BIOSTIMULANT EFFICACY OF *TETRASELMIS STRIATA* BUTCHER FOR IMPROVING PLANT HEALTH AND GROWTH

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

BOTANY

by

AYSWARYA KIREN K R

REG NO: AM21BOT009

DEPARTMENT OF BOTANY

ST. TERESA'S COLLEGE (AUTONOMOUS)

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CERTIFICATE

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Dr. LIZA JACOB

Supervising Teacher

Department of Botany

St. Teresa's College

(Autonomous)

Ernakulam

Dr. LIZA JACOB

Head of the Department of Botany

St Teresa's College

(Autonomous)

Ernakulam

External examiner

Valued.

1. Dr. Stephen Sequeira 18/4/2023

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

ST.TERESA'S COLLEGE (AUTONOMOUS) ERNAKULAM

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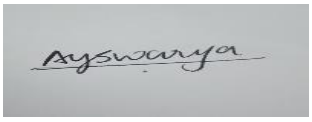
Signature of the Candidate: 

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Place : Ernakulam AYSWARYA KIREN K R

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ABBREVIATIONS

BLAST - Basic Local Alignment Search Tool

CTAB - Cetyltrimethylammonium Bromide

EDTA - Ethylenediamine Tetraacetic Acid

O. D - Optical Density

RRna - Ribosomal ribonucleic acid

16s rRNA - 16s ribosomal RNA

ABSTRACT

The arising demand of food and increasing population is a major issue and to provide an aid to that problem the present study was excecuted. Where *Tetraselmis striata* was studied to understand the biostimulant efficacy for the improvement of plant health and growth. Species were cultured, several growth parameters were studied and DNA was isolated and the microalgae was incorporated into plants and grown, several soil tests were also done. Seeds of *Vigna unguiculata* was used belonging to Leguminosae family. And the comparative studies were carried out by keeping one set of plants in normal soil without any additional inputs and another set of plants with the *T. striata* incorporated in soil. At the end of the study the plants with *T. striata* showed maximum growth on the basis of length, flowering and pods. While the plants in normal soil showed less growth and was slower in development.

Keywords: Biostimulant, Tetraselmis striata, plant growth, soil tests.

CHAPTER -1

INTRODUCTION

1. INTRODUCTION

The high demand for food and agricultural land per area is a result of an increasing population. To achieve maximum yield from plants nutrition is an important factor to control the production and the yield. So, fertilizers are mostly used to increase the production and to get the best yield from agriculture.

Fertilizer is any substance of natural or synthetic origin which is administered to soil or to plant to supply plant nutrient is known as fertilizer. There are numerous forms and types fertilizers available in markets which can be natural or artificial type. Fertilization mainly focuses on three types of macro nutrients like nitrogen, phosphorus and potassium with sporadic addition of micro nutrients like rock dust and many more. Farmers use these fertilizers in dry form or liquid form or through pellet form manually or with the help of machines. Fertilizers enhance and replace the soil nutrients which have been consumed and supply nutrients to the plants. Mostly chemical fertilizers are used since they are cheap and always available in market. There are many types of fertilizers; some of them are single nutrient fertilizer, multinutrient fertilizer, micronutrients, organic fertilizer etc.

In single nutrient fertilizer there will be a single main nutrient present. These include ammonia based like urea , ammonium nitrate, phosphate based like single superphosphate, triple phosphate, and potassium based like muriate of potash etc. Multinutrient fertilizer contains two more nutrients in them and these are mostly used in agriculures. Binary fertlizers consist two major nutrients like nitrogen and phosphorus. NPK fertilizers consist of three major components, which are nitrogen, phosphorus,potassium.

Organic fertilizer includes the use of manure and compost as fertilizer and it is one among the traditional farming practice which is still on going and give results in which chemical fertilizers fail.

The exponential rise in fertiliser use in recent years seems to have a negative impact on the environment. The deposition of heavy metals in the plant and soil systems may be impacted by fertilisation. Through the soil, plants take up the nutrients which allow them to move up the food chain. Thus, fertilising causes pollution of the water, air and soil.

The nitrogen and other chemicals reach the water body through various ways like leaching, flow, drainage. Where the nitrogen used in agriculture is not completely used by the plants

and they remain in the soil and gets into the ground water and surface water and other water bodies and pollute the water and make it unfit for further uses. The usage of nitrate mixed water leads to serious health issue in both humans and other living organisms. And another side effect is the eutrophication of the water body where nitrate and chemicals accumulate and enriches the water body and results in damaging water body and reduction in the living species, odour problems, emergence of unwanted species etc.

When chemical fertilizers are added in large amount to agriculture the compounds gets evaporated into the atmosphere and causes some major damage to the environment, vegetation and ecosystem. Many gases like nitrogen oxides, carbondioxide, methane, chloro fluro carbons etc. And these gases also contribute to the rise in greenhouse effect and ozone depletion and the chemical like NH_3 will change into acid and may cause acid rain which is harmful to all living organisms. In case of humans inhaling these chemicals lead to serious health issues.

Soil is one of the major elements in agriculture, the effect on soil leads to poor yield of crops. The effects on soil are seen as a result of continuous use of chemical fertilizers for a long time this will lead to depletion or alteration in soil composition, pH, and balance of nutrients in the soil and affect the microorganisms and humans. These chemicals will enter the soil and the plants absorb the fertilizer and into the crops and reaches the human body and in animals and this accumulation of chemical leads to serious health issues.

The chemical fertilizer can be replaced with the use of organic fertilizers or bio stimulants which will provide better yield and will not pollute or create problems to both the living organisms and the vegetation and ecosystem. Bio stimulant is any substance or microorganism that is administered into the plants or into the soil to improve the yield and provide tolerance to the abiotic factors, and enhance the growth of the plants and the intake of nutrients into the plants. There are many kinds of bio stimulant like Humic and fulvic acids ,Protein hydrolysates and other N-containing compounds, Seaweed extracts and botanicals, Chitosan and other biopolymers, Inorganic compounds, Beneficial fungi, Beneficial bacteria etc.

Bio stimulant follow certain characters like: The nature of bio stimulants is diverse, where a single or multiple compounds or organisms might be used. The physiological functions are diverse, which will be different for different plants.

It improves the efficiency of the plants metabolism, increases yield, quality of yield, increases tolerance against biotic and abiotic stresses, nutrient intake, translocation, enhances the soil properties, enhances the development of microorganisms in the soil, enhances the growth hormones etc.

Both macro algae and micro algae are being used as plant bio stimulants. More focus is on micro algae nowadays and micro algae are not that explored unlike macro algae. The micro algal bio stimulants show a great effect on the plants. Microalgae are a broad class of predominantly single-celled photosynthetic organisms that produce a wide variety of metabolites from CO2 and light. Micro algae are used in various fields such as bioremediation of waste, aquaculture, biofuels, pharmaceuticals etc. Microalgae provide a sustainable platform as a renewable source of energy and can be used in controlled condition unlike macro algae and cultivation is also easy and reliable. Considering the side effects caused by chemical fertilizer and the high price of fertilizers, increasing climate change, micro algal bio stimulants can be used in future years as it will reduce the usage of chemical fertilizer and the damage caused by it can be solved.

1.1***TETRASELMIS STRIATA***

CLASSIFICATION

Division:	Chlorophyta
Class:	Chlorodendrophyceae
Order:	Chlorodendrales
Family:	Chlorodendraceae
Genus:	*Tetraselmis*

Table 1.

Tetraselmis striata is a genus of phytoplankton. *T. striata* is a green algae it has highly coloured chloroplast, flagellated cell bodies, pyrenoid and eyespot within the chloroplast which is covered in a starch shell, scale-produced thecal-wall. These are some of the characteristic features of *T. striata*.

These species can be found in both freshwater and marine ecosystem. They are the primary producers in planktonic and benthic food webs. *T.striata* species are important as they are one

among the primary producers of aquatic ecosystem. *T.striata* species are free-living but some species are symbionts. Marine species grow fast and densely resulting in algal blooms in bay areas and shorelines. Due to their photosynthetic nature they can be found mostly in water. These species can be found in global waters and they are abundant. These species are now used in various research and fields like biofuels, to understand growth rates etc.

T. striata species have different types of cell shape and size. The cells may be elliptical, round, flattened, ovoid, compressed etc. The cells have four flagella which have equal length which emerge from a depression at the end. And the flagella appear in pairs and then move away from each other. Flagella are used for the motion in aqueous condition. Flagella are thick and covered of mucilage on it and are blunt at the end and its length is shorter than the cell. Numerous thylakoids and lamellae are present with in the chloroplast. The stigma is a concentration of pigment granules which is used by the cells to identify light and to position within the water. Nucleus is present and located at the centre. Golgi bodies are present between the chloroplast and the basal bodies. Mitochondria are present in cytoplasm. And cytoplasm contains vacuoles, organelles, lysosomes etc. Theca is present covers the cell in most species. Hairs are present in many species which are thick and curly. The scales present outside is mainly used to cover the bare membrane which is not covered by the inner scale which is larger in size.

Reproduction is mainly through cell division which occurs during non-motile stage and produces two daughter cells. And most species undergo a single division cycle. And during cell division organelles divide before nuclear division. Cell division is completed with the help of phycoplast which is a microtubule that helps the cell divide the nuclei into daughter cells. Depending on the environmental conditions the daughter cells can undergo and develop into flagellated cells or non- motile cells.

T.striata species have three life stages which include flagellated stage, a vegetative non-motile stage, and a cyst stage. The vegetative non-motile stage is the main stage of most species. The main difference between the vegetative and flagellated stage is the number of scale layers present in the thecal wall. The vegetative stage has two or more layer while flagellated stage has only one layer. The flagella are lost during the cyst stage in the cells and are known aflagellate and they produce a thick thecal shell for their protection. Among any stage it is possible that the species might undergo a complete transformation and develop flagella and become moti

CHAPTER 2

AIM AND OBJECTIVES

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AIM

Biostimulant efficacy of *T. striata* for improving plant health and growth.

OBJECTIVE

The current project is focussed with the following objectives:

1. To standardize culture parameters of the organism- *T.striata*
2. To isolate DNA in PCR amplification and the identification of the organism.
3. To find out the biostimulant efficacy of *T. striata* for improving the plant health and growth.

