day^{-1}$ and 0.497 g $L^{-1}$ $day^{-1}$ respectively, were achieved at high concentration of phosphate (0.115 g $L^{-1}$) and pH (9) at 21st day of cultivation. The elemental composition of optimized biomass has shown enhanced elemental accumulation of certain macro (C, O, P) and micronutrients (Na, Mg, Al, K, Ca and Fe) except for nitrogen and sulfur. The Fourier transform infrared spectroscopic analysis has revealed spectral peaks and high absorbance in spectral range of carbohydrates, lipids and proteins, in optimized biomass. The carbohydrates content of optimized biomass was observed as 58%, with 29.3 g $L^{-1}$ of fermentable sugars after acid catalyzed saccharification. The bioethanol yield was estimated as 51 % g ethanol/g glucose with maximum of 14.9 g/L of bioethanol production. In conclusion, it can be inferred that high specific growth rate and biomass productivity can be achieved by varying levels of phosphate concentration and pH during cultivation of *Closteriopsis acicularis* for improved yield of microbial growth, biomass and bioethanol production.



## 1. Introduction

Continuous crises in energy sector due to depletion and unsustainability of fossil reserves, climate changes associated with use of petro derived fuels and auxiliary environmental risks leads the attention of research towards green, sustainable and ecofriendly alternatives of fossil fuels (Ameen et al., 2021; Kim et al., 2021). Microalgae biomass is considered an important alternative tiny reserves of bioactive compounds with significant potential of producing biofuels (Khan et al., 2018). Bioethanol production and application as green fuel has been rising in the world (Chakraborty & Mukhopadhyay, 2020). The major raw materials being used at industrial scales for the bioethanol production

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.10.011
1319-562X/

Please cite this article as: F. Bibi, H. Yasmin, A. Jamal et al., Deciphering role of technical bioprocess parameters for bioethanol production using microalgae, Saudi Journal of Biological Sciences, https://doi.org/10.1016/j.sjbs.2021.10.011

# Appendix II

## Specific rate of growth (cellular dry weight)

| Run order | Zt (mg/L) | Z0 (mg/L) |
|---|---|---|
| 1 | 3600 | 10 |
| 2 | 7100 | 15 |
| 3 | 7900 | 20 |
| 4 | 3900 | 10 |
| 5 | 1980 | 10 |
| 6 | 3500 | 10 |
| 7 | 3700 | 10 |
| 8 | 3600 | 10 |
| 9 | 1000 | 5 |
| 10 | 3900 | 10 |
| 11 | 3900 | 10 |
| 12 | 875 | 5 |
| 13 | 3550 | 10 |

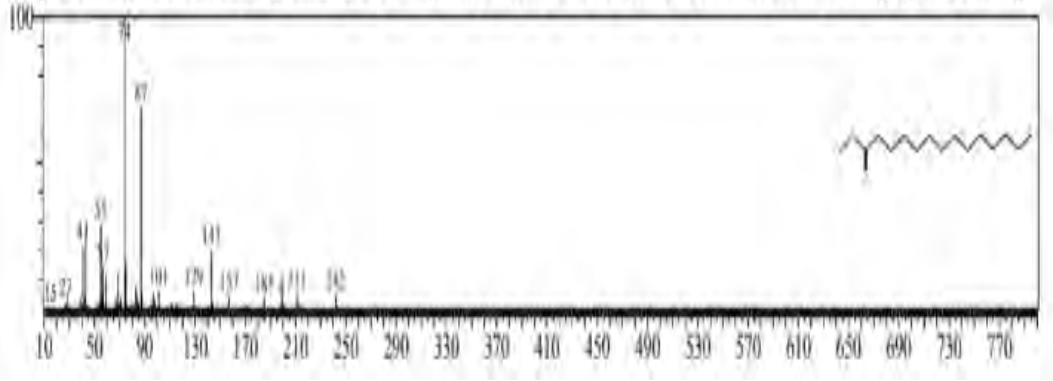
## Productivity of biomass (cellular dry weight)

| Run order | Z2 (mg/L) | Z1 (mg/L) |
|---|---|---|
| 1 | 4870 | 10 |
| 2 | 7470 | 15 |
| 3 | 6335 | 20 |
| 4 | 5425 | 10 |
| 5 | 3865 | 10 |
| 6 | 4405 | 10 |
| 7 | 5245 | 10 |
| 8 | 5365 | 10 |
| 9 | 2195 | 5 |
| 10 | 5980 | 10 |
| 11 | 5110 | 10 |
| 12 | 2720 | 5 |
| 13 | 5305 | 10 |

