STUDIES ON EFFICACY OF *CHLORELLA VULGARIS* FOR THE PRODUCTION OF BIOPLASTICS

Dissertation submitted in partial fulfillment of the requirements for the award of degree of **Master of Science** in

BOTANY

By

ARCHANA K T

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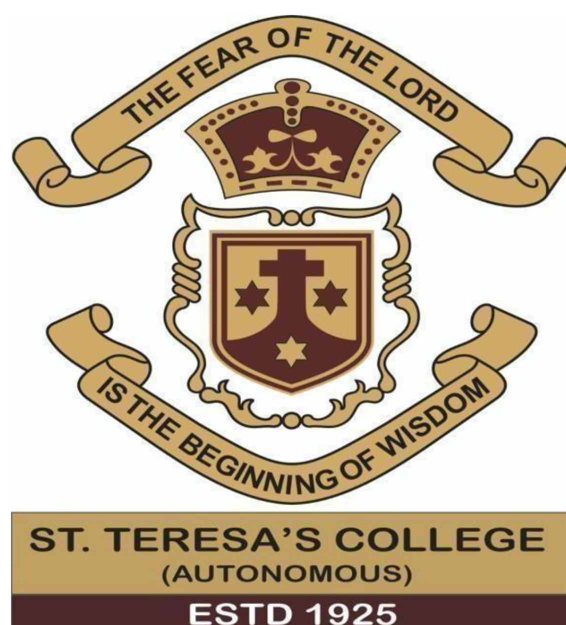

DEPARTMENT OF BOTANY

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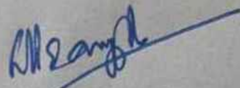
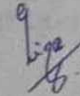


Dr. Elsam Joseph
Supervising Teacher
Department of Botany
St.Teresa's College (Autonomous)
Ernakulam



Dr. Liza Jacob
Head, Department of Botany
St.Teresa's College (Autonomous)
Ernakulam

Place: Ernakulam

Date : 12-04-2023

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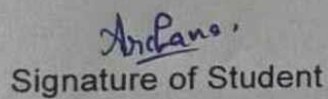

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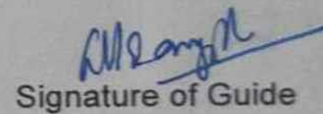

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I hereby declare that the work which is being presented in the dissertation, entitled **"STUDIES ON EFFICACY OF *CHLORELLA VULGARIS* FOR THE PRODUCTION OF BIOPLASTIC"**, in fulfillment of the requirements for the award of the degree of Master of Science 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.

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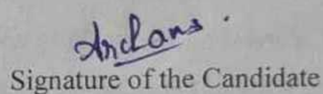

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ABBREVIATION

ml	-	Mililiter
µl	-	Microliter
µg	-	Microgram
nm	-	Nanometer
mg/l	-	Milligram per liter
g	-	Gram
rpm	-	Revolution per minute
OD	-	optimal density
PHB	-	Polyhydroxybutyrate
PHA	-	Polyhydroxyalkoantes
PLA	-	Polylactic acid
PP	-	Polypropylene
PVA	-	Polyvinyle alcohol
LDL	-	Low-density lipoprotein
FW	-	Food waste
FTIR	-	Fourier transform infrared
MTHF	-	Methyltetrahydrofuran
ISO	-	International organisation for standardisation
CTAB	-	Cetyl Trimethyl Ammonium Bromide
BG-11	-	Blue Green 11
DNA	-	Deoxyribonucleic acid
PCR	-	Polymerase Chain Reaction
rRNA	-	ribosomal Ribonucleic acid

ABSTRACT

Global plastics consumption has had a serious negative impact on the environment. To solve this problems, it is necessary to provide an alternative. Bioplastic is an alternative to traditional plastic. Bio plastic are plastic material made from natural sources like plants and other biological material instead of petroleum. Algal bio plastics have the potential to replace conventional plastics. The current study is conducted to findout the efficacy of *Chlorella vulgaris* for the production of bioplastic. *Chlorella vulgaris* has been identified as a major source for bioplastic production. The most extensively investigated biodegradable plastics is PHB that does not emit any toxins into the environment. PHB is a naturally occurring biopolymer in the cells of many bacteria and algae and is widely used in packaging and biomedical industries. *Chlorella vulgaris* was successfully cultured in BG-11 culture media under sterile laboratory condition. The bioplastic production was carried out by using PHB extracted from *Chlorella vulgaris*. The obtained bioplastic was thin, transparent and biodegradable

CHAPTER 1

INTRODUCTION

INTRODUCTION

Plastic are pervasive in the environment and become a major problem in many areas of our society. Production of plastics are globally increasing, due to the rising demand of plastic. Huge amount of plastics are being liberated in to the environment. Nowadays plastics are inevitable because it is being used in almost all the sectors. Starting from simple packaging material to complex automobile manufacturing process requires plastics. Such a huge production of plastics are menacing to the environment. Plastic are not easily degradable, they breakdown into fine nano-particle that will remain in the food chain causing harm to animals. Products made with plastic have chemical additions. Several of these substance have been linked to severe health issues, including tumours linked to hormones, infertility and neurodevelopmental disorders including ADHD and autism.

In the coastal and marine ecosystems all around the world, plastic pollution is acknowledge as a serious anthropogenic problem. Mega plastics, macro plastics, meso plastics and micro plastics are the four major levels that can be used to categorise the accumulated plastic waste in the ocean basins. Micro plastic are prevalent in marine and coastal system, while synthetic pollutants interact chemically with organic contaminants and metals. According to the U.S National Ocean and Atmospheric Administration (NOAA) and the European Chemical Agency, micro plastics are pieces of any type of plastic that are less than 5mm (0.20) in length. They pollute the environment by getting into the natural ecosystem through a variety of channels, such as clothes, food packaging, cosmetics, and industrial operations. Every sub-zone and layer of coastal and marine systems (pelagic and benthic) is densely populated with micro plastic pollution. One of the main elements influencing the chemical breakdown of plastics is salinity. In order to determine the nature of massive problem, scientific evidence on the distribution and permanence of micro plastic pollutants must concentrate on ocean basins and coastal ecosystem. Numerous marine creatures of different types consume micro plastics.

An additional rising issue is the chemical effects of swallowed macro and micro plastics. Heavy metals, plasticizers, and other plastic chemicals from the production process, as well as compounds that have adhered to plastic from the environment delivery vehicles for harmful contaminants (eg., heavy metals). For instance, it has been discovered that some micro plastics contain chemicals that some micro plastics contain chemicals that are known to be mutagenic, carcinogenic and reproductive toxic. The effect on food safety are not yet fully known,

although these chemicals may ingested at various trophic levels and cause bioaccumulation along the food chain.

During COVID-19 pandemic, the amount of plastic waste produced was increased due to the increased need for protective gear and packaging material. An increased amount of plastic, particularly from masks and medicinal waste, which results in ocean plastic pollution (Shams *et al.*, 2021) According to several media articles, the plastics industry is attempting to promote single use plastic production by capitalising on health concerns and consumer demand for disposable masks and packaging. Numerous studies have attempted to estimate how much plastic is leaking into the environment on a national and international scale, and these efforts have highlighted how challenging it is to identify the source and magnitudes of all plastic leakage.

Plastic can eventually decompose in the environment when released by a variety of processes, which would include thermal oxidation, photo degradation, hydrolysis and biodegradation (Andrady (2015); Cai *et al.*,2018). For instance, research has demonstrated that the most efficient method for the environmental plastic degradation is light-induced photo degradation (Cooper and Corcoran 2010). Studies have revealed that plastic are present in all terrestrial, aquatic and atmospheric systems (Do sul and Costa 2014). As a result of the various systems, distinctive properties, the pathways through which plastics entered different systems differed substantially. For instance, the principle source of plastic waste in the water habitat comprise wastewater treatment plants, home trash, urban pollution, industrial operations and storms (Koelmans *et al.*, 2019). According to Dris *et al.*, (2017), fibres are the type of plastic that is most abundantly found in air and is mostly caused by wind erosion, consumption of plastic trash, and urban dust. The two main forms of plastics found in soil are polythene (PE) and polypropylene (PP) (Lv *et al.*, 2019), mainly resulting from application of sewage sludge, plastic film, and waste water irrigation, and sewage sludge (Zhu *et al.*, 2019). Plastics can, however, not only penetrate into aquatic, atmospheric and terrestrial systems directly through a variety through a variety of paths, but they can also easily move between these different systems. Here, we suggest the "plastic cycle," which probably occurs in the environment and is comparable to the well-known carbon or nitrogen cycle. The phenomenon of plasticmigrating between water, the atmosphere, and the soil via various routes is known as the plasticcycle. Light plastics are far more easily collected and transported in diverse systems under theinfluence of various factors, which causes plastics to spread to some remote places of the environment. Studies have shown that plastics are present in remote places like the arctic

regions, the deep sea, and pristine mountain river basins (Allen *et al*., 2019; Woodall *et al*., 2014). Due to the long-distance transportation of plastic across many systems, plastic pollution is a worldwide concern that knows no geographic boundaries.

