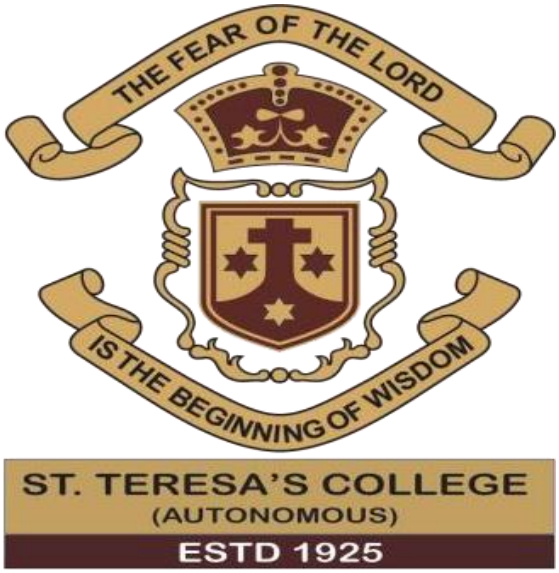
STUDIES ON THE SYNERGISTIC EFFECT OF LIPID FROM *CHLORELLA VULGARIS* AND *CITRUS LIMON* ESSENTIAL OIL FOR COSMECEUTICAL PRODUCT DEVELOPMENT

Dissertation submitted in partial fulfillments for the award of degree of
Master of science in Botany

By
ZAMEEA FARHA K K
REG NO:AM21BOT016



DEPARTMENT OF BOTANY

ST.TERESA'S COLLEGE (AUTONOMOUS)

ERNAKULAM

2022

CERTIFICATE

This is to certify that the dissertation entitled "**STUDIES ON THE SYNERGISTIC EFFECT OF LIPID FROM *CHLORELLA VULGARIS* AND *CITRUS LIMON* ESSENTIAL OIL FOR COSMECEUTICAL PRODUCT DEVELOPMENT**" is an authentic record of work curried out by **Ms.ZAMEEA FARHA K K** under my supervision and guidance on the partial fulfillment of the requirement of the M.Sc. Degree of Mahatma Gandhi University, Kottayam. I, further certify that no part of the work embodied in this dissertation work has been submitted for award of any other degree or diploma.

Dr. Elsam Joseph
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

Place: Ernakulam
Date :

External Examiners

1.
2.

DECLARATION

I hereby declare that the work which is being presented in the dissertation, entitled **"Studies on the synergistic effect of lipid from *Chlorella vulgaris* and *Citrus limon* essential oil for cosmeceutical development"**, 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. Elsam Joseph.

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:

Date : Signature of the Candidate

ACKNOWLEDGEMENT

I wish to express my sincere thanks and deep sense of gratitude to all the 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 pleasure and privilege to extend my profound sense of gratitude to Dr.Elsam Joseph, Associate 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 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 to me for successful completion of my work. I also express my deepest gratitude and appreciation for the guidance and encourage given by all the teachers.

I gratefully acknowledge to Dr. Alphonsa Vijaya Joseph, Principal, St.Tetesa's College, Ernakulam, for following me to do project in this institution.

Last but not least, I would like to thank the almighty God whose blessing have enabled me to accomplish my dissertation work successfully.

Sincere thanks also go to the non- teaching staff and my friends who have 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 to complete this work.

ZAMEEA FARHA K K

TABLE OF CONTENT

LIST OF TABLE

LIST OF PLATE

LIST OF FIGURE

ABBREVIATION

BG11- Blue-Green 11

BLAST- Basic local alignment search tool

CTAB- Cetyltrimethylammonium bromide

DNA- Deoxy ribonucleic acid

RNA- Ribonucleic acid

rRNA- ribosomal ribonucleic acid

DMSO- Dimethyl sulfoxide

EDTA- Ethylenediamine tetraacetic acid

ERK- Extracellular signal-regulated kinase

EO- Essential oil

g- Gram

mg- Milligram

Kg- Kilogram

mL- Milliliter

µl- Microliter

µmol- Micromole

kb- Kilobase

MSD- Microwave steam distillation

SD- Steam distillation

ORAC- Oxygen radical absorbance capacity

OD- Optical density

nm- Nanometer

PCR- Polymerase chain reaction

NCBI - National Center for Biotechnology Information.

MEGA X- Molecular Evolutionary Genetics Analysis

UV- Ultraviolet

UVR- Ultraviolet radiation

ABSTRACT

Microalgae are an intriguing source of bioactive compounds with potential industrial applications. Lipids from *Chlorella vulgaris* are a diverse group of beneficial compounds that are used in numerous industries. *Citrus* essential oils (EOs) provide a number of crucial qualities that may make them the best alternative to chemical-based antimicrobials in food, agriculture, medicine, and sanitary applications as well as an expensive ingredient in cosmetics and perfumes. *Chlorella vulgaris*, a microalgae, was extracted from fresh water and grown in BG11 media for this study. Genomic DNA was extracted from the algae using a CTAB buffer after they had been cultured. The ratio of 260 nm absorbance to 280 nm absorbance was used to calculate the purity of DNA. Running the material through a 1.0% Agarose gel allowed for the measurement of DNA concentration and purity. Polymerase chain reaction (PCR) was utilized to amplify the 16s region using certain primers. The lipid content of the *C. vulgaris* was extracted using the Bligh and Dyer method. With the process of steam distillation, *Citrus limon* peel essential oil was obtained. A separating funnel was then used to extract the solvent. The synergistic effect of the lipid extract and essential oil was investigated. The product underwent an organoleptic analysis, a stability test, and an irritancy test in order to evaluate its synergistic impact. The findings indicate that the products have greater antioxidant capabilities than artificial cosmetics.

CHAPTER 1
INTRODUCTION

INTRODUCTION

To protect consumers, cosmetics production is strictly regulated. In the past, natural substances have been employed for these purposes, but as time went on, the cosmetics industry gradually began to use more and more synthetic chemicals. One of the most intricate and substantial organs, the skin acts as a barrier against water loss and environmental stresses such ultraviolet radiation (UVR), infections, physical agents, and chemicals. A variety of acute and chronic illnesses are brought on by the persistent exposure of human skin to ultraviolet (UV) radiation. Additionally, this stressor can increase lipid peroxidation, harming cell membranes, activate or deactivate enzymes, and again, cause DNA damage that can cause cancer and other disorders when present in excess.

Metals included in cosmetic formulas have the potential to be absorbed via the skin, build up inside the body, and injure internal organs. The use of coal tar hair dyes in hair colorants can result in allergic responses, hair loss, and eye injury. The products, which are meant to improve our health and appearance, have a deep dark side. The amount of dangerous chemicals and harmful compounds utilized in cosmetics is excessive. These substances have the potential to have major negative effects on skin and to enter the body and cause cancer in the skin and other organs.

Topical cosmetic-pharmaceutical hybrids known as "cosmeceuticals" are cosmetic products with active chemicals that are claimed to offer therapeutic or drug-like advantages for skin health. Because of the trend of a modern lifestyle, the cosmeceutical sector expands annually on a global scale. Due to the inefficiency of synthetic cosmetics more recently, the cosmeceutical sector is gradually switching to natural bioactive components. People use cosmetic products often all throughout the world to shield their skin from external stimuli. As a result of the negative side effects created by synthetic cosmetics and the low absorption rate caused by the bigger molecular size of the chemicals, natural cosmetic products are now more popular with consumers.

In some cases, safety data are lacking for synthetic ingredients and they might cause hypersensitivity reactions, anaphylactic reactions, lethal poisonings or long term effects to users. Testing for genetic toxicity, phototoxicity, photo genotoxicity, toxicokinetics, and carcinogenicity of these drugs can be done in a systematic manner, and fresh studies may disclose different toxicity results. A constant exposure to various chemicals is also caused by the everyday usage of numerous cosmetic items. Because the same substances are present in many goods, there may be a synergistic interaction of various compounds and additive activity.These factors make the need to replace chemicals more and more urgent, driving the cosmetics industry to constantly search for new technologies. Finding natural ingredients to satisfy client demands, which are increasingly driven by awareness of the value of using high-quality products and environmental sustainability, is one of the current issues.

Macroalgae-based cosmetics are currently available on the market and are gradually replacing their synthetic equivalents. These substances may include pure macroalgal extracts or extracts with various beneficial components. Despite being mostly utilized as thickening and gelling agents, the active ingredients that cause macroalgae to be used in cosmetics are very varied. Even if some substances made from microalgae are already available on the market, the world of microalgae has yet to be fully discovered. Microalgae have a unique chemical makeup and contain a variety of compounds with great biological value. They are an unusual source of proteins and amino acids and can accumulate a high percentage of lipids. They often contain a lot of carbs in addition to other beneficial ingredients including vitamins, antioxidants, and minerals.

Recent advancements in the study of microalgae as a source of several value-added goods of interest, such as carotenoids, proteins, and vitamins, have been made in the quest for cosmetic and therapeutic products of natural origin. Carotenoids can soften the skin's surface, especially scars, and can greatly lessen the appearance of stretch marks. They are especially helpful in repairing any physically damaged skin tissue.

Microalgae represent an enormous biodiversity from which about 40.000 are already described or analyzed (Sommerfeld *et al* ., 2008). One of the most remarkable is the green eukaryotic microalga

Chlorella vulgaris, which belongs to the following scientific classification: Domain: Eukaryota, Kingdom: Protista, Division: Chlorophyta, Class: Tre-bouxiophyceae, Order: Chlorellales, Family: Chlorellaceae, Genus: Chlorella, Specie: *Chlorella vulgaris*. Hence, Martinus Willem Bei- jerinck, a Dutch researcher, first discovered it in 1890 as the first microalga with a well-defined nucleus (Beijerinck *et al.*,1890). The name Chlorella comes from the Greek word chloras (XÀ.rup6ç;), which means green, and the Latin suffix el/a referring to its microscopie size.

CLASSIFICATION

TABLE 1: CLASSIFICATION OF *C.VULGARIS*

Kingdom	Plantae
Division	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae

Chlorella vulgaris is a freshwater green algae. It is an unicellular spherical or ellipsoidal algae, which lack flagella. It is a good source of protein, fats, carbohydrates, fiber, vitamins and minerals. *Chlorella vulgaris* has an intriguing nutritional profile that includes lipids, carbs, fiber, vitamins, and proteins. Chlorella is particularly intriguing because it often contains up to 60% protein. By releasing bioactive peptides with a variety of potential bioactivities, hydrolyzing microalgae proteins can improve a number of other intriguing properties. With intriguing capabilities like

antioxidant, antihypertensive, anti-inflammatory, anti-cancer, and antibacterial, several bioactive peptides have been made from *Chlorella vulgaris*. Due to their useful qualities, such as solubility, emulsifying and foaming properties, as well as the potential for industrial use, microalgal peptides may also be of great interest.

Citrus limon (L.) Burm. f. is a tree with evergreen leaves and yellow edible fruits from the family Rutaceae. The fruit, in particular the juice and essential oil extracted from it, serves as the primary raw material for *Citrus limon*. Although the *Citrus limon* fruit stands out for having well-known nutritional qualities, it is important to note that modern phytotherapy and cosmetics undervalue its excellent biological activity.

Essential oils (EOs, also known as volatile or ethereal oils) are secondary metabolites that are produced by a variety of plant materials, including flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots. They are fragrant oily liquids with a distinct scent. EOs are typically soluble in organic solvents with a lower density than water and are liquid, volatile, limpid, and rarely colored. They are extremely intricate natural combinations of lipophilic compounds that can have anywhere between 20 and 60 different components at varying concentrations. In comparison to other components present in trace levels, they are characterized by two or three primary components (limonene, p-cymene, and ocimene) at relatively high concentrations (20–70%). The biological characteristics of the EOs are often determined by those key elements. Currently, there are over 3000 EOs known, of which 300 are commercially significant and employed in the pharmaceutical, agronomic, food, sanitary, cosmetic, and perfume industries.

