ENHANCEMENT OF LIPID PRODUCTION FROM MICROALGAE *DUNALIELLA SALINA* BY INDUCING NUTRIENT DEPRIVATION IN THE MEDIUM

Dissertation submitted in partial fulfillment of the requirements

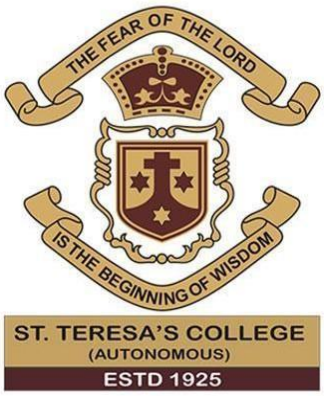
for the award of degree of "**Master of Science**" in

BOTANY

BY

ANNLIYA RAJU

REG NO: AM21BOT005



DEPARTMENT OF BOTANY

S.T TERESA'S COLLEGE

(AUTONOMOUS)ERNAKULAM

2023

CERTIFICATE

This is to certify that the dissertation entitled **ENHANCEMENT OF LIPID PRODUCTION FROM MICROALGAE *DUNALIELLA SALINA* BY INDUCING NUTRIENT DEPRIVATION IN THE MEDIUM** is an authentic record of work carried out by **Ms. ANNLIYA RAJU** under my guidance and supervision in the partial fulfilment of the M.Sc. Degree from Mahatma Gandhi University, Kottayam. I further certify that no part of the work embodied in this dissertation work has been submitted for the award of any other degree or diploma.

Dr. Arya P Mohan

Supervising Teacher

Department of Botany

St. Teresa's College (Autonomous)

Ernakulam

Dr. Liza Jacob

Head of the Department

Department of Botany

St. Teresa's College (Autonomous)

Ernakulam

St. Teresa's College (Auto nomous) DEPARTMENT OF BOTANY Ernakulam

Place: Ernakulam

Date: 18/4/23

External Examiner Valued.

1. Dr. Stephen Seymour 18/04/2023

2. Dr. Justin. R. Nayagam 18/04/2023

ST.TERESA'S COLLEGE (AUTONOMOUS) ERNAKULAM

Certificate of Plagiarism Check for Thesis

Author Name	ANNLIYA RAJU
Course of Study	MSc. Botany
Name of Guide	Ms. ARYA P. MOHAN
Department	Botany & Centre for Research
Acceptable Maximum Limit	20%
Submitted By	library@teresas.ac.in
Paper Title	ENHANCEMENT OF LIPID PRODUCTION FROM MICROALGAE DUNALIELLA SALINA BY INDUCING NUTRIENT DEPRIVATION IN THE MEDIUM
Similarity	7%
Paper ID	718636
Submission Date	2023-04-01 13:42:37

Signature of Student

Signature of Guide

Checked By
College Librarian

* This report has been generated by DrillBit Anti-Plagiarism Software

DECLARATION

I hereby declare that the work which is being presented in the dissertation, entitled "ENHANCEMENT OF LIPID PRODUCTION FROM MICROALGAE *DUNALIELLA SALINA* BY INDUCING NUTRIENT DEPRIVATION IN THE MEDIUM", in fulfillment of the requirements for the award of the degree of Master of Science in Botany and submitted to St. Teresa's College (Autonomous), Ernakulam is an authentic record of my own work carried out during M.Sc. period under the supervision of Dr. Arya P Mohan

The matter embodied in this dissertation has not been submitted by me for the award of any other degree of this or any other University/ Institute.

Place: Ernakulam

Date: 12/4/2023

Signature of the Candidate

ACKNOWLEDGEMENT

I wish to express my sincere thanks and deep sense of gratitude to Dr Jikku Jose director of Scire Science, Sreelakshmi V Pillai and all other members of Scire Science R & D laboratory, KINFRA Kalamassery, Kerala, India for introducing the present topic, their inspiring guidance and valuable suggestions throughout this work. Their constant supervision and willingness to share their vast knowledge made me understand this project and helped me to complete the assigned task on time.

I consider it as a privilege to extend my profound sense of gratitude to Dr.Arya P Mohan Assistant Professor of Department of Botany and Centre for Research, St.Teresa's College, Ernakulam for permitting me to carry out my project at Scire Science R&D laboratory, KINFRA Kalamassery, Kerala. I Wholeheartedly thank her for the heartfelt support and care she offered throughout the work.

I wish to express my sincere thanks to Dr. Liza Jacob, Head of the Department of Botany and Centre for Research, St.Teresa's College, Ernakulam for the guidance and intellectual support extended for successful completion of my work. I also express my deepest gratitude and appreciation for the guidance and encouragement given by all the teachers. I gratefully acknowledge Dr. Alphonsa Vijaya Joseph, Principal, St.Teresa's College, Ernakulam, for supporting me to do projects in this institution.

Last but not least, I would like to thank God almighty whose blessings have enabled me to accomplish my dissertation work successfully. I extend my sincere gratitude to the non-teaching staff and my dear friends who helped me to take this work to completion. I am extremely grateful to my beloved parents who have given their love, affection, encouragement and blessing which have guided me for the successful completion of this work.

ANNLIYA RAJU

TABLE OF CONTENTS

LIST OF TABLES 1

LIST OF IMAGES AND FIGURES 2

ABBREVATIONS 3

ABSTRACT 4

INTRODUCTION 5

AIM AND OBJECTIVES 11

REVIEW OF LITERATURE 13

MATERIALS AND METHODS 22

RESULT AND DISCUSSION 28

SUMMARY AND CONCLUSION 43

REFERENCES 46

APPENDIX 51

LIST OF TABLES

NUMBER OF TABLE	TITLE OF TABLE	PAGE NUMBER
1	Classification of *Dunaliella salina*	6
2	Culture parameters	23

LIST OF IMAGES AND FIGURES

FIGURE / IMAGE NUMBER	TITLE OF FIGURE	PAGE NUMBER
Image 1	Images of culture of *Dunaliella salina*	29
Image 2	Culture in nutrient deprivation.	30
Image 3	Microscopic observation of algal culture	31
Image 4	DNA visualisation in gel	35
Image 5	Lipid extracted from different medium	40
Figure 1	Graphical representation showing the absorbance	32
Figure 2	Graphical representation showing cell count	33
Figure 3	Graphical representation of chlorophyll content	34
Figure 4	DNA sequence of *Dunaliella salina*	35
Figure 5	Sequencing using blast	37
Figure 6	Graphic summary of alignment score	38
Figure 7	Phylogenetic tree showing relation with *D. salina* and *D. primolecta*	39
Figure 8	Graph showing crude lipid content	40

ABBREVATIONS

µg : microgram

µl : microlitre

BLAST : basic local alignment search tool

Ctab : cetyltrimethylammonium bromide

DNA : deoxyribonucleic acid

EDTA : ethylene diammine tetra acetate

Hiv : human immunodeficiency virus

Hrs : hours

Lux : unit of illumination

M : mole

Mg : milligram

Ml : millilitre

Nacl : sodium chloride

NCBI : national centre for biotechnology information

nm : nanometer

OD : optimal density

PCR : polymerase chain reaction

Ph : potential of hydrogen

Rpm : rotations per minute

Rrna : ribosomal ribonucleic acid

SrRNA : small ribosomal ribonucleic acid

TE buffer : tris edta buffer

Tris hcl : tris(hydroxymethyl)aminomethane hydrochloric acid

Uv : ultraviolet

D. Salina : Dunaliella salina

D. Primolecta: Dunaliella primolecta

ABSTRACT

This experiment was conducted to enhance the lipid production from microalgae *Dunaliella salina,* by inducing nutrient deprivation in the medium. Nutrients play a key role in the biochemical process and providing resistance to diseases, nitrogen and phosphate are the major components of DNA. Altering those nutrients will induce stress on the plant this may result in the enhancement of lipid production. In this experiment we used three mediums for the culture of microalgae *Dunaliella salina.* Nitrogen deprived F/2 medium, phosphate deprived F/2 medium, and F/2 medium. Nitrogen deprived F/2 medium showed an increased production rate of 69%, phosphate deprived F/2 medium showed 57% of lipid production and F/2 medium showed the least production rate of 18% lipid production.

INTRODUCTION

INTRODUCTION

The increased cost of fuel has not only affected a single nation, but the whole world. Non-renewable sources of fuel are being overexploited, leading to their gradual depletion. It is important to acquire more renewable sources of fuel production for sustainability. Lipid production is crucial for biodiesel production, but the high cost of raw materials and hardcore procedures make the process uneconomical. The need for lipid production in a cost-efficient manner is gaining importance, leading to the consideration of microalgae. Microalgae have a short lifespan and high productivity, which enables the completion of the procedure with less time and material cost. A variety of methods are used for lipid production, but most of them are costly and time-consuming. Algae are one of the easily available sources of lipids.

Dunaliella salina is one of the readily available marine algae that is rich in carotenoids and has the ability to synthesize lipids. The use of algae with a short lifespan and high reproductive capacity makes this method less time-consuming and cost-effective. Enhancement of lipid production by inducing nutrient deprivation can be utilized in producing a large amount of lipid with a short time span, which can be utilized in biodiesel production (Borovitzka, 1990). This reduces the use of fossil fuel, increases economic stability, and decreases the pollution rate caused by the burning of fossil fuels and their overexploitation.

Division	Chlorophyta
Class	Chlorophyceae
Order	chlamydomonales
Family	Dunaliellaceae
Genus	Dunaliella
Species	*D. Salina*

TABLE 1: Classification of *Dunaliella salina*

Algae are ubiquitous living organisms that can grow in various forms, sizes, and colors. They can exist independently or on the surfaces of other living organisms, soil, or rocks. Algae are crucial as they produce a significant amount of oxygen required by humans and other animals. They can thrive in diverse and extreme environments, leading to the production of unique bioactive and complex lipids that are not commonly found in terrestrial plants.

To make large-scale biodiesel production from microalgae economically feasible, significant improvements in productivity are necessary. While the maximum neutral lipid content of microalgae has received significant attention, other factors such as maximum algal cell densities and rates of biomass and lipid accumulation are equally essential. It is not yet clear how these factors interact to affect lipid production in *Dunaliella* species. Hence, this study aims to induce lipid production by implementing nutrient deprivation.

Lipids are crucial components of the cell membrane, consisting of a glycerol backbone, two hydrophobic fatty acid tails, and a phosphate group (hydrophilic) and are amphipathic. They have numerous commercial applications and the potential to replace fossil fuels in various industrial processes. While oils are already used as a sustainable alternative feedstock to produce a wide range of fundamental oleochemicals in the chemical industry, the primary global objective is to use them as a biofuel source. Biodiesel derived from plant, animal, or microbial oils is the most common biofuel used to replace fossil fuels in the transportation sector (Krishnan *et al.*, 2021). Triacylglycerols (TAG) from vegetable oil are trans-esterified with short-chain alcohols such as methanol or ethanol to create conventional biodiesel.

Due to excessive demands of contemporary society, natural resources have been depleted over the past centuries. Therefore, research efforts in both academic and corporate sectors have focused on exploring and developing new sustainable resources to meet increasing demand. Utilizing phototrophic organisms, which can utilize light energy to repair carbon dioxide and produce energy-rich products, has been a focus of research. Lipids from photosynthetic organisms such as microalgae and oleaginous yeast have been investigated as a potential supplement to current sources of energy and nourishment. Nutritional and environmental stress can cause growth arrests in photosynthetic organisms, leading to the build-up of storage metabolites, including lipids.