Bio plastic are plastic material made from natural sources like plants and other biological material instead of petroleum. Similar to traditional plastic, bio plastics have a variety of uses in everyday situation. When compared to conventional plastic, bio plastic is preferable .In several industries bio plastic have shown to be a successful alternative to plastic. Since they are made from organic natural substance including polysaccharides, proteins, and lipids, bio plastics can be used as safer substitutes for toxic petroleum-based polymers. Due to starch's abundance, renewability, sustainability, and biodegradability, starch-based bio plastics are a particularly attractive alternative. However, more study is needed to develop bio plastics so that they are both economically and practically practicable. Polylactic acid, also termed as PLA, which is usually synthesized from fermented plant starches, is the most widely used bio plastic. PLA is already widely used, frequently as single-use cups marked compostable in industrial facilities. To replace single-use items like food containers, a variety of bio plastic products are now available. Polyhydroxybutyrate (PHB) is a beneficial polymer because it can biodegrade in a variety of environments; nevertheless, the high price of high-quality PHB makes recycling an intriguing alternative.

According to The European Bio plastics Association states that "bio plastics are either bio based, biodegradable, or have both qualities". Biodegradable plastics are certified in accordance with a number of standards that specify the exact parameters for composting, including ISO 17088:2012, ISO 14855-2:2018, EN 14995:2006, EN 13432:2000, ASTM D6400-19, ASTM D5338-15 and AS 4736. Bio plastic are essential for reducing co2 emissions and combat pollution, our economy must use bio plastic. On the other hand, bio-based polymers, regardless of their biodegradability, are primarily certified in accordance with EN 16640:2015, EN 16785-1:2015, ISO 16620 4:2016 and ASTM 6866-18, that measure the renewable, "young" carbon fraction through 14C measurements or radiocarbon and elemental analysis. Since bio plastics don't need to be made using oil or crude oil- only 80% of the world's oil is required to generate plastic-fossil fuel.

Although they have been around for more than a century, biodegradable plastics have only recently gained significant scholarly and industry attention. Since the late 1800s, when the milk protein casein was used to create the biodegradable plastic Galalith TM, scientists have worked

to create bio based plastics. The first bio plastics, polyhydroxybutyrate (PHB), was developed in 1925 using bacteria. However, these bio based and biodegradable biopolymers were overtaken by inexpensive, resilient and adaptable petrochemical plastics since petroleum became the main source of fuel and chemicals in the early 1900s. Due to their appropriate disposal methods and associated environmental advantages, biodegradable plastics have once again come to the public's notice. In the early 2000s as a result of the environmental contamination caused by the improper use and disposal of such polymers.

Bio plastics can also be made from algae. Algal polymers with protein and carbohydrates bases can be used to create bio plastics. Genetic engineering can be used to increase the production of plastics (polymers) from algae. Algal bio plastics have the potential to completely replace conventional plastics in the past. Algae produce metabolites with a high content that can be converted into variety of products with additional value, providing them with a variety of commercial options. Bio plastics is also a products that extracted from algal metabolites, the most noteworthy being polyhydroxyalkanoates (PHA) and polylactic acid (PLA). Polyhydroxybutyrate (PHB) is one of the PHAs that is most frequently researched in bio plastic research. Without affecting cell division, sodium acetate in the growth media can promote PHB accumulation in cells. With the use of wastewater-cultured *Botryococcus braunii*, (kavitha *et al*; 2016) optimised the growth condition for PHB production. The data shown that the highest PHB production (247± 0.42 mg/L) was achieved using sewage water at a 60% concentration in a 40ºC , PH 7.5 growing medium (kavitha *et al.*, 2016). Utilizing algae provides a chance to increase economic efficiency through lower costs.

Various studies have conducted in several microalgae for the bio plastic production. Our environment are rich in micro algae, which can be easily cultivated, processed and used to produce biopolymers. Micro algae can grow in wastewater and have a quicker rate of growth while having no negative consequences. To create biodegradable plastic, algae's polysaccharides can be employed. Green algae belonging to the genus *Chlorella* are found in fresh water and have a protein content of about 58% (by weight). Due to its strong cell walls and greater thermal stability compared to *spirulina,* it has higher resistance to cracking. This species is frequently included in biomass-polymer mixtures. Blending is required for commercial applications after evaluating, comparing bio plastic manufacture from 100% microalgae biomass and mixtures incorporating additives and polymers. *Chlorella vulgaris*, as compared to spirulina, produced bio plastic for greater grade, according to test used to gauge product quality.

Numerous investigations have been done to determine the feasibility of using *spirulina* to produce bio plastic. *Spirulina* and *Chlorella* both have tiny cells, which makes them both desirable for the production of bio plastic blends. Considering their similarities, the varying amino acid concentrations of a *Chlorella* and *Spirulina* resulted in distinct behaviours and bio plastics characteristics when blended with PE. *Chlorella*-based bio plastics can have their product qualities enhanced by the introduction of compatibilizers.

TABLE: 1 CLASSIFICATION OF *CHLORELLA VULGARIS*	
SCIENTIFIC CLASSIFICATION OF *C.VULGARIS*	
Division	**Chlorophya**
Class	**Trebouxiophyceae**
Order	**Chlorellales**
Family	**Chlorellaceae**
Genus	***Chlorella***
Species	***C.vulgaris***

In this work, we make use of *Chlorella vulgaris*. It is a types of green unicellular microalgae in the chlorophyta division. It has existed since the Precambrian era on the earth. The majorityof *chlorella* species are freshwater, and they are particularly prevalent in environments with a lot of nutrients. They are often seen growing on soil. The microscopic, non-motile, unicellularor colonial microalgae of the genus *Chlorella* have spherical to ovoid shapes and only one chloroplast with a pyrenoid (Borowitzka *et al.*, 2018). Some *Chlorella* species have a high extracellular mucilage production capacity.

Chlorella has long been studied as a model microorganism for research on carbon assimilation and the photosynthetic apparatus because of its straightforward cell cycle, rapid growth rate, and comparable photosynthetic and metabolic pathways to those of higher plants. The cells lack flagella and have a spherical form with a diameter ranging from 2 to 10m. The chloroplast contains chlorophyll-a and chlorophyll-b, photosynthetic pigments.

Chlorella cells can reproduce under ideal circumstances with only water, sunlight, carbon dioxide and a small quantity of minerals. *C.vulgaris* is grown because it contains a lot of protein and other nutrients. *Chlorella* was thought to be the answer to the growing worldwide shortage of food in the late 1940s. These micro algae are widely used for the production of cosmetics, medicinal treatments, and even the detoxification of heavy metals in wastewater because they contain significant amounts of carbohydrates, intercellular proteins, lipids, vitamin C, carotenes and B vitamins (B1, B2 and B12), in addition to chlorophyll.

For many bioremediation procedures, *C.vulgaris* has been the preferred microalga. *C.vulgaris* has become a potential microorganism in bioremediation studies for a number of pollutants due to its capacity to remove them from wastewater and effluents, including inorganic nutrients (nitrate, nitrite, phosphate, and ammonium), fertilisers, heavy metals, detergents, pesticides, pharmaceuticals, and other emerging pollutants, as well as having high growth rates and easy cultivation requirements.

OBJECTIVES

1. Studies on efficacy of Chlorella vulgaris for the production of bioplastics.
2. To standardise the culture parameter of Chlorella vulgaris.
3. To isolate DNA , PCR amplification and the identification of Chlorella vulgaris.
4. Extraction of PHB content for bioplastic from Chlorella vulgaris.
5. Efficacy of Chlorella vulgaris for bioplastic production

CHAPTER 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

The influence of plastic pollution on both living and non-living elements of the ecosystem has been documented for more than 50 years, and it continues to be a problem on a globally. In view of our quest for sustainability, the growing worries about the short- and long-term effects of plastic matter incorporation into foods and water cannot be overemphasised (in terms of water, food, environment, and our health) (Iroegbu *et al*., 2021). Poorly reversible plastic pollution may have a variety of negative effects on the environment, such as alterations to the carbon and nutrient cycles, habitat changes in soils, sediments, and aquatic ecosystems, concurrent biological effects on endangered or keystone species, Eco toxicity, and associated societal effects (MacLeod *et al*., 2021). Primary plastic consumption as well as product lifespan have a significant impact on plastic trash formation. Only under bad management can plastic find its way into the seas and rivers (Ritchie and Roser; 2018).

Micro plastics (MPs), which are described as plastic particles ranging in size from 1 m to 5 mm, are increasingly omnipresent in both aquatic and terrestrial habitats. Concerns about the harmful impact of widespread micro plastic contamination on living things are increasing. (Ritchie and Roser; 2018). Micro plastics occur in a variety of shape and size, including spheres, pieces, and threads. Most come from the degradation of bigger polymers (macro plastics), with the exception of micro beads that are purposefully created. Over time, micro plastics break down into smaller and smaller pieces of trash, eventually turning into nanoplastics (Hale *et al*., 2020; Lambert & Wagner, 2016; Hartmann *et al*., 2019). As a result,micro plastics primarily represent a condition in transition between and nanomaterial and macro debris. Besseling *et al*., 2018 evaluated that Spherical micro plastics disintegration has the potential to produce $>10^{14}$ times more nanoparticles. Recent occurrences of micro plastics have been discovered in places thought to be free of pollution, such as the Antarctic, deep oceantrenches, distant mountain ranges, and Arctic sea ice (Hale *et al*., 2020). Many early studies concentrated on defining the degree of micro plastic pollution, as is typical with new environmental challenges, even if sampling and analytical techniques are not yet sufficient to the task. Additionally, research is also trying to expand on the creation, transport, destiny, different organism exposure, and ecological consequences of micro plastics (Hale *et al*., 2020).