Numerous scientific research have been conducted to support the usage of *Citrus limon* fruit extracts, essential oil, and the active components that have been extracted from these raw materials. Long believed to be beneficial for acne-prone skin that is susceptible to sunburn or mycosis, lemon-derived treatments are now widely available. Traditional usage of these basic materials exist all throughout the world in this regard.

Citrus limon essential oil's components have a depigmenting effect. Additionally, it has been demonstrated that the essential oil aids in the entry of lipids and water-soluble vitamins. It can be

used to encourage the skin's ability to absorb active chemicals. In addition, the essential oil can be utilized in cosmetic goods as a corrigent and a natural preservative in addition to its direct effect on the skin. Its effects are fungistatic and antibacterial, according to studies.

Using essential oils as natural preservatives in cosmetics is a novel and promising area of application. Industries have long been particularly interested in the microbiological safety of cosmetics since microbial decomposition can result in product degradation and pose a health concern to customers. Modern cosmetics have a rich composition that, when combined with their fluid formulation and direct contact with bacterial skin flora, creates an excellent habitat for the growth of microorganisms .The use of preservatives is essential due to the significant danger of contamination and subsequent risk to consumer health. In addition, bacterial contamination alters the physical and chemical composition of cosmetics, frequently leading to phase separation, discoloration, and odor release. Systems for preservation stop and restrict the development of microorganisms that could contaminate products during production, storage, or consumer use. Cosmetics are produced using sterile processes and appropriate packaging to be completely preservative-free and microbially stable. However, only specific formulas may produce excellent results, and they are subject to certain limitations, such as an earlier expiration date, a limited range of container types, and an unique protective atmosphere during production and packagingThe citrus limon fruit's chemical makeup is well recognized. It has been established not just for the entire fruit but also individually for the pericarp, juice, pomace, and essential oil. Additionally known are the chemical makes-up of the leaves and the fatty oil derived from *Citrus limon* seeds. Due to the huge variety of *Citrus limon* cultivars, variations, and hybrids, numerous research institutions take on the responsibility of examining the chemical makeup of the raw materials derived from them.

The understanding of substances obtained from microalgae in the field of cosmetology is currently limited and requires additional research. *Chlorella vulgaris*, however, has the potential to develop properties such as preventing skin blemishes, repairing skin damage, treating seborrhea, conditioning hair, repairing collagen, inhibiting inflammation, providing hydration, and providing

protection against damage from solar radiation, among other features (Mourelle; Gómez; Legido, 2017; Bhalamurugan; Valerie; Mark, 2018).

Currently, our understanding of *Citrus limon*'s cosmetic properties is constantly growing. *Citrus limon* fruit extracts are recommended for use in anti-aging cosmetics since research has demonstrated a high antioxidant impact. In addition, specialized dermocosmetics contain vitamin C from *Citrus limon* as a component. Utilizing it externally causes the skin to produce more collagen, smoothing and tightening the skin. When combined with vitamin E, it works as a synergistic antioxidant to decrease shallow wrinkles and slow the aging process.

Chlorella vulgaris, a species of green microalgae, is a eukaryotic microorganism with high photosynthetic ability. C. *vulgaris* is found in different environments, predominantly in fresh and saltwater, where there is exposure to high levels of UV radiation and extreme survival conditions. In response to these challenges, C. *vulgaris* produces several secondary metabolites that may have antioxidant properties (Cha *et al*., 2010; Priyadarshani; Rath, 2012; Wang *et al.,* 2015; Hynstova *et al*., 2017; Jiang; Kalodimos, 2017; Khan; Shin; Kim, 2018).

OBJECTIVE

- Standardize the culture parameter of *Chlorella vulgaris.*
- To isolate DNA , PCR amplification and the identification of the organism.
- Extraction of lipid from *Chlorella vulgaris* and essential oil from *Citrus limon.*
- To study the synergistic effect of lipid from *Chlorella vulgaris* and *Citrus limon* essential oil.

CHAPTER 2
REVIEW OF LITERATUR

REVIEW OF LITERATURE

The hazardous ingredients that are frequently contained in cosmetic items' compositions are blamed for persistent unpleasant effects and potential health hazards. Although the different systems in place across the world to regulate and oversee the quality of cosmetics are fairly intricate and thorough, they should be more stringent when adding new compounds with the potential to be harmful to the composition of cosmetics in order to prevent harm to human health. It is essential to implement a global cosmeto-vigilance in order to promote improvements in the production, marketing, and consumer usage of cosmetic products. This public health initiative is an effective way to learn about the safety of cosmetic products and their ingredients, preventing the hazards connected with cosmetic use from becoming a significant public health issue (Khan *et al*, 2019)

Chlorella vulgaris is a resilient species of chlorophyta, a group of unicellular green algae. Protein normally makes up more than half of its biomass (51%-58%), with the remainder being made up of carbohydrates, lipids, and other useful nutraceuticals like vitamins, antioxidants, and trace minerals (Becker, 1994). Numerous primary metabolites, including unsaturated fatty acids, polysaccharides, vitamins, and vital amino acids, are produced by algae (Thomas; Kim, 2013). Numerous studies have also revealed the anti-inflammatory, antioxidant, anticancer, antibacterial, and anti-aging properties of secondary metabolites generated from algae, including fucoidan, fucoxanthin, sulphated polysaccharide, polyphenol, and fucosterol (Peng *et al.*, 2011).

Chlorella vulgaris is a microalgae with a spherical shape that ranges in size from 2 to 10 micrometers; it lacks a flagellum because it is an immobile cell; its cytoplasm is made up of water, proteins, and minerals; and it has just one chloroplast with photosynthetic pigments like chlorophyll a and b in its interior. It is greenish in color, and it reproduces by binary partition through asexual (Raven; Evert; Eichhorn, 2007; Araújo, 2015b; Marino, 2018). Its wall is rigid due to the presence of glucosamine in its basic composition, however, its thickness and composition vary according to the growth phase (Safi et al., 2014; Marino, 2018).

The majority of DNA extraction methods for microalgae are based on the Doyle and Doyle (1990) hexadecyltrimethylammonium bromide (CTAB) approach, which necessitates crushing a sizable number of cells in liquid nitrogen. The fundamental CTAB approach has been published in several variations, however they are typically fairly time-consuming. On the other hand, some studies have totally eliminated the isolation phase and used whole cells in the PCR (Hoham et al. 2002), a technique that can only be used with particular organisms. Due to the mechanical strength of their cell walls and their extremely small size, some coccoid microalgae, like Chlorella vulgaris, can provide unique challenges for DNA isolation. Friedl created a method that can be utilized for some coccoid algae (Fridl, 1995).

DNA fragments ranging in size from 100 bp to 25 kb can be separated most successfully using agarose gel electrophoresis (Sambrook; Russell, 2001).

Agarose polymers form a network of bundles and non-covalently associate during the gelation process. The size of these bundles' pores affects the gel's ability to sieve molecules. DNA separation was completely changed by the use of agarose gel electrophoresis. Sucrose density gradient centrifugation was the primary method used to separate DNA before agarose gels were introduced; nevertheless, this method only offered an approximate size separation of the DNA molecules. When employing agarose gel electrophoresis to separate DNA, the DNA is placed into the gel's precast wells before a current is applied. DNA fragments will migrate to the positively charged anode in an electric field because the phosphate backbone of DNA (and RNA) molecules is negatively charged (Yun Lee *et.al.*, 2012).

Because of their powerful photosynthetic capacities and quick development rates, *chlorella* species are regarded as attractive biological resources. Due to its high protein content, *Chlorella vulgaris* is particularly grown for use in the food industry. It is also used to make specialty oils for the cosmetic and nutraceutical industries (Ahmad *et.al.*, 2020). Pharmaceutical and cosmetic sector has been forced to reinvent itself due to multiple factors based on economy changes. The latter gave a chance to cosmeceuticals. Products considered cosmeceuticals contain biologically active ingredients for aesthetic purposes. Simultaneously, these antioxidant metabolites are characterized

for a high biochemical capacity on the skin. This property causes the cosmeceutical to act in a more active and effective way on skin tissue. Convergence of the cosmetics and aesthetic medicine sector has been the reason for the rise of these products in recent years, to create a new sector focused on image. That is why, both beauty centers and aesthetic clinics are more and more expanded in global markets. The use of algae in cosmetic products has been in great demand due to its high content of trace elements, mineral salts, vitamins and amino acids that serve to maintain the skin good appearance, since they are directly assimilable by skin cells (Caicedo; Suarez; Gelves, 2020).

The use of microalgal lipids in the food, chemical, pharmaceutical, and cosmetic sectors has recently attracted a lot of interest. Different lipid classes serve as excellent moisturizers, emollients, and softeners. They also serve as surfactants and emulsifiers, give products consistency, transport color and fragrance, act as preservatives to preserve product integrity, and can be a component of a molecule's delivery system. Lipids and a wide variety of other bioactive compounds can be found in abundance in microalgae (De Luca *et al*., 2021).

One of the most intriguing species of microalgae is *chlorella*, which supports the production of collagen in the skin, tissue regeneration through joint healing for sports injuries, and cardiovascular protection, among other things (Ariede *et al.*, 2017). Additionally, it is well recognized that it helps lessen arterial collagen deterioration and shield against conditions like atherosclerosis. However, acquiring the microalga comes at a hefty expense. Chlorella vulgaris has primarily been treated using affordable and lucrative substrates.*Chlorella vulgaris* has primarily been treated using affordable and lucrative substrates (Chia *et al*., 2013; Albuquerque *et al*., 2014; Tiong *et al*., 2020). Depending on the conditions in which they were grown, microalgae might have different compositions. To put it simply, the microalgae biomass primarily consists of proteins, carbohydrates, and trace amounts of vitamins and antioxidants (Panahi *et al*., 2019). Proteins play a number of physiological roles in the cell, including development, repair, and maintenance. They also serve as chemical messengers, cellular activity controllers, and even a line of defense against intruders (Safi *et al*., 2014). Alanine and glutamic acid are the amino acids that are more prevalent

in *Chlorella vulgaris* species (Ursu *et al*., 2014). Glycolipids, waxes, hydrocarbons, phospholipids, and fatty acids are the main types of lipids, which are a class of molecules that are insoluble in water. Depending on the amount of nitrate and light intensity available during their cultivation, these chemicals can be adjusted (Liao *et al*., 2017). They are created by chloroplasts, and some organelles, such mitochondria, and cell walls also contain them (Safi *et al.,* 2014; Marino, 2018). Reduced sugars and polysaccharides like cellulose and starch are together referred to as carbohydrates. The most common polysaccharide in the chloroplast of the microalgae *Chlorella vulgaris* serves as a reservoir of energy for the cell in addition to carbohydrates. The structural polysaccharide cellulose, which serves as a barrier of defense, is found in the cell wall. Depending on the growing conditions, *Chlorella vulgaris* species overall composition might contain anywhere between 12 and 55% carbohydrates.(Lordan; Ross; Stanton, 2011; Safi *et a*l., 2014). Chlorophylls and carotenoids are the pigments that are most prevalent in *Chlorella vulgaris*. About 1-2% of the dry biomass of microalgae contains chloroplast thylakoids, where chlorophylls are found. Carotenoids, like -carotene, carry out important cellular tasks like capturing light and transferring it to the photosystem, which is a complex protein involved in cellular photosynthesis, and even shielding chlorophyll molecules from degradation when exposed to strong solar radiation (Solomon; Berg; Martin, 2011; Safi *et al*., 2014; Fernández Linares *et al*., 2017; Cezare Gomes *et al*., 2019).

The heterogeneous category of substances known as lipids is defined by their solubility in non-polar solvents and relative insolubility in water rather than by their structure (Bajguz,2000). During optimal growth conditions *Chlorella vulgaris* can contain 5 to 40% lipids by dry weight of biomass (Becker, 1994), with the majority of these being made up of glycolipids, waxes, hydrocarbons, phospholipids, and trace amounts of free fatty acids.(Lee,2008; Sommerfeld *et al*., 2008). The chloroplast synthesizes these elements, which are also found on the cell wall and on the membranes of organelles (chloroplast and mitochondria membranes). However, under unfavorable growth conditions, the amount of lipids (mostly triacylglycerols) might reach 58% (Mata *et al*., 2010).