CHAPTER 3 -
REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Regardless of the amount of nutrients present, a plant bio stimulant is any substance or microorganism administered to plants with the intention of improving nutrition efficiency, abiotic stress tolerance, and/or crop quality attributes (Kauffman *et al*.,2007). By application, commercial goods containing blends of these chemicals and/or microbes are also referred to as plant bio stimulants. Arguments based on scientific knowledge of the nature, mechanisms of action, and types of impacts of bio stimulants on crops and horticulture plants are used to support the definition presented in this article. Additionally, by defining the differences between bio stimulants and fertilisers, insecticides, and bio control agents, the proposed definition hopes to support the acceptance of bio stimulants by future legislation, particularly in the EU. Regardless of the food levels, many bio stimulants enhance nutrition. The proposed subclass of bio stimulants, bio fertilizers, improves the efficiency of nutrient usage and creates novel pathways for plant nutrient uptake.(Rose *et al*.,2014). In this respect, mycorrhizal and non-mycorrhizal fungi, bacterial endosymbionts (like Rhizobium), and plant growth-promoting rhizobacteria are all examples of microbial bio stimulants. As a result, microbes applied to plants can serve as both a bio control agent and a bio stimulant, and their purported agricultural effect will play a key role in determining how it should be regulated. The definition, idea, and key types of plant bio stimulants are all summarised in this review. The drivers, possibilities, and difficulties of the market development for bio stimulants are also briefly discussed in this article, with an emphasis on the legal and regulatory status of these substances in the EU and the US.(Du Jardin, 2015)

In this paper, the topic of plant bio stimulants is thoroughly and methodically examined in this review, which also takes into account the novel and basic guiding principles of this technology. Clarifying the underlying mechanisms of bio stimulant activity is necessary for the growth of a bio stimulant business based on science and the creation of effective regulations for these substances. The task of defining the basis in biology of bio stimulants as a class of compounds is made more difficult by the wide variety of industrial processes used in their preparation as well as the diverse sources of bio stimulants that are currently available on the market, including bacteria, fungi, seaweeds, higher plants, animals, and raw materials containing humates (Huang, 2007). This review offers a thorough and methodical analysis of the topic that suggests unique or emergent features of a complex of constituents, as compared to only the presence of identified essential plant nutrients, plant growth regulators, or plant protection chemicals. This term is significant because it underlines the idea that biological

function can be positively modified by applying compounds or mixtures of molecules without explicitly defining the mode of action. In this paper the focus of bio stimulant research and validation should be upon proof due to the difficulties in defining a "mode of action" for a bio stimulant and the requirement for the market in bio stimulants to gain legitimacy (Mayr (1982).It was proposed that rather than requiring the identification of a specific mode of action, the focus of bio stimulant research and validation should be on the demonstration of efficacy and safety as well as the identification of a broad mechanism of action. While there is a clear commercial imperative to justify bio stimulants as a distinct class of products, there is also a compelling biological case for the scientific development of, and experimentation with, bio stimulants in the hope that this may lead to the identification of novel biological molecules and phenomenon, pathways, and processes that would not have been discovered if the category of bio stimulants did not exist, or was not considered to be legitimate.(Yakhin *et al*., 2017)

In this paper, eco-friendly strategies are proposed to encourage plant development and improve agricultural output are top goals. Bio stimulants are a class of organic compounds that can increase crop growth and nutrient uptake while lowering the need for synthetic fertilizers (Yakhin *et al*., 2016). A path towards waste recycling and reduction is established by the creation of bio stimulants from by-products, which benefits growers, the food sector, registration and distribution companies, as well as users. The following factors are to be considered when choosing designated by-products for bio stimulant valorization: lack of pesticide residue, low cost of collection and storage, ample supply, and synergy with other valorization pathways. Over time, national and international programmes like NOSHAN, SUNNIVA, and Bio2Bio have been launched to investigate the potential value of by-products for the food and agriculture sectors. (ii) To examine about how bio stimulants made from organic waste streams work. Vermicompost, composted urban waste, sewage sludge, protein hydrolysate, and derivatives of chitin/chitosan have all been demonstrated to be effective in agriculture and horticulture (Sharma *et al*., 2014). Other classes of waste-derived bio stimulants or raw organic material with bio stimulant components have also shown efficiency. It is anticipated that additional research and development will increase the number of bio stimulants derived from by-products as the worldwide market for bio stimulants expands. The development of bio stimulants for a specific market is also necessary due to the global nutritional imbalance. The author examine some instances of bio stimulants made

from agricultural waste and talk about why agricultural biomass is such a useful resource for the creation of new agrochemicals.(Xu & Geelen, 2018)

This paper proposes that the use of bioeffectors, sometimes referred to as plant bio stimulants, is now standard practise in agriculture and offers several advantages for promoting growth and reducing stress. The goal of this review is to give a thorough summary of the effects that bio stimulants have been shown to have, including their capacity to increase abiotic stress tolerance. The application or inoculation of plant or algae extracts has advantageous effects on growth and stress tolerance. In addition to providing basic nutrients, algal extracts, protein hydrolysates, humic and fulvic acids, and other compounded mixes frequently promote growth and stress tolerance (Colla G *et al*., 2015). Higher yield, improved nutrient absorption and utilisation, increased photosynthetic rate, and resilience to biotic and abiotic stressors are a few of these impacts. This review focuses on the bioprotective effects against abiotic stress, even though the majority of bio stimulants have multiple and varied effects on plant growth (Rose M *et al*., 2014). Agricultural bio stimulants may help make agriculture more resilient and sustainable while providing a replacement for synthetic pesticides, which are becoming less and less popular with consumers. A thorough assessment of the literature demonstrates the important role that various bio stimulants play in providing protection against abiotic stress, but it also highlights the urgent need to address the underlying processes behind these benefits. (Van Oosten *et al*., 2017)

In order to assure optimal nutrient uptake, crop output, quality, and tolerance to abiotic stress, the use of natural plant bio stimulants is suggested as a creative option to handle the issues of sustainable agriculture. However, the procedure for choosing and characterising plant bio stimulant matrices is intricate and entails a number of exacting assessments that are tailored to the requirements of the plant (Eckardt *et al*., 2009). Here, it suggests a platform for the creation and production of highly distinctive plant bio stimulants that combines technology, procedures, and expertise. To explore and comprehend the particular mode(s) of action of bioactive compounds, chemistry, biology, and omic ideas are combined or integrated. The suggested method enables prediction and characterization of natural chemicals' role as bio stimulants. It is possible to manage and analyse vast amounts of complex data elaborating the use of the products and to cope up with the demands in agriculture.(Povero *et al*., 2016)

This paper proposes that the farming methods employed in integrated crop management can coexist with bio stimulants. Organic plant stimulants are critical for improving nutrient use

effectiveness, abiotic stress tolerance, and crop quality in sustainable agricultural systems. This paper explores the basic and novel principles that underlie this technology as it discusses the various formulation strategies for bio stimulants in crop production. It can't overstate the importance of bio stimulants in boosting crop productivity, lowering the cost of food, and raising food quality (Tejada M *et al*., 2016). This has highlighted the significance of bio stimulant compounds. For the lowest effective amount to be precisely administered to target locations, the bio stimulant formulation must be chemically stable and physically homogeneous under all likely storage circumstances. The objective is to maximise the effectiveness of bio stimulants while ensuring the protection during handling and administration.(Hazra & Purkait, 2020)

Bio stimulants or biostimulators are various compounds of natural origin that have advantageous effects on plant growth and development, abiotic stress, agricultural output, and grain quality. It comprises a wide range of natural and mineral components that plants can utilise as enzymes, growth hormones, and minerals; however, bio stimulants cannot be regarded as bio fertilizers (Pascual *et al*., 2018). Physiological effects depend on their composition. Bio stimulants used in plant development are currently one of the tools utilised in sustainable agriculture since they are commonly regarded as an environmentally benign agricultural practise. The findings of studies on the effects of bio stimulants carried out in Croatia on horticultural crops, including ornamentals like wild roses and wax begonias as well as edible plant species like bell pepper, tomato, strawberry, lettuce, garlic and basil and some ornamental plants. The examined bio stimulants were treated via seed treatment, foliar spray, or irrigation at all plant growth phases, from germination to full plant and fruit or flower commercial maturity. Morphological and anatomical, physiological, and qualitative parameters were examined to gauge the efficacy of bio stimulants. The evaluated bio stimulants primarily improved seed and transplant vigour, stimulated vegetative growth, improved nutrient uptake and circulation within the plant, enhanced antioxidative ability of plant tissues, resulting in better stress response, and enhanced plant yield and fruit/flower efficiency in this diverse range of studies. The research generally suggests potential advantages of bio stimulant use in horticulture production, particularly in stressful growth conditions like the transplant stage, low fertilisation rates, or prevalence of other abiotic stress. The effects on plants may vary depending on dosage, timing of treatment, environmental factors, and the type of plant. This is because there may be interactions

between the physiologically active chemicals present. Therefore, more investigation into the use of bio stimulants in horticulture production is advised.(Para\djiković *et al*., 2019)

Agriculture has been moving toward more eco-friendly, sustainable, or organic methods of cultivating crops. The goal of current agriculture is to cut back on resources without sacrificing quality and productivity. Farming practices can achieve these objectives, but they would be species-specific and time-consuming. Finding chemical compounds that can stimulate plant metabolism may make it possible to improve plant performance quickly and affordably. Plant extracts called bio stimulants are rich in a variety of bioactive substances, the majority of which are currently unidentified (BerlynGP *et al*., 1990). The plant's ability to absorb nutrients more effectively and its tolerance to biotic and abiotic stressors are typically improved by these compounds. The state of the art and potential applications of bio stimulants are discussed. Additionally, productive farming systems including horticultural and floricultural crops have received special attention (Canellas *et al*., 2015). When applied to vegetables, bio stimulants allowed the use of less fertiliser without sacrificing quality or production. Bio stimulants have proved successful in enhancing the quality and preserving the nitrate levels in green vegetables prone to nitrate buildup. Additionally, bio stimulants boosted the amount of leaf pigments (chlorophyll and carotenoids) and plant development in leafy vegetables by promoting root growth and boosting the antioxidant capacity of plants. In floriculture, bio stimulants used in the production of bedding plants promoted plant growth, causing plants to bloom and reach the commercial stage earlier and maximising space in the greenhouse.(Bulgari *et al*., 2015)

Abiotic stressors have a significant impact on plant growth, health, and quality; it can seriously reduce agricultural output if it occurs during the phenological periods of the plant when it is most vulnerable to stress (Traon *et al*., 2014). Additionally, extensive breeding operations and various process settings are needed for crop performance assessment in order to boost crop stress resistance through genetic improvements. The use of bio stimulants as agronomic techniques to combat abiotic stress has been suggested. By influencing primary or secondary metabolism, these compounds with bioactive chemicals do indeed benefit plants and increase their resilience to harsh climatic circumstances (Nardi *et al*., 2009). Many corporations are investing in the creation of novel bio stimulant products as well as the discovery of the most potent bioactive compounds found in various extracts that can trigger particular plant responses to abiotic stressors. Since the majority of these substances are

unknown and practically hard to characterise in terms of composition, they could be grouped according to how it function in plants. In this review, the main focus is on their usage to combat the most frequent environmental stresses. Bio stimulants have traditionally been used on high-value crops like fruits and vegetables.(Bulgari *et al*., 2019)

