Optimization of Lipid Production from Microalgae

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Optimization of Lipid Production from Microalgae" is based on the results of investigations carried out by me in the Microbiology Programme at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Judith M. Noronha and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation report **"Optimization of Lipid Production from Microalgae"** is a bona fide work carried out by **Miss. Deepisha Deepak Sawant** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in Microbiology, in the Discipline Microbiology, at the School of Biological Sciences and Biotechnology, Goa University.

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ABBREVATIONS USED

BLAST Basic local alignment search tool

DNA. Deoxyribonucleic

ITS. Internal transcribed spacer

MEGA Molecular evolutionary genetics analysis

NCBI National Centre for Biotechnology Information

NGS. Next-generation sequencing

SDS Sodium dodecyl sulphate

ABSTRACT

Isolation of a microalgal strain, which has high lipid content, which is the key to the 3rd generation biofuel. The lipid enhancement is done is a parameter of pH ranging from 5,6,7,8,9. And a NaNO3 concentration of 0.2,0.25,0.5,1.0 and 1.5.

For three cultures of *chlorella sp.1, chlorella sp. 2 and Asterarcys sp*.

The quantification of lipid was done using the sulfo-phospho-vanillin method, which is one of the colorimetric method used in determining algal lipid, which is a easier and faster and less labour intense and more accurate method in determining the lipid.

CHAPTER 1

INTRODUCTION

Aim and Objectives:

- To isolate, culture and identify microalgae from freshwater lakes.
- To explore the potential of microalgae for lipid production

Hypothesis:

In the search for alternatives to fossil fuels, microalgae are promising source of biofuels. One of the important properties of a given microalgal species /consortium, which determines its efficacy in biofuel production, is the intracellular lipid content. In the present study, we aim to isolate various microalgae from wetland ecosystems and measure their lipid content under conventional growth conditions and then after various interventions. The lipid accumulation is expected to be maximum at an optimum pH of growth. Limitation of an essential nutrient like nitrate. is expected to cause stress condition which further may increase the lipid accumulation.

1.Wetlands

Water bodies when considered to be very important for their "ecosystem services and biodiversity values for the local communities and society at large," they are designated as wetlands. Once an area has been titled as a wetland, any intrusion or pollution that poses a threat to this ecosystem is not permitted in that area. Examples of such activities include the initiation or growth of any industry, the handling or disposal of waste, from construction site or solid waste, or any untreated waste, etc. Nonetheless, the wetlands are opened for fishing

operations, and if necessary, the water will be given out for local usage. Native trees are or may be planted, as long as they support the surrounding environment. (https://timesofindia.indiatimes.com/city/goa/six-lakes-declared-official-wetlands/amp_articleshow/86693718.cms)

The lakes Xeldem and Nanda in Quepem, Toyyar in Chimbel, Dashi in Revora, Sarzora in Chinchinim, and Cottambi in Chimbel are the state wetlands. Amongst this the first Ramsar site of Goa is the Nanda lake. (https://timesofindia.indiatimes.com/city/goa/nanda-lake-in-curchorem-is-states-first-ramsar-site/amp_articleshow/93332412.cms)

1.1 Nanda Lake

Nanda lake that is situated in the taluka of Quepem has been designated as the first Ramsar site of the state of Goa. Nanda Lake passed nine requirement list for this designation which include providing a habitat for various bird species, and was therefore authorised as a Ramsar site. The lake serves as a habitat for several bird species, including the: lesser whistling duck, brahminy kite, intermediate egret, red-wattled lapwing, bronze-winged jacana, black-headed ibis, and common kingfisher, because of this ecological services and biological values it provides to the surrounding communities and society at large, Nanda Lake is regarded as being of critical significance. Most of the land of this lake is made up of sporadic freshwater marshes that are close to one of the Zuari River's main rivulets. Because of this, the people around this areas can conserve water which can be used during the non-monsoon season. The water stored can be used for supporting fishing and recreation, the water held in the lake can also used to plant paddy downstream mostly during off season. The lake turns into a swamp during the monsoon when the sluice gate is opened and water is released. Locals use the wetlands to cultivate paddy during this time as well.

1.2 Microalgae

One of the most common and prevalent groups of organisms on earth which are microscopic and photosynthesising are known as Microalgae. They are found living in the aquatic as well as terrestrial that is the soil surface, but are more dominant in the aquatic environment. They are found living in symbiotic relationship or can be found free living (Khan et al., 2018; Singh et al., 2015; Saad and Atia et al., 2014).

In their natural environment or surroundings, they frequently encounter stresses that can be biogenic (arising with the interactions with other species through different forms of parasitism or competition) or abiogenic (that can results from difference in temperatures, droughts, or change in light intensity, also food deficiencies, etc). The basics of microalgae's ability to survive in this hostile conditions or in settings, with greatly different parameters is very important.

Their remarkable physiological and biochemical plasticity can be seen in their physico-chemical parameters and/or in a highly competitive environment (Falkowski et al., 1997).

Furthermore, the variety of morphological alterations a cells can undergo to carry out specific tasks. Of these some are first the formation of spores and akinetes which are the thick-walled, non-dividing cells that are originated or formed in adverse environments and divide upon the arrival of favourable conditions.

ii) Heterocyst which are found in some cyanobacterial taxa are engaged in nitrogen fixation

iii) In cyanobacteria and microalgae, respectively, pili and flagella are used as a mode for motility.

These microbes have evolved a wide range of defence and adaptation strategies, from altering the proportion and makeup of the primary structural and functional elements of the cell.

Microalgae have similarities to the higher plants which include, photosynthetic processes. However, they can fix CO_2 biologically in a more efficient way than terrestrial plants, and can grow much faster as well (Dismukes et al., 2008. Williams and Laurens, 2010)

The different methods of growing microalgae in open ponds or closed photobioreactors are defined to reduced seasonality, low-cost culture media, and the use of the unused, unproductive or or non-arable land, (as noted by Moheimani and Borowitzka (2006).)

1.3 Areas of importance of Microalgae

1.3.1. Ecology and environmental research

Microalgae contribute ~50% to earth's primary photosynthetic productivity and thus are a crucial component in the food chain. Microalgal biological carbon fixation helps in the recycling of the carbon, some of which gets buried at ocean depth (Not et al., 2012). andMicroalgae are organisms that are highly impacted by changes in environment like temperature, light quality, and nutritional status (including pollution), making them ideal markers of ecosystem change. Microalgal populations can help in environmental impact assessment (Day et al., 1999).

1.3.2. Economic

Microalgae have the ability to biosynthesize, metabolise, accumulate, and produce a wide range of primary and secondary metabolites which sometimes or mostly are used as defence mechanisms or in the survival of the organism. These compounds are significant for/with current and future uses in agricultural, medicines, food, and cosmetics sectors.

1.4 Areas of Application

1.4.1. Agriculture and wastewater treatment

1)To enhance the soil for agricultural purposes or other relevant things, algal biomass or live cultures are employed (Nakao and Kuwazuka, 1991). 2)The capacity of *Nostoc muscorum* which is one of the cyanobacterial culture, the polysaccharides of which is used to retain water is advantageous when used as a soil stabiliser (De Caire et al., 1997). 3)According to Singh et al., (2014) and Abdel-Raouf et al., (2012), some cyanobacteria and green algae have effective antagonistic agents, which show effects against plant-pathogenic bacteria, fungus, and insects.