Triacylglycerols, in contrast to other lipids, do not play a structural role and instead build up as dense storage lipid droplets in the cytoplasm and inner thylakoid region of chloroplasts (Hu *et al*.,2008).

After each growth phase, Liu et al. improved a technique for detecting the accumulation of lipid droplets inside *Chlorella vulgaris* cells. The technique involves staining the cells with Nile red dye and using a fluorescence microscope to view the buildup of lipids via emitted blue light that displays the lipid droplets, particularly neutral lipids. Using this technique, it was demonstrated that the amount of neutral lipids that accumulated and the fluorescence intensity were related (Hu *et al.*, 2008). However, Chen et al. (Chen *et al*., 2011) claim that without cell disruption, this approach might not work since some microalgae have thick cell walls that might prevent the reagent from entering the cell completely. As a result, cell disruption is required to avoid inaccurate measurements and quantification.

Chlorella vulgaris total lipids are often extracted using the Bligh and Dyer method (a combination of chloroform and methanol), hexane, or petroleum ether (Zheng *et al*., 2011; Biedlingmaier *et al*.,1987). Following the evaporation of the extracting solvent, the remaining lipid extract is weighed, and column chromatography is used to separate the various lipid constituents. Finally, the residual lipid extract is subjected to gravimetric quantification of total lipids (Olmstead *et al*., 2013). In fact, these solvents are not employed on an industrial scale since they pollute the extract and are dangerous to the environment and humans (Mercer;Armenta,2011). Phospholipids (PL), glycolipids (GL), and neutral lipids (NL) make up the three main fractions of total lipids. Sequential elution of chloroform and acetic acid for NL, acetone and methanol for GL, and methanol for PL recovery is used to separate these fractions (Olmstead *et al*., 2013). Since it produces clean extracts free of contamination, supercritical carbon dioxide (SC-C02) extraction has been identified as a substitute for a greener extraction.

Additionally, a co-solvent to SC-C02 such ethanol can be utilized to boost the yield of extraction, or a preparatory cell disruption approach can be applied (Dejoye *et al*., 2011). It is important to note that the use of ethanol boosts the extraction yield of all lipophilic compounds (lipids and

pigments), but it may also avoid the labor-intensive but effective cell disruption method, which would result in a large decrease in manufacturing costs (Safi *et al., 2009).*According to growth conditions, the fatty acid profile changes and is suited for a variety of uses. For instance, according to Yeh and Chang (Yeh; Chang, 2012), Under conditions of mixotrophic development, *Chlorella vulgaris* can collect 60–68% of saturated and monounsaturated fatty acids made up of palmitic acid C16:0, stearic acid C18:0, palmitoleic acid C16:1, and oleic acid C18:1 (Zheng *et al*., 2011). Such a profile is more suitable for biodiesel production (Yeh;Chang, 2011). On the other hand, even under favorable growth conditions, its fatty acid profile makes it unsuitable for biodiesel production (Stephenson *et al*.,2009) but more suited for dietary purposes because it contains more polyunsaturated fatty acids like linoleic acid C18:2, linolenic acid C18:3, and eicosapentaenoic acid (Chen *et al*.,2011).

One of the most significant taxonomic components of the Rutaceae family is the genus Citrus. Most people are familiar with the beneficial nutritional, medicinal, and cosmetic benefits of citrus fruits (Mabberley, 2004).Due to its antibacterial and flavoring qualities, *Citrus limon* essential oil is utilized in the synthesis of shampoos, toothpastes, disinfectants, topical ointments, and other cosmetic products (González-Molina *et al.,* 2010). Citrus essential oils (EOs) have a number of significant qualities that make them potentially a perfect replacement for chemical-based antimicrobials in food, agriculture, pharmaceutical, and sanitary applications as well as an expensive ingredient in cosmetics and perfumes. These qualities include their bactericidal, virucidal, fungicidal, and medicinal properties associated with their fragrance (Palazzolo *et al*., 2013).

Since natural base products have no adverse effects when used properly, people are turning to them more and more these days. Additionally, the manufacture of natural products that are high-quality, functional, and free of chemical modifications, solvent residues, or additions is gaining appeal (Nurul Azlina,2015). Due to these factors, essential oils are utilized as base ingredients in a variety of goods, such as food flavorings, additives, and flavoring agents in the formulation of cosmetics and fragrances (Baserk, .Buchbauer, 2010). The majority of essential oils utilized come from

lemons. The *Citrus limon* essential oil is utilized all over the world. In many household products, such as soaps, polishes, furniture, air fresheners, and cleaning solutions, lemon essential oil is employed as an antimicrobial agent (Irshad *et al*., 2015).

It is commonly known that *Citrus limon* fruit has a specific chemical makeup. The pericarp, juice, pomace, and essential oil have all been independently determined in addition to the complete fruit. It is also known what the fatty oil derived from the seeds of *Citrus limon* contains. The challenge of studying the chemical makeup of the raw materials derived from the numerous *Citrus limon* types, cultivars, and hybrids is one that many research centers take on. When determining the biological activity of *Citrus limon* fruit and juice, flavonoids—flavonoids like eriodictyol, hesperidin, hesperetin, and naringin—flavones like apigenin and diosmin, flavonols like quercetin, and their derivatives—come in first place. Other flavonoids, including flavones like orientin and vitexin as well as flavonols like limocitrin and spinacetin, are also found in the entire fruit. Hesperidin, naringin, and neohesperidin are three flavonoids that are distinctive to *Citrus limon* fruit. *Citrus limon* contains the most eriocitrin when compared to other citrus species (Robards;Antolovich,1997). Another significant class of chemicals that can be present in both fruit and juice are phenolic acids. In the juice, ferulic acid, sinapic acid, and their derivatives make up the majority of these substances. On the other hand, the fruit's presence of p-hydroxybenzoic acid has been verified. Additionally, the fruit contains amino acids, a complex of B vitamins, sugars, coumarin compounds, carboxylic acids, and, most significantly, vitamin C. (ascorbic acid) (Goetz;Burm, 2014;Abad-García *et al.*, 2012).

Sahraoui et al. used a novel technique called microwave steam distillation to extract essential oil from citrus by-products (orange peels) (MSD). The results show that this innovative technique is effective when compared to the more traditional steam distillation (SD), which provides for significant time and energy savings throughout the extraction process. The extraction process was greatly accelerated by MSD without changing the makeup of the volatile oil. Significant benefits of MSD include a shorter extraction time; with MSD, yields are comparable to those obtained after 2 hours by SD, the standard method for isolating essential oils (Sahraoui *et al*., 2011).

Microwave steam distillation (MSD) equipment was utilized in the work by Ibtehal et al. to extract essential oils from three different types of fresh citrus peels: orange (*Citrus sinensis*), lemon (*Citrus limon*), and mandarin (*Citrus reticulate*). In terms of extraction time, yield, sample weight, and essential oil content, comparisons to traditional steam distillation (SD) have been performed. For a qualitative investigation, the concentration of limonene in each manufactured sample was determined using gas chromatography analysis of the essential oils (Ibtehal *et al.*, 2015).

The *Citrus limon* pericarp is composed of a thin, wax-coated exocarp and the flavedo, or exterior section of the mesocarp. Carotenoid dyes and oil vesicles are present in this component. A spongy, white parenchyma tissue makes up the inner portion of the mesocarp, commonly referred to as the albedo. The spongy, white tissue of the mesocarp separates the endocarp, or "fruit flesh," into segments (Mabberley, 2004). It is commonly known that *Citrus limon* fruit has a specific chemical makeup. The pericarp, juice, pomace, and essential oil have all been independently determined in addition to the complete fruit. It is also known what the fatty oil derived from the seeds of *Citrus limon* contains. The challenge of studying the chemical makeup of the raw materials derived from the numerous *Citrus limon* types, cultivars, and hybrids is one that many research centers take on (Klimek *et al*., 2020).

The general metabolism of green microalgae, like *C. vulgaris*, involves oxidant reactive molecules like H_2O_2, free radicals derived from molecular oxygen or chemically active oxygen species (ROS), and oxidized lipid derivatives produced at chloroplast, mitochondria, and peroxisome compartments, which has been previously discussed (Goiris *et.al.,* 2015). In a healthy cell, the production of oxidant and antioxidant molecules is balanced; however, when under stressful circumstances, the production of oxidizing agents may increase (Barsanti *et.al.*, 2008). In response, a variety of techniques have been used to increase the antioxidant machinery of microalgae by creating artificially demanding growing conditions such as high light intensity, high temperature, and osmotic stress (Skjanes *et.al.,* 2013). In this case, the cell will overproduce antioxidant molecules to counteract the potentially harmful effects of the excess oxidizing agents. The oxygen radical absorbance capacity (ORAC) assay was used to evaluate the hydrolysate's antioxidant potential. ORAC was performed in a black 96-well microplate (Nunc, Denmark) according to the method described by Coscueta et al (Coscueta *et.al.,* 2020). All the hydrolysates were analyzed in triplicate. The reaction was performed in a multi-detection plate reader (Synergy H1; BioTek Instruments, Winooski VT, USA) with excitation and emission wavelengths of 485 nm and 528 nm, respectively. Trolox (1–8 µM, final concentration in well) was used as the standard for the

calibration curve. The results were expressed in µmol TE (Trolox equivalent)/mL for DOE hydrolysate or in µmol TE/g hydrolysate and µmol TE/g protein for scaled-up hydrolysate (Cunha *et.al.,* 2020).

Hesperidin and hesperetin, two flavonoids derived from Citrus limon, have been demonstrated to have antioxidant activity that is not only limited to its capacity to neutralize free radicals but also enhances cellular antioxidant defenses via the ERK/Nrf2 signaling pathway (Parhiz *et.al.*, 2015). In addition, vitamin C shields DNA against mutations and stops the production of free radicals. Research have also revealed that pilocarpine can produce status epilepticus in adult rats, which results in a decrease in lipid peroxidation during seizures (Xavier *et.al.,* 2007). Hesperidin metabolites or their synthetic derivatives have been the subject of numerous in vitro and in vivo investigations to determine how well they inhibit inflammatory targets like NF-B, iNOS, and COX-2, as well as markers of chronic inflammation (Parhiz *et.al.,* 2015). In mice subjected to a formalin test, the essential oil from *Citrus limon* (30 or 10 mg/kg p.o.) demonstrated anti-inflammatory properties by lowering cell migration, cytokine production, and protein extravasation brought on by carrageenan. The same amounts of pure D-limonene were also used to achieve these results. The high concentration of D-limonene in *C. limon* essential oil is likely what causes it to have an anti-inflammatory effect (Amorim *et.al.,* 2016).

CHAPTER 3
MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Microalgal culture

Microalgal species selected for the current study was a strain of *Chlorella vulgaris* 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 and composition and culture parameter

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

TABLE 2: CULTURE PARAMETERS

Sl.no.	Parameter	Value
1	Working volume	200 ml
2	Temperature	24±1°C
3	Light intensity	700-800 lux
4	Photoperiod	16/18h (light/dark)
5	Time	18 Days

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 wavelengths such as 680nm, 686 nm and 750 nm in order to determine maximum absorbance using a spectrophotometer (LAB India).

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 were examined using 10X objective for distribution of the cells and refocused at 40X objective before counting cell in the four corner squares.

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

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 10 minutes at 3000 rpm (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 wavelengths, 480 nm (A480) and 700 nm (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 regions of the 16s 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 the room temperature. Equal volume of Phenol: Chloroform: Isoamylalcohol (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: Isoamylalcohol extraction was repeated and the aqueous layer was collected in eppendof 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 2/3rd 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 further pelleted 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 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 intensitics of band obtained by staining with (0.5µ/g mL) Ethidium bromide was compared with 250bp DNA marker from Chromous Biotech. The gel documentation system (BIORAD- Molecular image) was used for DNA visualisation on the gel.

3.2.2.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 react ion 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 1 minute, with a final extension at 72°C for 7 minutes.