A possible tactic is the improvement of microalgae as a sustainable source for biofuel through synthetic ecology. The robust biofuel production under various stresses as well as with the chosen two-stage cultivation modes were assessed for the co-cultivation model (*T. striata* and *Pelagibaca bermudensis*) (Cho *et al*., 2006). The function of metabolic exudates, including the precursors of quorum sensing, was evaluated.With a growth promotion of 1.2–3.6-fold and nutrient limitation, the co-cultivation model developed in this study promoted the biomass production of *T. striata* in a saline/marine medium at a wide range of pH, salinity, and temperature/light conditions. As a result, the created model may help *T. striata* cope with abiotic stress. Under various conditions, the growth-promoting bacterium *P. bermudensis's* quorum-sensing precursor dynamics displayed a distinctive pattern (Azizah et al., 2022). Targeted metabolomics revealed diverse stresses under different conditions (using liquid chromatography–mass spectrometry, LC–MS). *T. striata's* growth was supported by *P. bermudensis* and its metabolic exudates, which increased lipid productivity. *T. striata* growth was inhibited independently by hydroxy alkyl quinolones at higher concentrations. Particularly, in both axenic and co-cultivated circumstances, nitrate limitation caused a 1.5 times greater lipid content (30–31%) than control among the three two-stage culture modes (low pH, excessive salinity, and nitrate limitation).(Patidar *et al*., 2018)

Techniques for mass-producing the marine microalga *T.striata* for biofuel and a variety of by-products must be effective and sustainable. The phycospheric communities have the ability to significantly impact the quantity and quality of biomass and bioproducts because they influence microalgal growth and metabolism through a variety of allelochemical and nutritional interactions. In the current work, phycospheric communities of *T.striata*, a producer of biofuel (KCTC1432BP) were screened. From the phycosphere of *T. striata*, 26 different bacterial strains were recovered and identified. Then it was co-cultivated with each bacterial strain to test. In order to assess both its growth-promoting and growth-inhibiting effects (Chisti, 2007). Due to the maximal growth-promoting effects and mutualistic interactions, two promising strains (*P. bermudensis* KCTC 13073BP and *Stappia sp*. KCTC 13072BP) were chosen from among all of the others. Analysis of the growth rate, biomass

productivity, lipid and fatty acid content, and axenic growth of *T. striata* in O3 media were all done. Later, under both abundant and scarce nutrient conditions, growth-promoting mechanisms for these promising bacterial strains in the co-cultivation environment were examined (nitrate, phosphate, and vitamin B12). The two-fold increase in biomass productivity demonstrated *P. bermudensis'* capacity to promote growth. According to next generation sequencing study, these bacteria are promising for microalgal production without having any harmful effects on the natural seawater bacterial ecosystems. This study is the first to date to emphasise the function of bacteria that promote phycospheric growth in the promising biofuel feedstock *T. striata*.(Park *et al*., 2017)

Aquatic viruses, which number over 10 million per millilitre of water, are without a doubt one of the key elements influencing the ecology and evolution of the Earth's microbial community. Nevertheless, little is known about the diversity and functions of environmental viruses (Williamson *et al*., 2012). The virus (named TsV) that infects the coastal marine microalga *T.striata* is described here for the first time in detail. TsV, in contrast to other viruses known to infect microalgae, is a tiny (60 nm) DNA virus with a 31 kb genome. TsV was the only strain of the Chlamydomonadaceae family that could infect *T. striata* out of a total of eight distinct strains. One hour after infection, gene expression dynamics showed that viral transcripts were being up-regulated (p.i.). 24 hours after the injury. The emergence of viral factories inside the nucleus 24 hours post-infection was the first time an infection was clearly visible. The only method of TsV assembly was nuclear. The TsV-N1 genome revealed significant differences from known algal viruses (*Phycodnaviridae*). Only 9 of the 33 encoded ORFs could be identified based on putative function and/or homology. Among them were two genes with the highest resemblance to genes from human parasites of the urogenital tract, a surprising DNA polymerase type Delta (found only in Eukaryotes), and one other gene (Grimsley *et al*.,2011). Future research on the potential effects of microalgae viruses on human health is enabled by these findings, which support the notion that the variety of microalgae viruses extends well beyond the Phycodnaviridae family.(Pagarete *et al*., 2015)

This study's objective was to identify the ideal physical cultivation conditions for locally isolated strains of *T. striata* and *Nannochloropsis* sp. in order to achieve the fastest growth rate possible. Evaluation of biomass output at various agitation rates, light intensities, and temperatures was crucial. The experiment design and cultivation process optimization for

Nannochloropsis sp. and *T. striata* used central composite design and response surface methods. The verification tests supported the estimated values. The validity of the models for the culture of microalgal strains was validated by the good agreement between the predicted values and the experimental values. The interesting finding was that while T. striata's growth was heavily dependent on light exposure, temperature was a dominant factor in obtaining high chl-a content for *Nannochloropsis sp*.(Imamoglu *et al*., 2015)

T. striata's (Chlorophyta) highly purified flagella were extracted using Triton X114 phase partitioning. Only two prominent flagellar membrane proteins (Becker et al. 1990) (fmp) with apparent molecular weights of 145 and 57 kDa (fmp145 and fmp57) were enriched in the detergent phase, according to SDS-PAGE analysis, which showed that the majority of proteins were present in the aqueous phase. By using gel permeation chromatography, Fmp145 was purified. It was determined that fmp145 is a glycoprotein with 3-5 N-glycans of the high mannose and/or hybrid type using glycosidase treatment and lectin blot analysis. Using immunofluorescence and immunoelectron microscopy, a polyclonal antibody (anti-fmp136) that was produced against the deglycosylated version of fmp145 was utilised to locate the protein. Fmp145 was discovered to be present between the scale layers and the flagellar membrane by immunogold labelling (Roth *et al*., 1978). Fmp145 is swiftly and uniformly integrated into the growing flagella during flagellar regeneration. Antifmp136 could be a helpful immunomarker for the identification of *Tetraselmis* strains by fluorescence microscopy because it specifically cross-reacted with the flagella of only a subgroup of *Tetraselmis* strains that are distinguished by a specific flagellar hair type.(Gödel *et al*., 2000)

Tetraselmis tetrathele, Tetraselmis striata, Tetraselmis chuii, and *Tetraselmis gracilis* were four separate strains of marine algae that were isolated from saltpans in Kovelong, Chennai, Tamil Nadu, India. Through molecular identification, *T. striata* BBRR1's systematic location was verified. *T. striata* Butcher BBRR1 produced the most volumetric productivity, proteins, carbohydrates, and lipids when it was cultured in f/2 medium in a lab setting (Moheimani *et al*., 2015). The alga *T. striata* Butcher BBRR1 was grown in 10-m2 open raceway ponds using Modified CFTRI ABRR1 medium. Volumetric biomass productivity, specific growth rate, and lipid content were measured. *T. striata* Butcher BBRR1 had a fatty acid composition that included 33.14% palmitic acid, 22.64% 11-octadecenoic acid, and 21.94% heptadecanoic acid. The marine alga *T. striata* can be grown successfully in open raceway ponds to yield biomass that can be utilised to create biofuels, according to this study.(Boopathy *et al*., 2020)

Using fluorescence induced cell sorting and growth on norflurazon, two carotenoid-rich strains of the euryhaline microalga *T. striata* CTP4 were identified in this study. Independent of the growth circumstances, both strains, ED5 and B11, displayed a carotenoid content that was up to 1.5 times higher than the wildtype (Raja et al., 2013). In comparison to the wildtype, it was shown that ED5 and B11 cells had increased levels of the genes encoding phytoene synthase, phytoene desaturase, lycopene-cyclase, and -ring hydroxylase, which are all involved in the production of carotenoids. Comparing the amounts of eicosapentaenoic acid in both strains to those of the wildtype, they both revealed higher levels. The findings show how difficult it is to regulate carotenoid biosynthesis in a way that increases pigment content in microalgae.(Schüler *et al*., 2021)

There have been numerous studies on the development of various extraction methods to obtain algal extracts. The synthesis of algal extracts is being focused on at the moment without the use of harmful organic solvents or harsh extraction methods that might degrade the biologically active substances found in algal cell (Jeons *et al*., 2012). Critical overview of methods for extracting physiologically active chemicals from algae without causing them to degrade in this study is mentioned. Contrast between the benefits and drawbacks of both conventional extraction methods, including pressured liquid extraction, enzyme-assisted extraction, microwave-assisted extraction, ultrasound-assisted extraction, and supercritical fluid extraction. Algal products are safe for humans, animals, and plants. Therefore can be applied to modern agriculture (as bio stimulants, bioregulators, and feed additives), as well as to the food, cosmetics, and pharmaceutical industries.(Michalak & Chojnacka, 2014)

CHAPTER- 4

METHODOLOGY

3. MATERIALS AND METHODS

3.1 Microalgal culture

Microalgal species selected for the current study was a strain of *T. striata* 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 parameters

The algae culture broth was prepared (appendix 1) and the culture tubes were incubated under optimum conditions as given in the table 1.

SL. no	Parameters	Values
1	Working volume	250mL
2	Temperature	24±1 ℃
3	Light intensity	700-800 lux
4	Photo period	16/18 h (light/dark)
5	Time	20 days

Table 2: Microalgal culture parameters

3.1.2 Microscopic observation

After 5 days of incubation, the culture is 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 680 nm and 700 nm in order to determine maximum absorbance using spectrophotometer (LAB India)

3.1.4 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.

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

3.1.5 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 13000 rpm (Centrifuge HERMLE-Z 3242). The supernatant was discarded and the pellet resuspended in 90% methanol. Chlorophyll was then extracted from the sample during one hour incubation in a water bath (Rotek) at 50℃. 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 different wavelengths, using the UV spectrophotometer was blanked with ethanol.

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. Hence we explored the possibility of 16s forward and reverse primer for amplification.

3.2.1 DNA isolation

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

DNA isolation using CTAB

Freshly prepared CTAB (Cetyl Trimethyl Ammonium Bromide) buffer was preheated 65℃. 1 gm of the microalgae sample was ground in 16 mL of CTAB buffer and homogenized. The ground tissue incubated at 65℃ 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 eppendrof 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 2/3 rd volume of ice cold isopropanol and thoroughly mixed by inverting. The samples were kept for overnight incubation at -20℃. The supernatant was decanted off and the pellet 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.

3.2.2 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.