1.4.2. Food and nutraceuticals industries

1)Since ancient times, people have been using microalgal and cyanobacterial like, *chlorella*, *nostoc*, and *spirulina* as food source. Microalgae chemical cell makeup, which is a source of high protein content and vital minerals as well as vitamins, is what gives its biomass such a high nutritional value as well as health advantages. (Et al., Batista (2013). 2)Microalgae and cyanobacteria, have antibacterial and fungicidal properties, which enable them to be used in chemicals which are used in food preservatives (Guedes et al., 2011; Najdenski et al., 2013) .3)A recent method of consuming nutritious food and food supplements (nutraceuticals) is by use of microalgal biomass and/or metabolites (Gouveia et al., 2008)

CHAPTER 2

LITERATURE REVIEW

In todays ever growing world population, there is ever growing demand on the energy production supplies. The energy supplies are used in many ways considering the production and transportation at the highest (Singh et al., 2013). The total energy demand for transportation holds about 30% of the total cost production and is seen increasing at a rate of 2% every year (Atabani et al., 2013). The fuel that are derived from petroleum product are getting over as their demand for their consumption is high and they take thousands and millions of years to reform (Mofijur et al., 2013). Furthermore, the combustion of these products and matter after combustion that arise from the use of these fuels are regarded as environmentally harmful and also harmful to health. Due to the increasing cost of petroleum goods, climate change, and the reduction of fuel that are obtained from petroleum, all of these have all raised interest in finding an alternate energy sources which would be more cheaper, environment friendly and easily available(Palash et al., 2013) (Atabani et al., 2013) (Shahabuddin et al., 2013). Over the past few years all over the world scientists and researcher and other academic groups are working on finding an alternative which can replace fossil fuel derived energy sources with one that is readily available, affordable, practical in technical approach, and which can be accepted by the people (Liaquat et al., 2010). An alternate source of energy in comparison to fossil fuel is the Biodiesel. Biodiesel is regarded as one of the best alternative fuels for some of the following reasons – It is similar to diesel and is also environment friendly, non-flammable and renewable. With minor modifications, biodiesel can be applied to compressed engines (Tesfa and Mishra et al., 2013).Biodiesel can be obtained through process of trans-esterification which is one of the most method by which biodiesel is obtained (Costa and Almeida et al., 2013.)

Biodiesel can be extracted from three sources which are namely, the first-generation biofuel, the second-generation biofuel and the third-generation biofuel. (Mojifur et al., 2018).The first-generation biofuel: It is made up from edible oils like; peanut oil, soybean oil, sunflower oil, corn oil, rice bran oil, palm oil, coconut oil, olive oil (Atabani et al., 2012.)The second-generation biofuel: It is made up from non-edible oils like; Mahua, karanja, Jatropha, Castor and Neem. (Imdadul et al.,2016).The third-generation biofuel: It is made up from biomass from Microalgae and other sources which produce biomass.

However, there is now a serious worry about the sustainability of some first generation (1G) as it also is consumed as a food source and second generation (2G) biodiesel, due to the global development of biodiesel production (biodiesel from edible and non-edible oil source). Third-generation biodiesel's potential has been discovered as a result of the following: first the displacement of food crops; secondly the effects on the environment; and lastly the Climate change.The third-generation biodiesel can have a great impact on the energy infrastructure of the whole nation, even though first and second generation biodiesel is now only being used in modest amounts due it its availability. (Eisentraut A. 2010).The particular unique yield of biomass obtained from microalgae, of the third-generation biodiesel in comparison to other biomass of the third generation biodiesel category. Diesel and petrol are just two of the fuels that 3G biofuel can be combined with to reduce the pollution levels and better performance. Microalgae is a more energy-rich, environmentally friendly, and is a renewable feedstock as compared to others.Also due to the ability to create microalgae-derived biodiesel round the year is one of its main advantages. Next the oil yields about 20–50% dry weight of biomass, which is more than any other crop's output in biomass production used for biodiesel production. Also the unused land can be used for cultivation of microalgae. The cultivation of microalgae doesn't harm the environment, microalgae can develop quickly; and there is no need to use pesticides when growing microalgae (Rashid et al., 2014).

Oil/lipid extraction from microalgae.

Different methods can be used to extract oil or lipid from microalgae. Oil extraction is done using mechanical and chemical method which is the traditional way of extraction. The mechanical approach is further divided into three categories: ultrasonic assisted extraction, microwave assisted extraction, and oil expeller. Comparably, there are also three types of chemical extraction methods: Soxhlet extraction, supercritical fluid extraction, and rapid solvent extraction. (Mubarak et al., 2015).

The following are some difficulties in obtaining oil from microalgae: first in comparison to other sources, the efficiency is lower. Second the weak chemical interaction in wet biomass which is a huge problem. Third high drying expenses. Also use of too many chemicals in the extraction. Next lipids separation from the liquid media is a bit of a work, and lastly the dependency on the cell's physical characteristics.

Qualifications of lipid profile.

Solvent extraction and gravimetric determination are approaches in the traditional lipid determination process (Bligh and Dyer, 1959). Separating of the crude extracts and quantifying the lipid fractions using Thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), or gas chromatography (GC) are necessary in the further quantification of neutral lipids (Eltgroth et al., 2005). But there was a problem in using this method as it was seen that there was lipid oxidation or degradation. Also it was time consuming and labor intense and therefore only limited algal culture could be quantified at a time.

The other methods which are now known and are being used, help reduce this issues, such methods of quantification are of Nile red method. Nile red is a fluorescent dye which is lipid-souble and is employed to evaluate the lipid content of animal cells and microorganisms, such as mammalian cells (Genicot et al., 2005), bacteria (Izard and Limberger, 2003), yeasts (Evans

et al., 1985; Kimura et al., 2004), zooplankton (Kamisaka et al., 1999), and microalgae (McGinnis et al.,1997; Eltgroth et al., 2005; Elsey et al., 2007)

Some others had tried to develop quantification techniques which are faster in utilising the lipophilic fluorescent dyes with BODIPY or Nile Red because the extraction and drying processes are labour- and time-intensive (Bertozzini et al., 2011; Cooksey et al., 1987). DMSO which is a carrier solvents have been employed which helps in the staining process because microalgal cells have a strong/thick cell wall that stops the dye from getting inside the intracellular matrix of the microalgae (Chen et al., 2011; Cooper et al., 2010; Velmurugan et al., 2013). However, sometimes because of the association between fluorescence intensity and real lipid content varies among strains of different cultures, a broad spectrum lipid quantification assay using lipophilic dyes is not feasible as it gives different readings (Cooper et al., 2010). Due to the difference in strains of different cultures and the difference in their cell wall, thicker and tougher cell wall become more difficult to penetrate and therefore are not stained well and therefore cannot be properly quantified for the lipid profiling. The thinner cell wall are more easy to penetrate and gets stained darker and this can lead in the wrong lipid profiling. As a result, the fluorescent dye approach still can be used only for the qualitative analysis at best in the microalgae and is not effective for quantifying lipids.

Since it was introduced by Chabrol and Charonnat in 1937 (Chabrol and Charonnat, 1973). It was used for the quantification in humans lipids by (Vatassery et al., 1981). Drevon and Schmit in the year 1964 had introduced a better version which included a colorimetric method which would help in the quantification of lipids from a sample (Drevon and Schmit, 1964). Using the sulfo-phospho-vanillin reaction to quickly screen for wild type and mutant microalgal strains would be a major improvement over laborious or inaccurate previous methods. In comparison to other existing procedures, the suggested process is significantly simpler and faster, using

just small amounts of biomass orders of milli-grams or less and does not require a drying or extraction step.