Amplicons were checked for appropriate size by 2% agarose gel visualization. Amplicons were gel purified using commercial column based purification kit (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.

3.6. Crude Lipid extraction

3.6.1. Modified Bligh and Dyer method

Lipid content of the cultures A, B and C were determined based on 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 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 (%) stands for lipid content of cells, W (mgL^{-1}) for the dry cell weight and T stands for cultivation period in days.

ESSENTIAL OIL EXTRACTION

50g of *Citrus limon* were obtained. *Citrus limon* peels were transferred into a round bottom flask using a funnel. Added distilled water till half of the flask(1000mL). Assembled distillation apparatus. The oil was co-distilled with water and immicisible with it. First drop of distillation appears and vapour got condensed. Noted the early drops in the distillate that means the oil is being extracted properly. Distillation continued for 1 and half an hour after the first drop has appeared and the distillation process turned off.

For the separation liquid extraction was performed using Hexane as a solvent. Poured the extracted liquid into the separating funnel completely added 10mL hexane used as solvent into the 250mL of extracted lipid and replaced the cap and shook well for releasing the pressure that might from the funnel. After that kept it for a few seconds. Opened the stopper and collected the aqueous phase into the flask and stopped when the organic phase has reached at the tip and recovered it in separate container. Covered it using a cling film. Repeated the process with 5mL. Boiled Hexane at 62.5°C and continued the boiling till it reached 80°C.

MORPHOLOGICAL EVALUATION

It refers to the evaluation of the pack by its color, odor, appearance, touch, etc. The external characters of the samples were examined based on the method described by Siddiqui et al.

IRRITANCY TEST

Mark an area on the dorsal surface of the left hand. Definite quantities of prepared face packs were applied to the specified area and time was noted. Irritancy, redness, and swelling were checked and reported for regular intervals up to 24 hours if any.

STABILITY TEST

Stability testing of the prepared formulation was conducted by storing at different temperature conditions for the period of one month. The packed glass vials of formulation stored at different temperature conditions viz.., Room temperature and 45◦ c were evaluated for the physical parameters like color, odor, pH, texture and smoothness.

ANTIOXIDANT ACTIVITY

Superoxide scavenging activity

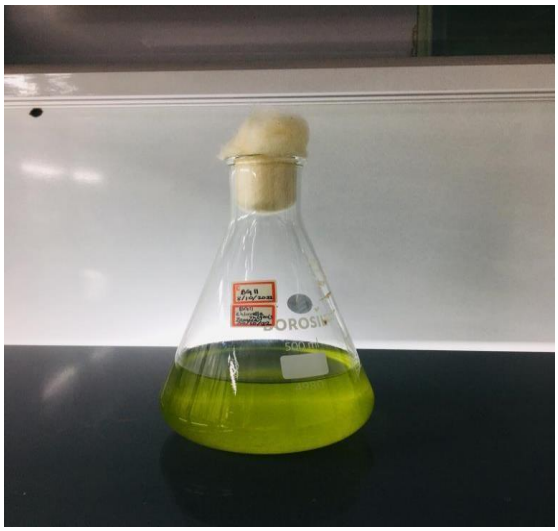
Superoxide scavenging activity (SOD) was carried out by using alkaline dimethyl sulfoxide (DMSO). Solid potassium superoxide was allowed to stand out in contact with dry DMSO for at least 24 hrs and the solution was filtered immediately before use. Filtrate (200mL) was added to 2.8mL of aqueous solution containing Nitro bluetetrazolium (56mM), EDTA (10mM) and potassium phosphate buffer (10Mm, pH 7.4). Sample extract (1mL) at various concentration (100mg/mL) in water was added and the absorbance was recorded at 560 nm against a control in which pure DMSO has been added instead of alkaline DMSO (Rice-evan *et al.,* 2014).

CHAPTER 4
OBSERVATION AND RESULT

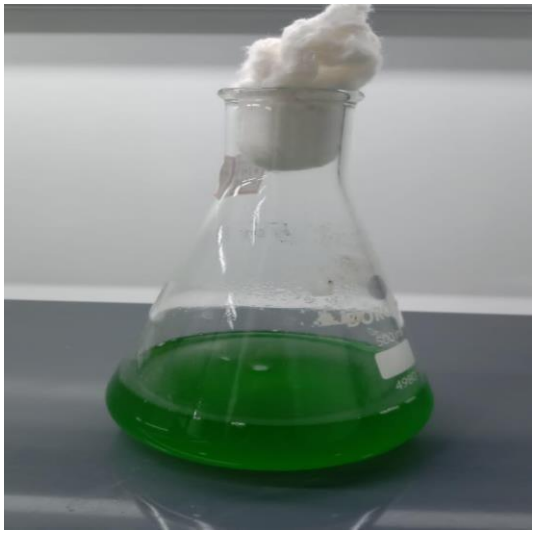
OBSERVATION AND RESULTS

4.1. MICROALGAL CULTURE

C.vulgaris has been cultured for 30 days in a conical flask. The culture in the conical flask showed a gradual change from the first day of inoculation up to the 15th day. The colour change from pale colour to dark green as indication of increase in chlorophyll content and cell count.



(a)



(b)

Plate .1. (a) *C.vulgaris* culture on the first day of inoculation

(b) *C.vulgaris* culture on the 15th day of inoculation

4.2. MICROSCOPIC OBSERVATION

The algae was observed under light microscope.The microscopic observation shows spherical shaped algal cells which were pale green in colour.

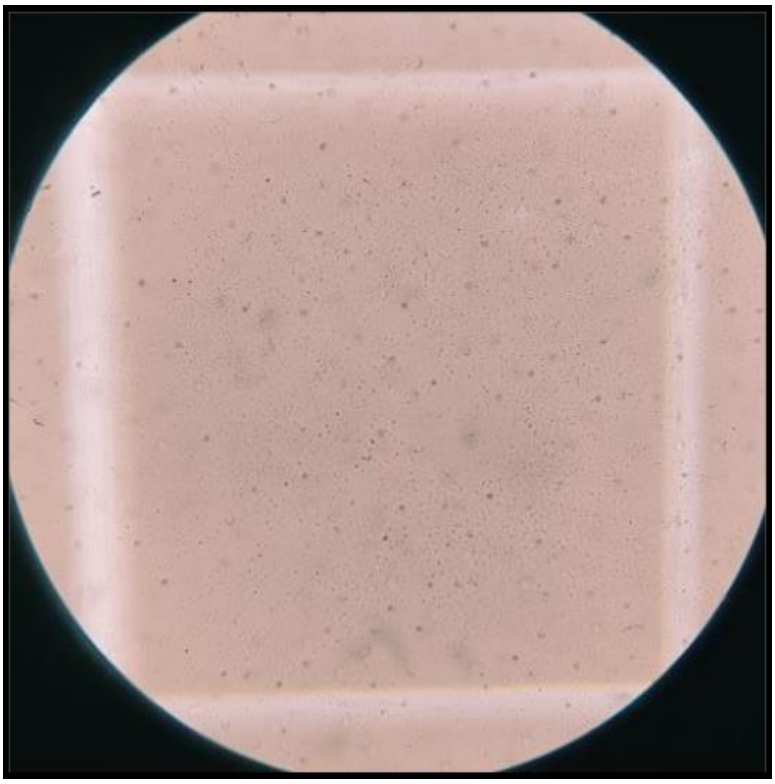


Plate.2. Microscopic observation of *C.vulgaris* (40X)

4.3. MAXIMUM ABSORBANCE DETERMINATION

The optical absorbance was measured at two wavelengths 620 nm and 700 nm. The maximum absorbance was observed on the 15^{th} day of inoculation.

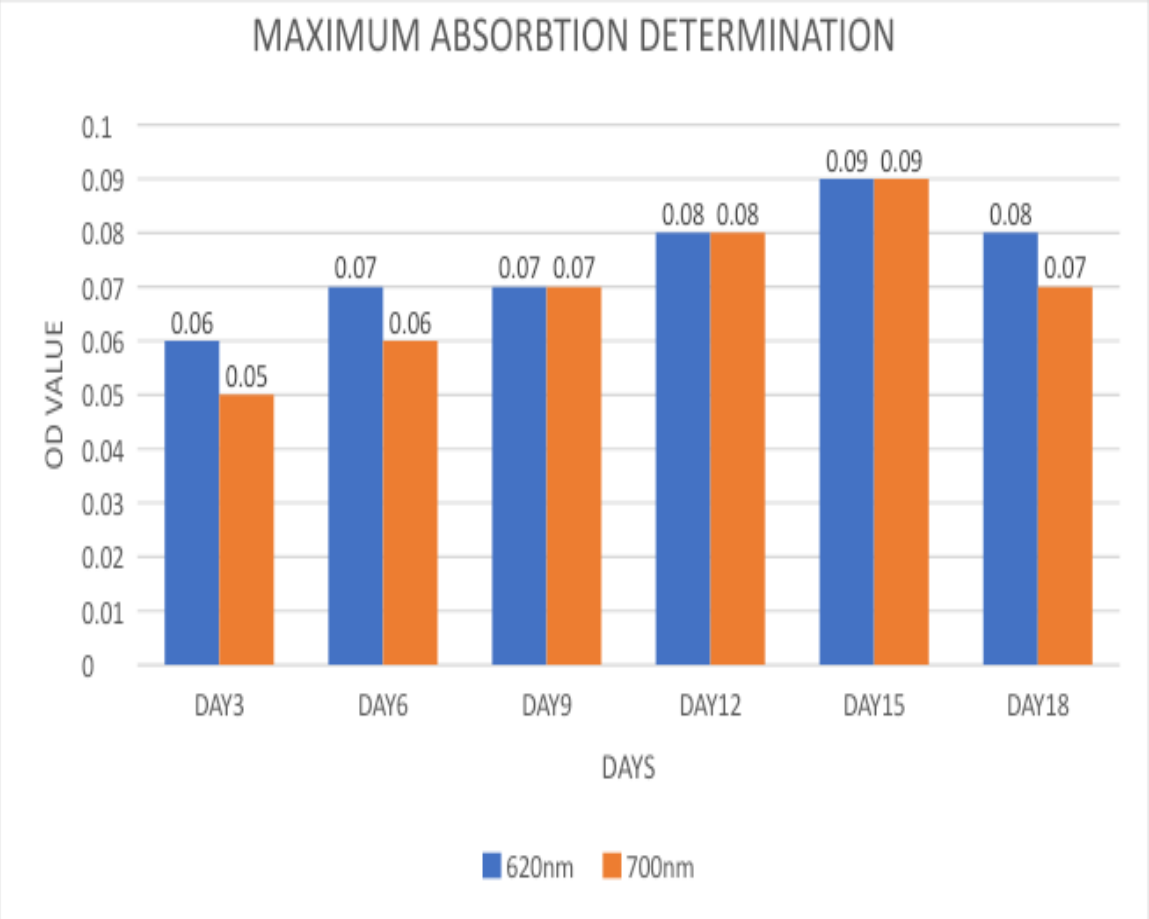


Figure.1. Maximum absorption determination

4.4. CELL COUNTING USING HAEMOCYTOMETER

Cell counting using a haemocytometer showed the cell density of the culture. The cell count was taken periodically. The maximum cell count was observed on the 15^{th} day of inoculation.