Environmental problems with plastic contamination are serious. Plastic waste, or plasticobjects found in natural settings without serving their original purpose, is tenacious, mobile,

and common in aquatic and terrestrial ecosystems, includes urban, rural, and distant areas (Hartmann *et al*., 2019). Large amounts of plastic waste are easily seen and have a negative impact on animal species via entrapment, consumption, and lacerations. Micro plastics, or minute pieces of plastic detritus, had, until recently, mostly been regarded as a source of plastic pollution. There is a growing amount of study being done today on how micro plastics originate, how they further fragment, how chemicals interact, how they end up in the environment, and what effects they could have.

The environmental issues brought on by leftover synthetic plastics, have made it possible to look for alternatives. Bio plastics, which are both functionally similar to synthetic plastics and environmentally sustainable, are touted as promising new materials to address these problems (Atiwesh *et al*., 2021). Bio plastics, which are renewable and biodegradable, can be utilised to lessen the issue of plastic waste, which is strangling the earth and polluting the ecosystem. Similar to any other polymeric material, the composition, degree of crystallinity, and environmental conditions all affect how quickly bio plastics degrade, with periods ranging from a few days to several years. These factors have made the creation of biodegradable bio plastics more popular recently. (Atiwesh *et al*., 2021). When the production of biopolymer (bio plastic) is compared to that of all other types of plastics, it is revealed that just 1% of the 320 million tonnes produced by other types of plastics are bio plastics (Mozaffari *et al*., 2019).

Bio plastic is also called as bio-based plastic. It can be produced either by removing sugar from plants including corn and sugarcane and converting that into polylactic acids (PLAs), or it may be produced using polyhydroxyalkanoates (PHAs) created using microorganisms. A wide range of base materials produced by algae can be used to assemble bio plastics. Carbohydratesand hydrocarbons are the two most important chemical substances. An interesting polymer forbio-derived and biodegradable polymers is polyhydroxybutyrate (PHB), which is a member ofthe polyester class. The biomass that is collected from the pond with lot of *Microcystis sp*, wereused for bio plastic production. Therefore, in this work it was clear that the algal bio plastic hasa good plasticizing potential (Abdo and Ali; 2019).

PHB (poly-hydroxybutyrate) is a thermoplastic that works well and shares many properties with common commercial polymers like polypropylene. PHB-based plastic alternatives are fully biodegradable and leave no residue, however they are less flexible than conventional plastics (Robert and Iyer; 2018). PHB is produced by numerous types of bacteria and algae as a food storage substance (Falcone, D. B; 2004). Due to its many benefits, including its high

yield and versatility in settings, algae make an excellent source for the manufacturing of plastic (Balaji *et al*., 2012). In a study conducted by Robert and Iyer (2018), algae are used for PHB production. The advantage of employing algae in industrial PHB production is that it can use solar energy to transform waste carbon dioxide, a greenhouse gas, into environmentally acceptable plastics.

Food waste (FW) is one of the biological sources of polymers that can be used to make bio plastic. A biodegradable plastic packaging material made from food waste such as coffee grounds, banana peel, rotten tomatoes, mouldy bread, and other food scraps that end up in landfills could be developed as a substitute for single-use plastics. About 15 million tonnes of food were wasted each year in the UK (Salemdeeb *et al*., 2017). Every year, the USA produces between 567 and 726 million tonnes of FW, which is up to 40% of all food production (US EPA, n.d.). This amount of FW is equal to 218 billion USD (Tsang *et al*., 2019). In France, the govt has already put in place a strategy to encourage the utilisation of FW through the recovery of energy (for example, biogas) and value-added products (for example, bio plastic), together with a penal law in the face of an FW epidemic (De Clercq *et al*., 2017). The majority of the publications focused on the production techniques for bio plastics, the operating circumstances, and novel bacterial/ archaeal species employed in the fermentation process, even though numerous papers indicated the simultaneous transformation of food waste into energy and bio plastics. The potential synthesis of polyhydroxyalkanoates (PHA) was only briefly mentioned in a small number of these research.

In a study done by Azieyanti *et al*., 2020 using banana peel for bio plastic production. This research's main goal is to create a bio plastic utilizing food scraps, banana peels. The bio plastic polymers were produced using two techniques. In the initial experimental processes, a paste made from banana peels was combined with chemical-based components and glycerol as a plasticizer. In the second test, glycerol was used as a plasticizer while combining a paste made from banana peels with natural-based components. Compared to chemical-based materials, natural-based materials were significantly less expensive. In contrast to chemical, which is difficult to locate and potentially dangerous, it was also simple to obtain. Bio plastic composites made from natural materials are robust and long-lasting, whereas those made from chemical components are extremely delicate and sensitive.

According to Bohmert *et al* (2000) describe transgenic *Arabidopsis thaliana (L.) Heynh*, plants that express the three enzymes necessary for the biosynthesis of polyhydroxybutyrate (PHB).

In the form of PHB, these plants stored more than 4percent of its fresh weight in thechloroplasts of their leaves. The amount of PHB formed was four times higher than previouslyreported levels, demonstrating plants' enormous potential to produce one such renewable resource. Numerous bacterial species accumulate the high molecular weight polyester polyhydroxybutyrate (PHB), which is also a biodegradable thermoplastic, as a form of carbon storage. Algae can increase economic efficiency by lowering expenses by using intracellular poly-hydroxybutyrate (PHB) accumulation for the synthesis of bio plastics. As a PHB accumulator with short generation cycles, the *cyanobacterium Nostoc muscorum* has a lot of potential as a raw material provider. In this study, a variety of experimental setups were investigated, including several phosphate-starved cell growth scenarios with the inclusion of outside carbon sources. The highest, absolute PHB accumulation was measured in a phosphate-starved medium with 1% (w/w) glucose and 1% (w/w) acetate. PHB accumulated inside algae cells (Haase *et al.*, 2012).

The most extensively investigated biodegradable plastics is polyhydroxybutyrate (PHB) that does not emit any toxins or residues into the environment, unlike petroleum-based plastics. PHB is one of the best derivative of PHA. A total of sixteen isolates were gathered and purified as part of a study to test PHB accumulating bacteria from marine sponges. The bio plastic was extracted using a chloroform and sodium hypochlorite solution dispersion, and its PHB identity was confirmed by FT-IR and 1H NMR analysis (Aryaraj and Pramitha; 2021)

PHBs are produced by a variety of bacteria from various environmental niches, and marine bacteria are infrequently found to synthesise PHBs. Most of the bacteria that produce PHB have been identified from soil and activated sludge. In general, marine habitats offer a special habitat for bacteria exposed to a wide range of environmental factors, such as temperature, salinity, nutrient constraint, and pressure extremes (Aryaraj and Pramitha; 2021). Vibrio was first identified as a PHA-producing bacterial genus and was isolated from several maritime environments (Aryaraj and Pramitha; 2021; Oliver and Colwell; 1973). In marine *haloarchaea*, the first PHB accumulation was reported in *Halobacterium sp*. from the Dead Sea and it was verified through the free-fracture technique (Aryaraj and Pramitha; 2021; Kirk and Ginzburg, 1972). This was followed by the genera *Halococcus*, *Halorubrum*, *Haloarcula*, *Haloquadratum*, *Haloterrigena*, *Haloferax*, *Natronococcus*, *Natrialba*, and *Natronobacterium*, they were also recognised as significant PHB producers (Aryaraj and Pramitha; 2021; Poli *et al.*, 2011). The sodium hypochlorite extraction technique was chosen for PHB extraction. To obtain PHB crystals, the precipitate was left to evaporate (Aryaraj and Pramitha; 2021).

Yabueng and Napathorn (2018) looked into a number of eco-friendly solvents for PHB extraction, including 2-MTHF, 1, 3-dioxolane, ethyl lactate, and 1, 3-propanediol. 1, 3-dioxolane, one of the green solvents tested, had the highest rate of PHB dissolution, while water and 1, 3-propanediol had no effect at all. This is the first report demonstrating the ability to dissolve PHB using 1, 3-dioxolane, which has a polarity index of 7.85 compared to chloroform's 4.1. As a result, based on this discovery, the solvent's polarity index is not a crucialvariable for PHB dissolution.

Microalgae could serve as a more advantageous biomass source for the manufacturing of bio plastics, because it can thrieve on waste resources, does not interfere with food sources, and may accumulate a lot of lipid (Rahman and Miller; 2017). Additionally, the production of bio plastics from microalgae can be more environmentally friendly and support both the circular economy and the biological economy (Mohan *et al*., 2019). Bio plastics are used in all industries like cosmetics, pharmaceuticals and food packaging. There are two main methodologies that have been studied in relation to the manufacture of bio plastics from microalgae sources. Microalgae biomass, bio- or petroleum-based polymers, and additives canbe combined to create composite materials that are known as bio plastics. Thermal mechanical processes, such compression moulding, are used to create these products. The alternate strategy relies on intracellular starch and polyhydroxybutyrates (PHBs) cultivation. The manufacturingof bio plastic can then be accomplished by extracting and processing these components. The microalgae cells in this case are not directly used. For the production of PHB, algae are employed. The advantage of employing algae in industrial PHB production is that it can use solar energy to transform waste carbon dioxide, a greenhouse gas, into environmentally acceptable plastics.

Microalgae have become a successful dual method for bio-valorising food processing sewage and food scraps hydrolysate, which encourages microalgae development into providing value-added by products primarily carbohydrates, lipids and proteins to the benefits of producing bio plastic. Additionally, before being released into the environment, several microalgae have effectively removed a significant proportion of organic contaminants from wastewater used in the food processing industry. When compared to the conventional approach, the development of microalgae cultivation in food processing sewage significantly reduced the cost of treatment of wastewater through reducing carbon emissions, energy use, and chemical utilisation while generating algal biomass that can be used to make low-cost fertiliser and bio plastics. According to a research on numerous microalgae species, they were all able to thrive on

hydrolysates of food waste and produced biomass that was rich in lipids, protein, carbs, and fatty acids. Food wastes may now be extracted using a variety of ways and used to the manufacturing of bio plastics (Chong *et al*., 2014).