Culturing of Microalgae

Culturing of algae dated back in the late 1800s. *Hematococcus sp*. is a *chlorophyte ,* is one of the culture that was cultured by Ferdinand Cohn in 1850 (Cohn 1850). The culture could not be maintained for an extended length of time, nor was it pure (i.e., free of other species); also, the culture media was not known. Afterwards, a specific medium that contained of a few salts, was used by Famintzin for the cultivation of green algae (Famintzin 1871).The Dutch microbiologist Beijerinck was the first one to report a axenic culture of algae which dated back in the 1890.Using a micropipette, Miquel (1890–1900) had invented the process of obtaining or isolating a single cells under a microscope. (Miquel 1893). Provasoli and other fellow-mates were successful in the making of an artificial medium which mimicked the conditions that were seen in the nature itself. They were also able to obtain and maintain a bacterial free culture by the use of antibiotics. (Provasoli 1960).

Important steps in microalgal culturing.

The process of isolating microalgae from their natural habitat, growing them, maintaining them and getting an unialgal or axenic laboratory cultures is a complex process. In process of this can be difficult and is seen to have many continuous failures. The failure in the isolation and maintenance can be due to various reasons of which some are: First Sample: Avoiding contamination is one of the precautions taken during sample collecting as this forms of cells are different from that those are attached to surface, and are prone to contamination. Second c hoice of media: For microalgae, a range of culture media have been employed; some popular ones include BG-11 (Rippka et al., 1979), f/2 (Guillard 1975), and Walne's medium (Walne

1970). Purification of microalgal cultures entails removing contaminating heterotrophic bacteria using a combination of streaking, microscopic observation, and the appropriate use of antibiotics (Andersen 2005; Waterbury 2006).

Molecular identification of Microalgae.

Morphological approaches have been the traditional method used for taxonomic identification of algae. Microalgae require a certain level of taxonomic competence and proper microscopic examination because the morphological distinctions between species are negligible. This explains the growing use of genetic identification techniques based on conserved gene sequences called barcodes (Krienitz and Bock 2012). However, due to the great variability in the genome sequence and organisation of both nuclear and organelle components, no single DNA barcode can be applied to all algae species. The internal transcribed spacer sequences (ITS) and the nuclear-encoded 18S rRNA gene are two examples of potential genes. There is broad applicability for the ITS2 sequence, which is located between the genes encoding the big and small ribosomal subunits (Caisová et al., 2013). The sole disadvantage is its lack of adequate conservation, which has been somewhat mitigated by supplementing secondary structure data with ITS2 sequence data to produce a significantly more accurate phylogenetic picture (Buchheim et al. 2011; Coleman 2003). Based on secondary structure data and the ITS2 sequence, green algae have been identified up to the species level in both wild samples and cultured (D'Elia et al. 2018; Ferro, Gentili, and Funk 2018; Hoda 2016). Moreover, this marker has been applied to Chlorophyceae members in order to identify taxa (Hegewald et al. 2010; Leliaert et al. 2014).

CHAPTER 3

MATERIALS AND METHODS

Materials

Media: Medium BG-11.

Chemicals: EDTA, phenol, chloroform, isoamyl alcohol, isopropanol, Tris chloride, sodium chloride, sodium dodecyl sulphate, agar, and sodium nitrate.

Antibiotic: Penicillin, streptomycin, and gentamycin

PCR Reagents: Taq polymerase, dNTP mix, 10X PCR assay buffer, forward and reverse primers.

Methods

Water sample collection.

Water samples were collected from 2 freshwater lakes namely Nanda Lake and Sarzora Lake from Goa, India. Water sample was collected in sterile containers, from surface of a depth of 0 to 2 meters. Samples were collected in 2 seasons; Rainy and Winter season.

Table 1: Water sample collection at Nanda and Sarzora lake.

	Site	Collection Date	Location
1	Nanda lake (Dry season)	7/7/23	15.241323,74.105460
2	Sarzora lake (Dry season)	7/7/23	15.218624,74.004720
3	Nanda lake (Wet season)	28/11/2023	15.241323,74.105460
4	Sarzora lake (Wet season)	28/11/23	15.218624,74.004720

Isolation of freshwater Microalgae

Water sample was collected from the lakes of Nanda and Sarzora. The water sample was pre-filtered through a 0.47µm filter membrane (so that larger organisms and mud particles are eliminated) using a filtration unit and a vacuum pump unit. The filtered water sample was again filtered through a 0.22µm-filter membrane and the concentrate retained on membrane was inoculated into BG-11 medium and incubated at 25°C, under a 16:8 hours light: dark cycle. Mixed culture of microalgae was purified by repeated serial dilution in a 6 wells plate.

Purification of Microalgal cultures.

In most cases, heterotrophic bacteria are present in algal cultures. An "axenic" culture is a unialgal culture that is free of bacteria. Axenic cultural establishment was attempted by:

i) **6 well micro-titer plate:** The algal culture from the BG-11 medium at its log phase was transferred into the 6 well plate containing 4.5ml BG-11 medium and later 0.5ml culture was inoculated into it. The plates were allowed to grow under light and dark condition for 16:8 hours at 25°C.

ii) **Streaking:** A loop full of liquid culture was streaked on BG-11 agar plates. The culture was then re-streaked till bacterial free Microalgae isolates were obtained.

iii) **Use of Antibiotics:** Antibiotics such as Penicillin, Streptomycin and Gentamycin was used in a cocktail mix (10, 2.5, 2.5mg/ml respectively). This was used at a concentration of 2% in accordance to the medium. After few days loop full was re-inoculated into BG-11 media(without antibiotics) to obtain bacterial free culture.

Maintenance of Microalgal culture.

Cultures of microalgae were maintained under standard conditions (25°C, 16:8 h light;dark cycle,) and were cultured into fresh BG-11 medium every 3-4weeks.

Identification of Microalgal cultures.

Morphological observation.

Microalgae cultures were mounted on and were first observed under 40X and checked with oil immersion at 1000X under a microscope.

Molecular identification of Microalgae.

DNA ISOLATION

DNA isolation procedure was carried out according to previously published protocols (Keshari et al.,2015; Newman et al.,1990; Radha et al.,2013). 15ml of microalgal culture was taken in a falcon tube and was pelleted at 8000rpm for 10mins. The cell pellet was resuspended in 500µl of TEN buffer (10mM Tris-Cl, 10mM EDTA, 150mM NaCl, pH 8) and later this is then centrifuged at 8000rpm for 5mins. The resulting cell pellet with the help of a thin plastic stick was crushed with glass powder and to this 600µl lysis buffer is added which is prepared using (2% SDS, 400mM NaCl, 40mM EDTA, 100mM Tris-Cl, pH 8) and this mixture was kept at room temperature for 5 minutes. Then centrifuged at 8000rpm for 5min, later to the supernatant, an equal amount volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, and the tube centrifuged at 10000rpm for 5 minutes. The aqueous layer from the tube was removed with a micropipette and was transferred to a fresh tube and an equal volume of chloroform: isoamyl alcohol (24:1) added. After centrifugation of this mixture at 10000rpm for 5mins, the aqueous layer was recovered, DNA was then precipitated by addition of 0.6X volumes of isopropanol, this mixture was then incubated at room temperature for 20 minutes, followed by centrifugation at 10,000rpm for 20mins. The DNA pellet was washed with 70% ethanol, air dried and then was resuspended in 20µl of 10mM Tris chloride and stored at –20°C

until further use. The isolated genomic DNA was run on a 0.8% agarose gel containing ethidium bromide, and observed under a UV transilluminator.