Algae, due to its high potential for producing biofuels and biocomponents, is an area of great interest for researchers. Chlorophyll-bearing autotrophic hydrophytes have found their place in the pharmaceutical and cosmetic industries, as well as in wastewater and sewage treatment, and in the extraction of anticancer compounds. They also play a role in satisfying nutrient demands. *Dunaliella salina* is a marine motile unicellular chlorophyte, and is important due to the highest concentration of natural beta-carotene found in the green unicellular flagellate *Dunaliella salina*. Additionally, the halophilic *Dunaliella* species produce extremely high levels of glycerol, making it a commercial source of β-carotene and glycerol. Currently, 29 species of *Dunaliella* with variations and morphologies are recognized. *Dunaliella* shares a similar morphology with *Chlamydomonas*, with the lack of a cell wall in *Dunaliella* being the primary distinction. *Dunaliella* has a single, cup-shaped chloroplast and two equal-length flagella, with a central pyrenoid in the halophilic and marine species. This genus has a wide range of cell shapes, including oval, spherical, cylindrical, elliptical, egg, pear, and spindle shapes with radial, bilateral, dorsoventral, or asymmetrical symmetry. Any kind of cell can alter shape in response to environmental changes, frequently becoming spherical in unfavourable circumstances. Cell size also varies with growth conditions and light intensity.

Renewable energy is energy produced from natural resources that regenerate themselves without depleting the planet's resources in less than a human lifetime. These resources, which include biomass, tides, waves, sunshine, wind, rain, and thermal energy stored in the earth's crust, have the advantage of being accessible in some capacity almost everywhere. They are practically endless. Even more crucially, they don't harm the climate or the ecosystem much. Fossil fuels like oil, coal, and natural gas, on the other hand, are only found in limited quantities and as we continue to extract them, they eventually run out. Even if they are created by natural processes, their replenishment takes time, and is not easy as the consumption it results from complex natural processes which lasts many decades. Fossil fuels are still highly used today, and they are even still substantially subsidized. In the meantime, the pollution they contribute to has risen to record levels, ranging from harmful particles to greenhouse gases that harm the environment. The combustion of fossil fuels for energy results in a significant amount of greenhouse gas emissions that contribute to global warming.

Most sources of renewable energy result in little to no emissions, even when considering the full life cycle of the technologies.

Renewable energy is beneficial for both mankind and the environment as it produces little to no air pollution. However, the increased use of fossil fuels in power production, industrial processes, transportation, and open burning of rubbish in cities has led to a rise in air pollution. Additionally, the use of charcoal and fuelwood for heating and cooking in many developing countries contributes to poor indoor air quality. The World Health Organization has conducted research on the negative effects of air pollution on human health (Schnurr *et al*., 2015). Lipids are valuable compounds that have potential as a replacement for fossil resources in various industrial processes. Oils can be utilized to generate a range of basic oleochemicals for organic synthesis, catalysis, or biotechnology in the chemical industry. A major global objective is to replace fossil fuels in the transportation sector, and currently, biodiesel made from plant, animal, or microbial oils is the predominant biofuel. Conventional biodiesel is produced by transesterification of triacylglycerols (TAG) from vegetable oil with short-chain alcohols such as methanol or ethanol.

Plants require various types of nutrients to grow and develop. These nutrients are classified into two categories: macronutrients and micronutrients. Nitrogen is a macronutrient that plants need, along with phosphorus (P), potassium (K), calcium (Ca), zinc (Zn), iron (Fe), boron (B), sulfur (S), and magnesium (Mg) (Tang *et al*., 2011). These nutrients play a critical role in regulating biochemical processes, offering resistance to illnesses, and ultimately improving the quality of crops. With the growing global population and declining crop yields, food safety has become a major concern. Altering the nutrients in microalgae can increase the production of lipids in response to stress conditions, which affect the photosynthetic rate of algae and induce lipid production.

In this experiment, we are using a nitrogen deprived F/2 medium phosphate-deprived F/2 medium and normal F/2 medium. Phosphate is vital for plant construction as it is used in photosynthesis as a catalyst. It’s also a critical component of DNA and RNA, which reads the genetic code. Phosphorus is a crucial part of ATP, which provides energy to the plant from seedling growth until it reaches maturity. Nitrogen is essential for plant health as it is a part of the plant structure and essential for metabolic functions such as photosynthesis.

Nitrogen is a critical component of all amino acids, which make up the building blocks of plant proteins and are essential for the growth of tissues such as cells, membranes, and chlorophyll. Therefore, plants with sufficient nitrogen levels will grow and develop rapidly and engage in higher rates of photosynthesis. Nitrogen also plays a role in the creation of DNA, which holds the genetic code for the plant. A deficiency in nitrogen and phosphate induces stress in algae, reducing the photosynthetic rate and resulting in a reduction of starch and protein production. This stress condition also stimulates lipid production, which can be extracted using various techniques. (Gao, *et al*., 2013).

AIM AND OBJECTIVES

AIM AND OBJECTIVES

This experiment mainly deals with the Enhancement of lipid production from microalgae *Dunaliella salina* by inducing nutrient deprivation. The objectives stressed on the experiment are listed below.

- To determine the growth parameters of algae in culture medium.
- To identify algal species by sequencing
- Isolate DNA of desired algae and multiply.
- Enhancement of lipid production of microalgae *Dunaliella salina* by inducing nutrient deprivation medium.
- Extract lipid from different mediums.
- Determination of crude lipid content from different mediums.
- Graphical analysis of the lipid content.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Microalgae have been rising as a feedstock for biofuel in response to the energy crisis. Due to a high lipid content, composed of fatty acids favourable for the biodiesel production, microalgae are still being investigated as an alternative to biodiesel. The quality and the quantity of lipid produced by microalgae may be affected by different Environmental factors and process conditions, which can be critical for the overall production of biodiesel. To maximize both the lipid content and the biomass productivity, it is necessary to start with robust algal strains and optimal physio–chemical properties of the culture environment in combination with a novel culture system. These accumulative approaches for cost reduction can take the algal process one step closer in achieving economic feasibility. (Shin, *et al*., 2018). The high photosynthetic capacity of microalgae is beneficial for lipid development. Numerous microalgae species like *Dunaliella salina* are grown under varied pressures for the manufacture of biofuel, industrial products, and pharmaceuticals. (Chi, *et al.*, 2017).

Microalgal lipids and related studies gain importance from past many years because they have many practical applications. Even though phototrophic development is the norm for microalgae, some species can also grow heterotrophically. Through straightforward adjustments to the physical and chemical characteristics of the culture medium, some species of microalgae can be made to overproduce specific fatty acids. Microalgae constitute a substantial source of uncommon and important lipids and fatty acids due to the striking variances in cellular architecture and development modes as well as the capacity to control their fatty acid content. Microalgal lipids and fatty acids must be generated at a large scale under regulated circumstances in order to be used as dietary components. Numerous growth systems have been created to produce phototrophic microalgae on a large scale under artificial or natural light, but they either lack control over the culturing parameters or are too expensive to use for the production of food products. Instead, large-scale heterotrophic microalgal production utilising traditional fermentation methods produces consistent biomass at minimal cost under extremely regulated circumstances. (Behrens and Kyle, 1996). Many of the main lipid groups and fatty acids found in other organisms are present in microalgae. However, they are also the main makers of several polyunsaturated fatty acids in the biosphere, particularly docosahexaenoic acid (DHA). Currently, infant formula is supplemented with some algae's high DHA content to make it more resemble human breast milk.

The most potent inducers of lipid accumulation in microalgae are deficiencies of nitrogen and phosphorus, but at the expense of biomass production. Therefore, modifying the culture medium with nitrogen, phosphorus, and carbon allowed *Dunaliella salina t*o produce as much biomass and lipid as possible. The benefits of bicarbonate augmentation, which served to sustain the health of the cells as seen by robust photosynthetic activity, may have contributed to the increase in biomass by reducing the impacts of stress. RuBisCO and ACCase, two crucial enzymes, had their expressions checked as well. The beta carboxyl transferase (accD) of the heteromeric ACCase is connected to the first phase of fatty acid biosynthesis, and RuBisCO has large and small subunits (rbcL and rbcS) responsible for incorporation of CO2. and improved biomass expression increase rbcL and rbcS gene expression in D. salina cells following NaHCO3 augmentation may be attributed. In order to concurrently maximise biomass and lipid content in phosphate-deficient *D. salina* cultures, the current work suggests a threshold level of nitrogen and bicarbonate. (Riyazat, *et al*., 2022).

This work became significant because Natural resources have been drained over many generations as a result of the excessive demands of modern society. Many researches are conducted in the field. As a result, modern research efforts in the academic and industrial sectors have been concentrated on discovering and creating new sustainable resources to meet rising demand. Research on the use of phototrophic organisms, which can repair carbon dioxide by using light energy and make products that are high in energy, has received some attention. Research has been done on using lipids from photosynthetic organisms in addition to current energy and nutrient sources. Microalgae and, to a lesser extent, oleaginous yeast have been predominantly the focus of industrial-scale efforts to generate lipid-derived commodities. Several strategies have been exploited to optimize lipid production in photosynthetic organisms ranging from exposure to environmental and nutrient stress to metabolic engineering using transgene expression as well as co-culturing and in silico investigations of flux distributions. These conditions result in nutritional and environmental stress-induced growth of microalgae and photosynthetic bacteria which result in accumulation of lipids in response to stress. (Levering, *et al*., 2015).

The green alga *Chlorella vulgaris* used a two-stage method for lipid generation that involved growth under nutrient-rich conditions followed by cultivation under nitrogen shortage and controlled settings of phosphate, light intensity, aeration, and carbon sources, and the growth is examined. Low nutrition levels, moderate aeration, and high light levels all aided in raising lipid productivity. (Mujtaba, *et al*., 2012). Researches proved many microalgae species are

capable of producing large amounts of lipids and nitrogen-limiting cultures can produce high concentration of lipids. Algal cells can rise concentration of lipid when cultivated in nitrogen-deficient media (Spoehr and Milner, 1949). Rate of microalgal biomass formation and lipid content affect the amount of biofuel that can be produced from algae. Numerous elements, including nutrition, have a significant role in limiting both biomass production and fat build-up. The marine microalgae *Dunaliella tertiolecta* was employed in this study as a model organism, and a profile of its dietary needs was established. For algae to develop at their best, inorganic phosphate and trace metals such as cobalt, iron, molybdenum, and manganese (Mn2+) were shown to be essential. Maximum biomass production needed nitrate, not ammonium (NH4+), as the form of inorganic nitrogen. Under nitrogen starvation growth conditions, lipids accumulated and this process was time-dependent. Results of this research can be applied to maximize production of microalgal lipids in optimally designed photobioreactors. (Chen, *et al., 2011*).

Microalgae are of particular most promising source of biomass for biodiesel production due to their potential to synthesize and accumulate large quantities of lipids in some species. *Dunaliella salina* unicellular flagellate *Dunaliella salina* is the richest natural source of the carotenoid β-carotene). The halophilic species of *Dunaliella* also accumulate very high concentrations of glycerol, cellular β-carotene accumulation in *D. salina* correlates with accumulation of specific fatty acid species rather than with total fatty acid content, production is high when a stress was introduced to the algae. (Lamers and Lorier, 2010). Financing for research into the manufacture of biofuels by photosynthetic microorganisms, this is currently thought to be impossible due to the inefficiency and high costs of fuel production. This can be solved by the implementation of algae to this field. The energy crisis in the middle of the 1970s, the U.S. Department of Energy (DOE) has been interested in the production of microalgal lipids that can be converted into biodiesel. (Weldy and Heusemann, 2007).