Microalgae are available in a wide variety of species and strains, and each has a unique chemical make-up and set of physical traits based on the market demands. Based on their highnutrient accumulation and quick growth rates, 4 strains were selected from among the many others. *Chlamydomonas*, *Chlorella*, *Botryococcus*, and *Spirulina* all have strong growth and survival rates in food waste medium, making them future possibilities for bio plastic manufacturing. Safi *et al*., 2014 found that the *Chlorella* has a significant capacity forcollecting lipids, proteins, and carbohydrates, up to 40%, 42-58%, and 12-55% of dry cell, etc.In addition, comparing to the other algae, *Spirulina* produces a high concentration of beneficialchemicals, including carotenoids, antioxidants, minerals, and vitamins. It also has the largest protein accumulation (46–63% dry cell).

The most popular species used in the production of biopolymers and plastic blends were *Chlorella* and *Spirulina*. An experiment (Zeller *et al*., 2013) concentrates mostly on thermo mechanical polymerization using microalgae protein biomass, including *Chlorella* and *Spirulina*, to create algal-based bio-plastic and thermoplastic blends. According to experimental findings, pressure, temperature, plasticizer concentration, and processing time areimportant factors in the polymerization and structural stabilisation of both algal proteinbiomass and thermoplastic blends incorporating polyethylene polymer during compression moulding. *Spirulina* microalgae performed better in blends than *Chlorella*, whereas *Chlorella* shows better bio plastic behaviour. (Khalis and Dianursanti 2018) In this investigation, PVA and *C.vulgaris* were utilised to make bio plastic. Because of its high biopolymer content, *C.vulgaris* is recommended as the new possible raw material (Protein, carbs). The downside of *Chlorella*/PVA is that it has poor physical-chemical qualities. Realizing the natural and synthetic materials have various qualities, a compatibilizer is required to enhance the uniformity and compatibility of combinations of the two types of materials. The objective of this work is to determine the optimal maleic anhydrates content as a compatibilizer for PVA-*Chlorella* plastic-based. This work concentrates on the thermal - mechanical polymerization from microalgae protein biomass, including *Chlorella* and *Spirulina*, to create algal-based bio plastics & thermoplastic mixtures (Zeller *et al*., 2013).

Chlorella vulgaris is a type of green unicellular microalgae belongs to division chlorophyta. In Japan, it is mostly utilised as a food additive that is high in protein or as a nutritional supplement. Martinus Willem Beijerinck found this single-celled algae in 1890 and it was the first microalgae to have a clearly defined nucleus. One of the most thoroughly studied microalgae is *Chlorella vulgaris*. Hence, a lot of research has been done on bio refinery using this microalga. Approximately 51% to 58% of Chlorella vulgaris' dry basis weight is made up of protein. Because of its rounded form and thick cell wall made of cellulose and pectin, Chlorella vulgaris is also resistant to cracking (Khalis and Dianursanti; 2018). Compared to other microalgae like *Spirulina plantesis*, *Chlorella vulgaris* has a higher heat stability (Zeller *et al*., 2013).

According to Daliry *et al*., 2017, One of the best accessible options for producing microalgae-based biodiesel is *C.vulgaris* microalgae, because to its availability and the adaptability of its cultivation conditions. Numerous studies have been done in this area. Since the cultivation of vulgaris for the production of fuel requires financial considerations to be made, it is crucial to first provide costs for the production of biomass and lipids. This study aims to identify the best conditions for the cultivation of this beneficial specie by reviewing all of the research done. The ideal environment for cultivating vulgaris, according to research, is a mixotrophic regime carried using a bubble column photobioreactor. Among nutrition circumstances, potassium nitrate and glucose are the most effective. The ideal operating conditions were indicated by an alkaline solution and 200 mL/min of aeration. The most effective rate of lipid synthesis was 66.25 mg/L/day.

Chlorella sp., *Spirulina sp.,* and *Nitzschia sp*. are the primary microalgae sources that are typically used to manufacture biodiesel, according to Abdullah *et al*., 2017; Chisti, Y; 2007). This is so that these three types of microalgae may produce the maximum oil. The sustainability of algal-based biodiesel is a fiercely disputed topic right now because oil extraction via algae is one of the more expensive procedures. The study conducted by Abdullah *et al*., 2017 choose *C.vulgaris* for bio diesel production. *C. vulgaris* seems to be the greatest option and the most appropriate species of algae for the work conducted. The purpose of this study is to determine the best conditions for oil extraction from *C.vulgaris*, a freshwater microalga, to generate the most oil. After making and extracting biodiesel, Das *et al*., 2018 chemically treated remaining microalgae biomass to create PHB-based bio plastic material.

Consuming *Chlorella* as a dietary supplement is popular. *Chlorella* product manufacturers exaggerate its alleged health benefits, especially its potential to treat cancer. *C.vulgaris* is a green, unicellular microalga with medicinal and biological benefits for health and wellbeing. A long-time source of food, *C.vulgaris* has a distinctive and varied composition of useful macro- and micronutrients, comprising proteins, omega-3 polyunsaturated fatty acids, polysaccharides, vitamins, and minerals. According to clinical studies, taking supplements containing *C.vulgaris* can lower blood sugar and lipid levels, as well as protect against cancer,oxidative stress, and chronic obstructive pulmonary disease (Panahi *et al*., 2016).

Chlorella is notable for its high protein content, which is one of its most notable nutritional qualities. That is about 60% of total protein contains the majority of the necessary amino acids. This quantity is three times greater than that found in beef, which is one of most concentrated sources of protein available. Low molecular weight of microalgae protein makes it easily digestible as long as the cells have been damaged (Rani *et al*., 2018). *C.vulgaris* peptides have a significant protective effect against cellular damage (Rani *et al*., 2018; Lordan *et al*., 2011). Lutein, which is produced by *Chlorella*, has been shown to have anti-cataract and anti-macular degeneration characteristics (Rani *et al*., 2018). *Chlorella* extracts were shown to have antioxidant, anti-inflammatory, and antibacterial effects on tumours. *Chlorella* has been found to improve the immune system, reduce blood pressure and cholesterol, and speed up wound healing. Low-density lipoprotein (LDL) cholesterol levels were significantly reduced and aortic atheromatous lesions were inhibited by *Chlorella*. Some of the micro algal species that are well-established in the skin care sector include *Spirulina* and chlorella. Dermochlorella®is one such product on the market that contains *C.vulgaris* and helps fight wrinkles by stimulating the production of collagen in the skin.

CHAPTER 3
MATERIALS AND METHODS

MATERIALS AND METHODS

4.1. 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.

4.2. Culture media and composition and culture parameter

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

TABLE: 2 Microalgal culture parameter		
Sl.no	Parameter	Value
1.	Working volume	200 ml
2.	Temperature	24p±10C
3.	Light intensity	700-800 lux
4.	Photo period	16/18 hrs. (light/ dark)
5.	Time	18 days

4.3. 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.

4.4. 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 620nm and 700nm in order to determine maximum absorbanceusing spectrophotometer (LAB India).

4.5. 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 fitting it.

After that, the counting chamber was allowed to stand on the bench for 2 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 squares.

Cell number of Cell density = (counted cells)/ (volume of square×Dilution factor)

4.6. Determination of chlorophyll content

The chlorophyll content of the microalgal cells was determined by using spectrophotometric techniques. Sample of the microalgal suspension was centrifuged for 10 minutes at 13000rpm (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, 405 nm (A 405) and 450 nm (A 450), using the UV spectrophotometer was blanked with methanol.

4.7. Identification of microalgae using molecular sequencing

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

4.8. DNA isolation

DNA isolation method by Doyle and Doyle (1987), Using CTAB yielded good quality DNA for PCR.

4.8.1. DNA isolation using CTAB

Freshly prepared CTAB (Cetyl Trimethyl Ammonium Bromide) buffer was preheated at 65o C, 1gm of the microalgae sample was ground in 16 ml of CTAB buffer and homogenized. The ground tissue incubated at 65°C in water bath for 30 minutes followed by incubation at the 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 (1 ml 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 sample were kept for overnight incubation at -20°C. The supernatant was decanted off and the pellets was washed with cold 70% ethanol. The DNA was further pelleted by centrifugation at 12,000 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.

4.8.2. Amount and purity of DNA

The yield of DNA extracted was measured using a UV spectrophotometer (Lab India) at 260 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 band obtained by staining with (0.5µg/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.

4.9. 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 20ng of

template DNA and primers using the PCR Master Mix (Fermentas, USA) and a thermal cycler.The reaction consisted of initial denaturation at 94ºC for 3 minutes followed by 40 cycles of denaturation at 94ºC for 30s, annealing at 55ºC for 30s, and extension at 72ºC for 1 minute, with a final extension at 72ºC 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 sequence alignment.

4.10. PHB CONTENT ANALYSIS TEST

3.10.1. Sudan black dye test

The algal culture was heat fix on glass slide and stained with 0.3% Sudan Black B stain in 60% ethanol for 10 minutes and rinsed with water and counter stained with 0.5% safranine for 5 minutes and observed under microscope.