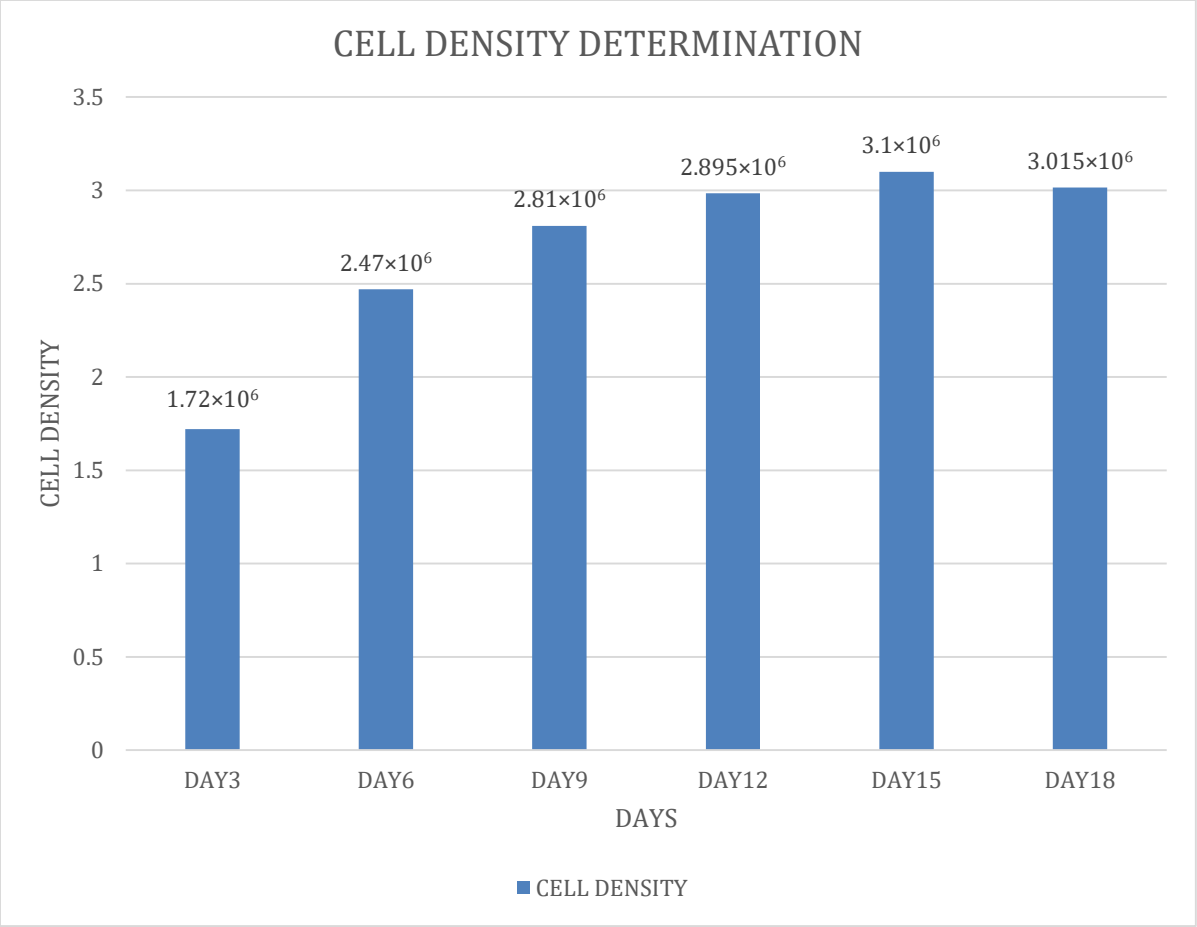


Figure.2. Cell density determination

4.5. DETERMINATION OF CHLOROPHYLL CONTENT

Chlorophyll content was determined from algal culture by using acetone method. The OD values was taken at different wavelengths (480 nm and 700 nm respectively). The chlorophyll content was maximum on the 15th day.

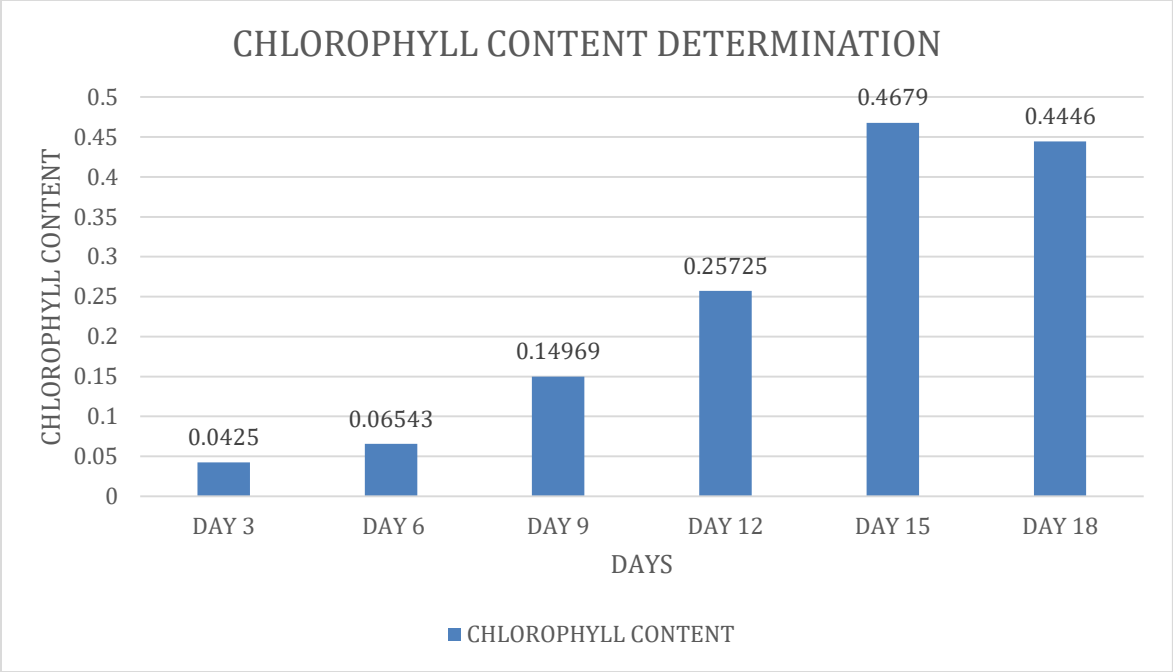


Figure.3. Chlorophyll content

4.6. IDENTIFICATION OF *C.VULGARIS* USING MOLECULAR SEQUENCING

Identification of organism using molecular sequencing method involves various steps. The very first step is the isolation of DNA. Isolation is done by Doyle and Doyle (1987), using CTAB yielded good quality DNA for PCR. DNA concentration and purity was also determined by running the sample in 1.0% Agarose gel. The gel documentation system was used for DNA visualization on the gel. Sequencing was performed with forward and reverse primers in ABI 3730 XL cycle sequencer.

MBT051 S1

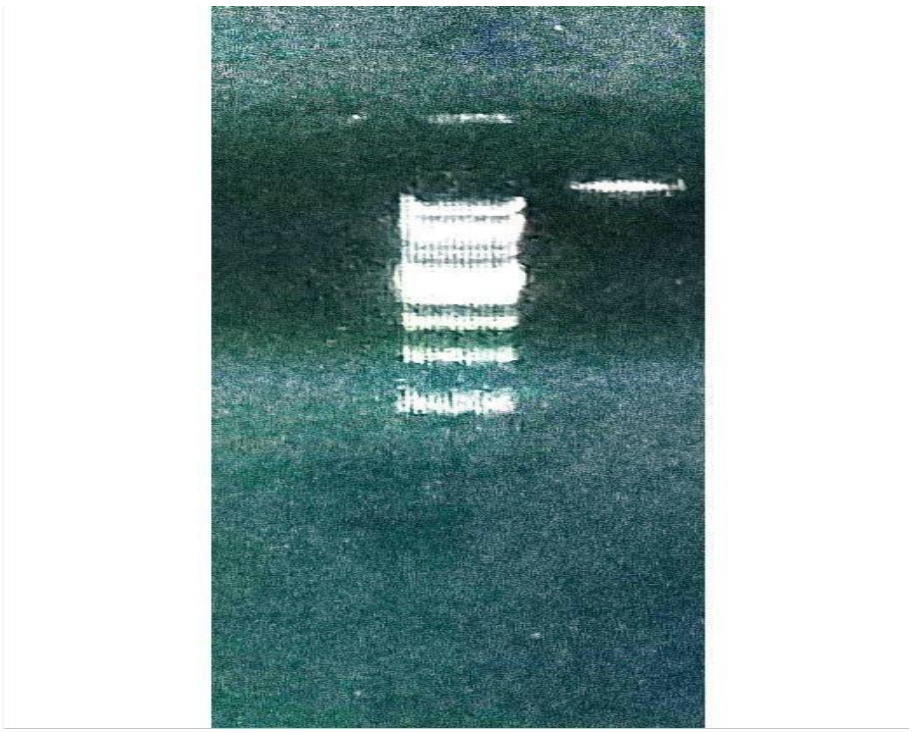


Plate.3. An Ethidium bromide stained 2% agarose gel showing 16s rRNA PCR amplification of DNA.

Lane (MBTO51): 100 bp marker.

Lane (S): 16s rRNA PCR amplicon of DNA.

>AF350260.1:1-586 Chlorella vulgaris 16S ribosomal RNA gene, partial sequence
AGGGACAACCATTGGAAACGATGGCTAATACCTCATAATACTGAGTAAGTTAAATGATGAATAATCGCCAAGAGA
TGGGC
TTGCGGCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCAGTATCTGGTCTGACCAGGATGA
TCACCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAACAGTGAGGAATTTTCCGCAATGGGCGAAAGC
CTGACGG
AGCAATGCCGCGTGAAGGATGAAGGCCTATGGGTTGTAAACTTCTTTTCTCAGAGAAGAAATTTTGACGGTATCT
GAGGA
ATAAGCATCGGCTAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGATGCAAGCGTTATCCGGAATGATTGGG
CGTAAAG
CGTCTGTAGGTGGCTTAAAAAGTCTCCTGTCAAAGATCAGGGCTTAACCCTGGGCCGGCAGGAGAAACTCTTAG
GCTAGA
GTTTGGTAGGGGCAGAGGGAATTCCCGGTGGAGCGGTGAAATGCGTAGAGATCGGGAGGAACACCAAAGGCG
AAAGCACT
CTGCTGGGCCACAACTGACACTGAGA