Energy and fuels like gasoline have primarily come from crude oils. However, since the 1970s, there have been major public worries about the long-term viability, price volatility, and damaging environmental effects of crude oil. As a result, in recent decades, biooils and biooil-based biodiesel fuels have come into use as substitutes for crude oils and crude oil-based petro-diesel fuels, respectively. Although petro-diesel fuels are still widely utilised today, the usage of biodiesel fuels in the power and transportation sectors is rising. As a result, the creation of algae biodiesel fuels as the fourth generation of biodiesel fuels has garnered considerable public interest. However, it is required to lower the cost of the feedstock by increasing the lipid

productivity of algal biomass in order to lower the overall cost of biodiesel synthesis. (Konur, 2021). Algae can therefore be utilised for extensive research on the generation of algal lipids for the production of algal biodiesel. Lipids are a significant algal biochemical with several uses, including the production of biodiesel. “Fatty acid methyl esters” (FAME), created by the transesterification of lipids, are what biodiesel is made of. Triacylglycerols (TAGs), which are true or neutral lipids found in algae, are crucial for producing high-quality, stable biodiesel fuel with a higher calorific value. For the purpose of enabling the selective synthesis of real lipids from algal biomass, new and enhanced physicochemical lipid extraction technologies are crucial. It can be used to produce biodiesel. (Krishnan, *et al*., 2021)

The most crucial nutrient sources for growth and development are nitrogen and phosphorus, which are influenced by a variety of circumstances. Studies have been done to determine how low levels of nitrogen and phosphorus affect the ability of *Chaetoceros calcitrans* and *Chlorella sp*. to produce lipids. These species' early stationary phase cultures were subjected to various nitrogen and phosphorus stresses, and the composition of the lipids and fatty acids was analysed using extraction and gas-liquid chromatography techniques. The findings showed that as nitrogen and phosphorus levels dropped in these two species, lipid synthesis considerably increased. (Adenan, *et al.,* 2016)

Under N, P, and Si starvation, the amount of two fatty acids remained largely constant. But there were clear species differences that contained a lot less, like in calcitrans of rT. pseudonana. Light restriction resulted in no discernible trend in the amount of lipid present in each cell. As a function of irradiance, the amount of N per cell—a marker of protein content—was essentially constant. These illustrations of protein, carbohydrate, lipid, and specific fatty acid changes in response to nutrient (N, Si, or P) or light limitation highlight the significance of understanding the growth curve's phase. because as cultures become dense and growth is halted by nutrient or light limitation, the nutritional value of the phytoplankters may change.

It is well known that microalgae can be used as a sustainable source for making biodiesel. The effectiveness of lipid generation and CO2 fixation by the native microalga *Chlorella vulgaris* is being studied in relation to nitrogen deprivation tactics and photobioreactor design. In order to increase microalgal lipid production, single-stage cultivation on basal medium with low starting nitrogen source concentration was carried out. This is demonstrated by a comparison of single-stage and two-stage nitrogen starvation procedures. When *C. vulgaris* was cultivated in a vertical tubular photobioreactor with a high surface to volume ratio, its lipid productivity

was shown to be improved. In addition to the significant lipid output, CO2 fixation occurred during the 10-day photoautotrophic development. Over 65% of fatty acids are found in the microalgal lipid, according to analysis of its fatty acid makeup. This lipid quality is suitable for biodiesel production. (Yeh and Chang, 2011)) For the generation of biodiesel, microalgae's lipid content is crucial. The photosynthetic efficiency, biomass composition, and lipid production of the marine unicellular microalgae *Chaetoceros muelleri* (Bacillariophyceae) and *Dunaliella salina* (Chlorophyceae) cultivated under various nutrient deprivation conditions were examined in this study. As a result of nitrogen and total nutrient deprivation, respectively, the greatest lipid content of *C. muelleri* and *D. salina* could be reached. Under those circumstances, analysis of the fatty acid methyl esters profile showed that the lipid collected by the two species was suitable for biodiesel production. These investigations led us to the conclusion that *C. muelleri* and *D. salina* would be excellent candidates for the manufacture of biodiesel. (Gao, *et al*., 2013).

If not controlled, rising levels of atmospheric carbon dioxide (CO2) may have unknown harmful impacts on the environment. Even with the tightest CO2 mitigation measures, the Intergovernmental Panel on Climate Change (IPCC) forecasts a 2°C to 4°C rise in global temperatures in its most recent assessment. Since burning fossil fuels directly affects the amount of CO2 in the atmosphere, burning fossil fuels can be blamed for a significant portion of global warming. Currently, the majority of biofuels that do not increase atmospheric CO2 are produced from terrestrial plants, such as ethanol from maize grain and biodiesel from soybean oil. The availability of fertile land greatly restricts the development of biofuels from terrestrial plants. (Weldy and Huesemann, 2007).

Dunaliella salina, a halotolerant green alga exhibits increased glycerol production, large -carotene build-up, and accelerated abscisic acid metabolism in response to stress, according to a study that focuses on the biochemical and physiological responses of *Dunaliella salina* to stress. Cellular responses in this regard are regulated and appear to rely on a variety of pathways that may be connected to a change in the abscisic acid balance. The cellular contents of Dunaliella are surrounded by an elastic plasma membrane, which enables quick volume changes in reaction to osmolarity changes outside the cell. *Dunaliella* lacks a stiff cell wall. (Cowan, *et al.*,1992). To make the large-scale manufacturing of biodiesel from microalgae economically viable, significant productivity increases are needed. There are other

characteristics that are equally crucial, despite the fact that the maximal neutral lipid content of microalgae has drawn a lot of attention as a target for optimization. These are the highest densities of algal cells that can be maintained during continuous cultivation and the rates of accumulation of both biomass and lipids. It has not been completely investigated how these parameters interact to affect lipid synthesis in *Dunaliella* species (Liu *et al*., 2013). Hence this study examines the rates of growth and lipid accumulation in *Dunaliella salina* in nitrogen and phosphate deprived medium. Microalgae-derived lipids and their related biodiesel production. *D. salina* grown in duplicate photobioreactors, the lipid production rates under high light, low light, as well as nitrogen sufficient and nitrogen deficient growing conditions were compared. The findings of cellular lipid production indicate that *D. salina*, which may be mass-cultivated in non-sterile outdoor ponds, has a great deal of potential to be a financially advantageous source for the production of renewable oil and biodiesel. (Weldy and Huesemann, 2007).

Due to their quick growth and high lipid content, microalgae have been suggested as producers of sustainable and carbon-neutral fuel feedstock. In this study, we focus at the impact of various nutritional stresses on the overall lipid productivity and physiology of a species of microalgae called *Dunaliella salina*. The dilution ratio was shown to only alter the harvest period and not the biomass or lipid production of the species in semi-continuous cultures. The productivity of biomass and lipids during distinct growth phases varied depending on the temporal nutritional status of the medium. In response to the diverse nutritional stressors, this species also displayed distinct cellular biochemistry and lipid metabolism. Other than nitrogen, most effective stimulators of lipid formation, lack of other elements such as trace elements can also be used in enhancing the lipid production rate. Nutritional shortage had a significant impact on lipid profiles, including chain length and degree of unsaturation, which are desirable traits for biodiesel generation. (Hosseini, *et al.*, 2018)

The effects of a deficient medium on the concentrations of oil content, fatty acid profile, and anticipated fuel qualities of *Dunaliella salina* were examined in this research study on nitrogen (N) and phosphorus (P). Studies in the past found that when Axenic *D. salina* cells were grown in F/2 growth medium with a sufficient level of salinity, growth dry weight, cell count, and their relationship were measured. Oils were then extracted by soaking after Soxhlet extraction. The concentrations of N and P had a significant impact on growth dry weight, with cultures grown at the recommended N and P concentrations achieving the highest growth dry weights. The pattern for oil content was the opposite: cultures grown in the absence of phosphorus (0.0P), under complete N/P famine, and under control had maximum oil concentrations of

24.86%, 22.85%, and 5.88%, respectively. Most fatty acid methyl esters were in the C14–C22 range. The American Society for Testing and Materials (ASTM) and European Committee for Standardization (EN) concluded that the estimated fuel characteristics of algal cells grown under NP stress met their requirements. (Almutairi, 2020). Under situations where there is enough nitrogen, *Dunaliella* sp. typically contain 25% lipids. With the exception of *Dunaliella* spp., which produced a larger quantity of carbohydrates, nitrogen shortage led to a significant rise in the lipid content in all species. (Ben-Amotz, *et al*., 1985).

A very intriguing green cell factory for the production of carotenoids and lipids in harsh environments is the unicellular marine alga *Dunaliella salina*. However, the main difficulties faced by researchers and those that still need to be resolved are the cultural circumstances and their productivity. The research looked at how the bicarbonate amendment affected *D. salina's* biomass, photosynthetic activity, biochemical components, nutrient uptake, and antioxidant response when there was a macronutrient deficit (N, P, and S). Significantly increased biomass, carotenoids like -carotene and lutein, lipid, and fatty acid content under nutrient deficit conditions, along with concurrent enhancement of the activities of nutrient assimilatory and carbonic anhydrase enzymes. In contrast to phosphate and sulphate deficiency, addition of bicarbonate during nitrate deficiency led to the greatest accumulation of carotenoid, especially -carotene, and lipids. In *D. salina* cultures under nutrient stress, bicarbonate supplementation decreased the oxidative stress brought on by ROS, decreased lipid peroxidation damage, and improved the activities of antioxidant enzymes (SOD, CAT, and APX). These findings demonstrated that macronutrient deficit cultures had a significant negative impact on *Dunaliella*'s ability to grow. However, by reducing the effects of oxidative stress during nutrient deficit conditions, the media amended with sodium bicarbonate demonstrated a significant recovery in the cell growth. The sulphate deficit medium supplemented with sodium bicarbonate showed the greatest cell growth recovery, followed by the phosphate deficit medium with sodium bicarbonate, and the nitrate deficit medium with sodium bicarbonate showed the least recovery. (Srinivasan, *et al.*, 2018). A potential source of lipids for the subsequent production of biodiesel is *Dunaliella salina*. However, the rate of biomass production and lipid content affect the amount of lipids produced by *D. salina*. Enhancing production efficiency is necessary to obtain more lipids. studies into the use of a two-stage culture strategy for better microalgal growth and lipid in order to produce a high yield of lipids, the culture was grown under four different nitrogen concentrations. The culture was also compared by varying the nitrogen concentration from high to low under high and low light

intensities. The results showed that the highest lipid contents (46%) and lipid per cell were obtained in the culture grown under low nitrogen concentration, whereas the highest lipid concentration and productivity were found in the culture grown under high nitrogen concentration respectively. and came to the conclusion that changing the nitrogen concentration of the *D. salina* culture from high to low while growing it under high light intensity was the best way to increase the overall lipid production in *D. salina*. This method can be applied to outdoor cultivation to produce algal biofuels effectively. (Akter, *et al*., 2016).

Many experiments in this field derived contrasting results like Lipid measurements are improved by using the FTIR signal at 2926 cm1 (rather than 1740 cm1), and the PCA of the full spectrum revealed a distinct separation between the nitrogen-rich and nitrogen-depleted cells. As anticipated, cells starved of nitrogen (N-depleted) grew much more slowly than N-replete cells. At 26 °C, N-depleted cells produced a final lipid content that was 78% higher than the N-replete samples, compared to a 28% difference at 16 °C. The total lipid production during the starvation period was lower for many samples due to slower growth rates brought on by the stress of nitrogen starvation. The highest cell density examined at 26 °C was the only stress condition that produced noticeably more total lipids. (Liu, *et al*., 2013). In batch or semi-continuous cultures, three marine phytoplankters—*Isochrysis galbana, Chaetoceros calcitrans* and *Thalassiosira pseudonana* that are frequently used in the culture of bivalve larvae were grown. As growth became constrained by nitrogen, silicon, phosphorus, or light, changes in protein, carbohydrate, lipid, and some fatty acids were measured. In comparison to cells growing under no nutrient limitation, under N starvation for 2 days, all 3 species showed a decrease in protein and an increase in carbohydrate while maintaining a relatively constant percentage of lipid. Lipid, protein, or carbohydrate levels did not change during the six hours of Si starvation. (Harrison, *et al., 1990)*

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Microalgal culture

Microalgal species selected for the current study was a strain of *Dunaliella salin*a which was previously isolated and preserved in Scire Science R & D laboratory, KINFRA Kalamassery, Kerala, India. The microalgal species were chosen due to their faster cell division, higher biomass index and the commercial importance of strains produced by species compared to other strains available in the laboratory.

3.1.1 Culture media, composition and culture parameter

The algal culture broth was prepared and the culture tubes were incubated under optimum conditions as given in the table 2.

S.NO	PARAMETER	VALUE
1	Working volume	200ml
2	Temperature	24±1ºC
3	Light intensity	700-800 lux
4	Photoperiod	16/18
5	Time	20 days
6	pH	20

Table 2: culture parameters

3.1.2 Microscopic observation

After 5 days of incubation, the culture was observed microscopically under 40X magnification using a light microscope (ZEISS primo star) on a daily basis in order to examine growth and multiplication of microalgal cells.