4.10.2. Extraction of polyhydroxybutrate (PHB)

100mL of sample was taken and centrifuged at 10,000 rpm for 15 minutes. The supernatant was discarded and the pellets was treated with 10mL of sodium hypochlorite and the mixture was incubated at 30ºC for two hours. After incubation, the mixture was centrifuged for 15 minutes and then washed with distilled water and methanol respectively.

After washing, the pellet was dissolved in 5mL of boiling chloroform. The chloroform solution was concentrated to a small volume. A volume of cold methanol was added and the sample was refrigerated overnight. The precipitate PHB was collected by centrifugation.

For the production of bioplastics 0.0334g of extracted PHB from *Chlorella vulgaris*, 0.0334g sorbitol, 0.0334g, gelatin, 1.1133 ml, 2% glycerol solution. All the ingredients were mix well, and the mixture was heated to 95ºC. The mixture was stirred continuously, while by heating, and once it is at a right temperature then the mixture was poured into a dried petri plate. The time required for the separation of plastic is depending on the temperature of the room; it may take several days. After complete drying, the bio plastic was separated from the petri plate using a scalpel.

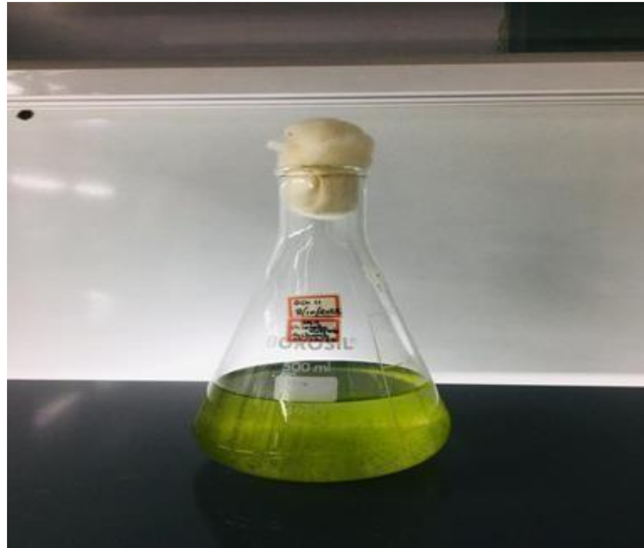
CHAPTER 4

OBSERVATIONS AND RESULTS

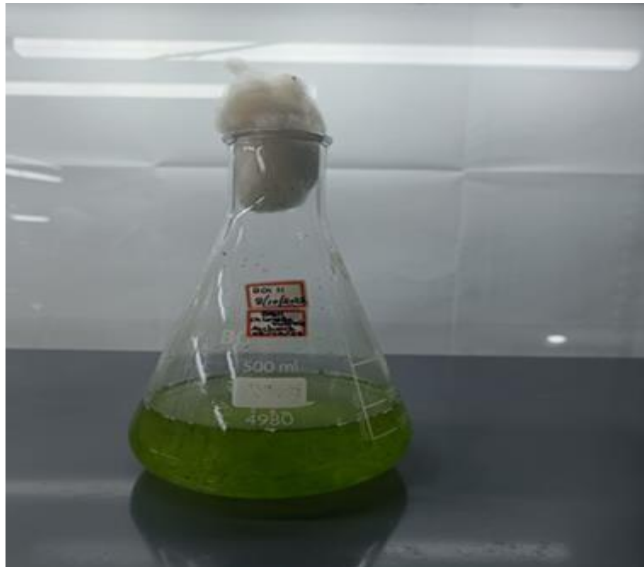
RESULTS

5.1. MICROALGAL CULTURE

The culture of *Chlorella vulgaris* was carried out in a conical flask. A gradual colour change was observed from the first day to the 15th day of inoculation. The colour change from pale green to dark green was an indication of the increase in the chlorophyll content and also the cell count.



(a)



(b)

PLATE : 1(a &b). *C.vulgaris* culture

a : *C.vulgaris* culture on the 1st day of inoculation

b : *C.vulgaris* culture on the maximum growth at 15th day

5.2. MICROSCOPIC OBSERVATION

Microscopic observation of the culture of *Chlorella vulgaris* was observed under the light microscope (40x). It showed algal cell as small and rounded in structure and also which were pale green in colour. The observation also showed the increase in growth and maturation of the culture.

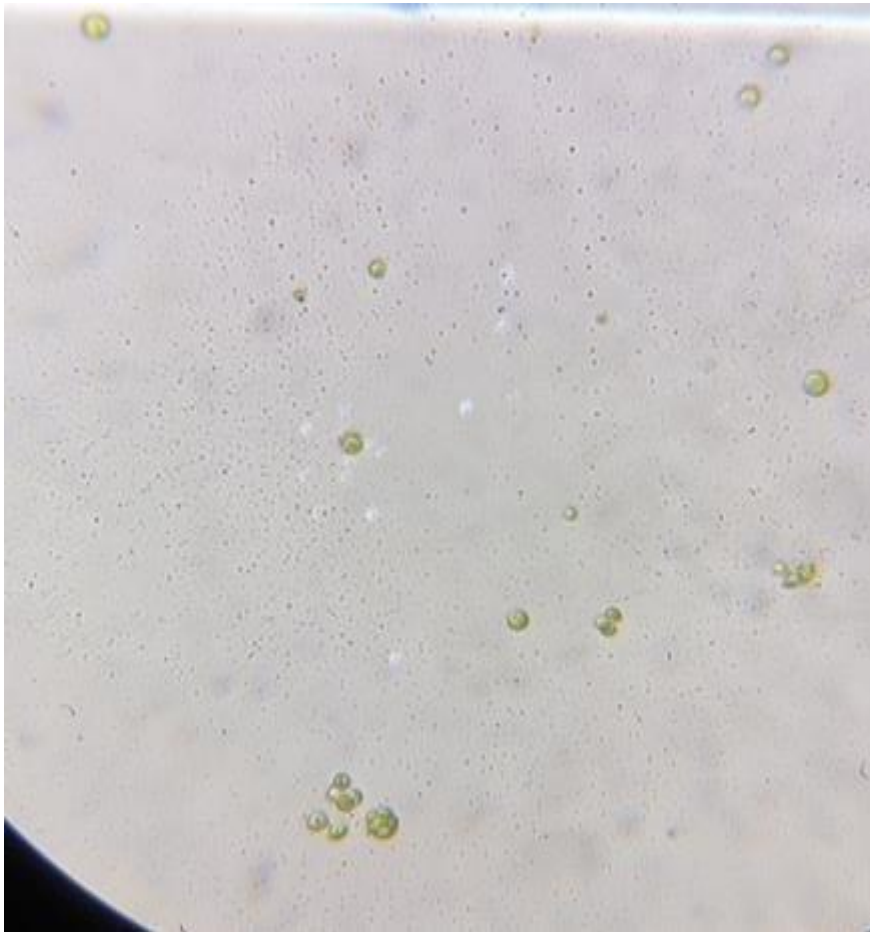


Plate: 2. microscopic observation of *C.vulgaris* under light microscope 40x

5.3. MAXIMUM ABSORBANCE DETERMINATION

The optical density of the *Chlorella vulgaris* was measured at two wavelength, 620nm and 700nm respectively. The maximum absorbance was observed at 700nm. The maximum growth were observed during the 15th day.