# GC-MS Peaks Identification

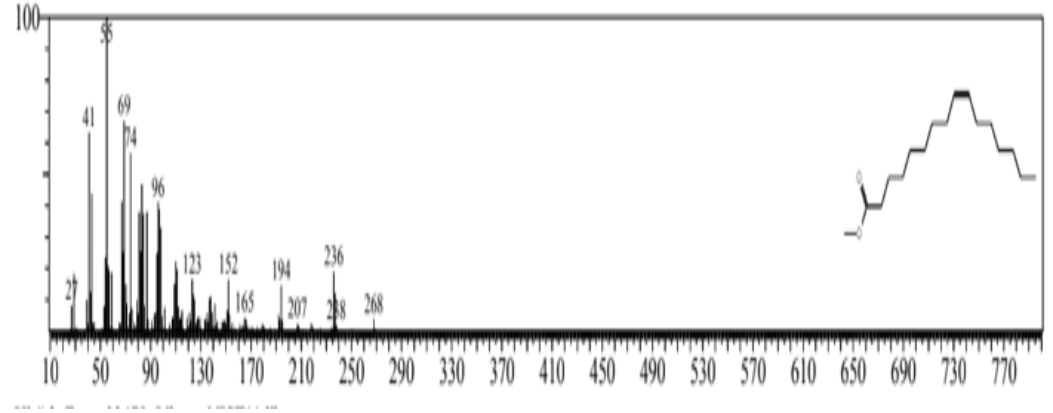
SI:97 Formula:C15H30O2 CAS:124-10-7 MolWeight:242 RetIndex:1680

CompName:Methyl tetradecanoate $$ Tetradecanoic acid, methyl ester $$ Myristic acid, methyl ester $$ Methoheneat 2495 $$ Methyl myristate $$ Methyl n



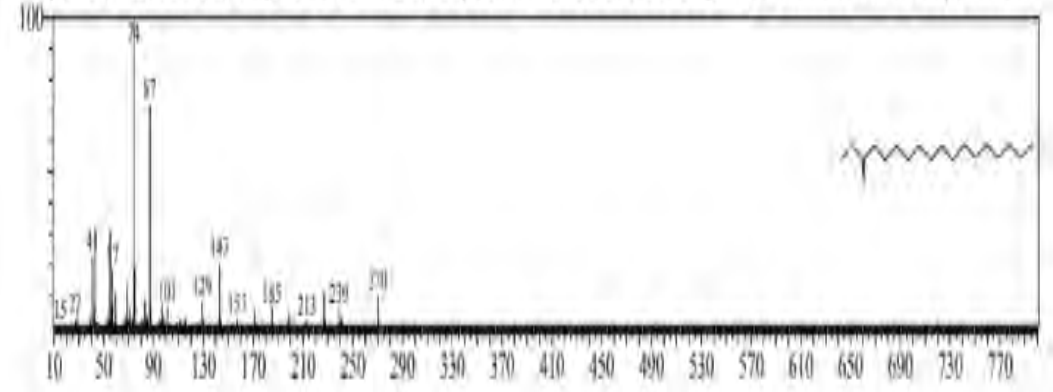

SI:96 Formula:C17H32O2 CAS:1120-25-8 MolWeight:268 RetIndex:1886

CompName:9-Hexadecenoic acid, methyl ester, (Z)- $$ Methyl palmitoleate $$ Methyl palmitoleinate $$ Palmitoleic acid, methyl ester $$ Methyl (9Z)-9-he