Plate.4. DNA sequence of *C.vulgaris*

Following sequencing, the sequence that was acquired is compared to the database to determine whether there are any similarities. Word-by-word similarity searches are employed in BLAST to locate the matched sequence from the database.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Chlorella vulgaris 16S ribosomal RNA gene, partial sequence; chloroplast gene for chloroplast product	Chlorella vulgaris	1083	1083	100%	0.0	100.00%	586	AF350260.1
Chlorella vulgaris strain MSU-AGM 14 16S ribosomal RNA gene, partial sequence; chloroplast	Chlorella vulgaris	1050	1050	100%	0.0	98.98%	609	KM189121.1
Chlorella sp. ArM0029B chloroplast, complete genome	Chlorella sp. Ar…	1042	1042	100%	0.0	98.81%	119989	KF554427.1
Micractinium singularis strain MM0003 plastid, complete genome	Micractinium sin…	1037	1037	100%	0.0	98.63%	139597	MN894287.1
Micractinium pusillum strain CCAP 232/1 chloroplast, complete genome	Micractinium pus…	1031	1031	100%	0.0	98.46%	115638	MN649872.1
Micractinium sp. LBA 32 chloroplast, complete genome	Micractinium sp. …	1026	1026	100%	0.0	98.29%	109688	MH983006.1
Uncultured Streptophyta clone UVmas1_53 16S ribosomal RNA gene, partial sequence; chloroplast	uncultured Strep…	1026	1026	100%	0.0	98.29%	701	JQ701246.1
Uncultured bacterium clone AD05 16S ribosomal RNA gene, partial sequence	uncultured bacte…	1024	1024	100%	0.0	98.29%	990	KC009751.1
Uncultured Streptophyta clone UV-2_3 16S ribosomal RNA gene, partial sequence; chloroplast	uncultured Strep…	1020	1020	100%	0.0	98.13%	706	JQ700677.1
Uncultured cyanobacterium clone Gap-2-18 16S ribosomal RNA gene, partial sequence	uncultured cyan…	1018	1018	100%	0.0	98.12%	870	EU642172.1
Chlorella sp. SUN-2 16S ribosomal RNA gene, partial sequence; plastid	Chlorella sp. SU…	1014	1014	100%	0.0	97.96%	1454	EF114678.1
Auxenochlorella pyrenoidosa isolate FACHB-5 chloroplast, complete genome	Auxenochlorella …	1009	1009	100%	0.0	97.78%	107442	MN128434.1
Uncultured phototrophic eukaryote clone NV1_CYA_1_29 16S ribosomal RNA gene, partial sequence; plastid	uncultured photo…	1007	1007	100%	0.0	97.78%	666	FJ204892.1
Uncultured bacterium clone CK-86 16S ribosomal RNA gene, partial sequence	uncultured bacte…	1003	1003	100%	0.0	97.61%	1453	KM200526.1
Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: Sa75_M_3	uncultured bacte…	1002	1002	100%	0.0	97.61%	1415	LC065717.1
Uncultured bacterium clone N06Jun-31 16S ribosomal RNA gene, partial sequence	uncultured bacte…	996	996	100%	0.0	97.44%	803	EU442895.1
Uncultured bacterium clone P1O-64 16S ribosomal RNA gene, partial sequence	uncultured bacte…	996	996	100%	0.0	97.44%	809	EU375419.1
Uncultured cyanobacterium clone XZNMC45 16S ribosomal RNA gene, partial sequence	uncultured cyan…	990	990	100%	0.0	97.27%	1371	EU703214.1
Chlorella variabilis clone DT025 chloroplast, complete genome	Chlorella variabilis	985	985	100%	0.0	97.10%	118106	MZ647689.1
Chlorella sp. ATCC 30562 plastid, complete genome	Chlorella sp. AT…	983	983	100%	0.0	97.10%	124881	KY629617.1
Chlorella variabilis isolate NC64A chloroplast, complete genome	Chlorella variabilis	983	983	100%	0.0	97.10%	124793	KJ718922.1
Chlorella variabilis plastid, complete genome	Chlorella variabilis	983	983	100%	0.0	97.10%	124579	HQ914635.1
Uncultured bacterium gene for 16S rRNA, partial sequence, clone: Hados.Water.Eubac.3	uncultured bacte…	979	979	100%	0.0	96.93%	1452	AB355044.1
Chlorella variabilis strain YTU, ANTARCTIC.001 small subunit ribosomal RNA gene, partial sequence; chloropl…	Chlorella variabilis	974	974	100%	0.0	96.76%	1359	MN372092.1
Prochlorococcus sp. EGEMACC 6 isolate A 16S ribosomal RNA gene, partial sequence	Prochlorococcus…	974	974	100%	0.0	96.76%	975	JQ726701.1
uncultured bacterium partial 16S rRNA gene	uncultured bacte…	972	972	100%	0.0	96.76%	1412	LR638220.1
uncultured bacterium partial 16S rRNA gene	uncultured bacte…	972	972	100%	0.0	96.76%	1413	LR636755.1
Chlorella heliozoae plastid, complete genome	Chlorella heliozo…	972	972	100%	0.0	96.76%	124353	KY629616.1
Pseudochlorella pringsheimii 16S ribosomal RNA gene, partial sequence; tRNA-Ile and tRNA-Ala genes, com…	Pseudochlorella …	970	970	100%	0.0	96.59%	4781	MF683077.1
Uncultured bacterium clone AG09 16S ribosomal RNA gene, partial sequence	uncultured bacte…	970	970	100%	0.0	96.59%	936	KC009731.1
Uncultured Chlorella clone BF 009 16S ribosomal RNA gene, partial sequence	uncultured Chlor…	970	970	100%	0.0	96.59%	1455	KC994689.1
Uncultured Chlorella clone RFLP band pattern 11 16S ribosomal RNA gene, partial sequence	uncultured Chlor…	970	970	100%	0.0	96.59%	1452	KC994673.1
Uncultured bacterium clone AF06 16S ribosomal RNA gene, partial sequence	uncultured bacte…	965	965	100%	0.0	96.42%	895	KC009761.1
Chlorella sorokiniana isolate 1230 chloroplast, complete genome	Chlorella sorokin…	961	961	100%	0.0	96.42%	109803	KJ742376.1
Chlorella sorokiniana chloroplast, complete genome	Chlorella sorokin…	961	961	100%	0.0	96.42%	109811	KJ397925.1
Chlorella sp. JC183 plastid partial 16S rRNA gene, strain JC183	Chlorella sp. JC…	957	957	100%	0.0	96.25%	1449	HF536585.1
C.sorokiniana plastid DNA small subunit (16S-like) ribosomal RNA	Chlorella sorokin…	957	957	100%	0.0	96.25%	1493	X65689.1
Uncultured bacterium clone LB532 16S ribosomal RNA gene, partial sequence	uncultured bacte…	955	955	100%	0.0	96.25%	1453	MG238532.1
Chlorellaceae sp. M7 16S ribosomal RNA gene, partial sequence; plastid	Chlorellaceae sp…	955	955	100%	0.0	96.25%	1175	HM573453.1
Uncultured cyanobacterium clone LPSB10 16S ribosomal RNA gene, partial sequence	uncultured cyan…	955	955	100%	0.0	96.25%	1321	FJ901770.1
Uncultured bacterium clone 3-1 16S ribosomal RNA gene, partial sequence	uncultured bacte…	953	953	100%	0.0	96.08%	1461	KX823759.1
Uncultured bacterium clone CK-111 16S ribosomal RNA gene, partial sequence	uncultured bacte…	953	953	100%	0.0	96.08%	1466	KM200540.1
Uncultured eukaryote clone Ke0219r46 16S ribosomal RNA gene, partial sequence; plastid	uncultured eukar…	953	953	100%	0.0	96.08%	610	GQ422918.1
Cyanobacterium G1 16S ribosomal RNA gene, partial sequence	cyanobacterium …	950	950	100%	0.0	96.08%	798	KT156639.1
Uncultured bacterium clone PAS3_C01 16S ribosomal RNA gene, partial sequence	uncultured bacte…	948	948	100%	0.0	95.90%	721	DQ830612.1
Chlorella vulgaris strain UTEX259 plastid, complete genome	Chlorella vulgaris	946	946	100%	0.0	95.90%	176851	MK948102.1

Plate.5. Blast search

The graphic summary of alignment represents the sequence similarity of the query sequence with that of sequences in the databases. The horizontal lines on each graph reflect sequences that have matches in the database. Here, the alignment score is higher than 200, which is represented by red lines, signifying excellent or good matches.

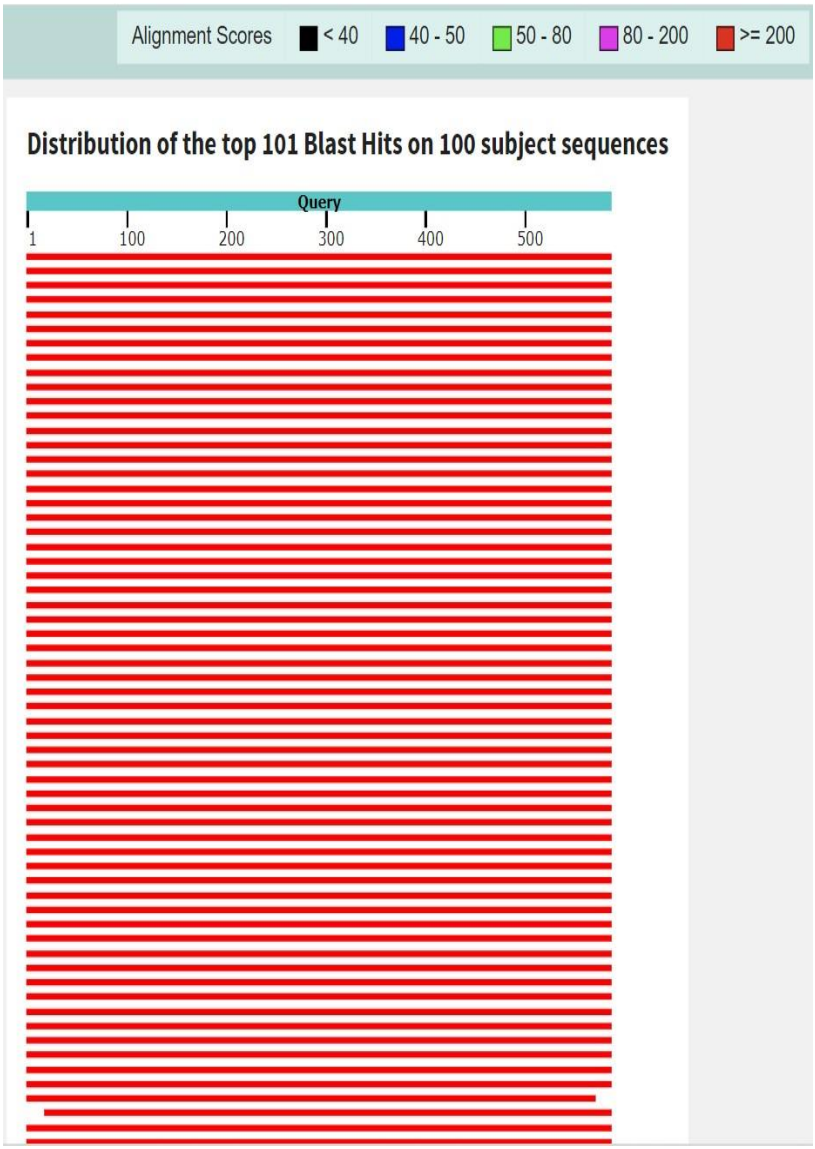


Plate.6. Graphic summary of alignment score

Phylogenetic analysis of algae was conducted using MEGA X. The phylogenetic tree shows that the *Chlorella vulgaris* 16s ribosomal RNA gene partial sequence shows close relationship with *Chlorella vulgaris* strain MSU-AGM 14 16s Ribosomal RNA gene partial sequence chloroplast.

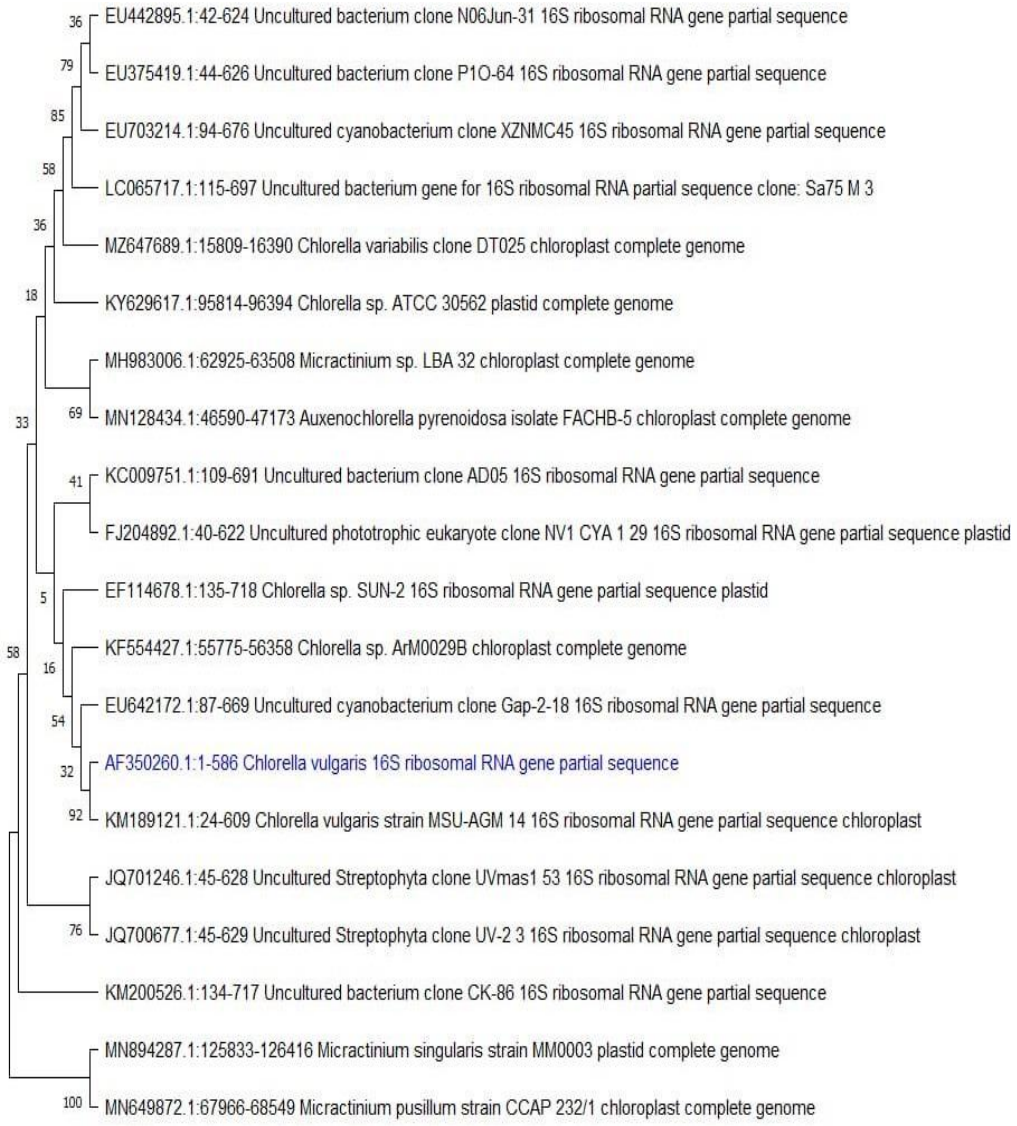


Plate.7. Phylogenetic tree

4.7. ESSENTIAL OIL EXTRACTION

The essential oil from *Citrus limon* was extracted by steam distillation process using distillation apparatus. For the separation liquid extraction separating funnel was used. The aqueous phase is collected into the flask and the organic phase into the container.