3.1.3 Maximum absorbance determination

The efficiency of biomass growth was controlled by measuring the optimal density (OD), which is defined as the absorption of visible radiation. The optical absorbance was measured at various wavelength such as 680nm, 686 nm and 750 nm in order to determine maximum absorbance using spectrophotometer.

3.1.5 Cell counting using Neubauer Haemocytometer.

The microalgae concentration in the mixed culture was counted using the improved Neubauer haemocytometer counting chamber. A clean Neubauer cover glass was attached to the counting chamber by pressing it carefully in place. Then the sample was collected using a pipette and gently allowed to run quickly through the leading edge of the coverslip into the chamber, exactly filling it. After that, the counting chamber was allowed to stand on the bench for two minutes before counting using the light microscope (ZEISS primo star). The grid under the microscope was examined using 10X objective for distribution of the cells and refocused at 40X objective before counting cells in the four corner squares.

$$\text{Cell number or Cell density} = \frac{\text{Counted cells per ml}}{\text{Volume of square} \times \text{Dilution factor}}$$

3.1.6 Determination of chlorophyll content

The chlorophyll content of the microalgal cells was determined by using spectrophotometric technique. Sample of the microalgal suspension was centrifuged for 10minutes at 3000rpm (Centrifuge HERMLE-Z 3242). The supernatant was decanted and the pellet resuspended in 90% methanol. Chlorophyll was then extracted from the sample during one hour of incubation in a water broth (Rotek) at 50°C. The sample was again centrifuged for 10 minutes at the same speed. For the spectrophotometric determination of chlorophyll, the absorbance of light green supernatant was measured at two wave lengths, 480nm (A480) and 700nm (A700), using the UV Spectrophotometer was blanked with methanol.

3.2 Identification of microalgae using molecular sequencing

At the molecular level, the rRNA genes are the most widely used markers for the identification of algae due to their conserved function and universal presence. Several researchers have exploited the conserved the conserved regions of the 16 s rRNA gene for phylogenetic analysis. Here we explored the possibility of 16s forward and reverse primer for amplification.

3.2.1 DNA isolation

DNA isolation method by Doyle and Doyle (1978), using CTAB yielded good quality DNA for PCR.

DNA isolation using CTAB

Freshly prepared CTAB (Cetyl Trimethyl Ammonium Bromide) buffer was preheated at 65°C. 1gm of the microalgae sample was ground in 16 mL of CTAB buffer and homogenised. The ground tissue incubated at 65°C in a water bath for 30 minutes followed by incubation at room temperature. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed thoroughly to form an emulsion. The aqueous layer was collected after centrifugation at 10,000 rpm for 10 minutes and transferred to a new 50 mL centrifuge tube using a cut tip. Phenol: Chloroform: Isoamyl alcohol extraction was repeated and the aqueous layer was collected in eppendorf tubes (1mL in each tube). 3M sodium acetate (pH 5.2) was added to the aqueous phase and DNA was precipitated by the addition of 2/3 rd volume of ice- cold isopropanol and thoroughly mixed by inverting. The samples were kept for overnight incubation at -20°C. The supernatant was decanted off and the pellet was washed with cold 70% methanol. The DNA was separated by centrifugation at 12000 rpm for 10 minutes. The ethanol was decanted off and the pellet was air dried to remove all traces of ethanol and resuspended in a 100µl TE buffer.

3.2.2 Amount and purity of DNA

The yield of DNA extracted was measured using a UV spectrophotometer (Lab India) at 620 nm. The purity of DNA was also determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the sample in 1.0% Agarose gel. The intensities of the band obtained by staining with (0.5µ/g mL) Ethidium bromide was compared with 250 bp DNA marker from Chromous Biotech. The gel documentation system (BIO RAD- Molecular images) was used for DNA visualisation on the gel.

3.2.3 PCR amplification

16s region was amplified by polymerase chain reaction (PCR) with specific primers (forward and reverse primers). Amplification of the conserved regions of the 16s rRNA gene was conducted in a reaction mixture with a final volume of 20µl that contained about 20mg of template DNA and primers using the PCR Master Mix (Fermentas, USA) and a thermal cycler. The reaction consisted of initial denaturation at 94°C for 3 minutes followed by 40 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 1minute, with a final extension at 72°C for 7minutes. Amplicons were checked for appropriate size by 2% agarose gel visualization. Amplicons were gel purified using commercial column based

purification kits(Invitrogen, USA) and Sequencing was performed with forward and reverse primers in ABI 3730 XL cycle sequencer. Forward and reverse sequences were assembled and contig was generated after trimming the low- quality bases. Sequence analysis was performed using online tool BLAST of NCBI database and based on maximum identity score E value top most sequences was utilized for multiple sequences alignment.

3.4. Nutrient Deprivation Study on *Dunaliella salina*

The influence of macronutrients like phosphate and nitrate on the growth and lipid content of the microalgal cells were determined by using 3 types of F/2 medium with altered composition

3.4.1 Basic F/2 Medium containing both nitrate and phosphate (control)-A

500 mL F/2 media was prepared by the addition of 0.5 mL of nitrate and 0.25 mL vitamin solution directly to sterile seawater and the preparation was autoclaved at 121°C, 15 lbs pressure for 20 minutes. Aliquots of 0.5 phosphate and 0.5 ml trace metals were also sterilised using autoclave and it was added aseptically to the media once it cooled to room temperature.

3.4.2 Nitrate Deprived F/2 medium (Nitrate limited)-B

500 mL F/2 media was prepared by the addition of 0.25 mL Vitamin solution directly to sterile seawater and the preparation was autoclaved at 121°C, 15 lbs pressure for 20 minutes. Aliquots of 0.5 mL phosphate and 0.5 mL trace metals were also sterilised using autoclave and it was added aseptically to the media once it cooled to room temperature.

3.4.3 Phosphate Deprived F/2 medium (Phosphate limited) -C

500 mL F/2 media was by the addition of 0.5 mL of nitrate and 0.25 mL vitamin solution directly to sterile sea water and the preparation was autoclaved at 121°C, 15 lbs pressure for 20 minutes. Aliquots of 0.5 mL trace metals were also sterilised using autoclave and it was added aseptically to the media once it cooled to room temperature.

3.5 Comparison of growth characteristics of 3 culture media

Growth of microalgae on different media (A, B and C) were determined by methods such as macroscopic observation of cultures, microscopic observation under 40X magnification. Determination of chlorophyll content at 480 nm and 700 nm, cell count using Neubauer haemocytometer.

3.6 Crude Lipid extraction

3.6.1 Modified Bligh and Dyer method

Lipid content of the cultures A, B and C were determined based on a modified method adapted from Bligh and Dyer (1959). This method extracted lipids from the microalgal cells by using a mixture of methanol, chloroform, and water. Microalgal sample was centrifuged at 3500 rpm for 10 min. The pellet obtained was mixed was mixed with methanol and chloroform in 1:2 ratio. After overnight stay, the mixture was re- centrifuged and the lower layer that contained lipid and chloroform was extracted and dispensed into pre-weighed vials. All vials were placed in a water bath at 65°C for 8 h or kept in an oven at 80°C for 4 h to evaporate the chloroform and lipids, before weighing (Shah et al., 2014). Dry weight of the cultures A, B and C were determined by centrifugation of the cultures at 5000 rpm for 10 minutes and the pellets formed were dried using hot air oven (161°C for 1 hour) Crude lipid content was determined by dividing the residue weight with the dry cell weight. The crude lipid content was determined by using the equation (1)

$$\text{Crude Lipid Content} = \frac{\text{Residue weight}}{\text{Dry cell weight}}$$

$$\text{Crude lipid content in percentage (\%)} = \frac{\text{Residue weight}}{\text{Dry cell weight}} \times 100$$

Crude lipid productivity was determined using following equation 2)

$$P = C \times W/T$$

Here P denotes the lipid productivity and it was expressed in mgL -1 d -1. C (%) stand for lipid content of cells, W(mgL $^{-1}$) for the dry cell weight and T stands for cultivation period in days.

RESULT AND DISCUSSION

RESULT

MICROALGAE CULTURE

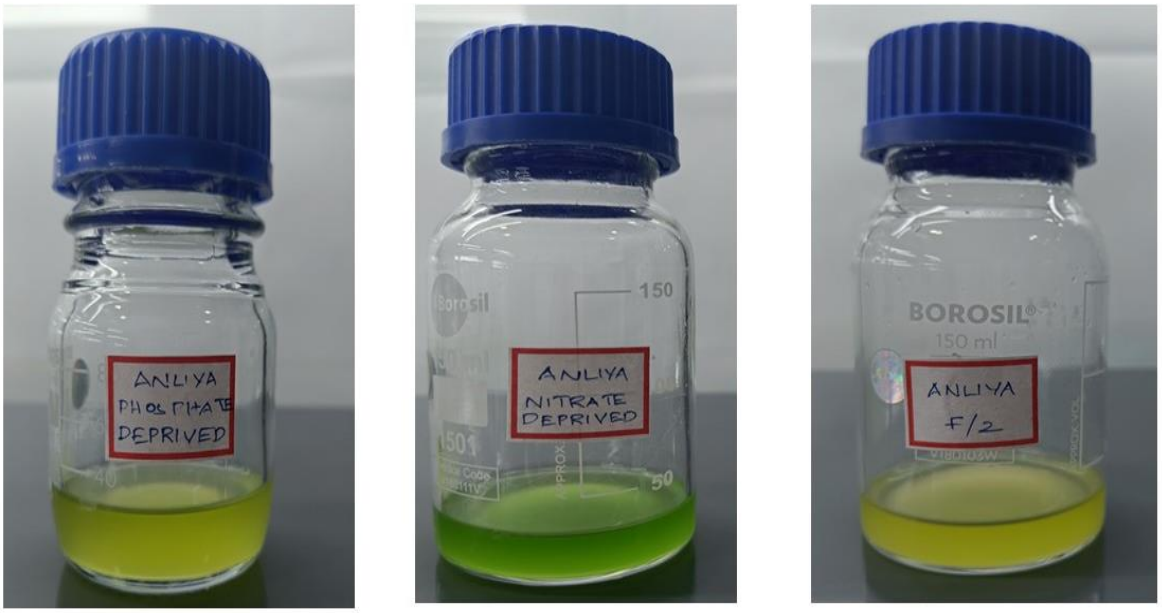
The marine microalgae *Dunaliella salina* was cultured in conical flask by maintaining the growth conditions. The colour change was noted for 15 days. On the first day of inoculation a pale green colour was observed, gradually the intensity of colour increased and found to be dark green at the 15th day of inoculation (Image1).

a **b**

Image 1: **a**) 1st day of culture of *Dunaliella salina*, **b**) Culture of *Dunaliella salina* on 15th day

NUTRIENT DEPRIVED CULTURE

Algae Dunaliella salina was cultured in three conditions among which first one was nitrogen deprived F/2 medium, second in phosphate deprived F/2 medium and finally in F/2 medium (Image 2). The nutrient deprivation was done to enhance the lipid production.



a b c

Image 2: a) culture in phosphate deprivation **b)** culture in nitrate deprivation **c)** Culture in F/2 medium

MICROSCOPIC OBSERVATION

The micro algae *Dunaliella salina* was observed under light microscope (Image 3). The microalga cell appeared in pale green, round to ovel in shape. Microscopic observation showed an increased growth of algal cell.