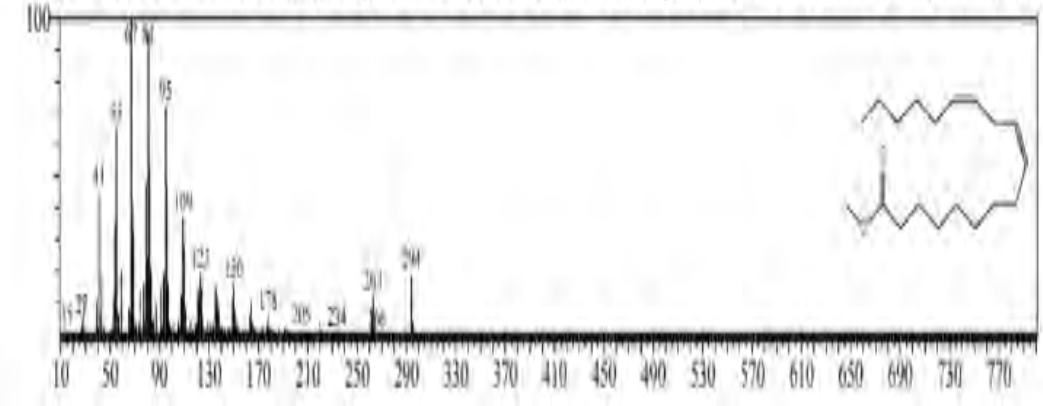

SI:93 Formula:C17H34O2 CAS:112-39-0 MolWeight:270 RetIndex:1878

CompName:Hexadecanoic acid, methyl ester $$ Palmitic acid, methyl ester $$ n-Hexadecanoic acid methyl ester $$ Methofene 2216 $$ Methyl hexadecano



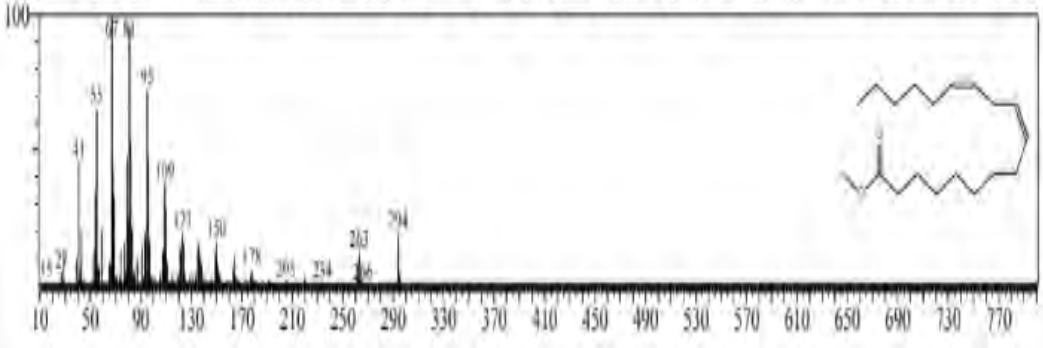
SI 93 Formula:C19H34O2 CAS:112-63-0 MolWeight:294 RetIndex:2093

CompName:9,12-Octadecadienoic acid (Z,Z)-, methyl ester $$ Linoleic acid, methyl ester $$ Methyl cis,cis-9,12-octadecadienoate $$ Methyl linoleate $$ M