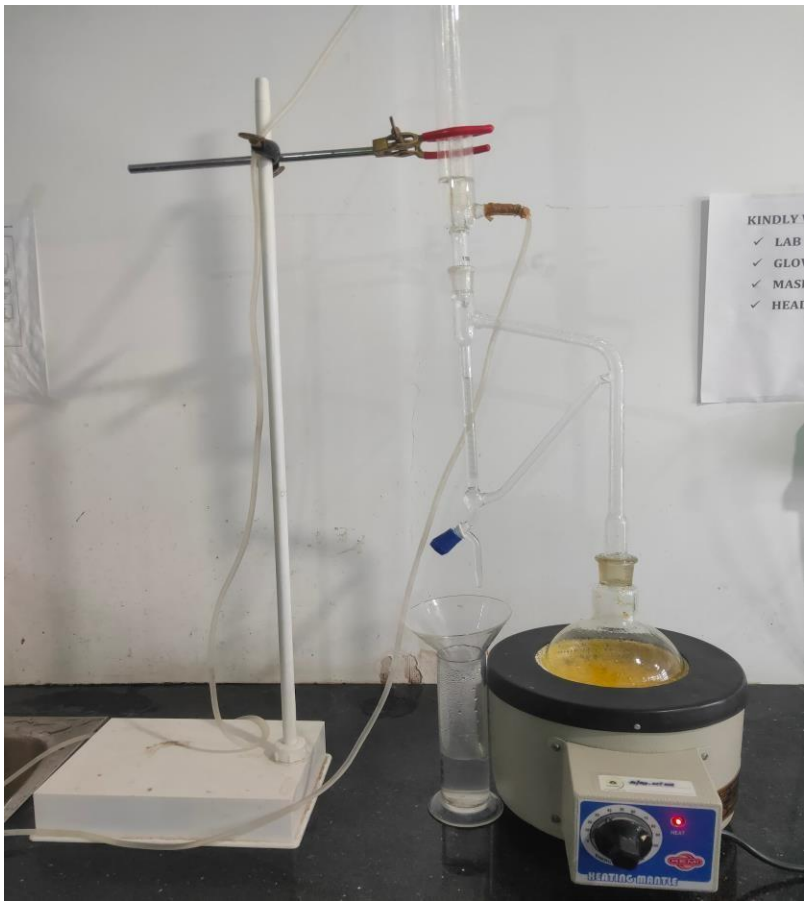


Plate.8. Steam distillation apparatus

Plate.9. Separation funnel

The lipid extract and essential oil was blended together and stored in a glass vial. The product was colourless at room temperature with pleasant odour.

Plate.10.The product in glass vial

4.8. ORGANOLEPTIC ANALYSIS

Organoleptic analysis was done by 10 panelists and took the overall acceptance. Majority of the panelists marked as 7 and 6 for the odour, gloss level, smoothness and stickiness.

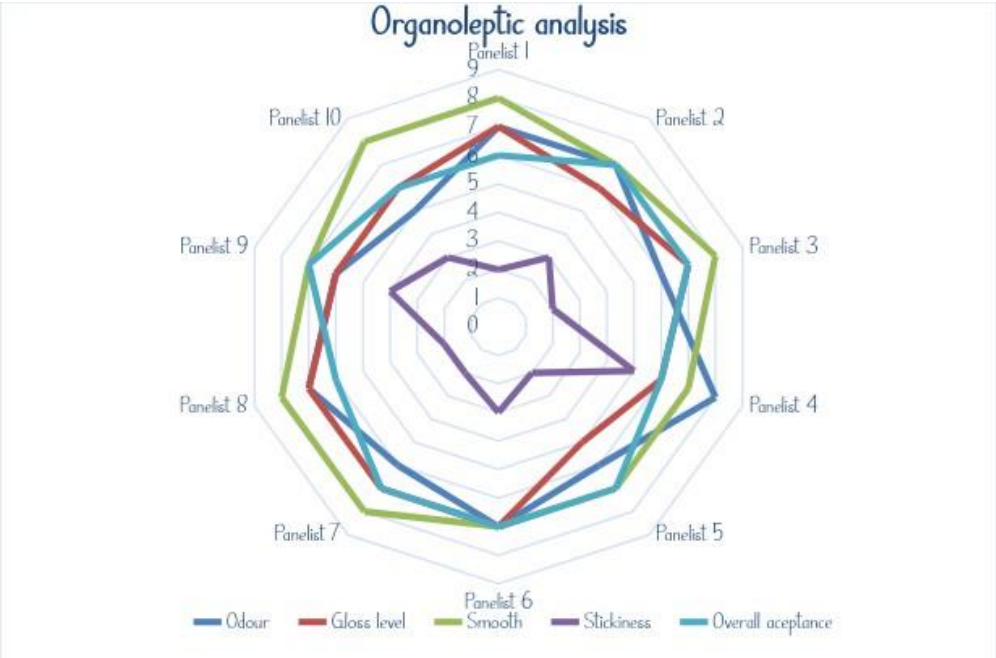


Figure.4. Organoleptic analysis

4.9MORPHOLOGICAL EVALUATION

Morphological evaluation of the pack was examined by testing its color, odor, appearance, touch, etc. There was no change in the colour and has a pleasant odour.

TABLE 3: TABLE FOR MORPHOLOGICAL EVALUATION		
S.NO:	**PARAMETER**	**OBSERVATION**
1.	Colour	No change
2.	Odor	Pleasant
3.	Appearance	Smooth, Fine

4.10. STABILITY TEST

Stability test of the pack was examined by testing its color, odor, appearance, touch, etc. at room temperature. There was no change in the colour and odour of the extract. The pH of the extract was 6.62 and it has fine texture.

TABLE 4: TABLE FOR STABILITY TEST		
SL.NO:	**PARAMETER**	**ROOM TEMPERATURE**
1.	Colour	No change
2.	Odor	No change
3.	pH	6.62
4.	Texture	Fine
5.	Smoothness	Smooth

4.11. IRRITANCY TEST

The presence of irritation, redness, or swelling was monitored and reported at regular intervals for up to 24 hours and the result was negative. There is no irritation, redness and swelling.

TABLE 5: TABLE FOR IRRITANCY TEST		
S.NO:	**PARAMETER**	**OBSERVATION**
1.	Irritation	Negative
2.	Redness	Negative
3.	Swelling	Negative

The antioxidant properties of the product was tested by applying into the skin and analyzed using skin moisture analyzer. The moisture content of the skin before applying the product was 11% and after applying was 33%.

Plate.11. Skin Moisture analyzer

Moisture content of skin before applying the product = 11%
Moisture content of skin after applying the product=33%

CHAPTER 5
DISCUSSION

DISCUSSION

In this study the microalgae *Chlorella vulgaris* was collected from fresh water and cultured in BG11 media. After culturing the algae the genomic DNA was isolated using a CTAB buffer. 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. Using specific primers, polymerase chain reaction (PCR) was used to amplify the 16s region. The amplified products were visualized using a gel documentation system. Using Bligh and Dyer method the lipid content from the *C.vulgaris* was extracted. The essential oil from *Citrus limon* peel was extracted by steam distillation. Then the solvent extracted using a separating funnel. The lipid extract and essential oil blended together and analyzed the synergistic effect. To analyze the synergistic effect of the product performed the organoleptic analysis, stability test and irritancy test. The results shows that the products has an augmented antioxidant properties than synthetic cosmetic products.

According to the study of Chen *et al* and Yoo *et al,* Blue green-11 (BG-11) has been the most popular culture medium of option for cultivating *C. vulgaris*, even if the ideal conformation of culture medium for doing so has remained debatable (Chen *et.al.*, 2009). In the study of Kates and Volcani the extraction of total lipid from the biomass was performed in triplicate according to the modified Bligh and Dyer method (Kates; Volcani, 1965). Ruchi *et al* reported extraction of essential oil was made by steam distillation process using Clevenger apparatus for about 4-5 hours (Ruchi *et.al.,* 2015).

De Luca *et al* reported that some types of microalgae are classified as oleaginous because they can accumulate appreciable amounts of lipid. Lipids and their derivatives are one of the key components in cosmetic compositions (De Luca *et.al.,* 2021). *Because* of their abundant supply of carotenoids, vitamins, and phenolics, microalgae are considered a source of nutraceutical antioxidants. Many studies have focused on increasing the output of antioxidants by introducing various stress situations. In specialized dermocosmetics, vitamin C from *Citrus limon* is a component. The skin becomes smoother and tenser as a result of its external application since it causes an increase in collagen formation. It functions as a synergistic antioxidant when used with vitamin E in anti-aging products, reducing shallow wrinkles.

CHAPTER 6

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Microalgae are a fascinating source of bioactive substances with potential industrial uses. Lipids are a diverse group of beneficial compounds that are used in numerous industries. The use of microalgae in cosmetics is an interesting way to expand the search for additional natural components from biomass that is environmentally friendly. Lipid products offer formulas for cosmetics a beneficial perspective. *Chlorella vulgaris* is a freshwater green algae. It is a unicellular spherical or ellipsoidal algae, which lacks flagella. A fascinating nutritional profile of lipids, carbohydrates, fiber, vitamins, and proteins may be found in *Chlorella vulgaris*. Citrus essential oils (EOs) offer a variety of important characteristics that could make them the ideal solution for chemical-based antimicrobials in food, agriculture, medicinal, and sanitary applications as well as a costly component in cosmetics and perfumes.

Chlorella vulgaris, a microalgae, was extracted from fresh water and grown in BG11 media for this study. The ratio of 260 nm absorbance to 280 nm absorbance was used to calculate the purity of DNA. Genomic DNA was extracted from the algae using a CTAB buffer after they had been cultured. Running the material through a 1.0% Agarose gel allowed for the measurement of DNA concentration and purity. Polymerase chain reaction (PCR) was utilized to amplify the 16s region using certain primers.The lipid content of the *C. vulgaris* was extracted using the Bligh and Dyer method. With the process of steam distillation, *Citrus limon* peel essential oil was obtained. A separating funnel was then used to extract the solvent. The synergistic effect of the lipid extract and essential oil was investigated. The product underwent an organoleptic analysis, a stability test, and an irritancy test in order to evaluate its synergistic impact. The findings indicate that the products have greater antioxidant capabilities than artificial cosmetics.