3.2.3 PCR amplification

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

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.

3.3 Soil Sample Collection

Agro waste soil and normal soil are collected from Ernakulam, Kerala, India and KINFRA green house kalamassery, Kerala, India. These samples were chosen due to Nutrient content in different areas.

3.3.1 Sample Collection Procedure

1. Find the field for sample collection.

2. Remove the surface litter at the sampling spot.

3. Make a “V” shaped cut to a depth of 15cm in the sampling spot using spade.

4. Remove thick slices of soil from top to bottom of exposed face of the “V” shaped cut and place in a clean container.

5. Mix the sample thoroughly and remove foreign materials like roots, stones, pebbles, and gravels.

3.4 Field Preparation

3.4.1 Inoculum preparation

The species was collected from lab. After harvesting, the biomasses were lyophilized and stored at -20˚C. Then, the lyophilized microalgae were separated and stored in airtight envelopes.

Field is prepared by mixing powdered *T. striata* with soil.

These samples are collected in a sampling kit for further test.

The remaining soil were filled in growbags collected from KINFRA kalamassery kerala, India.

3.5 Selection of Seed

Healthy *Vigna unguiculata* seeds were collected from Ernakulam.

The seeds were seeded (4 in each bags) in bags and observe the growth parameter at different interval of days.

3.6 SOIL TEST

3.6.1 DETERMINATION OF SOIL-pH

Reagents;

1. pH Reagent 1 (pH-1)

2. pH Reagent 2 (pH-2)

3. Decolourizer (D-1)

4. pH colour chart (Chart No-1)

Test Method

1. Measure 10c.c of soil (take 2 times in the soil measuring tube (No.1) and transfer into soil mixing tube (No.2).

2. Add 25 ml of pH reagent-1 (pH-1) into the soil and shake well for 5 minutes. Then add a pinch of Decolourizer (D-1) into the soil mixture, again shake well. Then filter into the colour developing bottle (NO.3) by using a funnel and filter paper.

3. To the clear filtrate, add 4-5 drops of pH-Reagent-2 (pH-2) and mix well. Wait 2-3 minutes for colour to develop. The colour that forms id compared with the pH colour chart (Chart No.1).

3.6.2 ESTIMATION OF AVAILABLE NITROGEN IN SOIL

Reagent;

1. Nitrogen Reagent-1 (N-1)

2. Nitrogen Reagent-2 (N-2)

3. Decolourizer (D-1)

4. Nitrogen Colour Chart (Chart No.-2)

Test Method

1. Measure 5c.c of soil, in the soil measuring tube (No-1) and transfer into soil mixing tube (No.2)

2. Add 25 ml of Nitrogen Reagent-1 (N-1) into the soil and shake for 5-10 minutes. Add a pinch of Decolourizer (D-1) into the soil mixture and again mix well. Then filter into the colour developing bottle (No.3) by use a funnel and filter paper.

3. To the clear filtrate, add 2 drops of Nitrogen Reagent-2(N-2) and mix well. Wait 1-2 minutes for colour to develop. The colour that forms is compared with Nitrogen colour chart(chart No-2) and record as Low (L1 & L2), Medium (M1 & M2) or High (H1 & H2). Discard the solution and wash all the tubes well.

3.6.4 ESTIMATION OF AVAILABLE PHOSPHOROUS IN SOIL

Reagents;

1. Phosphorous Reagent-1 (P-1)

2. Phosphorous Reagent-2 (P-2)

3. Decolourizer (D-1)

4. Phosphorus colour chart (chart No-3)

Test Method

1. Measure 5c.c of soil in the soil measuring tube (No.1) and transfer into soil mixing tube

(No.2)

2. Add 25 ml of phosphorous reagent-1 (P-1) into the soil and mix well for 15 minutes. Add a pinch of Decolourizer (D-1) into the soil mixture and again mix well. Then filter into the colour developing bottle (No.3) by using a funnel and filter paper.

3. To the clear filtrate, add 2 ml of phosphorous reagent-2 (P-2) and mix well. Wait for 1-2 minutes for colour to develop. The colour that forms in compared with Phosphorous colour chart (Chart No-3) and record as Low (L1&L2), Medium (M1&M2) or High (H1&H2). Discard the solution and wash all the tubes well.

3.6.5 ESTIMATION OF AVAILABLE POTASSIUM IN SOIL

Reagents:-

1. Potassium reagent-1 (K-1)

2. Potassium Reagent-2 (K-2)

3. Decolourizer (D-1)

Test Method

1. Measure 5c.c of soil in the soil measuring tube and transfer into the soil mixing tube.

2. Add 25ml of Potassium reagent-1 into the soil and mix well for 10-15 minutes. Add a pinch of Decolourizer (D-1) into the soil mixture and again mix well. Then filter into the colour developing bottle by using a funnel and filter paper.

3. To clear filtrate add 1ml of Potassium reagent-2 (K-2) and mix well. Wait 1-3 minutes for colour to develop. The cloudiness that forms is compared with Potassium colour chart and record as Low, Medium or High. Discard the solution and wash all the tubes well.

3.6.6 ESTIMATION OF ORGANIC CARBON IN SOIL

Reagents:-

1. Organic carbon reagent-1 (OC-1)

2. Organic carbon reagent -2 (OC-2)

3. Organic colour chart

Test Method

1. Transfer one full spoon of soil into the soil mixing bottle.

2. Put the gloves and add 5ml of Organic carbon reagent (OC-1) into the soil and mix.

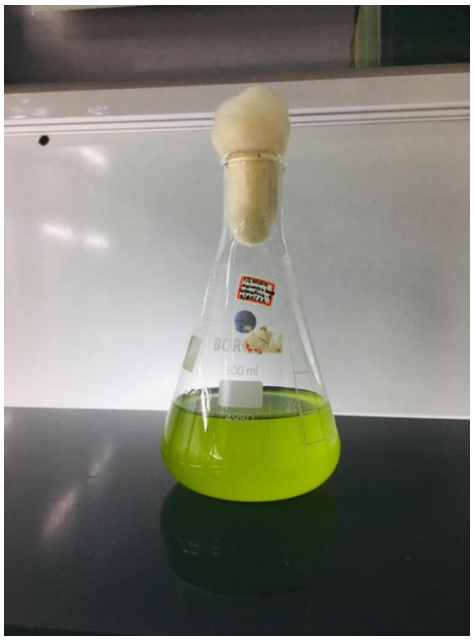
3. Add very slowly 5ml of Organic carbon reagent-2 (OC-2), mix well and allow to stand for 10 minutes to complete the reaction. Then transfer the supernatant liquid carefully into a glass test tube. The colour that form is compared with Organic carbon colour chart and record as low, medium or high. Discard the solution carefully and clean all the tubes well.

CHAPTER-5

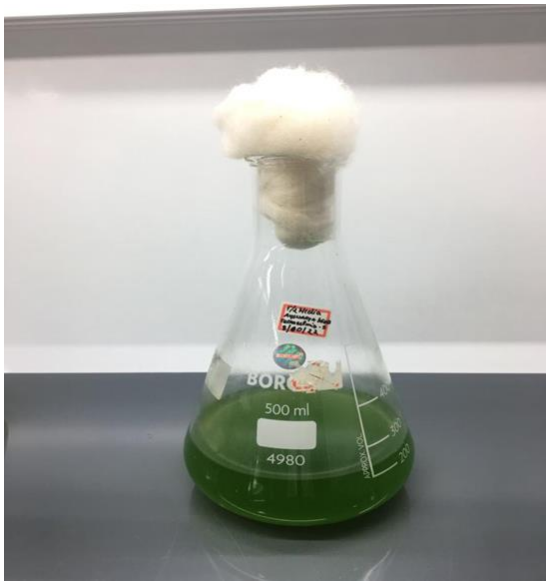
RESULT AND DISCUSSIONS

4. RESULT

4.1 MICROALGAL CULTURE



(A)



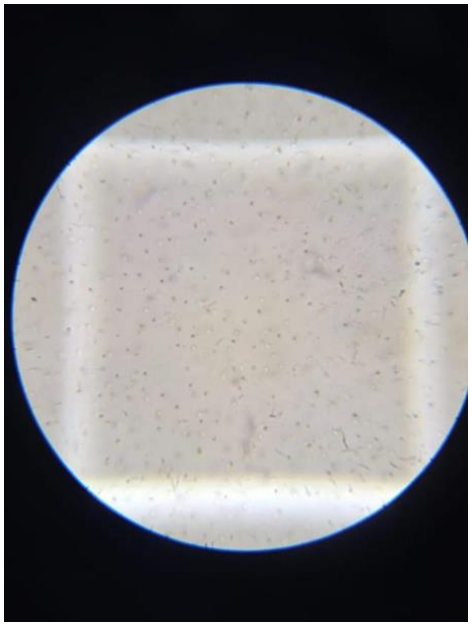
(B)

PLATE 1

The marine algae *T. striata* was cultured in a conical flask with under optimum conditions. The culture in the conical flask showed a gradual colour change

from the first day of inoculation upto the 15 th day. The colour changes from pale green to dark green and this is an indication in the increase in cell count and chlorophyll content.

4.2 MICROSCOPIC OBSERVATION



(A)

PLATE 2

Microscopic observation of *T. striata* under the light microscope 45X was taken. Microscopic observation shows a gradual increase in the cell count.

4.3 MAXIMUM ABSORBANCE DETERMINATION

The optical absorbance was measured at two wavelengths 620nm and 700nm using UV spectrophotometer at an interval of three days. As the number of days increases the OD value also increases gradually that indicates the growth.

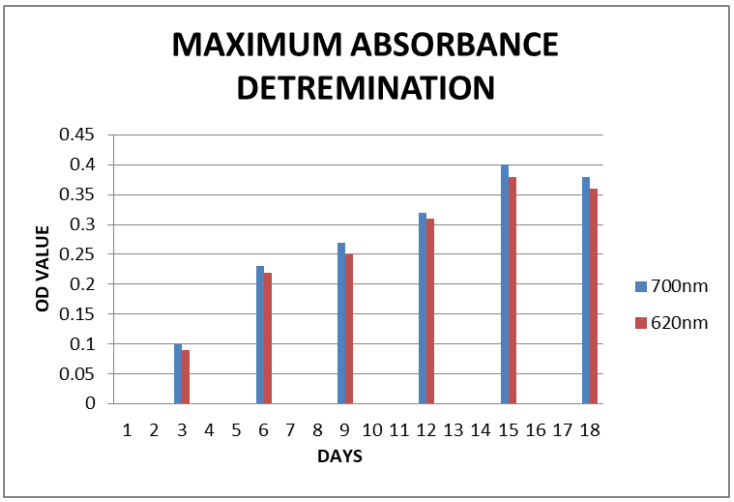


Figure 1. Graph for maximum absorbance determination

The maximum OD is obtained at 700 nm. OD value shows the speed at which the light travels through the sample. Maximum growth is seen during the 15th day, after that there is slight drop in the OD value.