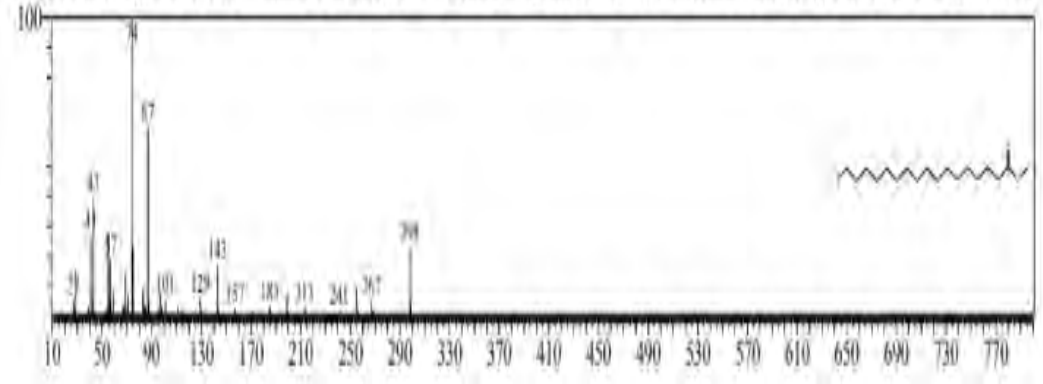

SI 87 Formula:C19H34O2 CAS:112-63-0 MolWeight:294 RetIndex:2093

CompName:9,12-Octadecadienoic acid (Z,Z)-, methyl ester $$ Linoleic acid, methyl ester $$ Methyl cis,cis-9,12-octadecadienoate $$ Methyl linoleate $$ M

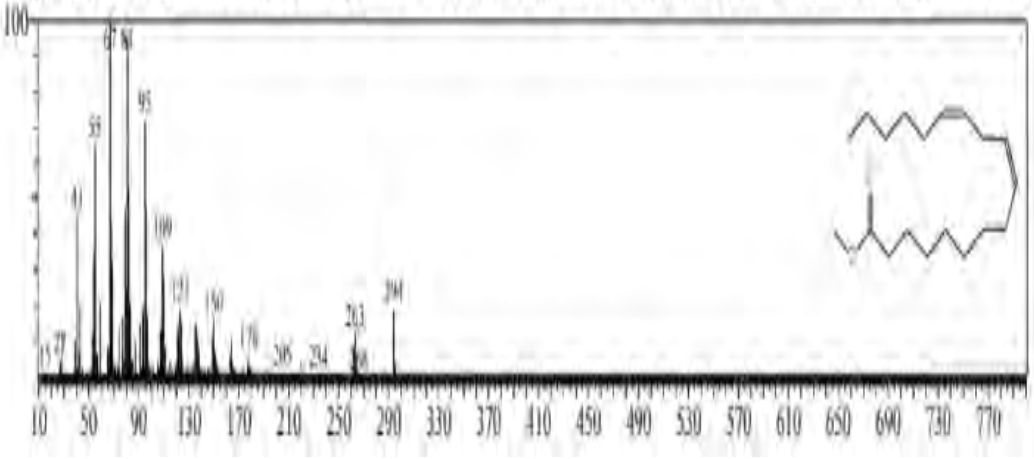



SI 91 Formula:C19H38O2 CAS:112-61-8 MolWeight:298 RetIndex:2077

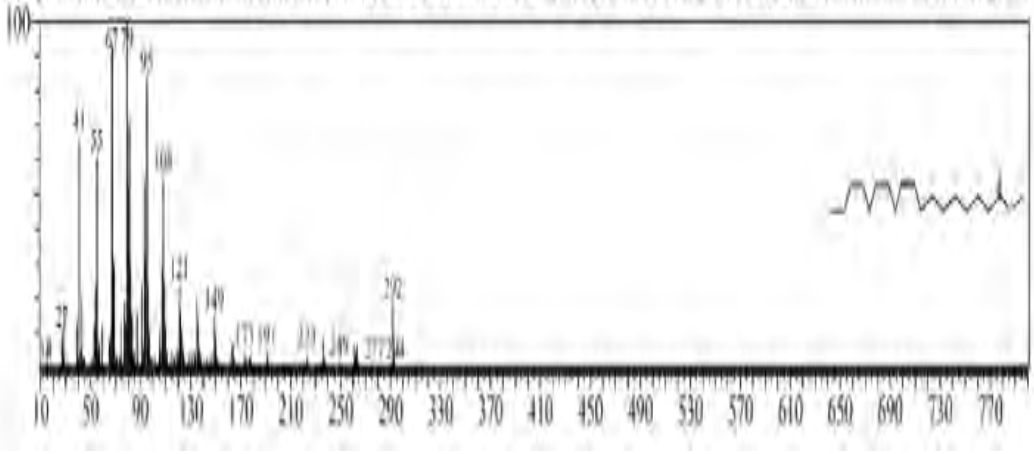
CompName:Methyl stearate $$ Octadecanoic acid, methyl ester $$ Stearic acid, methyl ester $$ n-Octadecanoic acid, methyl ester $$ Kemester 9718 $$ Me