PCR Amplification

The amplification of Microalgal DNA was done using the universal primers for microalgal cultures. The internal transcribed spacer 2 (ITS-2) sequence, this was amplified using primers of ITS-f and ITS-r (Liu, Gerken, and Li 2014).

Amplification was carried out using a thermocycler. After addition of PCR reaction components i.e sterile distilled water, PCR buffer, dNTPs, ITS forward and reverse primers and the template DNA (excluding Taq polymerase), the initial denaturation was carried out at 95°C for 1 minutes, after which Taq polymerase was later added and the amplification reaction cycle was started again(30cycles). After the whole process the PCR product was loaded on a 0.8% agarose gel, and checked on a UV transilluminator.

Later the sample was handed over to companies to get the DNA sequence (Here the sample was purified according to the company). Later Sanger sequencing was used to sequence purified fragments automatically. The resulting forward and reverse sequences as provided by the company were concatenated to create a contig, which was then compared to other known sequences in the NCBI-GenBank database using BLAST (Altschul et al., 1990) to determine which organism match was the closest to the isolated sample. Using the CLUSTALW, the sequences which displayed greater than 97% of similarity to the query sequence and belonged to related genera were chosen and aligned accordingly. Using the Maximum Composite Likelihood model, the 1000 bootstrap replications, the Neighbour-Joining heuristic, and MEGA software (Tamura et al., 2011), a phylogenetic tree was created showing the relation

with other related species. The tree was constructed, checking that the branch values were more than 60 to the isolated sample.

Table 2: PCR Parameters

PCR Parameters	Time	Temperature
Initial denaturation	5 min	95°C
Denaturation	30 s	95°C
Annealing	30 s	50°C
Extension	1 min	72°C
Final extension	10 min	72°C

Table 3: Reaction components of PCR mix.

Components	Volume in µl
Distilled Water	27.2
Buffer	5.0
dNTPs	3.0
Forward primer	5.0
Reverse primer	5.0
Template	5.0
Taq. Polymerase	0.8
Total	50.

LIPID ENHANCEMENT IN MICROALGAE

Different parameters were tested for to see lipid content accumulation and to compare the lipid content in each of the test preformed. Lipid production, of different microalgae at varying concentration of sodium nitrate and pH was checked after inoculation for a period of three week.

In 50ml of BG-11 medium 5ml of microalgal was inoculated to check and compare the lipid content at varying concentration of; 0.1, 0.25, 0.5, 1.0, 1.5 g/l of $NaNO_3$. Similarly, to check and compare the lipid content of microalgae under different pH, in 50ml of BG-11medium 5ml of culture was added at varying pH of; 5, 6, 7, 8, 9 (Sharma et al.,2018). The culture was allowed to grow in light: dark condition and checked for lipid content by the SPV method for 3 weeks.

SPV METHODS (SULFO-PHOSPHO-VANILLIN)

SPV REAGENT

The first step in preparation of the phospho-vanillin reagent is to dissolve 0.06 g of vanillin in 1ml of ethanol and 9ml of distilled water and mixed thoroughly (Mishra et al.,2013). This mixture was then mixed with 40ml of concentrated phosphoric acid, and the resulting reagent was kept in the dark until needed. Freshly prepared phospho-vanillin reagent was made just before the experiment to give maximum accurate results.

STANDARD LIPID PROFILE

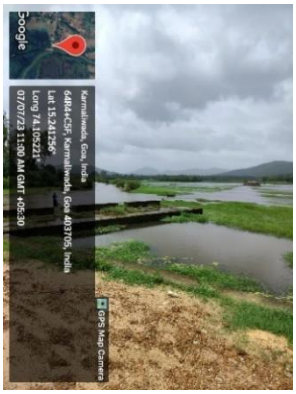
Commercial canola oil was used in order to get the standard lipid stocks at a concentration of 2mg/ml made when 20mg of canola oil in 10ml of chloroform was added. The mixture was then stored at -20 C until further use. The empty glass vials having Teflon covers was filled with varying microliters (25, 50, 75, 100, 125, 150, 175, 200) of standard canola oil stock

solution of 2mg/ml. This concentration mix was heated in a water bath for ten minutes at 60 degrees Celsius to allow the solvent to evaporate from this stock, 100 microliters of distilled water was added to the lipid standard in the glass vials after evaporation then 2ml of concentrated sulfuric acid to the mixture along with freshly prepared 5ml of phospho-vanillin reagent, this mixture is then heated in water bath for 10mins at 100 degrees Celsius, this vials were then cooled in ice bath for five minutes . After this the glass vials were kept for incubation on a shaker incubator at 37 degrees Celsius for 15mins under shaking conditions of 150rpm. The sample was calibrated for its lipid content at 530nm (Knight et al.,1972) using a spectrophotometer.

CHAPTER 3

RESULTS AND DISCUSSION

Sample collection



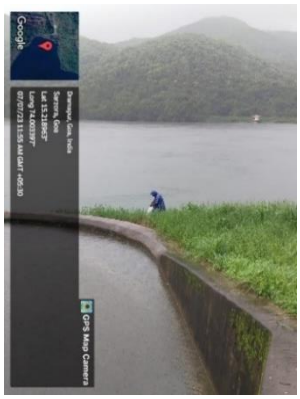


Fig1a Fig1b Fig1c

Fig1a: Nanda lake

Fig1b: Sarzora lake

Fig1c: Water sample collection.

Table 4:: Physicochemical parameters

Sample	Date	pH	Salinity
Nanda lake (wet season)	7/7/23	6.49	0
Sarzora lake (wet season)	7/7/23	6.74	0
Nanda lake (dry season)	28/11/23	6.38	0
Sarzora lake (dry season)	28/11/23	6.64	0

The physicochemical parameters of the water sample were checked. Microalgal cultures were isolated from the freshwater lakes of Nanda and Sarzora. These cultures were in the mixed form (Fig 2)

Fig 2: Mixed microalgal culture.

Purification of Microalgal cultures

Various methods were employed, to obtain unialgal culture. Serial dilution was carried out in 6 well plate, containing 4.5ml of BG-11 medium and 0.5ml of algal culture (Fig:). And this was kept in light: dark condition for 16:8 hours duration.

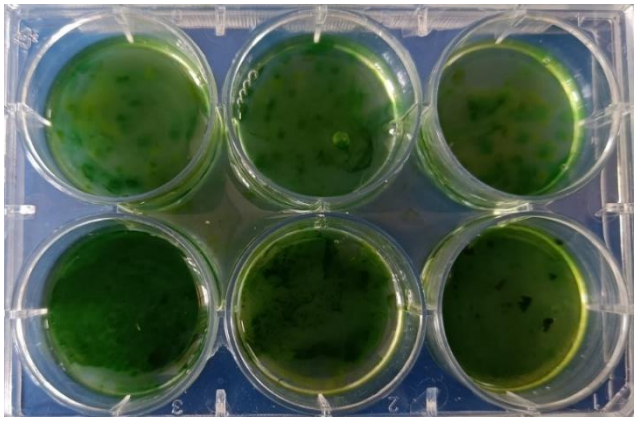


Fig 3: 6 Well micro-titer-plate method.