The present approach provided an innovative methodology for cosmetic application, presenting a synergistic effect between the microalgae *Chlorella vulgaris* and *Citrus limon* essential oil. The results show that the products are more effective antioxidants than synthetic cosmetics. Due to public demand and the fact that synthetic cosmetic products are clearly having adverse effects on individuals, the cosmetic industry is researching novel compounds derived from natural materials. The presented study reveals the synergistic effect of lipid and essential oil. The study confirms that the product have greater antioxidant effects than synthetic cosmetics. Antioxidant and anti-inflammatory properties of this product are very high, research into these ingredients and their uses must be promoted and extended.

.

CHAPTER 7

REFERENCE

REFERENCE

1. Abad-García, B., Garmón-Lobato, S., Berrueta, L. A., Gallo, B., & Vicente, F. (2012). On line characterization of 58 phenolic compounds in Citrus fruit juices from Spanish cultivars by high-performance liquid chromatography with photodiode-array detection coupled to electrospray ionization triple quadrupole mass spectrometry. Talanta, 99, pp:213–224.
2. Ahmad, M., Shariff, M., Yusoff, F. M., Goh, Y. M., & Banerjee, S. (2020c). Applications of microalga *Chlorella vulgaris* in aquaculture. Reviews in Aquaculture, 12(1), pp:328– 346.
3. Amorim, J. H., Simas, D. L. R., Pinheiro, M. M. G., Moreno, D., Alviano, C. S., Da Silva,C. O., & Fernandes, P. D. (2016c). Anti-Inflammatory Properties and Chemical Characterization of the Essential Oils of Four Citrus Species. PLOS ONE, 11(4), e0153643.
4. Ariede, M. B., Candido, T. M., Jácome, A. L. M., Velasco, M. V. R., Carvalho, J., & Baby,
5. R. (2017). Cosmetic attributes of algae - A review. Algal Research-Biomass Biofuels andBioproducts, 25, pp:483–487.
6. Bajguz, A. (2000). Effect of brassinosteroids on nucleic acids and protein content in cultured cells of Chlorella vulgaris. Plant Physiology and Biochemistry, 38(3), pp:209– 215.
7. Bajguz, A., & Piotrowska-Niczyporuk, A. (2014b). Interactive effect of brassinosteroids and cytokinins on growth, chlorophyll, monosaccharide and protein content in the green alga Chlorella vulgaris (Trebouxiophyceae). Plant Physiology and Biochemistry, 80, pp:176–183.
8. Barsanti, L., Coltelli, P., Evangelista, V., Frassanito, A. M., Passarelli, V., Vesentini, N., & Gualtieri, P. (2008). Oddities and Curiosities in the Algal World. NATO Science for Peace and Security Series, pp:353–391.
9. Becker, E. W. (1994b). Microalgae: biotechnology and microbiology. Choice Reviews Online, 32(02), 32–0920.

10. Chen W, Sommerfeld M., And Hu Q (2011). Microwave-assisted nife red method for in vivo quantification of neutral Iipids in microalgae. Bioresour Technol 102:135-41.
11. Cunha, S. C., Coscueta, E. R., Nova, P. C. C. V., Silva, J., & Pintado, M. R. (2022). Bioactive Hydrolysates from Chlorella vulgaris: Optimal Process and Bioactive Properties. Molecules, 27(8), 2505.
12. Dejoye C., Vian M.A., Lumia G., Bouscarle C., Charton F.,& Chemat F. (2011). Combined extraction processes of Iipid from Chlorella vulgaris microalgae: microwave prior to supercritical carbon dioxide extraction. !nt J Mol Sei 12: 9332-41.
13. De Luca M., Pappalardo I., Limongi A.R., Viviano E., Radice, R.P.; Todisco S., Martelli G., Infantino V., & Vassallo, A.(2021). Lipids fromMicroalgae for Cosmetic Applications. Cosmetics 8,
14. Evangelista, V., Barsanti, L., Frassanito, A. M., Passarelli, V., & Gualtieri, P. (2008). Algal Toxins: Nature, Occurrence, Effect and Detection. NATO Science for Peace and Security Series.
15. Fernando, I.S., Nah, J.W., &Jeon, Y.J. (2016).Potential anti-inflammatory natural products from marine algae.Environ. Toxicol. Pharm 48, pp:22–30.
16. Friedl, T. W. P. (1995b). Inferring taxonomic positions and testing genus level assignments in coccoid green lichen algae: a phylogenetic analysis of 18S ribosomal RNA sequences from *Dictchloropsis reticulata* and from members of the genus Myrmecia (Chlorophyta, Trebouxiophyceae cl. nov.)1. Journal of Phycology, 31(4), pp:632–639.
17. Goetz, P. (2014). *Citrus limon* (L.) Burm. f. (Rutacées) citronnier. Phytothérapie, 12(2), 116–121.
18. Goiris, K., Muylaert, K., & De Cooman, L. (2015). Microalgae as a Novel Source of Antioxidants for Nutritional Applications. Handbook of Marine Algae.
19. González-Molina, E., Schmeda-Hirschmann, G., Moreno, D., & García-Viguera, C. (2010). Natural bioactive compounds of *Citrus limon* for food and health. Journal of Pharmaceutical and Biomedical Analysis, 51(2), pp:327–345.

20. Hoham, R. W., Bonome, T. A., Martin, C., & Leebens-Mack, J. (2002b).A combined 18S rDNA and rbc-L phylogenetic analysis of Chloromonas and Chlamydomonas (Chlorophyceae, Volvocales) emphasizing snow and other cold-temperature habitats. Journ of Phycology. 38: pp:1051–64.
21. Hu, Q., Sommerfeld, M. R., Jarvis, E. E., Ghirardi, M. L., Posewitz, M. C., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant Journal, 54(4), pp:621–639.
22. Irshad, M., Subhani, M. I., Ali, S., & Hussain, A. (2019). Biological Importance of Essential Oils. IntechOpen EBooks.
23. Lee, P. C., Costumbrado, J., Hsu, C. C., & Kim, Y. J. (2012). Agarose Gel Electrophoresis for the Separation of DNA Fragments. Journal of Visualized Experiments, 62.
24. Liao, Q., Sun, Y., Huang, Y., Murphy, J. D., Fu, Q., & Zhu, X. (2017). Simultaneous enhancement of Chlorella vulgaris growth and lipid accumulation through the synergy effect between light and nitrate in a planar waveguide flat-plate photobioreactor. Bioresource Technology, 243, pp:528–538.
25. Liu, Z., Wang, G., & Zhou, B. (2008). Effect of iron on growth and lipid accumulation in *Chlorella vulgaris.* Bioresource Technology, 99(11), pp: 4717–4722.
26. Lordan, S., Ross, R. P., & Stanton, C. (2011b). Marine Bioactives as Functional Food Ingredients: Potential to Reduce the Incidence of Chronic Diseases. Marine Drugs, 9(6), pp:1056–1100.
27. Mabberley, D. J. (2004). Citrus (Rutaceae): A Review of Recent Advances in Etymology, Systematics and Medical Applications. Blumea, 49(2), pp:481–498.
28. Martins., Caetano., &Mata. (2010). Microalgae for biodiesel production and other applications: A review, v. 14, pp: 217–Mercer P, Armenta RE. Developments in oil extraction from microalgae. Eur J Lipid Sei Technol 2011 ;113:539-47.
29. Nurul Azlina Binti Mohamed, (2005), "Study on important parameters affecting the Hydro-distillation for Ginger oil production", M.Sc. thesis, chemical engineering, university of Malaysia.

30. Olmstead IL, Hill DR, Dias DA,Jayasinghe NS, Callahan DL, Kentish SE, et al. (2013).A quantitative analysis of microalgal Iipids for optimization of biodiesel and omega-3 production. Biotechnol Bioeng .110:2096-104.
31. Panahi, Y., Khosroushahi, A. Y., Sahebkar, A., & Heidari, H. (2019b). Impact of Cultivation Condition and Media Content on Chlorella vulgaris Composition. Advanced Pharmaceutical Bulletin, 9(2), pp:182–194.
32. Parhiz, H., Roohbakhsh, A., Soltani, F., Rezaee, R., & Iranshahi, M. (2015). Antioxidant and Anti-Inflammatory Properties of the Citrus Flavonoids Hesperidin and Hesperetin: An Updated Review of their Molecular Mechanisms and Experimental Models. Phytotherapy Research, 29(3), pp:323–331.
33. Peng, J., Yuan, J., Wu, C., & Wang, J. (2011). Fucoxanthin, a Marine Carotenoid Present in Brown Seaweeds and Diatoms: Metabolism and Bioactivities Relevant to Human Health. Marine Drugs, 9(10), pp:1806–1828.
34. Pribyl P., Cepak V., & Zachleder V. (2012).Production of lipids in 10 strains of Chlorella and Parachlorella, and enhanced lipid productivity in Chlore/la vulgaris. Appl Microbiol Biotechnol ,94, pp:549-61.
35. Robards, K. (1997). Analytical chemistry of fruit bioflavonoids. Analyst, 122.
36. Safi C., Camy S., Frances C., Varela M., Badia E., Pontalier P-Y., et al. (2013). Extraction of Iipids and pigments of *Chlorella vulgaris* by supercritical carbon dioxide: influence of bead milling on extraction performance. J Appl Phycol.
37. Safi, C., Zebib, B., Merah, O., Pontalier, P., & Vaca-Garcia, C. (2014). Morphology, composition, production, processing and applications of Chlorella vulgaris: A review. Renewable & Sustainable Energy Reviews, 35,pp: 265–278.

38. Sahraoui, N., Vian, M. A., Maataoui, M. E., Boutekedjiret, C., & Chemat, F. (2011). Valorization of citrus by-products using Microwave Steam Distillation (MSD). Innovative Food Science and Emerging Technologies, 12(2), pp:163–170.
39. Shakir, I. K., & Salih, S. (2015). Extraction of Essential Oils from Citrus By-Products Using Microwave Steam Distillation. Journal of Chemical and Petroleum Engineering, 16(3), 11–22.
40. Skjånes, K., Rebours, C., & Lindblad, P. (2013). Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. Critical Reviews in Biotechnology, 33(2), 172–215.
41. Stephenson AL., Dennis JS., Howe CJ., Scott SA., & Smith AG.(2009). Influence of nitrogen- Iimitation regime on the production by *Chlorella vulgaris* of Iipids for biodiesel feedstocks. Biofuels,1 pp:47-58.
42. Thomas, N. F., & Kim, S. (2013). Beneficial Effects of Marine Algal Compounds in Cosmeceuticals. Marine Drugs, 11(12), pp:146–164.
43. Ursu, A., Marcati, A., Sayd, T., Santé-Lhoutellier, V., Djelveh, G., & Michaud, P. (2014). Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*. Bioresource Technology, 157, pp:134–139.
44. Xavier, S., Barbosa, C. C. F., Barros, D., Silva, R., Oliveira, A., & De Freitas, R. M. (2007b). Vitamin C antioxidant effects in hippocampus of adult Wistar rats after seizures and status epilepticus induced by pilocarpine. Neuroscience Letters, 420(1), pp:76–79.
45. .Yeh, K. L., & Chang, J. S. (2012). Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga *Chlorella vulgaris* ESP-31. Bioresource Technology, 105, pp:120–127.
46. Yeh, K.L., & Chang, J. S. (2011). Nitrogen starvation strategies and photobioreactor design for enhancing Iipid content and Iipid production of a newly isolated microalga *Chlorella vulgari*s ESP-31: implications for biofuels. Biotechnol J, 6:1358-66.
47. Zheng, H., Yin, J., Gao, Z., Huang, H., Ji, X., & Dou, C. (2011). Disruption of *Chlorella vulgaris* Cells for the Release of Biodiesel-Producing Lipids: A Comparison of Grinding, Ultrasonication, Bead Milling, Enzymatic Lysis, and Microwaves. Applied Biochemistry and Biotechnology, 164(7), pp:1215–1224.

APPENDIX

APPENDIX 1

CULTURE MEDIUM

BG 11 : 0.3254 g

Distilled water : 200mL

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