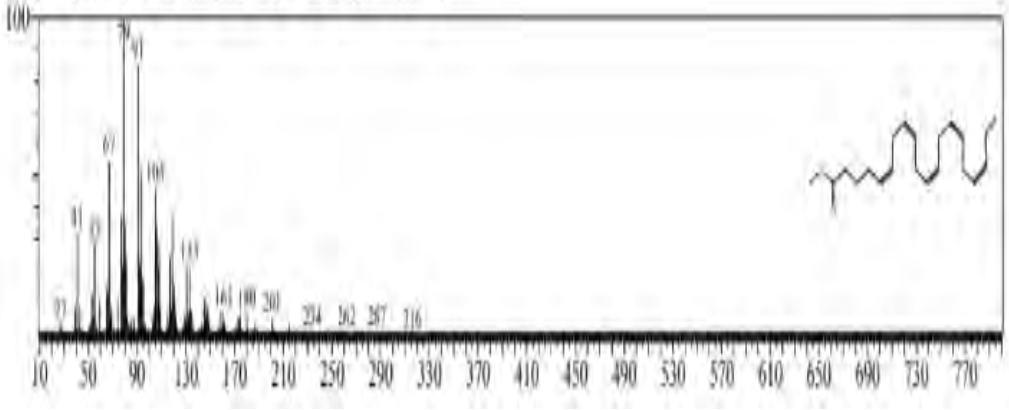
SL90 Formula:C19H34O2 CAS:112-63-0 MolWeight:294 RetIndex:2093
CompName:9,12-Octadecadienoic acid (Z,Z)-, methyl ester $$ Linoleic acid, methyl ester $$ Methyl cis,cis-9,12-octadecadienoate $$ Methyl linoleate $$ M



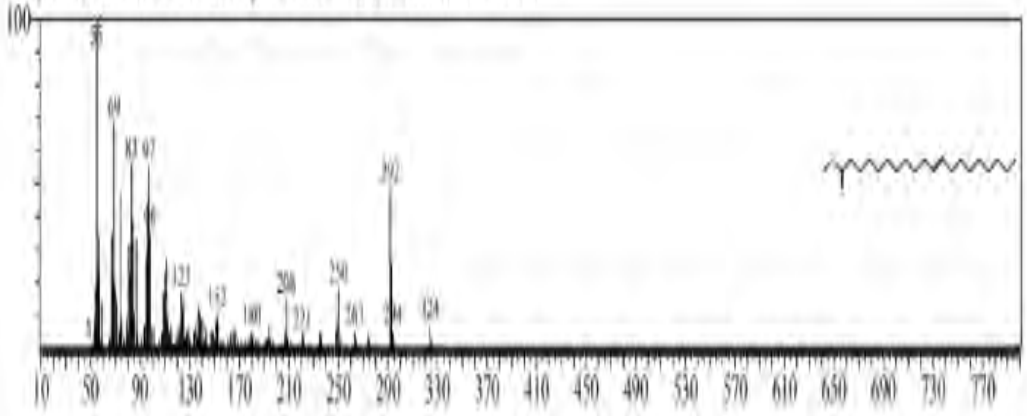

SL63 Formula:C19H32O2 CAS:301-00-8 MolWeight:292 RetIndex:2101
CompName:9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- $$ Linolenic acid, methyl ester $$ Methyl all-cis-9,12,15-octadecatrienoate $$ Methyl lino