Treatment with an antibiotic cocktail helped in elimination of bacterial contaminant from the microalgal culture. The cultures were streaked on BG-11 agar, to determine their axenic stage.

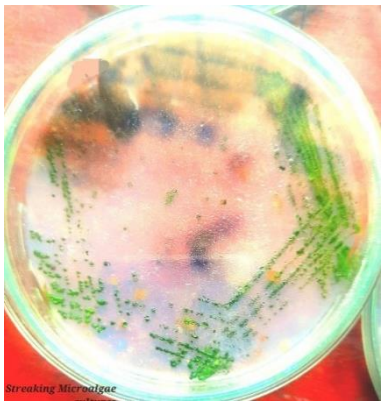
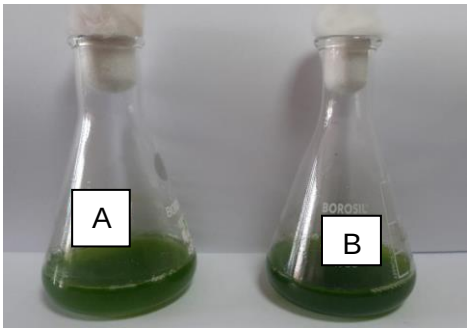


Fig 4: Microalgal colonies growing on a BG-11 agar plate

Maintenance of culture.

Maintenance of culture under 16:8 hours light: dark cycle



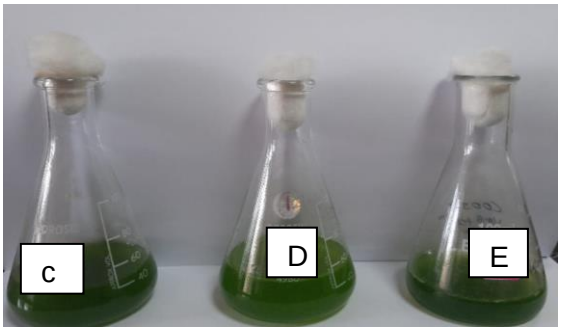


Fig 5A: Microalgae from Nanda Lake and Fig 1B: Sarzora lake

Fig 5C: *Asterarcys sp*. Fig1D: *Chlorella sp. 1* Fig1E: *Chlorella sp. 2*

Identification of Microalgal culture.

The microscopic images of the isolates of Nanda (Fig 5d) and Sarzora (Fig 5e) lakes and other microalgal species of *chlorella sp.1 (Fig 5a)., Chlorella sp.2 (Fig 5b) and Asterarcys sp.* (Fig 5c:)

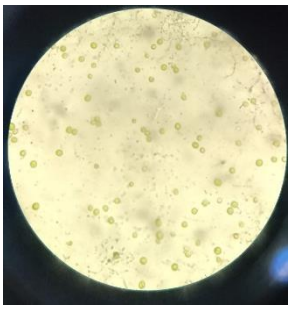


Fig 6a

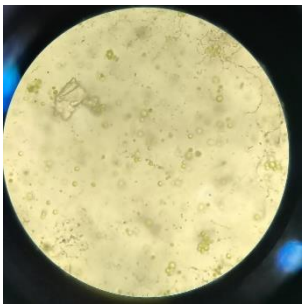


Fig 6b

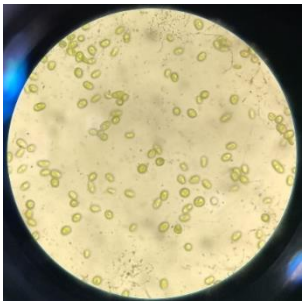


Fig 6c

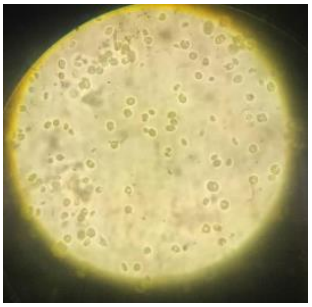


Fig 6d

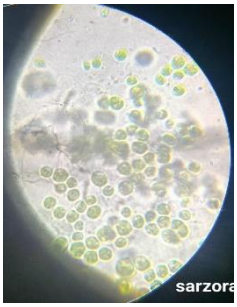


Fig 6e

Fig 6a: Microscopic image of *Chlorella sp.1*

Fig 6b: Microscopic image of *Chlorella sp. 2*

Fig 6c: Microscopic image of *Asterarcys spp*.

Fig 6d: Microscopic image of microalgal isolate NMG from Nanda lake.

Fig 6e: Microscopic image of microalgal isolate SMG from Sarzora lake.

DNA Isolation

DNA isolation of NMG was carried out successfully. Lane 3 showed the isolated DNA

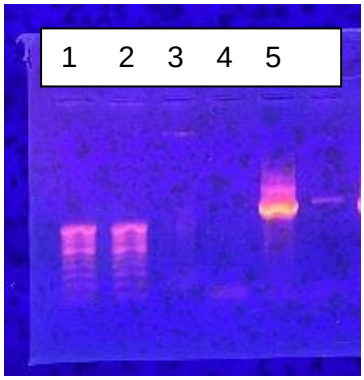


Fig 7: Lane 3 contains the genomic DNA of culture NMG

PCR Amplification

PCR amplification of the ITS 2 sequence was carried out from the genomic DNA of NMG