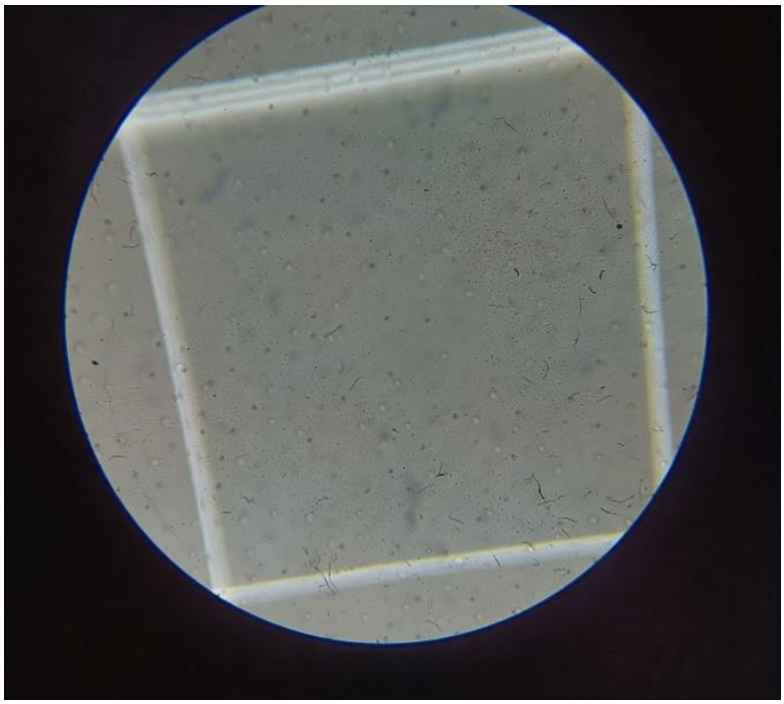


Image 3: Microscopic observation of algal culture

MAXIMUM ABSORBANCE DETERMINATION

The optical density of algal culture of microalgae *Dunaliella salina* was determined periodically at 700nm and 620nm wavelength. Algal culture showed maximum absorbance in 15th day. After day 15 the absorbance found to be declined.

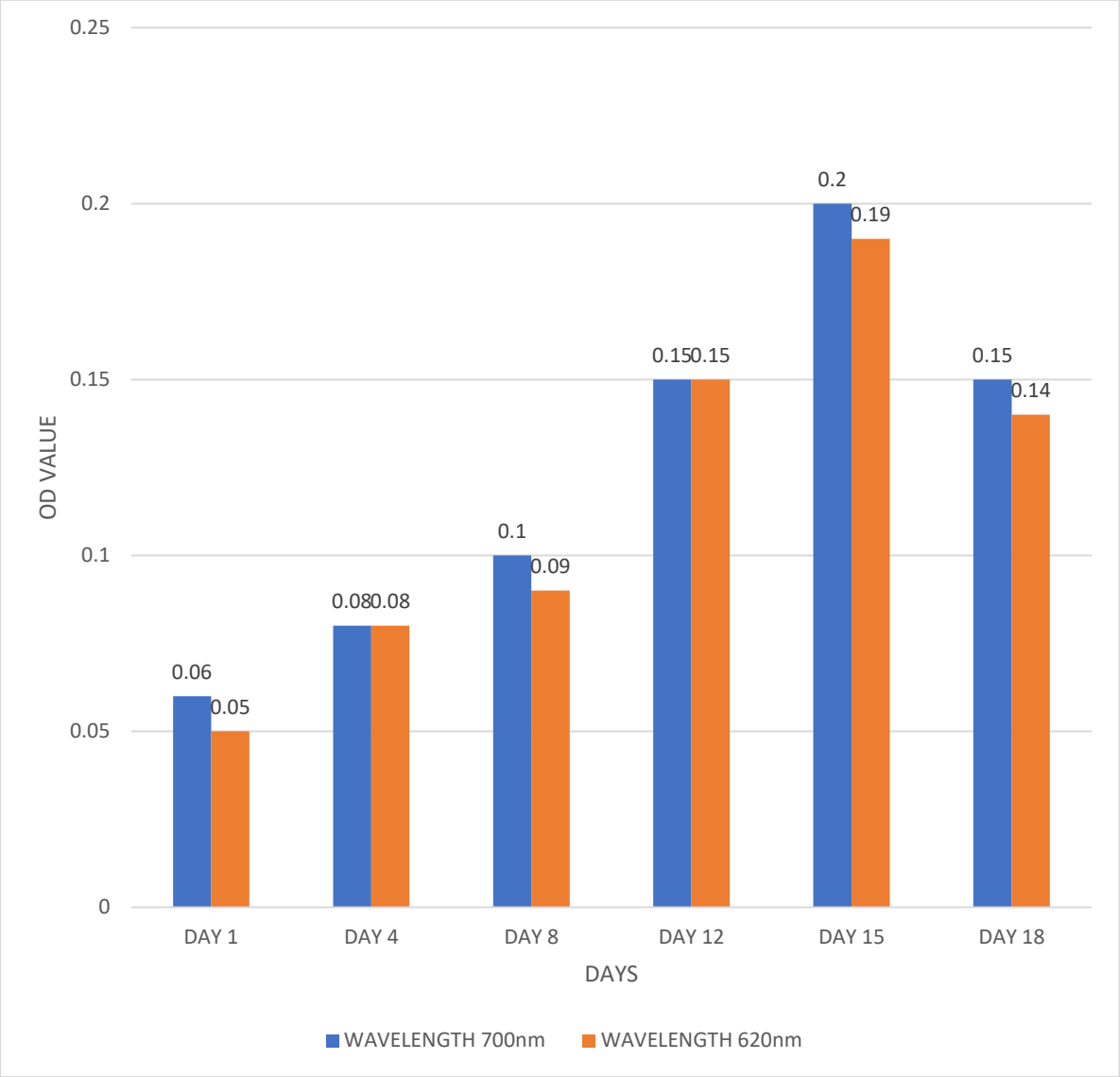


Fig. 1: Graphical representation showing the absorbance

CELL COUNT USING HAEMOCYTOMETER

Cell count was measured periodically by using the Neubauer haemocytometer which shows the cell density of culture. The cell count was found to be increasing with days of culture. The graphs (Fig. 2) were constructed using the data.

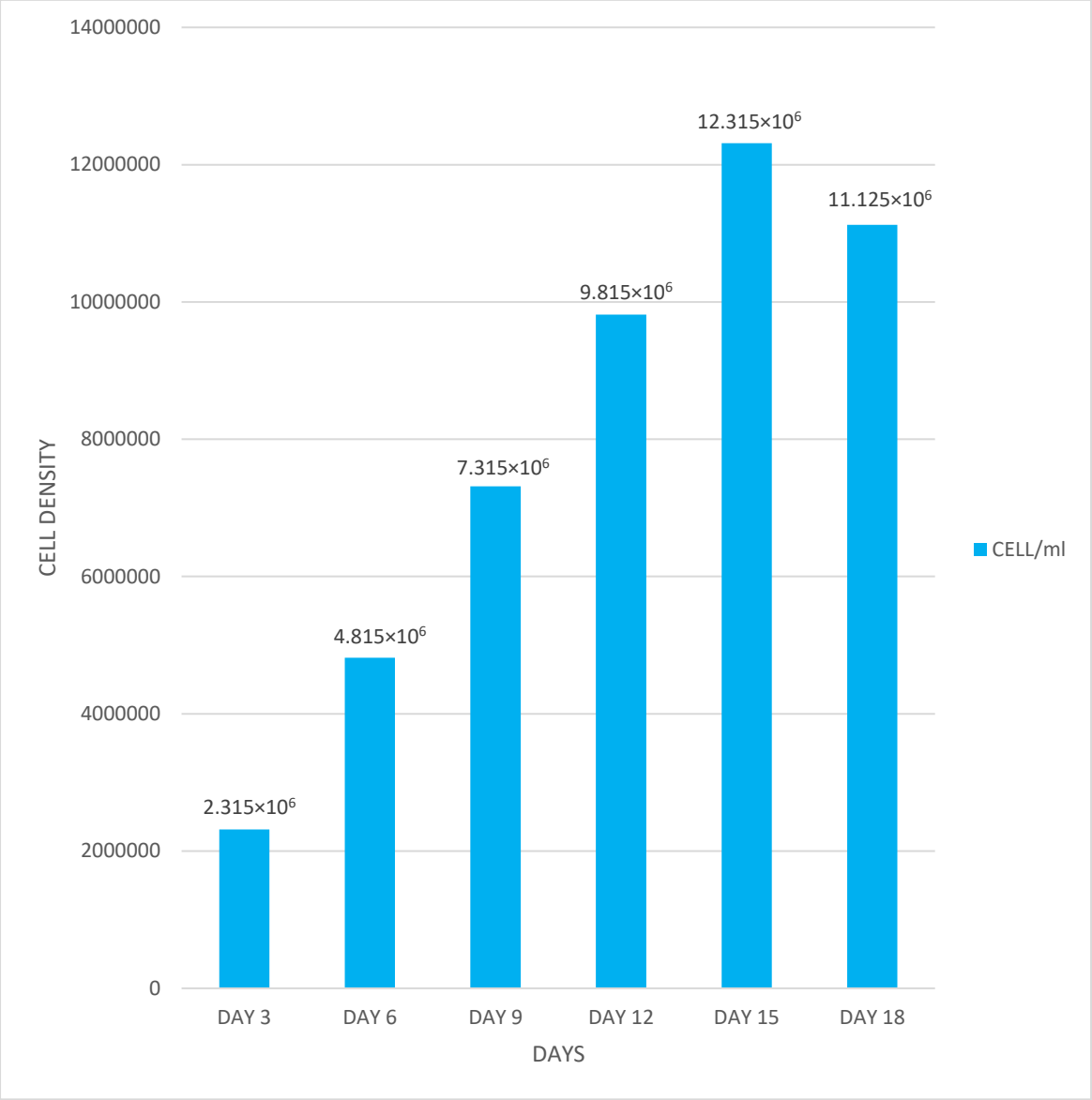


Fig. 2: Graphical representation showing cell count/ml of solution

DETERMINATION OF CHLOROPHYLL CONTENT

Chlorophyll content of algal culture of *Dunaliella salina* was measured using acetone method the OD values are taken at different wavelengths. This was done periodically and the values were tabulated and graph was plotted (Fig. 3).

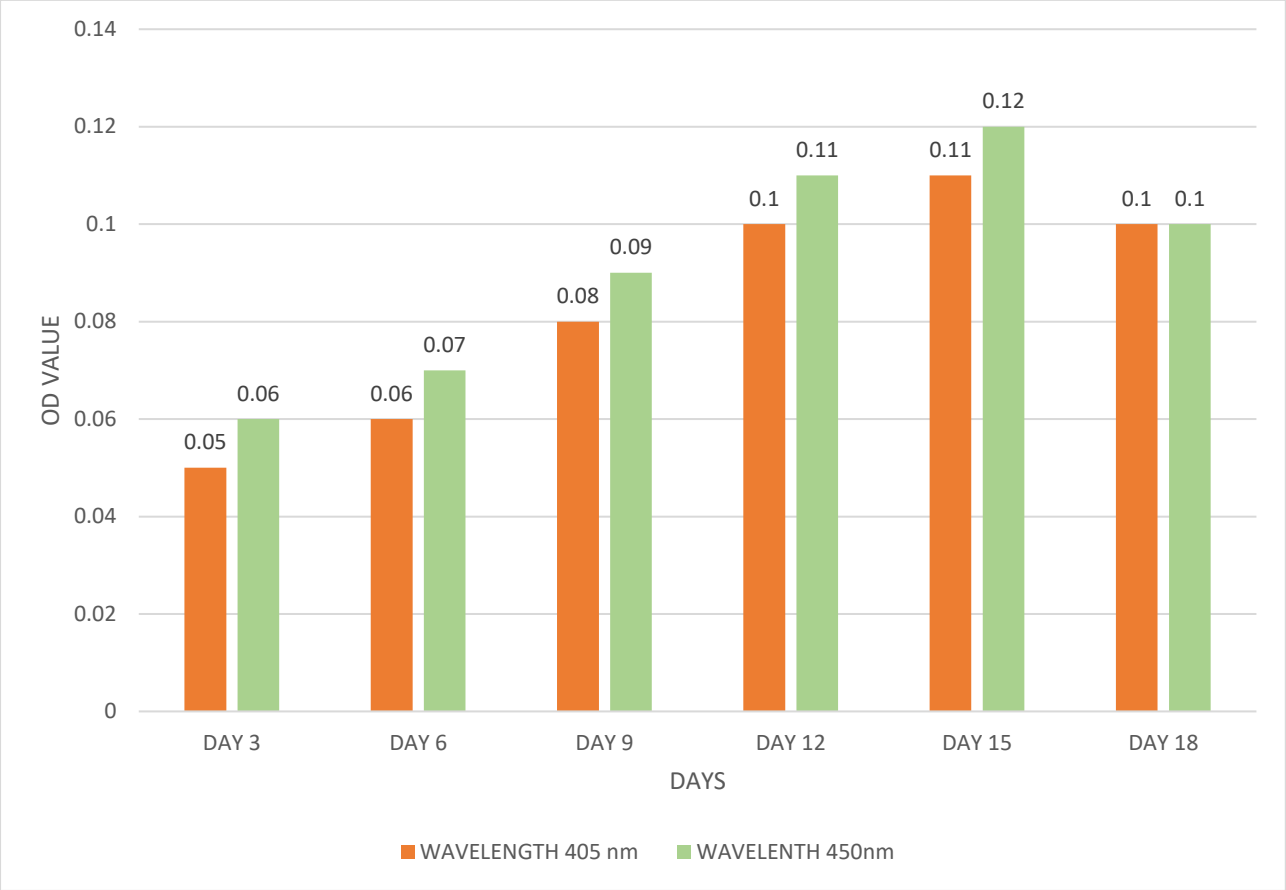


Fig. 3: Graphical representation of chlorophyll content in periodic analysis

IDENTIFICATION OF *DUNALIELLA SALINA* BY MOLECULAR SEQUENCING

DNA isolation was done by Doyle and Doyle method using CTAB buffer and the DNA pellets were obtained. DNA extracted is measured using a UV spectrophotometer. The purification and separation of DNA is done by running the sample in 1.0% Agarose gel. The intensities of band obtained by visualising in the gel documentation system by staining with Ethidium bromide and compared with DNA marker. The image obtained is shown in image 4