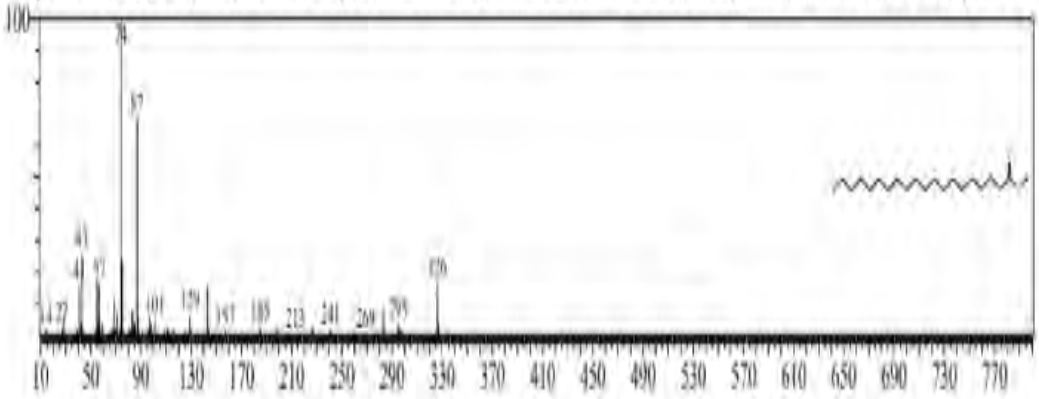

SL94 Formula:C21H32O2 CAS:2734-47-6 MolWeight:316 RetIndex:2316
CompName:5,8,11,14,17-Eicosapentaenoic acid, methyl ester, (all-Z)-



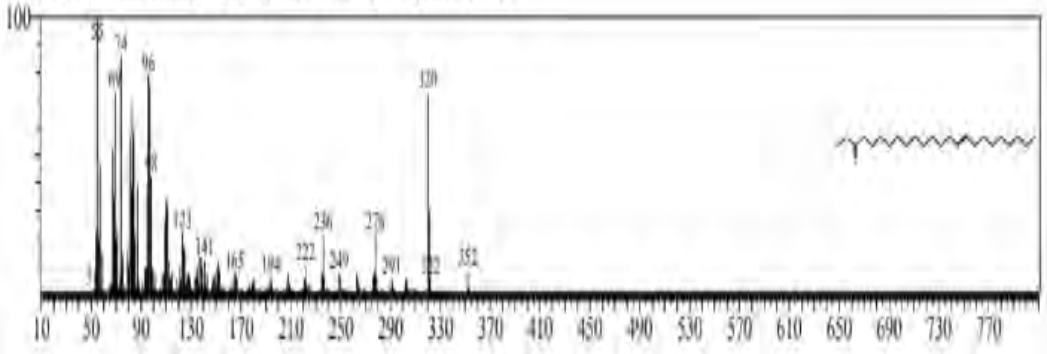
SI.89 Formula:C21H40O2 CAS:3946-08-5 MolWeight:324 RetIndex:2284
CompName:11-Eicosenoic acid, methyl ester $$ Methyl (11E)-11-icosenoate # $$