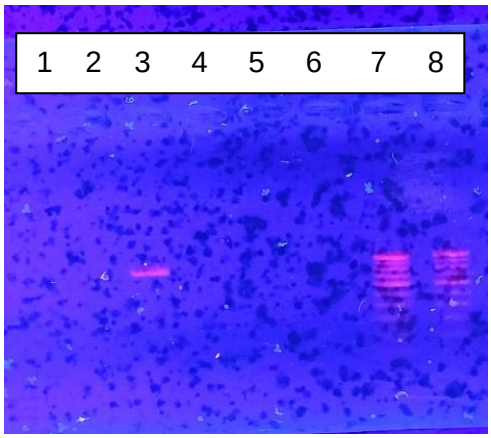


Fig 8: Lane 3 contains PCR amplified product of isolate NMG

Sequences producing significant alignments Download Select columns Show 100

select all *0 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Poterioochromonas malhamensis strain SAG933-1c internal transcribed spacer 1, partial sequence; 5.8S ribosoma…	Poterioochromon…	1039	1039	95%	0.0	99.15%	725	MH549414.1
Poterioochromonas malhamensis strain CMBB008 small subunit ribosomal RNA gene, partial sequence; internal tr…	Poterioochromon…	1039	1039	95%	0.0	99.15%	2517	MH536660.1
Poterioochromonas malhamensis strain CCMP2740 small subunit ribosomal RNA gene, partial sequence; internal t…	Poterioochromon…	1039	1039	95%	0.0	99.15%	2518	MH536658.1
Poterioochromonas sp. strain DO-2004 small subunit ribosomal RNA gene, partial sequence; internal transcribed s…	Poterioochromon…	1039	1039	95%	0.0	99.15%	2517	MH536655.1
Poterioochromonas malhamensis strain P03 small subunit ribosomal RNA gene, partial sequence; internal transcri…	Poterioochromon…	1039	1039	95%	0.0	99.15%	792	PP212032.1
Poterioochromonas malhamensis strain P01 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g…	Poterioochromon…	1039	1039	95%	0.0	99.15%	802	PP212031.1
Poterioochromonas malhamensis strain CCMP2144 internal transcribed spacer 1, partial sequence; 5.8S ribosoma…	Poterioochromon…	1039	1039	95%	0.0	99.15%	803	PP212030.1
Poterioochromonas malhamensis 18S rRNA gene (partial), ITS1, 5.8S rRNA gene and ITS2 (partial), strain DS	Poterioochromon…	1039	1039	95%	0.0	99.15%	2009	FN662745.1
Poterioochromonas malhamensis strain CMBB-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal R…	Poterioochromon…	1034	1034	95%	0.0	98.98%	725	MH549417.1
Poterioochromonas malhamensis strain SAG933-9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal …	Poterioochromon…	1034	1034	95%	0.0	98.98%	725	MH549415.1
Poterioochromonas malhamensis strain SAG933-1d small subunit ribosomal RNA gene, partial sequence; internal …	Poterioochromon…	1034	1034	95%	0.0	98.98%	2516	MH536659.1
Poterioochromonas malhamensis strain SAG933-1a small subunit ribosomal RNA gene, partial sequence; internal …	Poterioochromon…	1034	1034	95%	0.0	98.98%	2518	MH536656.1
Poterioochromonas malhamensis strain B-JZ internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA …	Poterioochromon…	1034	1034	95%	0.0	98.98%	792	PP212033.1
Poterioochromonas malhamensis strain SAG933-8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal …	Poterioochromon…	1030	1030	95%	0.0	98.81%	725	MH549416.1
Poterioochromonas malhamensis strain CMBB010 small subunit ribosomal RNA gene, partial sequence; internal tr…	Poterioochromon…	1030	1030	95%	0.0	98.81%	2517	MH536661.1
Poterioochromonas malhamensis strain 22-6-Y15 small subunit ribosomal RNA gene, partial sequence; internal tra…	Poterioochromon…	1007	1007	95%	0.0	97.96%	762	OQ405058.1
Mallomonas sp. NF-WJ05 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete seq…	Mallomonas sp. …	1005	1005	95%	0.0	98.30%	756	DQ223654.1
Poterioochromonas malhamensis strain CCMP3181 internal transcribed spacer 1, partial sequence; 5.8S ribosoma…	Poterioochromon…	965	965	100%	0.0	95.45%	732	MH549413.1
Poterioochromonas stipitata strain CCMP1862 small subunit ribosomal RNA gene, partial sequence; internal transc…	Poterioochromon…	813	813	99%	0.0	90.47%	2531	MH536657.1

Fig 9: BLAST alignment table for the ITS-2 sequence of culture NMG

The amplified PCR product was submitted for Sanger sequencing and the forward and reverse sequencing reads obtained. The BLAST results of ITS-2 contig sequence of NMG showed 99.15% identity with several strains of *Poterioochromonas malhamensis*. Thus, the tentative identification of this isolate is *Poterioochromonas* sp.

Lipid enhancement

Table 5: Standard lipid profile using canola oil

Stock of canola oil in µl (total volume: 350 µl)	Concentration (mg / ml)	Absorbance 530nm
25	0.1	0.11
50	0.3	0.12
75	0.4	0.29
100	0.6	0.34
125	0.7	1.20
150	0.9	1.31
175	1.0	1.13
200	1.1	1.41
Blank	0.0	0.00

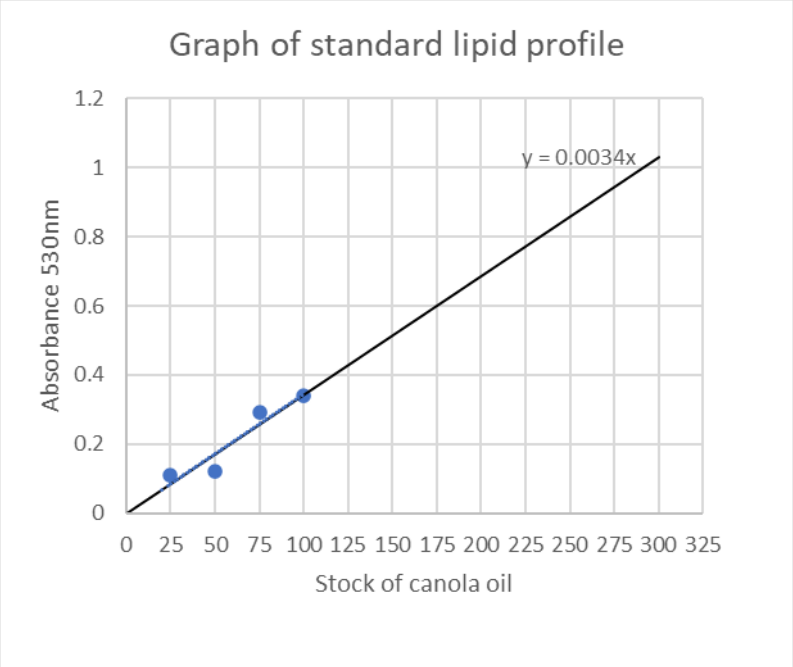


Fig 10: Graph of standard lipid profile

Fig 11: Standard canola oil stock.

Fig 11A: Blank, Fig 11B: 25µl, Fig 11C: 50µl, Fig 11D: 75µl, Fig 11 E: 100µl, Fig 11 F: 125µl, Fig 11G: 150µl, Fig 11H: 175µl, Fig 1 I: 200µl.

The lipid profile of 3 cultures of microalgae: *Chlorella spp.1, Chlorella sp. 2 and Asterarcys sp*. was checked for the lipid production under two parameters namely, pH and $NaNO_3$ concentration.

The lipid production of each of the culture under pH of 5,6,7,8,9 was checked, for 3 weeks.

The result of each pH is given in a bar graph form, in which it depicts the graph of Absorbance at 530nm v/s concentration at each pH

The lipid production of each of the culture under $NaNO_3$ concentration of 0.1,0.25,0.50,1.0 and 1.5g/200ml was checked, for 3 weeks.

The result of each $NaNO_3$ concentration is given in a bar graph form, in which it depicts the graph of Absorbance at 530nm v/s concentration at each $NaNO_3$ concentration.

Table6: Effect of pH on *Asterarcys spp.* Over 3 weeks

Effect of pH on *Asterarcys spp.*	Concentration mg/ml		
pH	W1	W2	W3
5	38.82	557.35	513.82
6	59.41	470	648.24
7	83.24	696.76	567.94
8	46.47	644.12	796.18
9	37.94	790.59	560.59

Figure 12: Effect of pH on lipid accumulation in *Asterarcys sp*. over 3 weeks

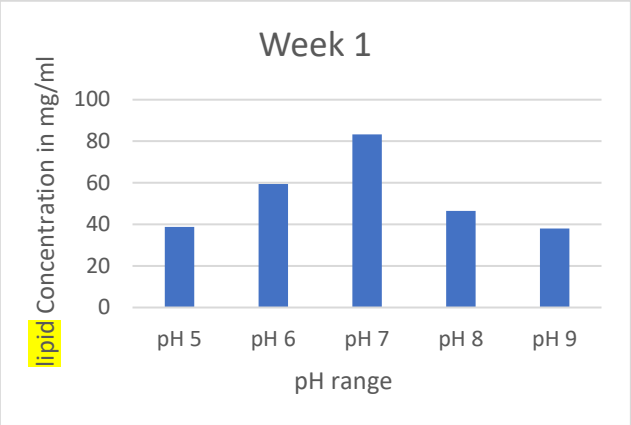


Fig 12 A:

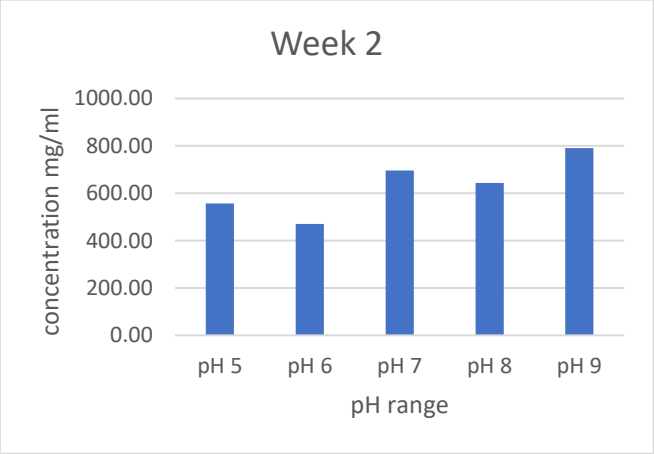


Fig 12 B:

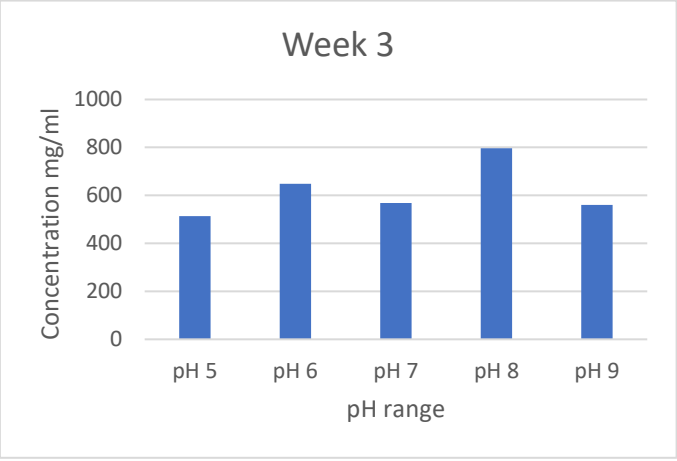


Fig 12 C:

The lipid accumulation in the *Asterarcys* sp. culture after 1 week of incubation was maximum when the pH of the growth medium was 7. After 2 weeks of incubation, the maximum lipid accumulation was seen at pH 9. After 3 weeks of incubation, the maximum lipid accumulation was seen at pH 8.

Table 7: Effect of $NaNO_3$ on *Asterarcys spp*.

Effect of NaNO3 on *Asterarcys spp*.			
$NaNO_3$ concentration g/l	W1	W2	W3
0.1	7.94	143.82	406.47
0.25	20.59	404.41	742.94
0.5	22.94	627.65	671.47
1.0	42.35	121.76	712.35
1.5	37.94	288.24	127.94
C	31.18	345	545

Fig 13: Effect of $NaNO_3$ on lipid accumulation in *Asterarcys sp*. over 3 weeks

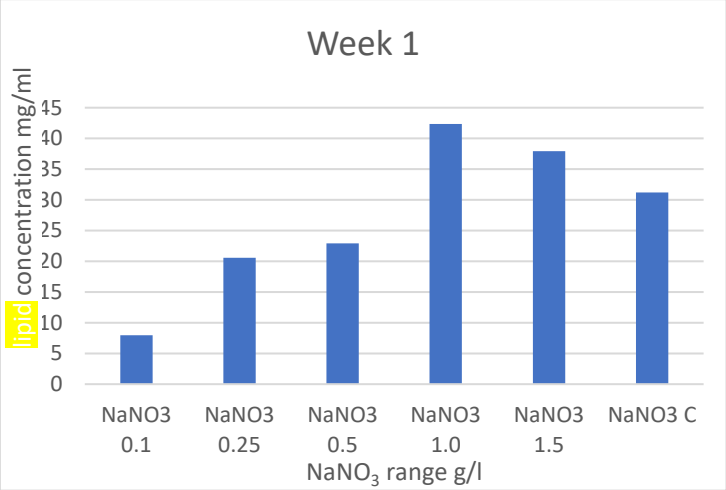


Fig 13 A:

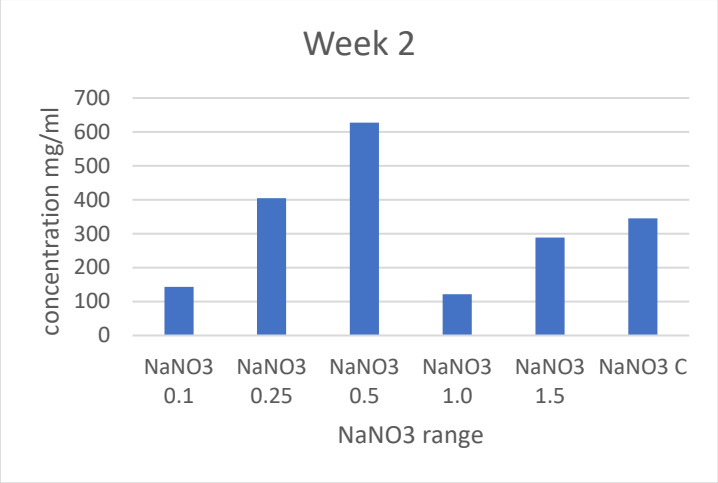


Fig 13: B:

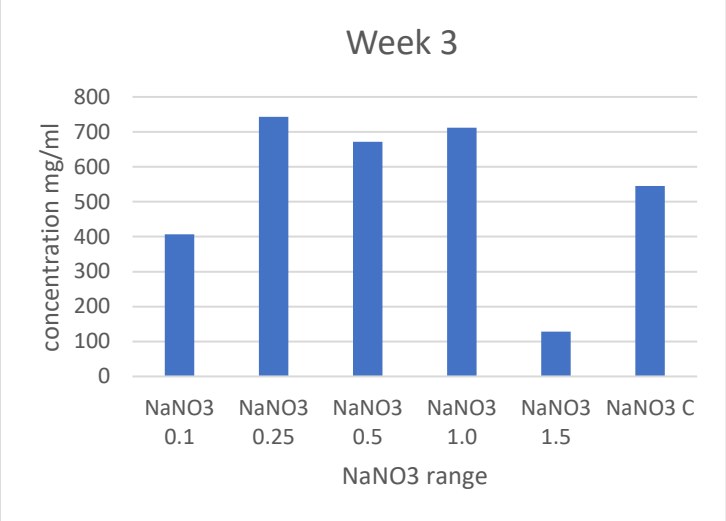


Fig 13C:

The lipid accumulation in the *Asterarcys sp.* culture after 1 week of incubation was maximum when the $NaNO_3$ concentration of the growth medium was 1.0 g/l. After 2 weeks of incubation, the maximum lipid accumulation was seen at 0.5g/l $NaNO_3$ concentration. After 3 weeks of incubation, the maximum lipid accumulation was seen at 0.25g/l $NaNO_3$ concentration.