4.4 CELL COUNTING USING NEUBAUER HAEMOCYTOMETER

Cell count was measured periodically by using Neubauer Haemocytometer which shows the cell density of culture. The cell number density is calculated.

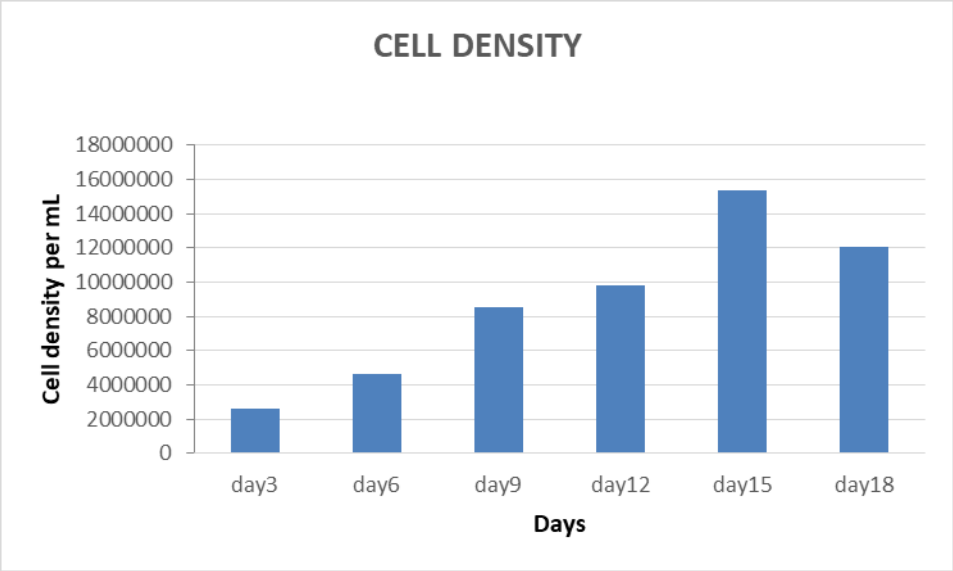


Figure 2. Graph for cell density

There is a gradual increase in cell growth from day 1 to day 15. It is indicated by the increase in cell number density per ml. After attaining the maximum growth the cell number density there is sudden drop in the cell density. The maximum growth is observed at 15^{th} day.

4.5 DETERMINATION OF CHLOROPHYLL

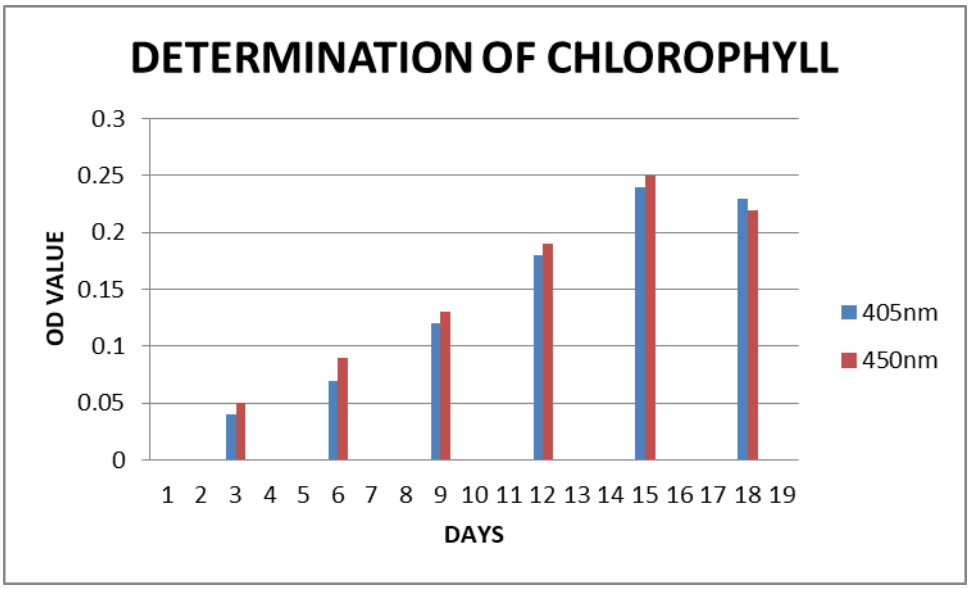


Figure 3. Graph for Chlorophyll content determination

The graph shows a gradual increase in the chlorophyll content which indicates the growth of the culture. The graph shows the chlorophyll content of the culture during different days. The chlorophyll content is maximum at the 15th day and after that there is a slight decrease in the chlorophyll content. The maximum value is obtained under 450nm.

4.5 IDENTIFICATION OF ORGANISM USING MOLECULAR SEQUENCING.

4.5.1 DNA isolation and agarose gel electrophoresis

The identification of organism is mainly done with the help of many steps. The first step is isolation of DNA . DNA isolation method used is by Doyle and Doyle (1987), using CTAB yielded good quality DNA for PCR.

MBT051 S1

(A)

PLATE 3

Lane (M1): 100 bp marker.

Lane (S1): 28s rRNA PCR amplicon of DNA

The DNA gets separated after the gel electrophoresis according to their molecular size. The band pattern is very specific for each organism and therefore this can be used for the identification and confirmation of the selected organism. The band obtained from electrophoresis is viewed with the help of gel documentation system.

>KY549667.1:1-580 Tetraselmis striata 28S ribosomal RNA gene, partial sequence
AGACTAACTAGGATTCCCTTAGTAACGGCGAGTGAAACGGGATAAGCCCAACTTGAAAATCTGCAGGCCTTGTCTGCCGA
ATTGTAGTCTATAGAAGCAACCTCTGAGGCGCGCCTGGATTAAGTTCCCTGGAAGGGGACGTCAGAGAGGGTGAGAACCC
CGTCATCTATGGCTGCCGTCTCTTCACGAGATGCTCTCGCAGAGTCGGGTTGGTTGAGAATCCAGCCCTAATTGGGAGGT
ACATCCCTTCTAAGGCTAAATACCGGTAAGAGACCGATAGCGAACAAGTACCGCGAGGGAAAGATGAAAAGGCCTTTGAA
AAGAGAGTTAAAAAGTGCTTGAAATTGTTGAGGGGGAAGTGTATCAGAGGCGTGTGTGCTCCTAGGCTGTATGTAGACTC
TAACCGGTCTGCCCCTCAGTGCTAGGAGCTGGTCAGCATGGGTTAGCTTAGTGGGACAAAAGCAGAGATTGCTACTTTGC
CGATGCCACTTCGCTGTCCGAGGATTAAGAGCGCTTCGCCTTCGGAGCTGCGTTCTCATGATGCTGGCAGAGCACCTGAT
ACAGCCCGTCTTGAAACAGG

(A)

PLATE 4

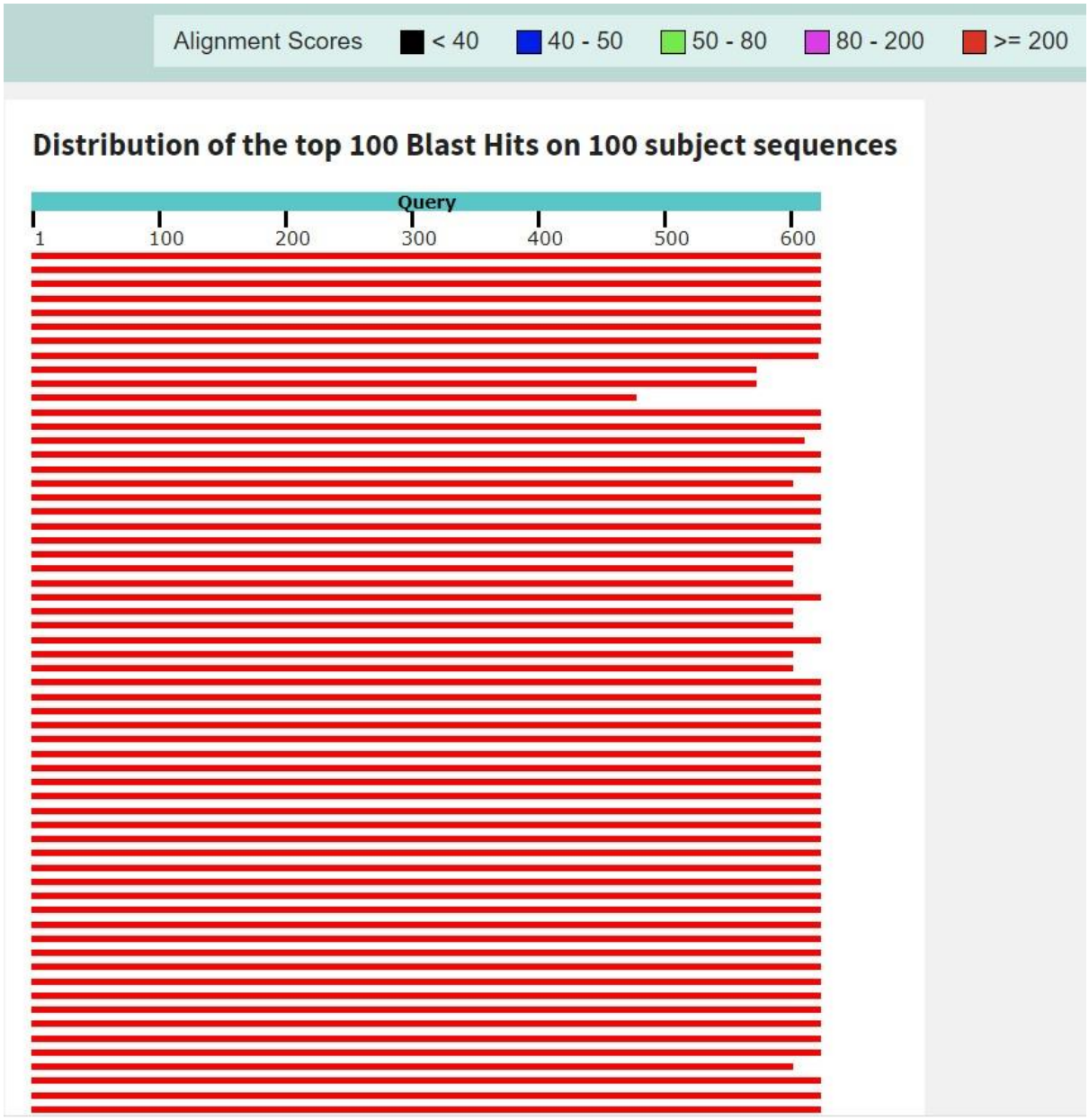
After sequencing the obtained sequence is then compared with the database to identify the similar sequences. Blast has been used to identify the similar sequence from the database with the help of word by word similarity search.