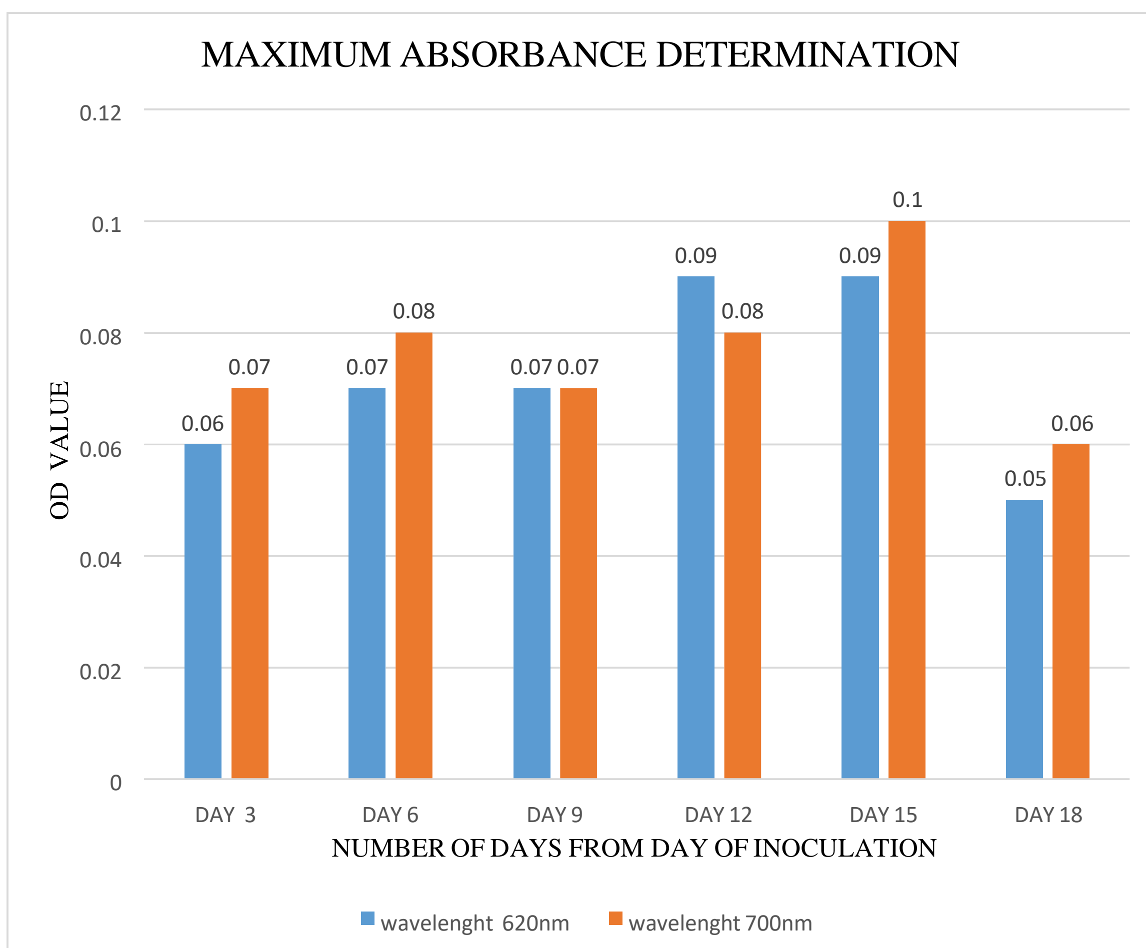


Figure : 1. Graph for maximum absorbance determination at 620nm and 700nm

5.4. CELL COUNTING USING HAEMOCYTOMETER

Cell counting using haemocytometer showed the cell density of the culture. The cell count was taken periodically. The cell counted with respective days which has been given in the graph. The cell growth were observed from the 3rd day to 18th day. The cell growth is indicated by an increase in cell density per ml. The maximum cell growth were observed at 15th day.

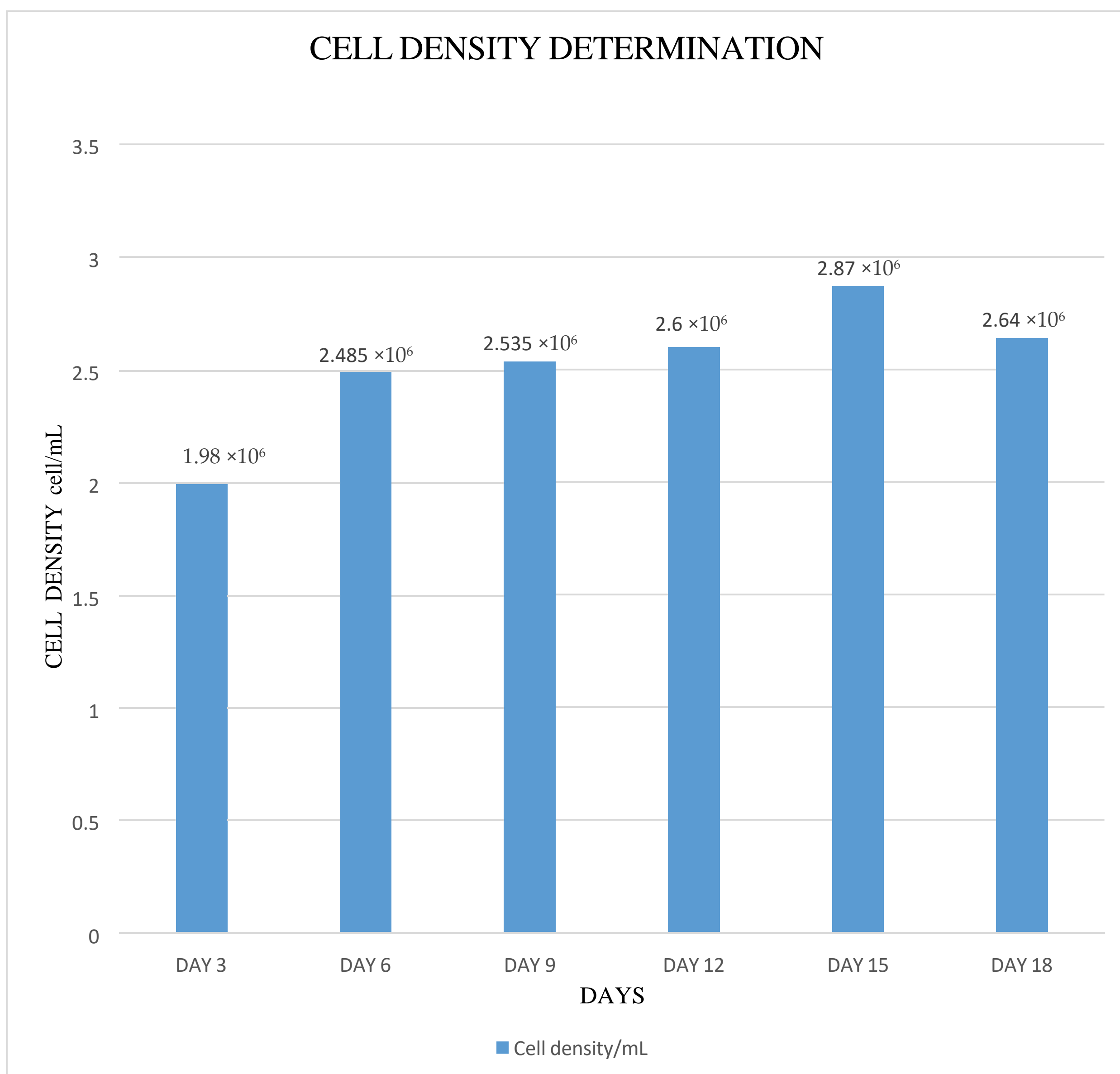


Figure : 2. Cell density determination graph

4.5. CHLOROPHYLL CONTENT DETERMINATION

The chlorophyll content was determined from the algal culture by using acetone method. The OD values was taken at two wavelengths (405nm and 450nm). By using spectrophotometer, the chlorophyll content of the algal culture were determined. The maximum chlorophyll content was observed at the 15th day.

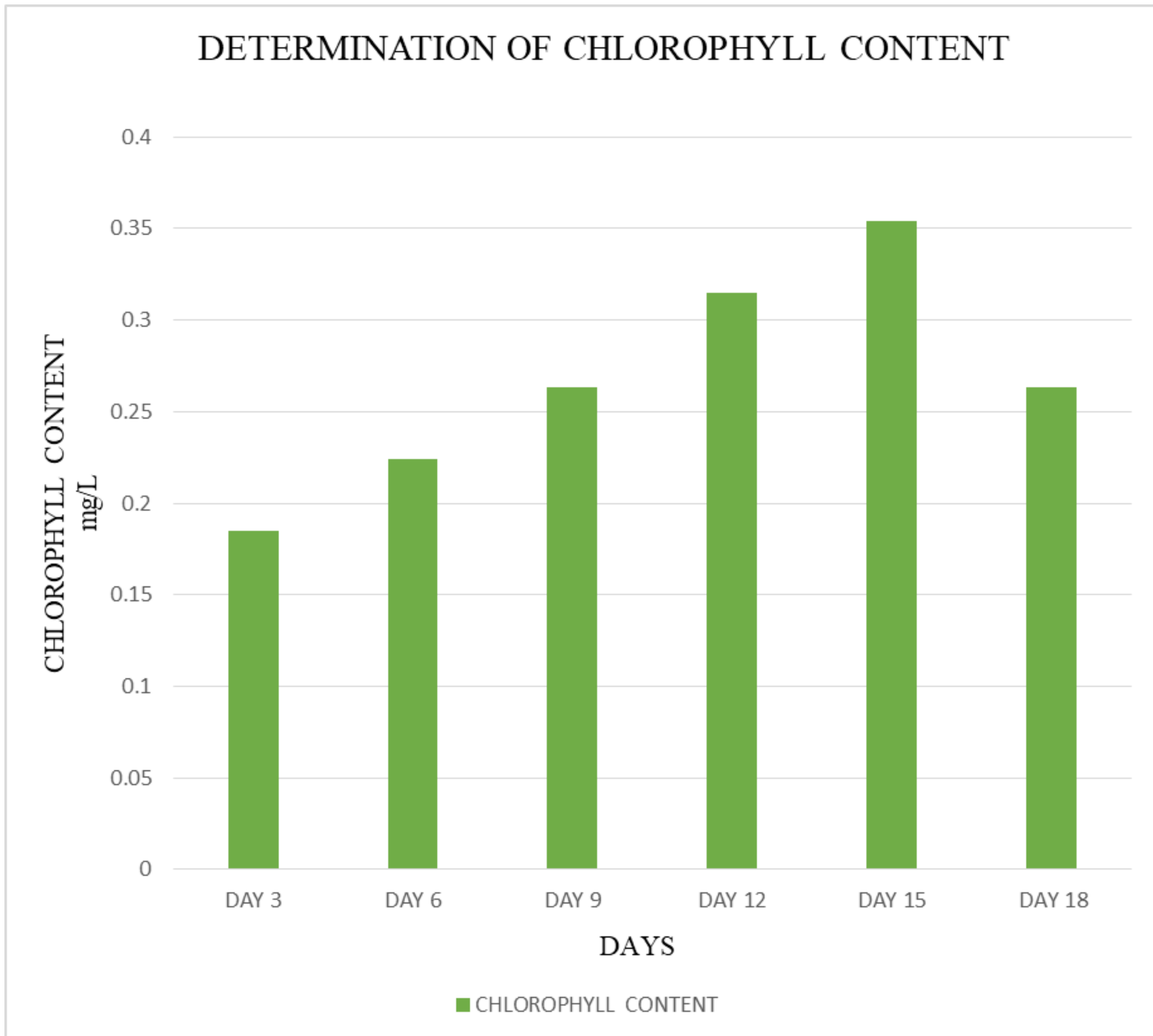


Figure: 3. Determination of chlorophyll content at 405nm and 450nm

4.6. IDENTIFICATION OF ORGANISM USING MOLECULAR SEQUENCING

For the identification of microalgae using molecular sequencing involves several steps. The first step for the identification involves DNA isolation. DNA isolation is done by using Doyle and Doyle (1987), by using CTAB yielded good quality DNA for PCR.