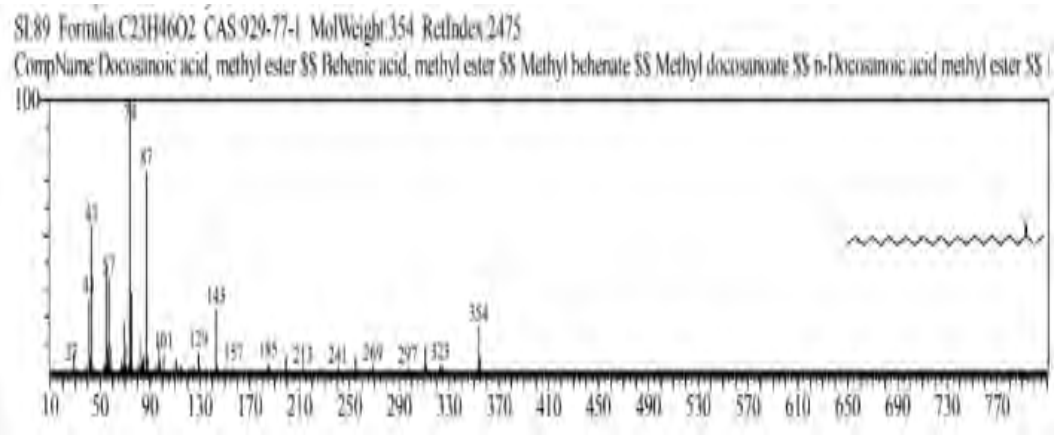



SI.91 Formula:C21H42O2 CAS:1120-28-1 MolWeight:326 RetIndex:2276
CompName:Eicosanoic acid, methyl ester $$ Methyl arachisate $$ Methyl eicosanoate $$ Arachidic acid methyl ester $$ Kemester 2050 $$ Methyl arachidat

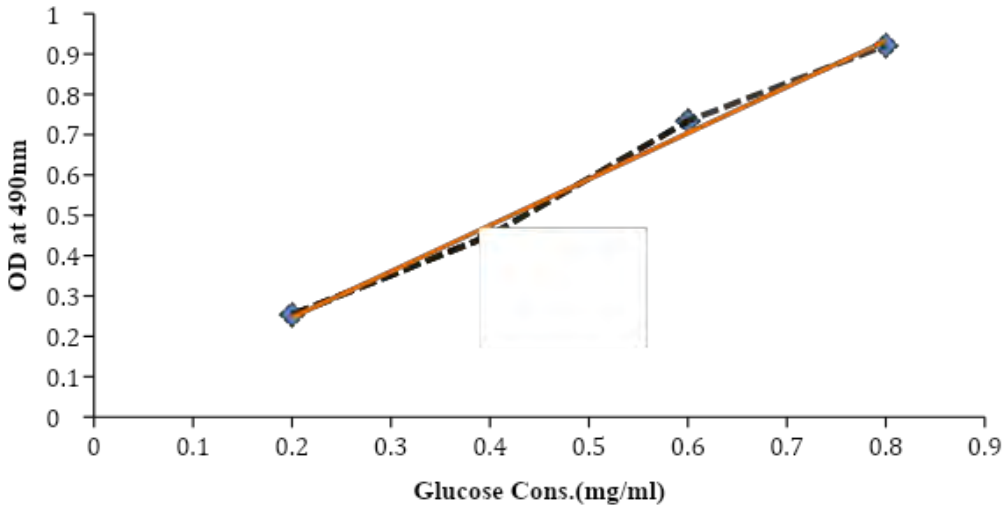



SI.89 Formula:C23H44O2 CAS:56630-69-4 MolWeight:352 RetIndex:2483
CompName:13-Docosenoic acid, methyl ester $$ Methyl 13-docosenoate $$

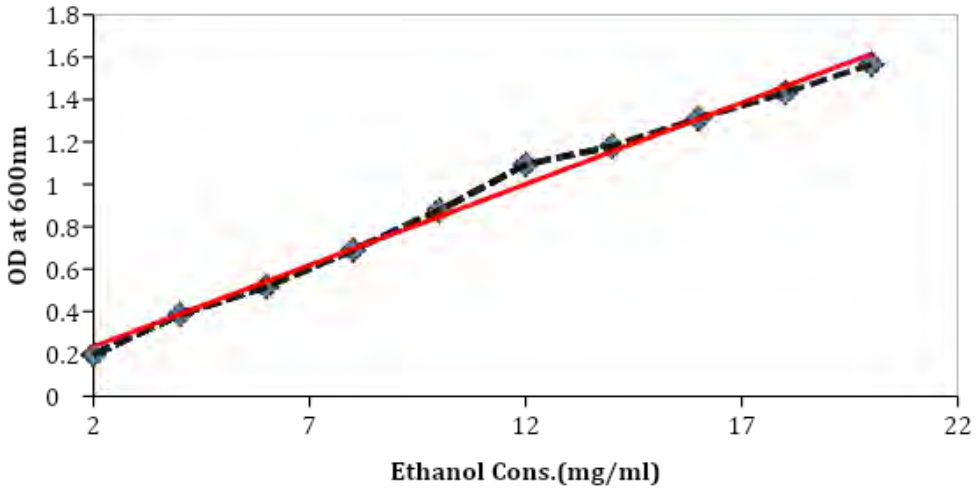





## Carbohydrates estimation curve



## Bioethanol estimation (Standard curve)




Statistical analysis in the form of mean values of duplicate experimentation of microalgae screening and characterization;