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Tetraselmis striata 28S ribosomal RNA gene, partial sequence	Tetraselmis striata	1149	1149	100%	0.0	100.00%	622	KF021315.1
Tetraselmis striata 18S rRNA gene (partial), 5.8S rRNA gene, 28S rRNA gene (partial), ITS1 and ITS2, strain SA…	Tetraselmis striata	1149	1149	100%	0.0	100.00%	4027	HE610129.1
Tetraselmis sp. SMS19 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S…	Tetraselmis sp. S…	1072	1072	100%	0.0	97.75%	3575	MT489380.1
Symbiodinium sp. strain SMS5 strain SMS19 internal transcribed spacer 2 and large subunit ribosomal RNA gen…	Symbiodinium sp…	1072	1072	100%	0.0	97.75%	1435	MT489354.1
Tetraselmis marina isolate MP0060 large subunit ribosomal RNA gene, partial sequence	Tetraselmis marina	1029	1029	100%	0.0	96.47%	1238	KY655021.1
Tetraselmis marina 18S rRNA gene (partial), 5.8S rRNA gene, 28S rRNA gene (partial), ITS1 and ITS2, strain C…	Tetraselmis marina	1029	1029	100%	0.0	96.47%	5630	HE610131.1
Scherffelia dubia 18S rRNA gene (partial), 5.8S rRNA gene, 28S rRNA gene (partial), ITS1 and ITS2, strain SAG…	Scherffelia dubia	1011	1011	100%	0.0	95.98%	3981	HE610128.1
Tetraselmis sp. NK 28S ribosomal RNA gene, partial sequence	Tetraselmis sp. NK	963	963	99%	0.0	94.68%	626	KF021318.1
uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge…	uncultured fungus	939	939	91%	0.0	96.32%	1523	OU942927.1
uncultured fungus genomic DNA sequence contains 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA ge…	uncultured fungus	933	933	91%	0.0	96.15%	1516	OU942254.1
Tetraselmis marina isolate MP0018 large subunit ribosomal RNA gene, partial sequence	Tetraselmis marina	765	765	76%	0.0	95.61%	807	KY655018.1
Oogamochlamys zimbabwiensis strain UTEX 2214 26S ribosomal RNA gene, partial sequence	Oogamochlamys…	734	734	100%	0.0	88.02%	3253	DQ015758.1
Oogamochlamys zimbabwiensis strain UTEX 2213 26S ribosomal RNA gene, partial sequence	Oogamochlamys…	734	734	100%	0.0	88.02%	2029	DQ015729.1
Chlorogonium euchlorum strain SAG 12-3 voucher SAG:12-3 26S ribosomal RNA gene, partial sequence	Chlorogonium eu…	730	730	97%	0.0	88.46%	2104	AF395507.1
Chlamydomonas frankii strain SAG 18.72 voucher SAG:18.72 26S ribosomal RNA gene, partial sequence	Chlamydomonas…	730	730	100%	0.0	87.92%	2105	AF395499.1
Carteria crucifera strain UTEX 432 28S ribosomal RNA gene, partial sequence	Carteria crucifera	730	730	100%	0.0	87.92%	2132	AF183454.1
Chlorococcum diplobionticum strain UTEX 950 26S ribosomal RNA gene, partial sequence	Chlorococcum di…	723	723	96%	0.0	88.60%	2088	AF395505.1
Lobochlamys segnis strain SAG 9.83 voucher SAG:9.83 26S ribosomal RNA gene, partial sequence	Lobochlamys se…	723	723	100%	0.0	87.70%	2089	AF395504.1
Lobochlamys segnis strain SAG 17.72 voucher SAG:17.72 26S ribosomal RNA gene, partial sequence	Lobochlamys se…	723	723	100%	0.0	87.70%	2090	AF395500.1
Pseudoschroederia antillarum culture SAG:15.86 strain SAG B 15.86 28S ribosomal RNA gene, partial sequence	Pseudoschroede…	723	723	100%	0.0	87.84%	2028	AF277656.1
Chlamydomonas pulsatilla strain UTEX 410 26S ribosomal RNA gene, partial sequence	Chlamydomonas…	717	717	100%	0.0	87.58%	3248	DQ015732.1
Chlorococcum oleofaciens gene for 26S rRNA, partial sequence, strain: SAG 213-11	Chlorococcum ol…	712	712	96%	0.0	88.25%	2083	LC066336.1
Chlorococcum sp. AREC1 28S ribosomal RNA gene, partial sequence	Chlorococcum s…	712	712	96%	0.0	88.26%	626	KF021310.1
Chlamydopodium vacuolatum strain UTEX 2111 28S ribosomal RNA gene, partial sequence	Chlamydopodiu…	712	712	96%	0.0	88.26%	2087	AF183468.1
Gymnomonas nepalensis strain Nepal-Gonzalez 26S ribosomal RNA gene, partial sequence	Gymnomonas ne…	712	712	100%	0.0	87.38%	1994	DQ015741.1
Chlorococcum oleofaciens strain UTEX 105 26S ribosomal RNA gene, partial sequence	Chlorococcum ol…	712	712	96%	0.0	88.25%	2019	DQ015724.1
Chlorosarcinopsis bastropiensis strain Ind-Jiht-4 large subunit ribosomal RNA gene, partial sequence	Chlorosarcinopsi…	706	706	96%	0.0	88.10%	1209	MW485748.1
Tetraedron bitridens strain UTEX 120 26S ribosomal RNA gene, partial sequence	Tetraedron bitrid…	706	706	100%	0.0	87.28%	2050	AY779900.1
Ascochloris multinucleata strain UTEX 2013 26S ribosomal RNA gene, partial sequence	Ascochloris multi…	706	706	96%	0.0	88.10%	2106	AF395492.1
Ascochloris multinucleata strain UTEX 2013 28S ribosomal RNA gene, partial sequence	Ascochloris multi…	706	706	96%	0.0	88.10%	2105	AF277652.1
Oogamochlamys gigantea strain SAG 44.91 26S ribosomal RNA gene, partial sequence	Oogamochlamys…	706	706	100%	0.0	87.26%	3228	DQ015757.1
Chloromonas chlorococcoides SAG 72.81 gene for 26S ribosomal RNA, partial sequence	Chloromonas chl…	704	704	100%	0.0	87.32%	1801	LC438806.1
Chloromonas reticulata SAG 26.90 gene for 26S ribosomal RNA, partial sequence	Chloromonas reti…	704	704	100%	0.0	87.32%	1801	LC438803.1
Chloromonas reticulata strain UTEX LB 1970 26S ribosomal RNA gene, partial sequence	Chloromonas reti…	704	704	100%	0.0	87.32%	2091	AF395508.1
Chlamydomonas macrostellata strain SAG 72.81 26S ribosomal RNA gene, partial sequence	Chlamydomonas…	704	704	100%	0.0	87.32%	2031	DQ015725.1
Stephanosphaera sp. UTEX-LB 2409 strain UTEX LB 2409 26S ribosomal RNA gene, partial sequence	Stephanosphaer…	702	702	100%	0.0	87.12%	2107	AF395512.1
Stephanosphaera pluvialis gene for 26S rRNA, partial sequence, strain: UTEX 771	Stephanosphaer…	701	701	100%	0.0	87.12%	2085	LC066339.1
Caespitella pascheri 5.8S rRNA gene, ITS2 and partial 28S rRNA gene, strain SAG 410-1	Caespitella pasc…	701	701	100%	0.0	87.12%	1858	HF920667.1
Stephanosphaera pluvialis isolate UTEX 771 28S ribosomal RNA gene, partial sequence	Stephanosphaer…	701	701	100%	0.0	87.14%	2018	KC589698.1
Schroederia setigera strain UTEX LB 2454 28S ribosomal RNA gene, partial sequence	Schroederia seti…	701	701	100%	0.0	87.14%	2108	AF277657.1
Ourococcus multisporus strain UTEX 1240 28S ribosomal RNA gene, partial sequence	Ourococcus mult…	701	701	100%	0.0	87.24%	2092	AF277655.1
Chloromonas reticulata SAG 32.81 gene for 26S ribosomal RNA, partial sequence	Chloromonas reti…	699	699	100%	0.0	87.16%	1801	LC438805.1
Chloromonas chlorococcoides SAG 15.82 gene for 26S ribosomal RNA, partial sequence	Chloromonas chl…	699	699	100%	0.0	87.16%	1801	LC438801.1
Chloromonas chlorococcoides gene for 26S ribosomal RNA, partial sequence, strain: SAG 15.82	Chloromonas chl…	699	699	100%	0.0	87.16%	2013	AB906359.1
Dunaliella sp. SPMO 201-3 26S ribosomal RNA gene, partial sequence	Dunaliella sp. SP…	699	699	100%	0.0	87.10%	1992	DQ324028.1
Lobochlamys culleus isolate SAG 18.72 26S ribosomal RNA gene, partial sequence	Lobochlamys cul…	697	697	100%	0.0	87.22%	2060	KC196730.1

(A)

PLATE 5

The sequence shows the similarity with *T. striata* 28S ribosomal RNA gene, partial sequence.



(A)

PLATE 6

The graphic summary of alignment shows the sequence similarity of query sequence with that of sequences in the databases. Each horizontal line is the representation of sequence that is in the database matches. Here the alignment score is greater than 200, that is why it is represented by red lines which indicates that there is good matches.

Phylogenetic analysis of microorganism is carried out using Mega X, which is a highly efficient tool in evolutionary studies.

(A)

PLATE 7

The phylogenetic tree show that the *T. striata* 28S ribosomal RNA gene, partial sequence shows close relationship between *T. striata* 18S rRNA (partial) 5.8S rRNA gene 28S rRNA gene (partial) ITS1 and ITS2 strain SAG 41.85 and with *Tetraselmis* sp. SMS19 small subunit ribosomal RNA gene partial sequence internal transcribed spacer 15.8S ribosomal RNA gene and internal transcribed spacer complete sequence and *Symbiodinium* sp. Strain SMS5 strain SMS19 internal transcribed spacer 2 and large subunit ribosomal RNA gene partial sequence.