MBT051 S1

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

Lane (MBTO51): 100 bp marker.

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

DNA get separated based on the molecular size after the gel electrophoresis. Shorter fragments of DNA will move more faster than the larger fragments. The band pattern of the DNA will be specific for each organism thus it can be used for identification of the microalgae. The band obtained were viewed under gel documentation system.

>AF350260.1:1-586 Chlorella vulgaris 16S ribosomal RNA gene, partial sequence

AGGGACAACCATTGGAAACGATGGCTAATACCTCATAATACTGAGTAAGTTAAATGATGAATAATCGCCAAGAGATGGGC

TTGCGGCTGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGATGATCAGTATCTGGTCTGACCAGGATGATCACCC

ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAACAGTGAGGAATTTTCCGCAATGGGCGAAAGCCTGACGG

AGCAATGCCGCGTGAAGGATGAAGGCCTATGGGTTGTAAACTTCTTTTCTCAGAGAAGAAATTTTGACGGTATCTGAGGA

ATAAGCATCGGCTAACTCTGTGCCAGCAGCCGCGGTAAGACAGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAG

CGTCTGTAGGTGGCTTAAAAAGTCTCCTGTCAAAGATCAGGGCTTAACCCTGGGCCGGCAGGAGAAACTCTTAGGCTAGA

GTTTGGTAGGGGCAGAGGGAATTCCCGGTGGAGCGGTGAAATGCGTAGAGATCGGGAGGAACACCAAAGGCGAAAGCACT

CTGCTGGGCCACAACTGACACTGAGA

Plate: 5. *C.vulgaris* 16s rRNA gene partial sequence

After sequencing, the sequence that was acquired is compared to the database to see whether there are any similarities. By doing a word-by-word similarity search, BLAST is utilised to locate the matching 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 sequence display the similarity with the 16s rRNA gene of Chlorella vulgaris, as well as the chloroplast gene for chloroplast product.

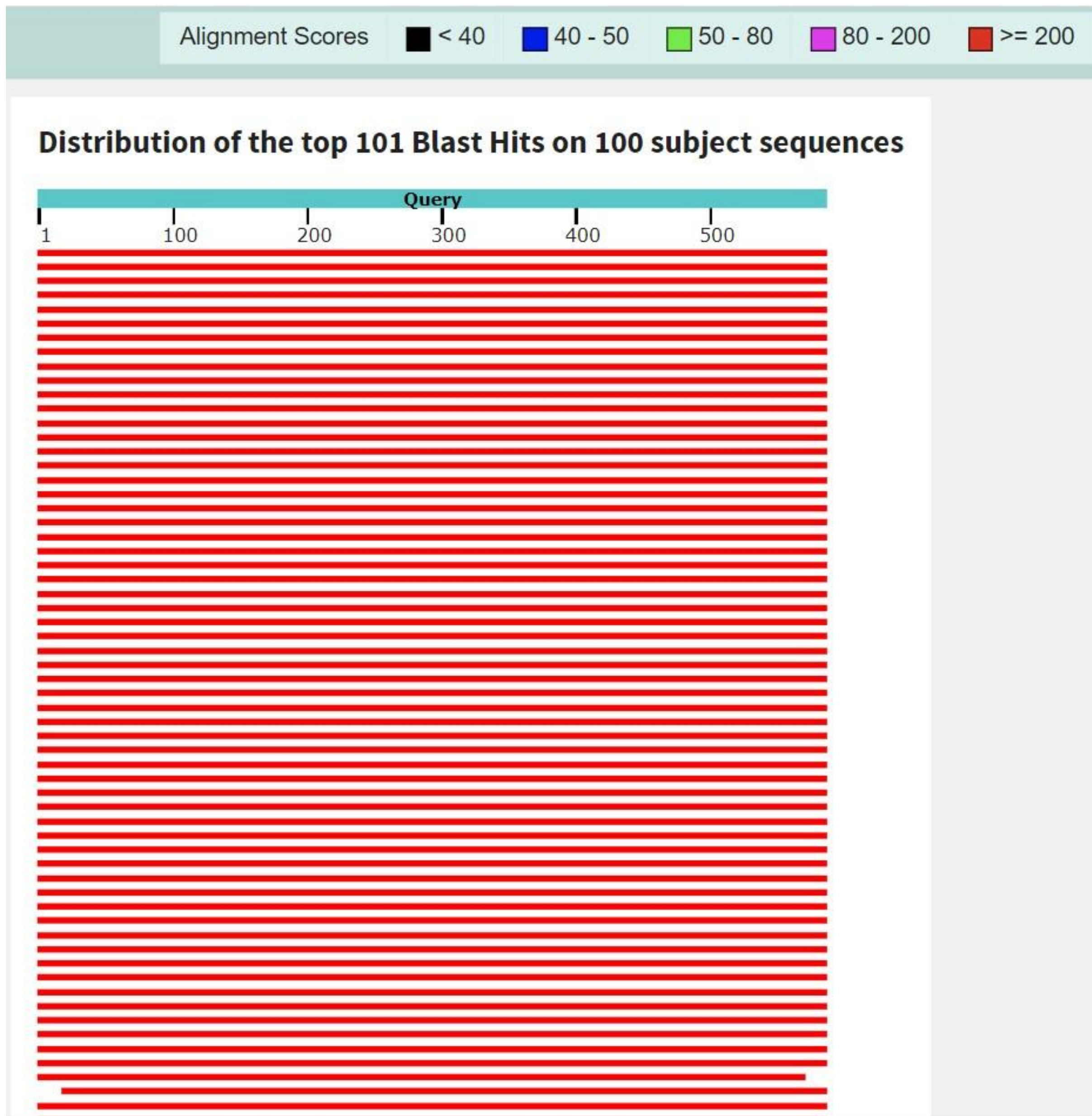


Plate: 6. Graphic summary on alignment score

The graphic summary alignment shows how closely the query sequence matches the sequences in the databases. More than 500 nucleotides make up the query length. Each one of the horizontal lines represents a sequence that is present in the database. Here, the alignment score is higher than 200, which is indicated by red lines, signifying excellent or good matching.

Mega X, a very effective tool in evolutionary studies, which is used to do phylogenetic analysis of algae.