| | F2 | | FSL | | MSB | | MRA | |
|---|---|---|---|---|---|---|---|---|
| Rate of growth (g $d^{-1}$ $L^{-1}$) | 0.168 | 0.170 | 0.174 | 0.176 | 0.133 | 0.131 | 0.141 | 0.141 |
| Productivity of biomass (mg $d^{-1}$ $L^{-1}$) | 121.3 | 120.8 | 186.9 | 187.1 | 96.7 | 97.2 | 102.2 | 101.9 |
| Lipids Content % | 17.2 | 16.9 | 29.6 | 30.3 | | 11 | 14.4 | 13.7 |
| Carbohydrates Content % | 37.9 | 38.1 | 27 | 27 | 21.3 | 20.7 | 18.9 | 19.1 |

Statistical analysis in the form of mean values of duplicate experimentation of cumulative biogas production yield and methane content;

| Batch | Biogas (mL) 1 | Biogas (mL) 2 | Mean Biogas (mL) | Methane content (mL) 1 | Methane content (mL) 2 | Mean Methane content |
|---|---|---|---|---|---|---|
| Control | 270.3 | 269.8 | 270 | 133 | 133 | 133 |
| *Closteriopsis acicularis* | 691.4 | 690.7 | 690 | 460.2 | 459.8 | 460 |
| *Tetradesmus nygaardii* | 665.8 | 666.6 | 666 | 466.5 | 465.6 | 466 |
| *Closteriopsis* sp. + Eucalyptus leaves | 1182.1 | 1181.9 | 1182 | 884 | 884 | 884 |
| *Tetradesmus* sp. + Eucalyptus leaves | 960.3 | 959.8 | 960 | 688.9 | 689.2 | 689 |

The experimental data on ethanol fermentation and yield calculations;

**Experimental calculations of fermentation**

Amount of $CO_2$ collected during fermentation = 3.85 L
Moles of gas produced = 0.162 moles of $CO_2$
Therefore, moles of ethanol produced = 0.162 moles of ethanol
Volume of fermentation medium = 0.5 L
Concentration of ethanol = 0.162 moles / 0.5 L = 0.324 moles/L
**Yield of ethanol** = 0.324 mol/L x 46 g/mol = **14.9 g/L**

Figure: Fermentation reactor and collected CO2

## List of Publications

- Farhana Bibi, Humaira Yasmin, Asif Jamal, Mohammad S. AL-Harbi, Mushtaq Ahmad, Muhammad Zafar, Bashir Ahmad, Bassem N. Samra, Atef F. Ahmed, and Muhammad Ishtiaq Ali. 2021. “Deciphering Role of Technical Bioprocess Parameters for Bioethanol Production Using Microalgae.” Saudi Journal of Biological Sciences. https://doi.org/10.1016/j.sjbs.2021.10.011, IF=4.219
- Farhana Bibi, Asif Jamal, M. Ishtiaq Ali. Optimization of lipids synthesis in novel microalgae as a feed stock for biodiesel production using response surface method. Journal of Biochemical Engineering. (Research article Submitted), IF=3.9
- Farhana Bibi, Asif Jamal, Zaixing Huang, M. Ishtiaq Ali. Advancement and role of abiotic stresses in microalgae biorefinery. International Journal of Fuel. (**Review article accepted**), IF=6.6
- Rozina, Mushtaq Ahmad, Muhammad Zafar, Zainab Yousaf, Sher Aman Ullah, Shazia Sultana, and Farhana Bibi. n.d. “Identification of Novel, Non-Edible Oil Seeds via Scanning Electron Microscopy as Potential Feedstock for Green Synthesis of Biodiesel.” Microscopy Research and Technique, https://doi.org/10.1002/jemt.23942 , IF=2.79

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