Image 4: An Ethidium bromide stained 2% agarose gel showing 16s rRNA PCR amplificationof DNA**.**

In Fig. 7 Lane (MBT049) represents100 bp marker and Lane (S) represents 16s rRNA PCR amplicon of DNA was obtained.

Amplification of the 16s region was done by polymerase chain reaction (PCR) with specific primers using reaction mixture of template DNA and primers using the PCR Master Mix and a thermal cycler. The amplified amplicon is then sequenced to identify the organism with accuracy. The sequence was exactly similar with *Dunaliella salina* 16s rRNA gene which is the most conserved sequence in *Dunaliella salina*.

>AF506698.1 Dunaliella salina 18S ribosomal RNA gene, partial sequence
ACCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCAT
GTCTAAGTATAAACTGCTTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTT

ATAGTTTATTTGATGGTACCTTTACTCGGATAACCGTAGTAATTCTAGAGCTAAT
ACGTGCGTAAATCCCGACTTCTGGAAGGGACGTATTTATTAGATAAAAGGCCAG
CCGGGCTTGCCCGACTCTTGGCGAATCATGATAACTTCACGAATCGCACGGCTTT
ATGCCGGCGATGTTTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGGATA
GAGGCCTACCATGGTGGTAACGGGTGACGGAGGATTAGGGTTCGATTCCGGAGA
GGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAATT
ACCCAATCCCAACACGGGGAGGTAGTGACAATAAATAACAATACCGGGCATTTT
TGCCTGGTAATTGGAATGAGTACAATCTAAATCCCTTAACGAGTATCCATTGGAG
GGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTTAA
GTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTCGGGTGGGTTGTAGCGGTCAGC
CTTTGGTTAGTACTGCTACGGCCTACCTTTCTGCCGGGGACGAGCTCCTGGGCTT
AACTGTCTGGGACTCGGAATCGGCGAGGTTACTTTGAGTAAATTAGAGTGTTCAA
AGCAAGCCTACGCTCTGAATACATTAGCATGGAATAACACGATAGGACTCTGGC
TTATCTTGTTGGTCTGTAAGACCGGAGTAATGATTAAGAGGGACAGTCGGGGGC
ATTCGTATTTCATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGACGAACTTCT
GCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTGGGGGCT
CGAAGACGATTAGATACCGTCGTAGTCTCAACCATAAACGATGCCGACTAGGGA
TTGGCAGGTGTTTCGTTGATGACCCTGCCAGCACCTTATGAGAAATCAAAGTTTT
TGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAA
GGGCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACT
TACCAGGTCCAGACACGGGGAGGATTGACAGATTGAGAGCTCTTTCTTGATTCTG
TGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGGTTGCCTTGTCAGGTTGATTCC
GGTAACGAACGAGACCTCAGCCTGCTAAATAGTCACGTCTACCTCGGTAGGCGC
CTGACTTCTTAGAGGGACTATTGGCGTTTAGCCAATGGAAGTGTGAGGCAATAAC
AGGTCTGCGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATGCATT
CAACGAGCCTATCCTTGGCCGAGAGGTCCGGGTAATCTTTGAAACTGCATCGTGA
TGGGGATAGATTATTGCAATTATTAGTCTTCAACGAGGAATGCCTAGTAAGCGCG
AGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTC
CTACCGATTGGGTGTGCTGGTGAAGTGTTTGGATTGGTATCATTGGGGGGAAACC
TCCGTTGGTACTGAGAAGAACATTAAACCCTCCCACCTAGAGGAAGGAGAAGTC
GTAACAAGGTTTCCGTAGGTGAACCTGCAGAAGGATCA

Fig. 4: DNA sequence of 16srRNA of *Dunaliella salina*

Sequence analysis was performed using online tool BLAST of NCBI database. The result is shown in Fig. 5

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Dunaliella salina 18S ribosomal RNA gene, partial sequence	Dunaliella sal…	3301	3301	100%	0.0	100.00%	1787	AF506698.1
Dunaliella salina isolate CCAP 19/25 small subunit ribosomal RNA gene, partial sequence	Dunaliella sal…	3267	3267	99%	0.0	99.78%	2160	MG022673.1
Dunaliella primolecta genome assembly, chromosome: 12	Dunaliella pri…	3256	5344	100%	0.0	99.55%	10042981	OU611762.1
Dunaliella tertiolecta strain CCAP 19/6B 18S ribosomal RNA gene, partial sequence; internal t…	Dunaliella tert…	3253	3253	99%	0.0	99.55%	2627	KJ756820.1
Dunaliella tertiolecta strain CCAP 19/27 small subunit ribosomal RNA gene, partial sequence;…	Dunaliella tert…	3253	3253	99%	0.0	99.55%	2442	MW471050.1
Dunaliella primolecta genome assembly, chromosome: 8	Dunaliella pri…	3251	4386	100%	0.0	99.50%	11327755	OU611758.1
Dunaliella sp. CCAP19/15 strain CCAP 19/15 small subunit ribosomal RNA gene, partial sequ…	Dunaliella sp.…	3243	3243	99%	0.0	99.55%	2585	MW471055.1
Dunaliella quartolecta strain CCAP 19/8 18S ribosomal RNA gene, partial sequence; internal t…	Dunaliella qu…	3221	3221	98%	0.0	99.55%	2401	KJ756824.1
Dunaliella polymorpha strain CCAP 19/7A 18S ribosomal RNA gene, partial sequence; interna…	Dunaliella pol…	3221	3221	98%	0.0	99.55%	2430	KJ756821.1
Dunaliella polymorpha strain CCAP 19/7B small subunit ribosomal RNA gene, partial sequenc…	Dunaliella pol…	3221	3221	98%	0.0	99.55%	2693	MW471057.1
Dunaliella tertiolecta strain CCAP 19/7C small subunit ribosomal RNA gene, partial sequence;…	Dunaliella tert…	3221	3221	98%	0.0	99.55%	2727	MW471056.1
Dunaliella bioculata strain CCAP 19/4 small subunit ribosomal RNA gene, partial sequence; in…	Dunaliella bio…	3216	3216	98%	0.0	99.49%	2638	MW471058.1
Dunaliella salina 18S ribosomal RNA gene, partial sequence	Dunaliella sal…	3201	3201	98%	0.0	99.38%	1780	EF195157.1
Dunaliella tertiolecta strain Dtsi 18S ribosomal RNA gene, partial sequence	Dunaliella tert…	3193	3193	97%	0.0	99.60%	1750	EF473729.1
Dunaliella salina isolate RKA2 18S ribosomal RNA gene, partial sequence	Dunaliella sal…	3171	3171	98%	0.0	99.09%	1778	KU212877.1
Dunaliella primolecta strain CCAP 11/34 18S ribosomal RNA gene, partial sequence; internal t…	Dunaliella pri…	3169	3169	97%	0.0	99.54%	2516	KJ756819.1
Dunaliella salina strain JR101 18S ribosomal RNA gene, partial sequence	Dunaliella sal…	3164	3164	97%	0.0	99.37%	1747	EU589199.1
Dunaliella salina strain JR102 18S ribosomal RNA gene, partial sequence	Dunaliella sal…	3162	3162	97%	0.0	99.37%	1745	EU589200.1
Dunaliella pseudosalina isolate MAH 18S ribosomal RNA gene, partial sequence; internal tran…	Dunaliella ps…	3155	3155	96%	0.0	99.54%	2428	KU641615.1
Dunaliella sp. SAS11133 18S ribosomal RNA gene, partial sequence	Dunaliella sp.…	3133	3133	95%	0.0	99.71%	1722	KF054056.1
Dunaliella sp. ABRIINW M1/1 18S ribosomal RNA gene, partial sequence	Dunaliella sp.…	3129	3129	95%	0.0	99.59%	2080	EU522092.1
Dunaliella salina strain CCAP 19/12 18S ribosomal RNA gene, partial sequence	Dunaliella sal…	3101	3101	95%	0.0	99.59%	1700	KJ756842.1

Fig. 5: Sequencing using BLAST

The graphic summary of the sequence shows the quality of sequence similarity. The red coloured line shows that alignment score is greater than 200 that can indicate the quality of the sequence similarity. The graphic summary is represented in Fig. 6

Fig. 6: Graphic summary of alignment score

The sequence is then analysed for its phylogenetic relationship by using MEGA X tool. MEGA X give the phylogenetic tree which shows the phylogenetic relationship of 16srRNA gene of *Dunaliella salina* and *Dunaliella primolecta* sequence. The phylogenetic tree is represented in Fig. 7

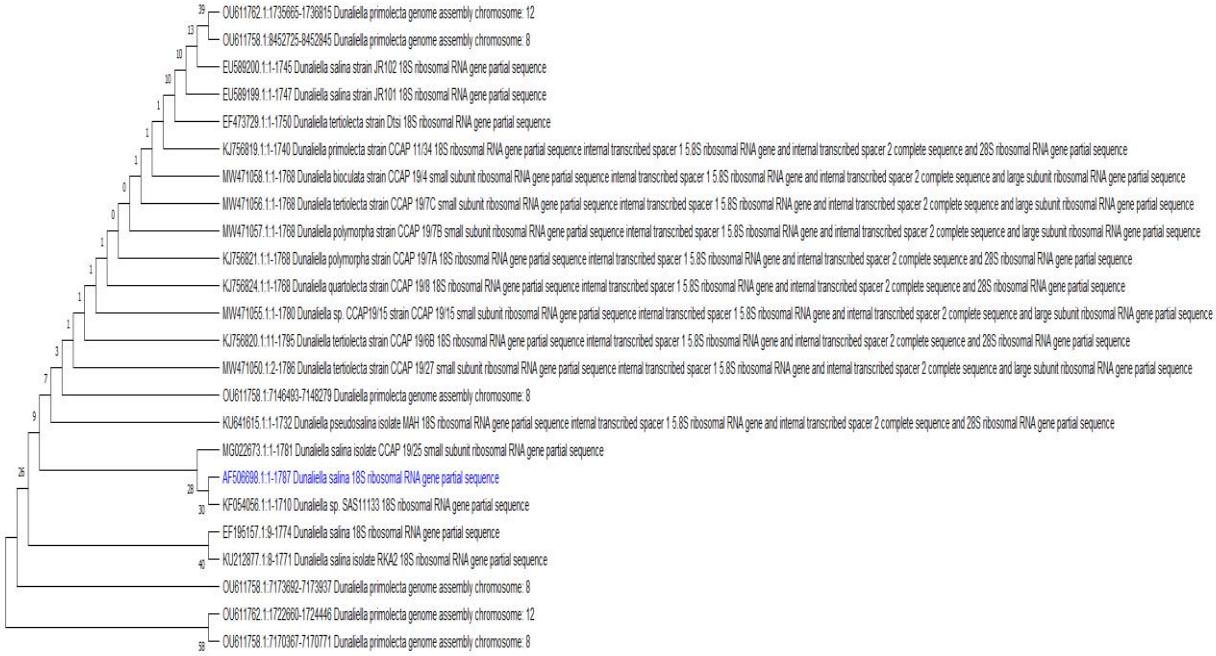


Fig.7: Phylogenetic tree showing relationship with *D. salina* with *D. primolecta*