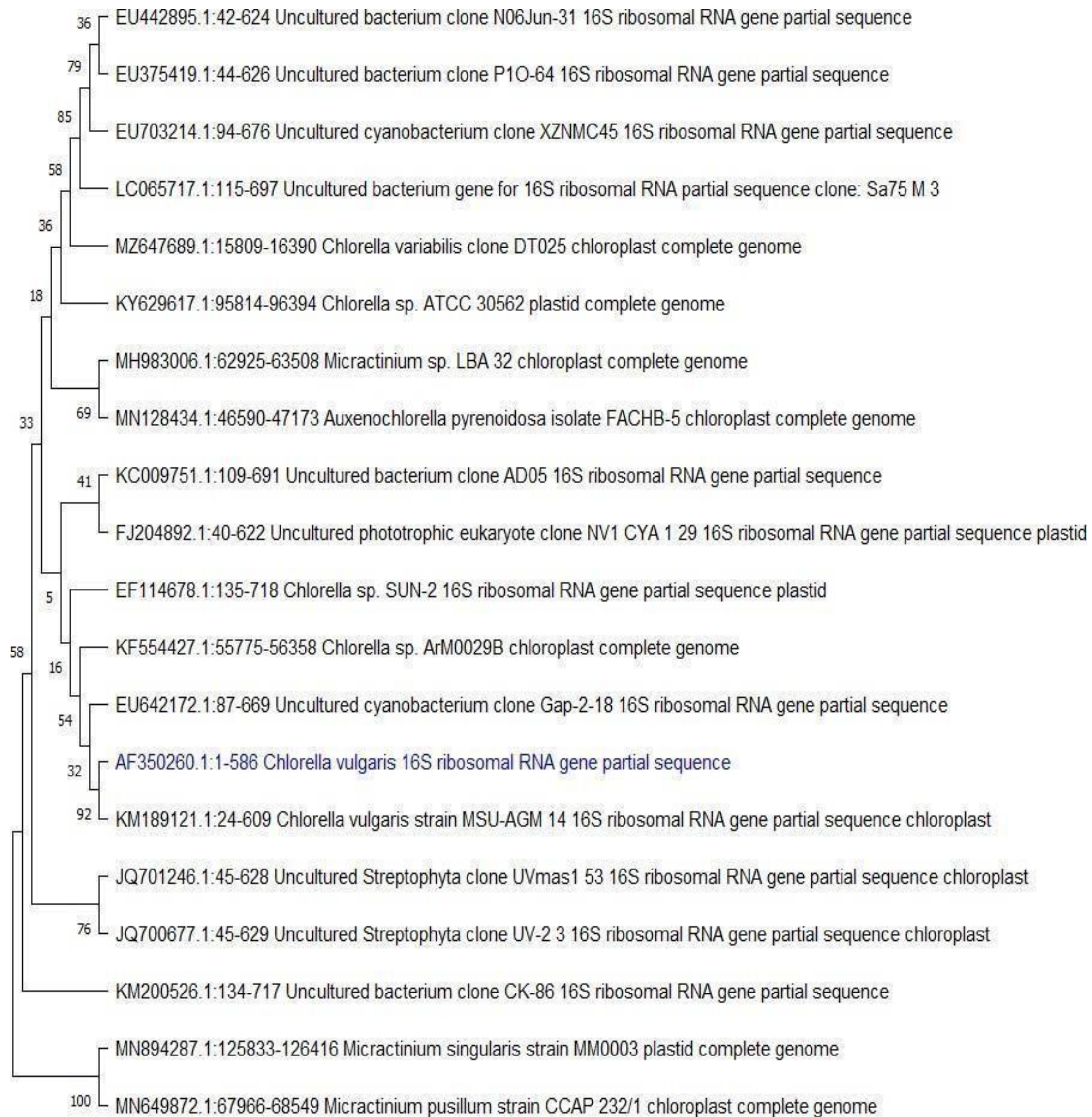


Plate: 10. Phylogenetic tree

The result of phylogenetic analysis reveals that the C.vulgaris 16s ribosomal RNA gene partial sequence show close relationship with C.vulgaris strain MSU-AGM 14 16s Ribosomal RNA gene partial sequence chloroplast and also with uncultured Cyanobacterium clone Gap -2-18 16s Ribosomal RNA gene partial sequence.

4.7. PHB CONTENT ANALYSIS TEST

4.7.1. SUDAN BLACK DYE TEST

The presence of PHB content was analysed using Sudan Black Dye test. The test is conducted for the presence of PHB content in the cultured algae. Colour change was observed in the algal culture on the glass slide.

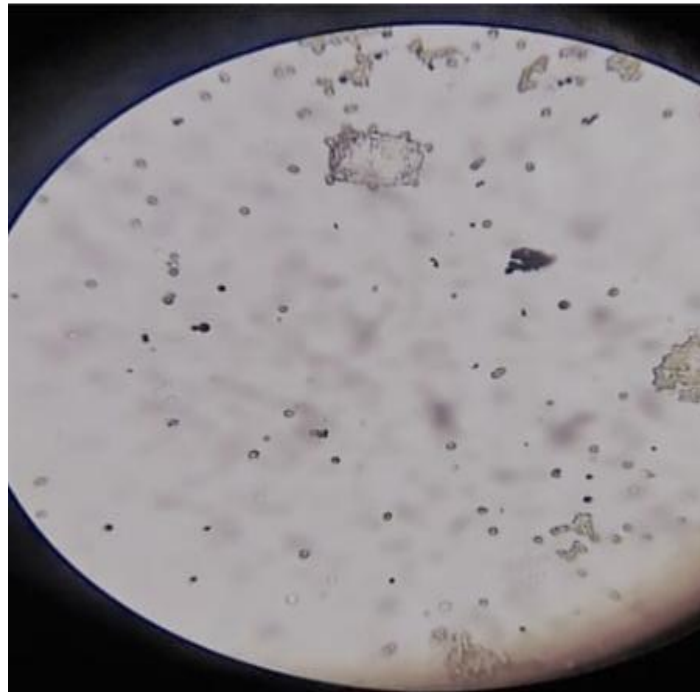


Plate: 11. Microscopic observation using sudan black dye test

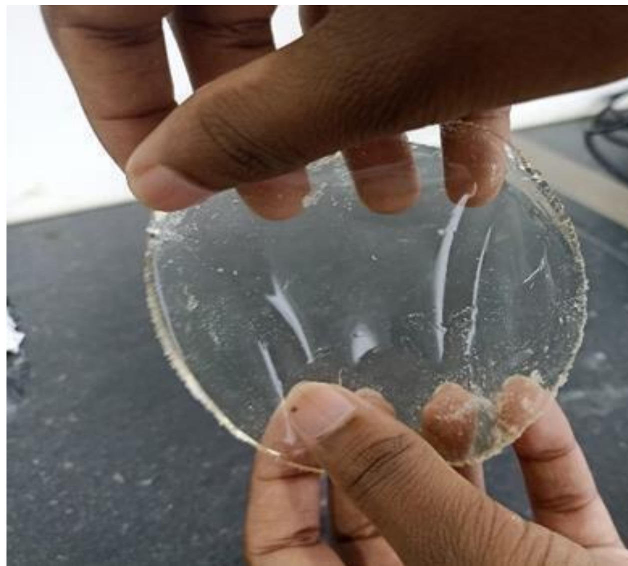
The figure 12 shows the microscopic observation of the algal culture using Sudan Black dye test. It shows a slightly pink colour to the algal culture. Presence of PHB content in the algal culture thus confirmed.

4.7.2. EXTRACTION OF PHB CONTENT

PHB content was extracted from the sample of algal culture. From 100ml of sample, 0.0334g of PHB was taken for the production of bioplastics. The obtained bioplastics was a thin biofilm, which was transparent in appeareance.

(a)

(b)



(c)

Plate: 12.(a,b&c). Bioplastic obtained from *C.vulgaris*

a : Drying of bioplastic in petridish

b : Bioplastic film separated from petridish

c : Bioplastic

CHAPTER 5

DISCUSSION

DISCUSSION

Chlorella vulgaris has been identified as a major source for bioplastic production. *C.vulgaris* is a unicellular green microalga having medicinal and biological benefits for people's health. Which is mainly found in freshwater ecosystems. 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.

The algae has been successfully culture in BG-11 culture media under sterile laboratory condition for about 18 days. The cell growth and cell count was examined periodically by using spectrophometer and by haemocytometer. Chlorophyll content of algal culture was also examined. Identification of this microalgae was performed by using molecular sequencing analysis. Bioplastic was also produced by extracting the PHB content from the *C.vulgaris.*

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. Chlorella is one of the most popular species used in the production of biopolymers and plastic blends. *Chlorella vulgaris* shows better bio plastic behaviour (Zeller *et al*; 2013). Thus it is utilized to make bioplastic.

PHB is produced by numerous types of bacteria as well as algae as a food storage substance (Falcone, D. B; 2004). Due to its many benefits, including its high yield and versatility in settings, algae make an excellent source for the manufacturing of plastic (Balaji *et al*; 2012).

PHB (poly-hydroxybutyrate) is a thermoplastic that works well and shares many properties with common commercial polymers like polypropylene. PHB-based plastic alternatives are fully biodegradable and leave no residue, however they are less flexible than conventional plastics (Robert and Iyer; 2018). PHB is a biodegradable polymer that is widely used in the packaging and biomedical industries

The most extensively investigated biodegradable plastics is polyhydroxybutyrate (PHB) that does not emit any toxins or residues into the environment, unlike petroleum-based plastics. PHB is one of the best derivative of PHA. The PHB content was extracted using a chloroform and sodium hypochlorite solution. The precipitate of PHB was collected by centrifugation. The collected PHB are then utilized for the production bioplastic.

CHAPTER 6
SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Bio plastic are plastic material made from natural sources like plants and other biological material instead of petroleum. Similar to traditional plastic, bio plastics have a variety of uses in everyday situation. Bio plastics can also be made from algae. Algal bio plastics have the potential to completely replace conventional plastics in the past. In this study, efficacy of *Chlorella vulgaris* for the production of bioplastics was studied. The microalgae has been successfully cultured in BG-11 medium under sterile environmental condition. After about 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. The observations showed increase in growth and maturation of the culture.

The maximum absorbance was also observed at 700nm wavelength using spectrophotometer. The microalgal concentration in the culture was counted by using the improved Neubauer haemocytometer counting chamber. Cell counting was taken periodically. The maximum cell growth was observed at the 15th day. The chlorophyll content of the algal culture was also examined by using acetone method. By all these observations conducted it was clear that the *Chlorella vulgaris* was highly efficient for bioplastic production.

Identification of *Chlorella vulgaris* was performed using molecular sequencing analysis involving several steps. The first step involves DNA isolation, done by using Doyle and Doyle (1987), using CTAB yielded quality DNA for PCR. DNA get separated based on the molecular size after gel electrophoresis. DNA bands obtained were viewed under gel documentation system.The sequence that was acquired is compared to the database to see any similarity. BLAST is utilized to located the matching sequence from the database.

Finally PHB content analysing test was conducted and it takes about two days to complete the procedure. Sudan Black dye test was conducted to examine the presence of PHB, It showed slightly pink colour to algal culture under microscopic observation. The PHB is then extracted from the 100ml sample by using sodium hypochloride and chloroform solution. The precipitate of PHB was collected by centrifugation. The collected PHB (about 0.0334g) is then mixed with 5mL sorbitol, 0.0334g of gelatin, 2mL of 2% glycerol were mixed well and heated (95^{0}C). At optimal room temperature, mixture were poured into a petriplate for drying. After complete dry, bioplastic film was formed and was separated from the petriplate using a scalpel. The obtained bioplastic was thin, transparent and biodegradable.

CHAPTER 7
REFERENCE

REFERENCE

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APPENDIX

APPENDIX 1

CULTURE MEDIA

BG-11 : 0.3254g

Distilled water : 200ml

APPENDIX 2

CTAB BUFFER

1M Tris HCl : 1.576 g

4M NaCl : 2.3376 g

0.5M EDTA : 1.8612 g

2% CTAB : 0.2 g

APPENDIX 3

PREPARATION OF MASTER MIX

Molecular biology grade water : 15µl

10X assaybuffer : 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