4.6 SAMPLE COLLECTION

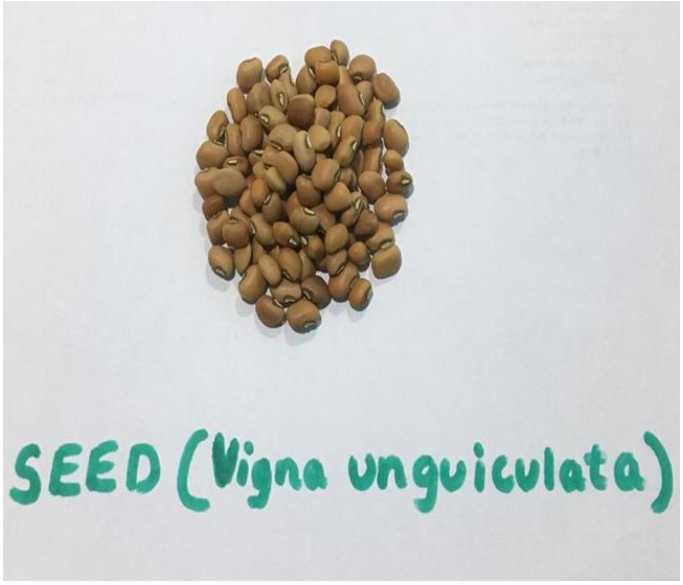
(A)

Appropriate soil was taken by following the soil sample collection methods. And later on proper field was prepared for the growth and comparative study of plants.

(B)

PLATE 8

The soil collected is mixed with the test microorganism (*T. striata*). And it is used for comparative and growth analysis of the plant health. To these soils the plants are grown and studies are carried out separately.



(A)

PLATE 9

Seeds of *V. unguiculata* belongs to Leguminosae family. Appropriate seeds are collected to grow and analyse the study. As these seeds are selected because they can be easily grown and easy to understand the growth. And later on further studies was carried out.

(A)

Further studies and growth of the plants were carried out in the green house.

(B)

PLATE 10

Two sets of soil are taken in grow bags, first SST (Test) which consist of the algae with soil and SSC (Controlled) which consist of the normal soil. And both the sets have equal number of seeds are planted (4 in number). And seeds are planted in both the sets.

(A)

(B)

(C)

(D)

PLATE 11

(A)

(B)

(C)

(D)

(E)

(F)

PLATE 12

(A) (B)

PLATE 13

The above given photos are taken after the seeds are sown and during the beginning of the germination of the plants in the soil. And more number of seeds are germinated in the SST sets mainly and thus indicating the growth rate when compared with normal soil and *T. striata* incorporated one.

DAYS	SSC1	SSC2	SSC3	SST1	SST2	SST3
1	planted	planted	Planted	Planted	planted	Planted
2	-	-	-	-	+	+
3	-	+	+	+	+	+

Table 3: Germination table

The table above gives the germination rate of the plants and it shows that the SST plants have higher germination rates when compared to SSC plants.

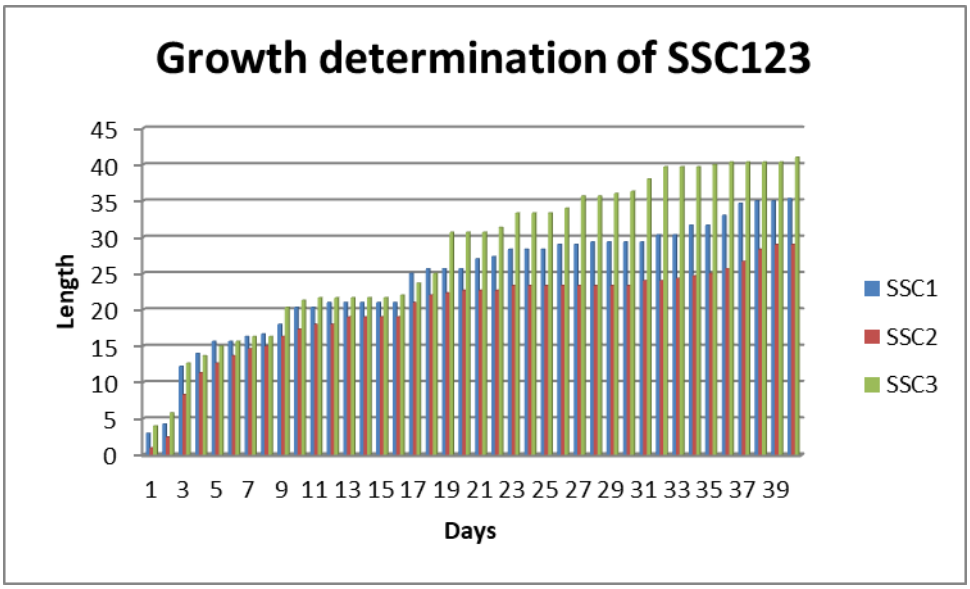


Figure 4. Graph of growth determination of SSC123

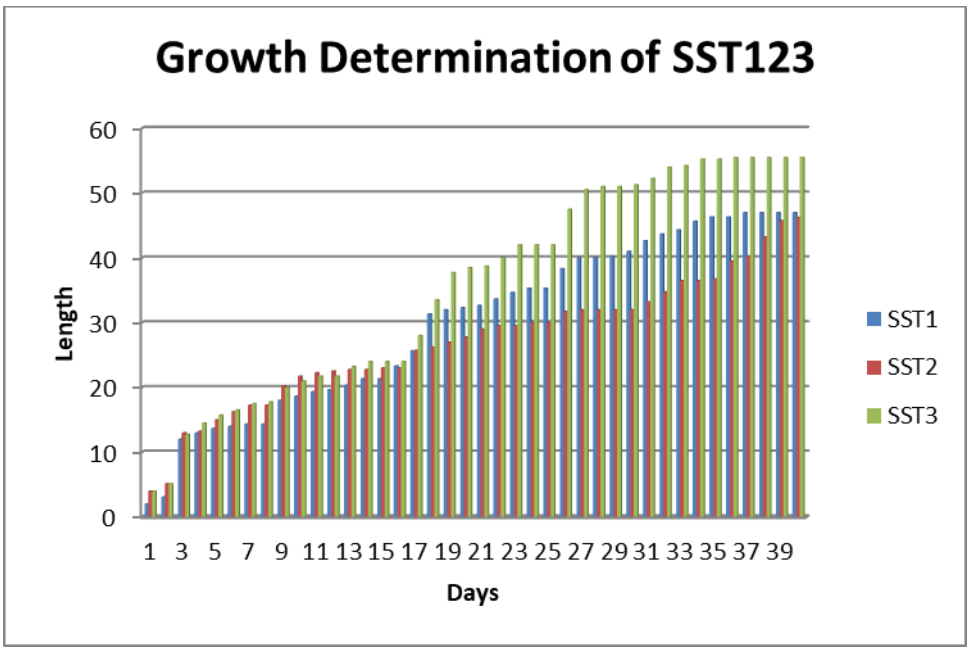


Figure 5. Graph of the growth determination of SST123

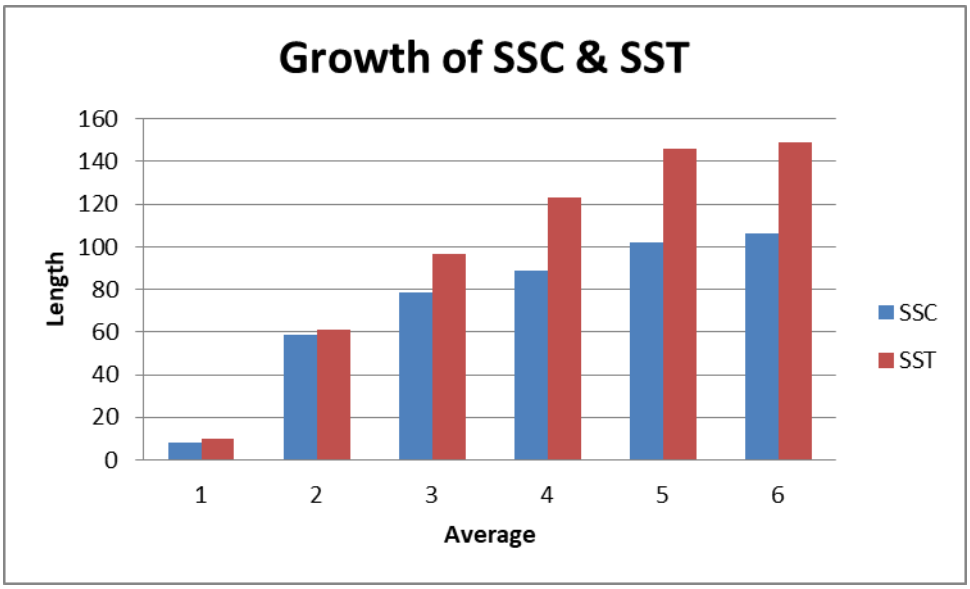


Figure 6. Graph of average growth of SSC and SST

(A)

(B)

(C)

PLATE 14

(A)

(B)

(C)

(D)

(E)

PLATE 15

(A)

(B)

(C)

(D)

(E)

PLATE 16

(A)

(B)

(C)

PLATE 17

(A)

(B)

PLATE 18

(A)

(B)

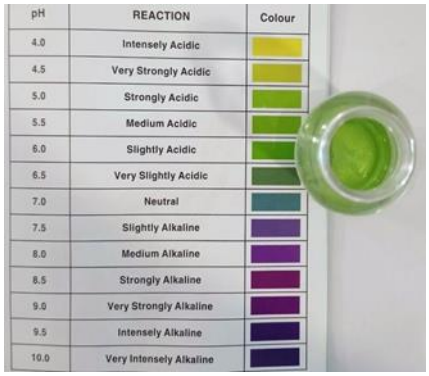
PLATE 19

BUD, FLOWER AND FRUIT DEVELOPMENT

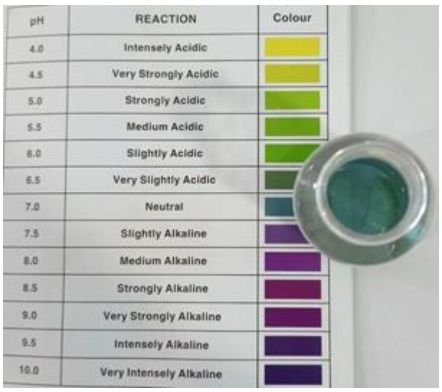
DAY	SSC1	SSC2	SSC3	SST1	SST2	SST3
46	-	-	-	2 Buds	2 Buds	2 Buds
48	-	-	-	2 Buds	2 Buds	2 Buds
50	-	-	-	2 Buds	2 Buds	2 Buds
52	1 Bud	-	-	2 Buds 2 flowers	2 Buds 1 Flower	3 Buds
54	2 Buds	1 Bud	1 Bud	1 Bud 3 Flowers	2 Flowers	3 Flowers
56	2 Flowers	1 Flower	1 Flower	4 Flowers	2 flowers	3 Flowers
58	2 Flowers	1 Flower	1 Flower	4 Flowers	2 Flowers	3 Flowers
60	2 Pods	1 Pod	1 Pod	4 Pods	2 Pods	3 Pods
62	2 Pods	1 Pod	1 Pod	4 Pods	2 Pods	3 Pods
64	2 Pods	1 Pod	1 Pod	4 Pods	2 Pods	3 Pods
66	2 Pods	1 Pod	1 Pod	4 Pods	2 Pods	3 Pods

Table 4

The above table shows the development of bud, flowers and legumes. The table gives a clear idea about the development. It shows that the SST plants have higher growth and production rates when compared with SSC plants. First bud, flower and legume was developed on SST and on SSC plants the development was slow and growth was less.