EXTRACTION OF LIPID

Lipid content of the cultures in nitrogen deficient F/2 medium, phosphate deficient F/2 medium and F/2 medium were extracted based on a modified method adapted from Bligh and Dyer. Crude lipid content was determined by dividing the residue weight with the dry cell weight. The lipid content of cultures was observed. The nitrogen deprived F/2 medium showed maximum production rate of 69%, and the phosphate deficient F/2 medium showed 57% which is less production rate than nitrogen deprived medium and the F/2 medium showed less production rate of 18%. The crude lipid content was determined by using the equation. The image extract of lipid is given in image 5. Graph is constructed using the value which is given as Fig. 8

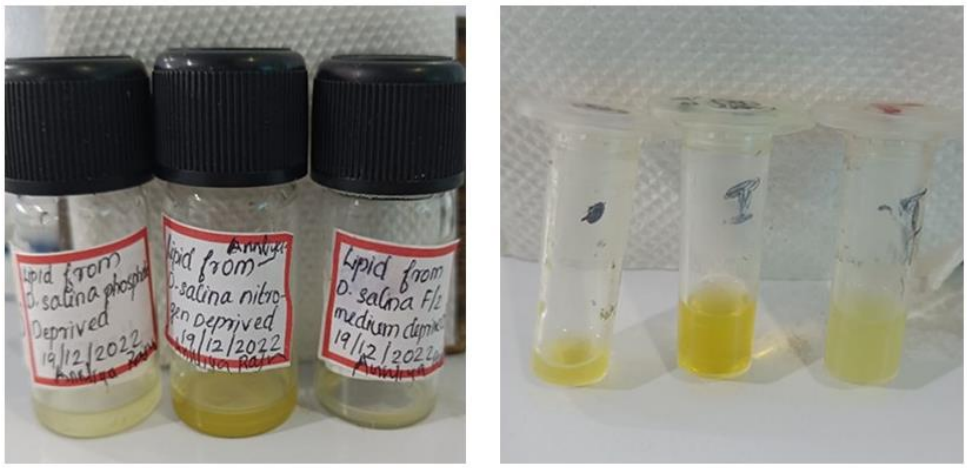


Image 5: a) and **b)** shows Lipid extracted from different mediums

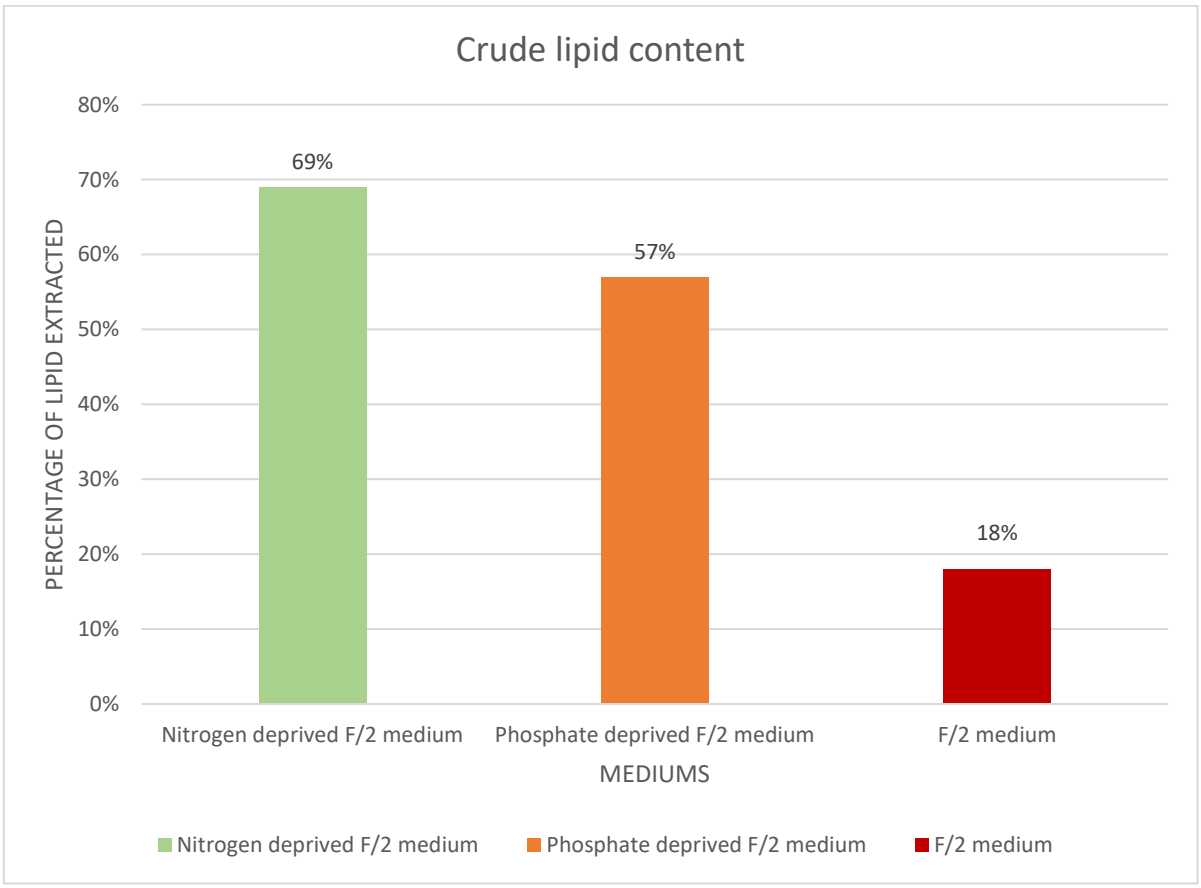


Fig. 8: Graph representing the crude lipid content

DISCUSSION

A review on Depletion of fossil fuels and anthropogenic climate changes done by Hook and Tang (1990) discussed the Significant increases in the global output of fossil fuels, the main source of CO2 emissions, are another indicator of anthropogenic climate change. Depletion of fossil fuels has also been noted as a potential problem in the future. Fossil fuels are one of the major CO2 emitters which is the greatest input of global warming. It is certainly about time to change this and stop seeing anthropogenic release of CO2 as something detached from future energy supply questions. Energy cannot be seen as a limitless input to economy. To overcome the crisis implication of biofuel gaining much importance, biodiesel is one among the important biofuels; the production of biodiesel from algae is still under research.

Microalgae are still being researched as a biodiesel substitute because they have a high lipid content that is made up of fatty acids that are advantageous for the manufacture of biodiesel. Shin, Choi, *et.al*, (2018) through their work explained the lipid production from microalgae. The quantity and quality of lipid produced by microalgae might vary depending on environmental factors and manufacturing settings, which can be important for the entire generation of biodiesel. Starting with resilient algal strains and ideal physio-chemical features of the culture environment in combination with a unique culture system is required to enhance both the lipid content and the biomass production. These cumulative cost-cutting strategies can move the algae process one step closer to becoming economically viable. In this experiment on culturing the algae *Dunaliella Salina* for checking the lipid production ,the culture was done on three different medium by inducing nutrient deficient condition in them, nitrogen deficient condition we got the maximum rate of lipid production and showed consequent reduction in phosphate deficient environment and F/2 medium, all the culture condition except the nutrient deficiency are kept same even though there was a difference in the rate of lipid this clear proof that the quality and quantities in culture condition will affect the lipid production

To make the large-scale production of biodiesel from microalgae economically viable, significant productivity improvements are needed. Despite receiving a lot of attention as a target for optimization, the maximum neutral lipid content of microalgae is just one of many variables that should be taken into consideration. These are the rates of biomass and lipid accumulation, and the highest algal cell densities that can be maintained during continuous cultivation are equally significant. Therefore Liu, Mukherjee*, et.al*., (2013) conducted the study using Fourier transform infrared spectroscopy (FTIR) to analyse the rates of growth and lipid

accumulation in *Dunaliella salina* under various temperature and cell density conditions. Compared to N-replete cells, cells that had been subjected to nitrogen starvation (N-depleted) exhibited very little growth. The final lipid content of the N-depleted cells was higher than that of the N-replete samples. Srinivasan, Mageswari, *et.al*., (2018) Studied the lipid production by *Dunaliella salina* by bicarbonate supplementation and reducing oxidative stress induced during macronutrient deficit conditions on the addition of sodium bicarbonate dramatically enhanced the biomass, carotenoids, including -carotene and lutein, lipid, and fatty acid content when nutrients were in short supply, while also enhancing the activities of the carbonic anhydrase and nutrient assimilatory enzymes. In contrast to phosphate and sulphate deficit, administration of bicarbonate during nitrate deficiency led to the greatest accumulation of carotenoid, notably -carotene, and lipids. In my study the three mediums were selected, nitrogen deprived medium, phosphate deprived medium and f/2 medium among which the nitrogen deprived medium give the best output and phosphate deprived medium was better than F/2 medium. F/2 medium show least amount of lipid production.

SUMMARY AND CONCLUSION

CONCLUSION

In this experiment, we aimed to enhance the lipid production of *Dunaliella salina* by inducing nutrient deprivation in the medium. *Dunaliella salina* is a microalgae. Dunaliella salina is one of the easily available marine algae which are rich in accumulation of carotenoids and have the ability to synthesize lipids. Use Lipids Produced from algae are currently gaining importance due to the scarcity of renewable fossil fuels and the rate of carbon emission and pollution caused by them are nowadays in high concerned biofuel are one of the best alternatives, biodiesel is one among them. Many studies are happening on usage of algal lipid for biodiesel.

Many methods and raw materials for lipid production are costly. To overcome the crisis a cost efficient method should be implemented to sort out the cost so in this experiment we used algae which are cosmopolitan in distribution and are widely available in large amounts. In this experiment we used microalgae *Dunaliella salina* is a marine motile unicellular chlorophyte, and is important due to the highest concentration of natural beta-carotene found in the green unicellular flagellate *Dunaliella salina*. Additionally, the halophilic *Dunaliella* species produce extremely high levels of glycerol, making it a commercial source of β-carotene and glycerol. Currently, 29 species of *Dunaliella* with variations and morphologies are recognized.

Micro algae *D. salina* is cultured in F/2 medium. The culture was maintained for 20 days by providing suitable growth conditions ph was maintained in 20 throughout the culture. Growth parameters like cell count per ml of sample, chlorophyll content of sample, maximum absorbance, etc were analysed periodically throughout the culture period the culture showed maximum growth parameters in 15 the day after 15^{th} day the growth parameters of culture later found to be declined. DNA isolation is done by Doyle and Doyle method using CTAB method and DNA pellets were obtained. Yield DNA was measured in spectrophotometer and the purity of DNA obtained was analysed using agarose gel and the band is visualised by the dye, ethidium bromide. The PCR amplification in the 16s region was amplified by polymerase chain reaction (PCR) with specific primers (forward and reverse primers). and the molecular sequencing and sequencing using BLAST, a computer tool maintained by NCBI. The graphic summary of the sequence was analysed which showed the quality of sequence similarity. The phylogenetic tree was constructed by a MEGA X tool. The red coloured line shows that alignment score is greater than 200 that can indicate the quality of the sequence similarity

The algae was cultured in three different mediums, nitrogen deprived F/2 medium and phosphate deprived F/2 medium and controlled medium. The nutrients are vital part of a living organism nitrogen and phosphorous play an important role in the life activities of algae. The deprivation causes a stress to plant cell which will increase the lipid production. Lipid was extracted from all three mediums by using Bligh and dyer method and the amount of lipid extracted from each medium is weighed and the Crude lipid content was determined by dividing the residue weight with the dry cell weight. The crude lipid content was determined by using the equation. The maximum production rate observed in nitrogen deprived F/2 medium and phosphate deprived F/2 medium show production rate less than that of nitrogen deprived medium and F/2 medium show less rate of production. The nitrogen deprived F/2 medium showed maximum production rate of 69%, and the phosphate deficient F/2 medium showed 57% and the F/2 medium showed less production rate of 0.18%.

From this experiment it is evident that inducing nutrient deprivation can be used as an effective tool for the lipid production. Among which the nitrogen deprived F/2 medium gives the best result. Hence this method can be used as a cost efficient and reliable method for lipid production in large scale.

REFERENCES

REFERENCES

1. Adenan, N. S., Yusoff, F. M., Medipally, S. R., & Shariff, M. (2016). Enhancement of lipid production in two marine microalgae under different levels of nitrogen and phosphorus deficiency. Journal of Environmental Biology, 37(4), 669.
2. Akter, R., Hossain, S. M. M., Zhe, W., Kermanee, P., & Juntawong, N. (2016). Enhanced lipid production in *Dunaliella salina* grown under high light intensity by shifting the culture from high to low nitrogen concentration. Advances in Environmental Biology, 10(7), 18-29.
3. Almutairi, A. W. (2020). Effects of nitrogen and phosphorus limitations on fatty acid methyl esters and fuel properties of *Dunaliella salina*. Environmental Science and Pollution Research, 27(26), 32296-32303.
4. BEHRENS, P. W., & KYLE, D. J. (1996). Microalgae as a source of fatty acids. Journal of Food Lipids, 3(4), 259-272.
5. Ben-Amotz, A., Tornabene, T. G., & Thomas, W. H. (1985). Chemical profile of selected species of microalgae with emphasis on lipids 1. Journal of Phycology, 21(1), 72-81.
6. Benemann, J.R. and Tillett, D.M.: 1987, 'Effects of Fluctuating Environments on the Selection of High Yielding Microalgae', Final Report to the Solar Energy Research Institute Roessler, P.G.: 1990, 'Environmental Control of Glycerolipid Metabolism in Microalgae: Commercial Implications and Future Research Directions', Journal of Phycology, 26: 393-399.
7. Bharte S, Desai K (2019) The enhanced lipid productivity of *Chlorella minutissima* and *Chlorella pyrenoidosa* by carbon coupling nitrogen manipulation for biodiesel production. Environ Sci Pollut Res 26:3492–3500
8. Bligh, E.G. and Dyer, W.J.: 1959, 'A Rapid Method of Total Lipid Extraction and Purifi cation'. Canadian Journal of Biochemistry and Physiology 37 (8) 911-917. [14]
9. Pak, J.H., S.-Y. Lee, Y.-N. Kim, and H.-Y. Lee.: 1993, 'The Production of Algal Hydrocarbons in Outdoor Cultivations of *Dunaliella salina* 1650', Journal of Microbiology and Biotechnology, 3(1): 46-50.
10. Bonnefond, H., Moelants, N., Talec, A., Mayzaud, P., Bernard, O., & Sciandra, A. (2017). Coupling and uncoupling of triglyceride and beta-carotene production by

Dunaliella salina under nitrogen limitation and starvation. Biotechnology for Biofuels, 10, 1-10.

11. Borowitzka, M. A. (1990, August). The mass culture of *Dunaliella salina*. In Technical Resource Papers. Regional Workshop on the Culture and Utilisation of Seaweeds (Vol. 2, pp. 63-80).
12. Chen, M., Tang, H., Ma, H., Holland, T. C., Ng, K. S., & Salley, S. O. (2011). Effect of nutrients on growth and lipid accumulation in the green algae *Dunaliella tertiolecta*. Bioresource technology, 102(2), 1649-1655.
13. Chi, N. T. L., Anto, S., Ahamed, T. S., Kumar, S. S., Shanmugam, S., Samuel, M. S., & Pugazhendhi, A. (2021). A review on biochar production techniques and biochar based catalyst for biofuel production from algae. Fuel, 287, 119411.
14. Cowan, A. K., Rose, P. D., & Horne, L. G. (1992). *Dunaliella salina*: a model system for studying the response of plant cells to stress. Journal of experimental botany, 43(12), 1535-1547.
15. Gao, Y., Yang, M., & Wang, C. (2013). Nutrient deprivation enhances lipid content in marine microalgae. Bioresource Technology, 147, 484-491.
16. Harrison, P. J., Thompson, P. A., & Calderwood, G. S. (1990). Effects of nutrient and light limitation on the biochemical composition of phytoplankton. Journal of Applied Phycology, 2(1), 45-56.
17. Hosseini, N. S., Shang, H., & Scott, J. A. (2018). Biosequestration of industrial off-gas CO2 for enhanced lipid productivity in open microalgae cultivation systems. Renewable and Sustainable Energy Reviews, 92, 458-469.
18. Huesemann, M.H.: 2006, 'Can Advances in Science and Technology Prevent Global Warming?', Mitigation and Adaption Strategies for Global Change 11, 539-577.
19. Huntley, M.E., Redalje, D.G.: 2006, 'CO2 Mitigation and Renewable Oil from Photosynthetic Microbes: A New Appraisal', Mitigation and Adaption Strategies for Global Change, Available Online First, May 22, 2006.
20. Jiminez, C., Cossio, B.R. and Niell, F.X.: 2003, 'Relationship Between Physiochemical variables and productivity in open ponds for the production of Spirulina: A predictive model of algal yield', Aquaculture 221 (1-4), 331-345.
21. Konur, O. (2021). Algal lipid production for biodiesel production: A review of the research. Biodiesel Fuels Based on Edible and Nonedible Feedstocks, Wastes, and Algae, 741-765.

22. Krishnan, S., Jeevanandam, J., Acquah, C., & Danquah, M. K. (2021). Extraction of algal neutral lipids for biofuel production. In Biodiesel Fuels Based on Edible and Nonedible Feedstocks, Wastes, and Algae (pp. 785-802). CRC Press.
23. Lamers, P. P., van de Laak, C. C., Kaasenbrood, P. S., Lorier, J., Janssen, M., De Vos, R. C.,& Wijffels, R. H. (2010). Carotenoid and fatty acid metabolism in light-stressed *Dunaliella salina*. Biotechnology and bioengineering, 106(4), 638-648.
24. Levering, J., Broddrick, J., & Zengler, K. (2015). Engineering of oleaginous organisms for lipid production. Current opinion in biotechnology, 36, 32-39.
25. Liu, J., Mukherjee, J., Hawkes, J. J., & Wilkinson, S. J. (2013). Optimization of lipid production for algal biodiesel in nitrogen stressed cells of *Dunaliella salina* using FTIR analysis. Journal of Chemical Technology & Biotechnology, 88(10), 1807-1814.
26. Metzger, P. and Largeau, C.: 2005, 'Botryococcus braunii: a rich source for hydrocarbons and related ether lipids', Applied Microbiology and Biotechnology 66: 486-496.
27. Mujtaba, G., Choi, W., Lee, C. G., & Lee, K. (2012). Lipid production by *Chlorella vulgaris* after a shift from nutrient-rich to nitrogen starvation conditions. Bioresource technology, 123, 279-283.
28. N.G. and Chisholm, S.W.: 1981, 'Phytoplankton Lipids: Interspecific Differences and Effects of Nitrate, Silicate and Light-Dark Cycles', Journal of Phycology, 17: 374-384.
29. Pak, J.H., S.-Y. Lee, W.C. Shin, and H.Y. Lee.: 1991, 'Optimization of Producing Liquid Fuel from Photosynthetic Algal Growth', Journal of Microbiology and Biotechnology, 111-115.
30. Pak, J.H., S.-Y. Lee, W.C. Shin, and H.Y. Lee.: 1991, Optimization of Producing Liquid Fuel from Photosynthetic Algal Growth', Journal of Microbiology and Biotechnology, 111-115.
31. Park, D.-H., H.-W. Ruy, K.-Y. Lee, C.-H. Kang, T.-H. Kim, and H.-Y. Lee.: 1998 'The Production of Hydrocarbons from Photoautotrophic Growth of *Dunaliella salina* 1650', Applied Biochemistry and Biotechnology, 70-72: 739-746.
32. Riyazat Khadim, S., Mohanta, A., Singh, P., Maurya, P., Kumar Singh, A., Kumar Singh, A., & Asthana, R. K. (2022). A Study on *Dunaliella salina* Under Selected Nutrient Manipulation with Reference to the Biomass, Lipid Content Along with Expression of ACCase and RuBisCO Genes. BioEnergy Research, 1-16.

33. San Pedro, A., González-López, C. V., Acién, F. G., & Molina-Grima, E. (2013). Marine microalgae selection and culture conditions optimization for biodiesel production. *Bioresource technology*, *134*, 353-361.
34. Schnurr, P. J., & Allen, D. G. (2015). Factors affecting algae biofilm growth and lipid production: A review. Renewable and Sustainable Energy Reviews, 52, 418-429.
35. Sheehan, J., Dunahay, T., Benemann, J. and Roessler, P.: 1998, 'A Look Back at the U.S. Department of Energy's Aquatic Species Program-Biodiesel from Algae, Golden, CO, National Renewable Energy Institute, NREL/TP-580- 24190, 328 pp.
36. Shin, Y. S., Choi, H. I., Choi, J. W., Lee, J. S., Sung, Y. J., & Sim, S. J. (2018). Multilateral approach on enhancing economic viability of lipid production from microalgae: a review. Bioresource technology, 258, 335-344.
37. Spoehr, H.A. and Milner, H.S.: 1949, 'The Chemical Composition of *Chlorella:* Effect of environmental conditions', Plant Physiology 24: 120-149.
38. Srinivasan, R., Mageswari, A., Subramanian, P., Suganthi, C., Chaitanyakumar, A., Aswini, V., & Gothandam, K. M. (2018). Bicarbonate supplementation enhances growth and biochemical composition of *Dunaliella salina* V-101 by reducing oxidative stress induced during macronutrient deficit conditions. Scientific reports, 8(1), 1-14.
39. Tornabene, T.G., Holzer, G. and Peterson, S.L.: 1980, 'Lipid Profi le of the Halophilic Alga, *Dunaliella salina'*, Biochemical and Biophysical Research Communications 96(3): 1349-1356.
40. Weldy, C. S., & Huesemann, M. I. C. H. A. E. L. (2007). Lipid production by *Dunaliella salina* in batch culture: effects of nitrogen limitation and light intensity. Journal of Undergraduate Research, 7.
41. Yeh, K. L., & Chang, J. S. (2011). Nitrogen starvation strategies and photobioreactor design for enhancing lipid content and lipid production of a newly isolated microalga *Chlorella vulgaris* ESP-31: implications for biofuels. *Biotechnology Journal*, *6*(11), 1358-1366.
42. Yilancioglu, K., Cokol, M., Pastirmaci, I., Erman, B., & Cetiner, S. (2014). Oxidative stress is a mediator for increased lipid accumulation in a newly isolated *Dunaliella salina* strain. PloS one, 9(3), e91957.
43. Yuan, Y., Li, X., & Zhao, Q. (2019). Enhancing growth and lipid productivity in *Dunaliella salina* under high light intensity and nitrogen limited conditions. Bioresource Technology Reports, 7, 100211.

APPENDIX

APPENDIX 1

CULTURE MEDIUM

$NaNO_3$: 0.2ml
NaH_2PO_4 : 0.2ml
Trace metal solution : 0.2ml
Vitamin solution :0.1ml

APPENDIX 2

CTAB BUFFER

1M Tris HCl : 1.576 g
4M NaCl : 2.3376 g
05 M EDTA : 1.8612 g
2% CTAB : 0.2 g

APPENDIX 3

PREPARATION OF MASTER MIX

Molecular biology grade water : 15µl
10X assay buffer : 2.5µl
Template DNA : 1µl
Forward primer : 0.5µl
Reverse primer : 0.5µl
$MgCl_2$: 2.5µl
DNTPS : 2.5µl
Taq DNA polymerase : 0.5µl