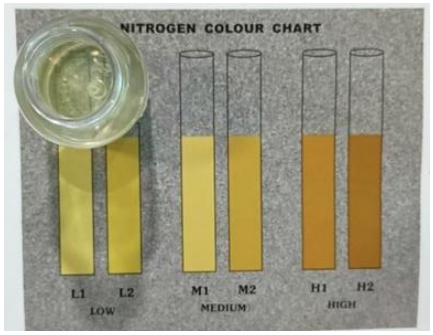


(A)

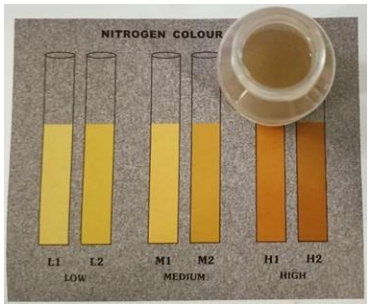


(B)

(A) Is the result of the soil taken for SSC (Controlled), (B) Is the result of the soil taken for SST (Test). The SST soil is found as neutral in Ph. And the SSC soil is found as acidic.



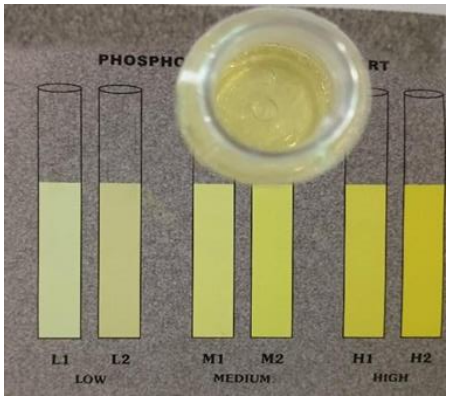
(C)



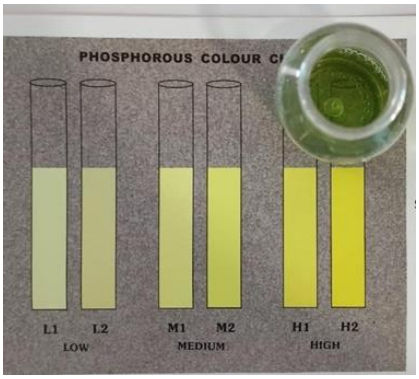
(D)

(C) Is the result of the soil taken for SSC (Controlled), (D) Is the result of the soil taken for SST (Test). In the SST soil it is found that nitrogen content is high and in SSC soil the nitrogen content is found low.

PLATE 20

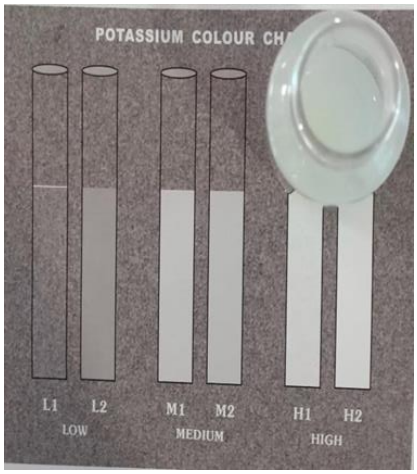


(A)

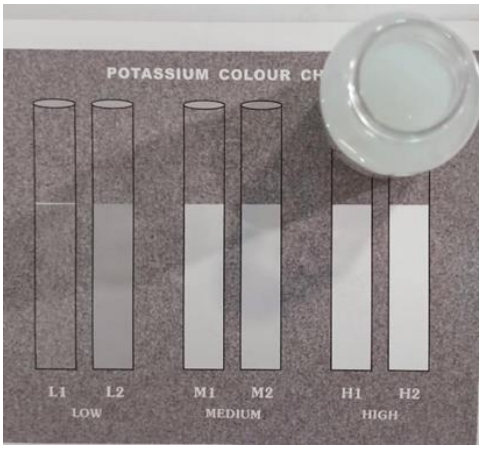


(B)

(A) Is the result of the soil taken for SSC (Controlled), (B) Is the result of the soil taken for SST (Test). In the SST soil it is found that the phosphorous content is high and in the SSC soil is medium.



(C)

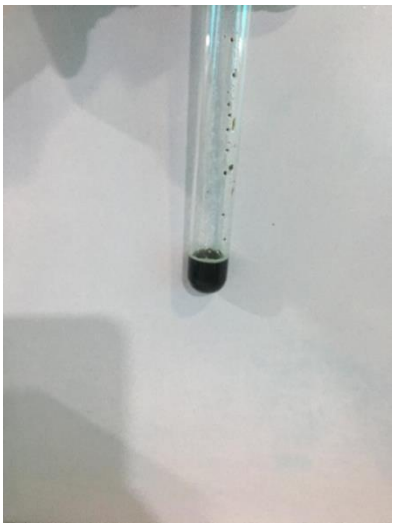


(D)

(A) Is the result of the soil taken for SSC (Controlled), (B) Is the result of the soil taken for SST (Test). It is found that the SST soil is high in potassium content and the SSC soil is also high.

PLATE 21

(A)



(B)

(A) Is the result of the soil taken for SSC (Controlled), (B) Is the result of the soil taken for SST (Test). It is found SST soil is high in carbon content while the SSC soil is medium.

PLATE 22

DISCUSSION

Tetraselmis is a genus of phytoplankton. *T. striata* is a green algae. These species can be found in both freshwater and marine ecosystem. They are the primary producers in planktonic and benthic food webs. *Tetraselmis* species are important as they are one among the primary producers of aquatic ecosystem. Marine species grow fast and densely resulting in algal blooms in bay areas and shorelines. Due to their photosynthetic nature they can be found mostly in water. These species can be found in global waters and they are abundant.

The experiment provides a new insight into the relationship between the plant growth and the efficacy of *Tetraselmis* as a biostimulant. When compared with the data that is obtained from the graphs represent a tremendous increase in growth of those plants which have been planted in the field with *Tetraselmis*. While the ones which have been planted in normal soil without any other supplements shows much less growth.

The data contributes a clearer understanding when we compare the average of both SSC and SST graphs. It clearly gives the idea about the impact of *Tetraselmis* on the plants. It shows a steady increase in the growth of SST plants and the growth of the SSC plants are way less and very slow when compared with the *Tetraselmis* incorporated SST.

The analysis supports that the *T. striata* as biostimulants shows a very good potential for the improvement of plant growth and overall features. It gives a high ratio of growth in terms of germination, growth, flowering and crop production. Environmental factors, soil, water etc mainly influence plants and their growth but with the help of *Tetraselmis* as a biostimulant the plants can grow and give optimal results.

As a matter of fact these results should be taken into account when considering about plant growth, health and economical factors. Because we can minimise the use of harsh chemicals, growth regulators that provide side effects as well as which are damaging the ecosystem and we can replace it with the biostimulant produced from *Tetraselmis* as it does not degrade the ecosystem and it can be produced in large quantity. There are many more possibility that can be considered for further research based on the usage of Tetraselmis in the field of agriculture and other fields. As they are abundant in nature and can be cultivated easily.

CONCLUSION

This study envisaged researching and the sustainability and feasibility of marine microalgae *T. striata* as a biostimulant for better improvement of plant health and growth. In the present attempt, *T. striata* was collected from Scire Science, which was collected from sea. Then it was purified and cultured in f/2 medium under aseptic conditions. And later it was screened to find out its growth performance and it was found that it has a maximum biomass and absorbance and cell density etc when compared with other strains. Hence it was selected for the study. And its DNA isolation was done using Doyle and Doyle method. And the identification and confirmation of species was done by PCR and molecular sequencing. Then the microalga was converted into powder form for further study. Then soil samples were collected for testing and the soil was mixed with powdered form of *Tetraselmis* and it was made into one set (which comprises of three growbags each) called SST and another set (which also comprises of three growbags each) with normal soil called SSC.

Later seeds of Leguminosae family (*V. ungiculata*) was selected to carry out the study as these are way easier to cultivate and to obtain results fast and they are easily available in the market and these have a short duration to complete life cycle, hence they are used in the study. To these SSC and SST, seeds were added and allowed to grow under optimal conditions with all the necessary requirements like water, humidity, light etc in a green house. And in each set four seeds were grown. When the plants started growing the germination and the growth of the plants were observed and noted periodically and photos were also taken to support the study and observe the growth of both set of plants and to compare and understand the effect of biostimulant using a microalagae. And the length of the plants were measured and calculated. After the growth period of the plants the measurements or the data taken were converted into graphical data with the help of graphs for better understanding.

First the average length of each set of SSC and SST were taken and separate graphs were plotted. In SSC there was three grow bags and in each it had four plants and their length measurement was converted into average and added it into the graph the same was done in SST set. And there by two graphs were plotted and then the average of both the graphs were again calculated and a final graph was plotted for better understanding and it shows the results of the study where it can be clearly observed that the plants grown in the SST in which the *Tetraselmis* was incorporated has a high growth rate when compared with SSC with much

less growth among plants. Both the set of plants were given equal amount of water and other factors except for the microalgae.

It has also been observed that the plants growing in the SST were the first to germinate while the plants in the SSC were slow and growth was delayed when compared with the SST plants. Hence from all this data obtained from the study points out that *T. striata* has all the potential to improve the plant health as biostimulant. And the hence the study has shown positive results when analysed with the objectives and further more possibilities lie ahead in the field of *Tetraselmis* in agriculture and crop production.

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APPENDIX

APPENDIX 1

F/2 MEDIUM

NaNO3 : 0.6mL

NaH2PO4.H2O :0.6mL

Trace metal element : 0.6mL

Vitamin solution : 0.3mL

APPENDIX 2

CTAB BUFFER

1M Tris HCL :1.576 g

4M NaCl :2.3376 g

05M 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 : 1 µL

Forward primer : 0.5µL

Reverse primer :0.5 µL

MgCl2 : 2.5 µL

DNTPS : 2.5 µL

Tac DNA polymerase :0.5 µL