Table 8: Effect of pH on *Chlorella spp.*

Effect of pH on *Chlorella* spp 1.			
pH	W1	W2	W3
pH 5	38.82	775.9	405.29
Ph 6	59.41	776.8	549.71
pH 7	83.24	790.6	564.71
pH 8	49.41	735.9	771.47
pH 9	72.35	736.5	603.53

Fig 14: Effect of pH on lipid accumulation in *Chlorella sp.* over 3 wee

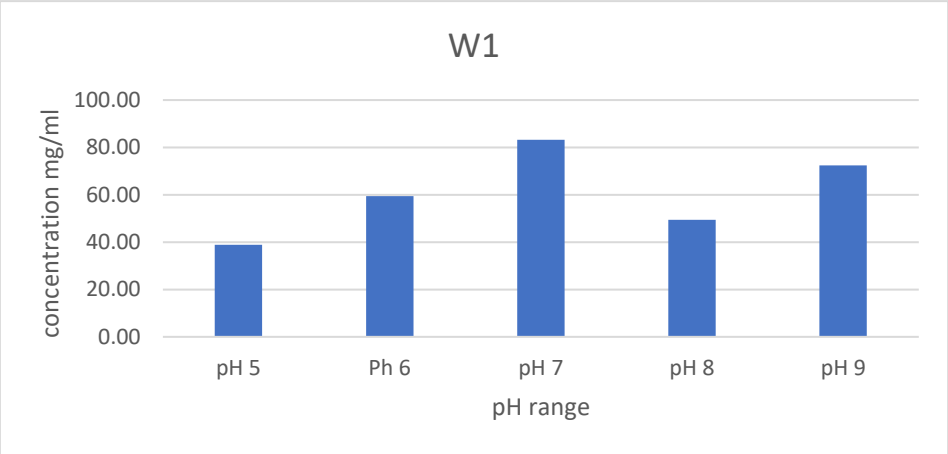


Fig14 :

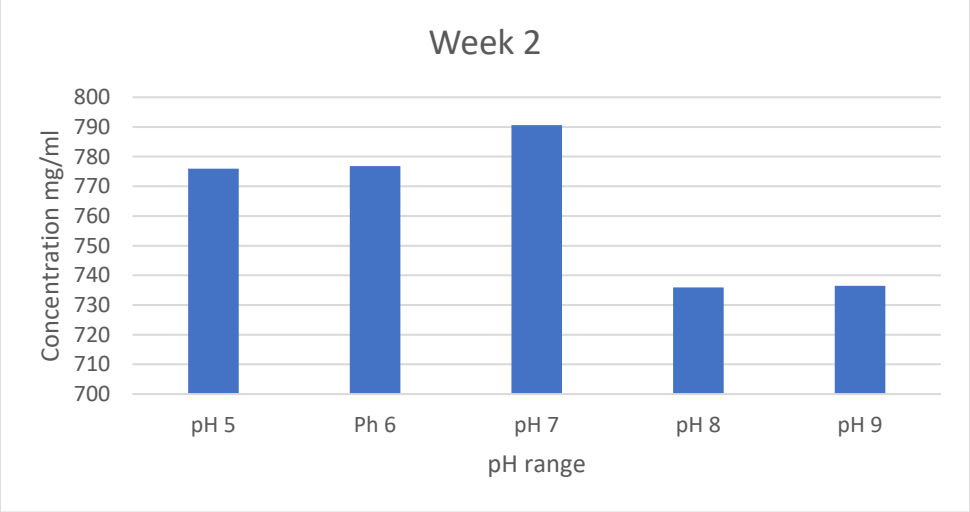


Fig 14 B:

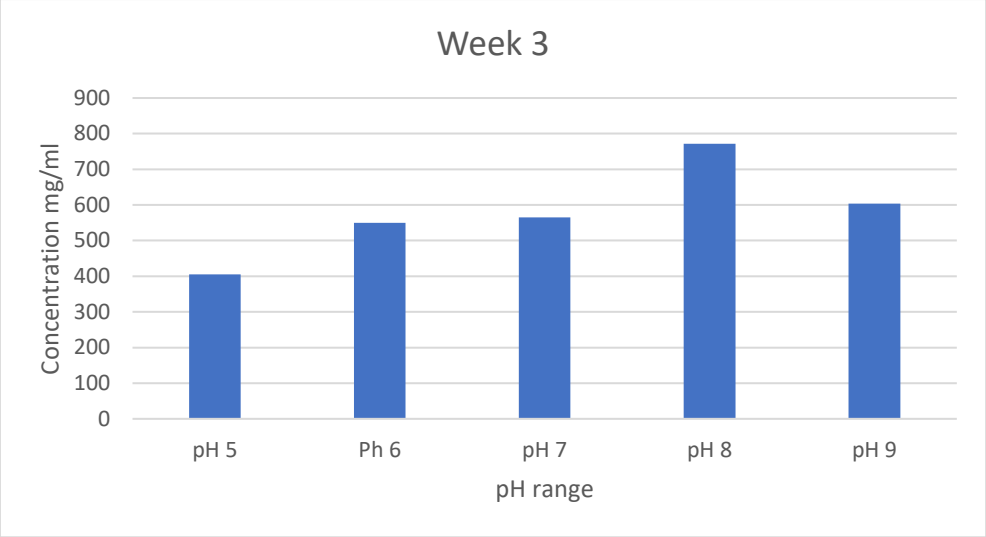


Fig14 C:

The lipid accumulation in the Chlorella sp. culture after 1 week of incubation was maximum when the pH of the growth medium was 7. After 2 weeks of incubation, the maximum lipid accumulation was seen at pH 7. After 3 weeks of incubation, the maximum lipid accumulation was seen at pH 8.

Table 9: Effect of $NaNO_3$ on *Chlorella spp.1*

Effect of NaNO3 on *Chlorella spp.1*			
NaNO3 Concentration	W1	W2	W3
0.1	8.24	106.18	455.88
0.25	104.12	720.88	458.24
0.5	12.35	689.71	726.47
1.0	80.29	748.82	526.76
1.5	115	382.06	455
C	74.12	203.53	551.47

\

Fig 15: Effect of $NaNO_3$ on lipid accumulation in *Chlorella sp*. over 3 weeks

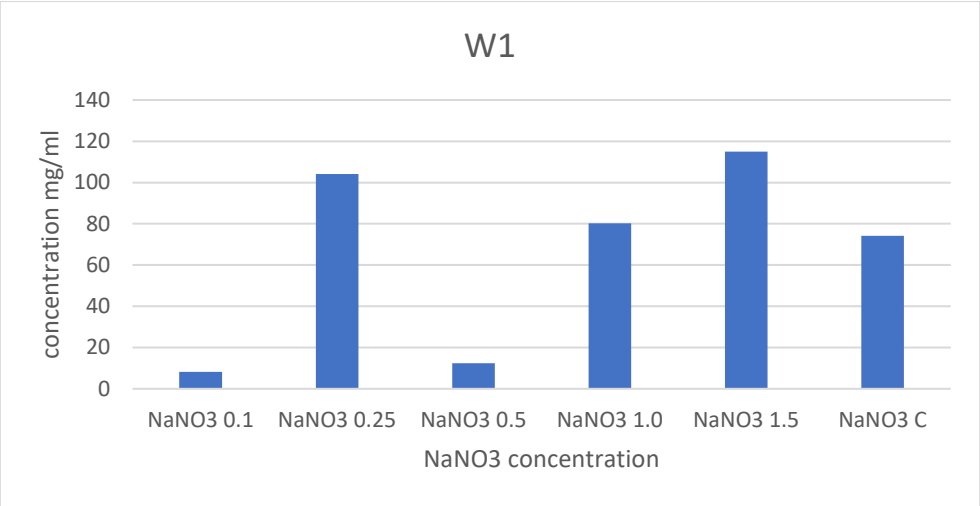


Fig 15 A:

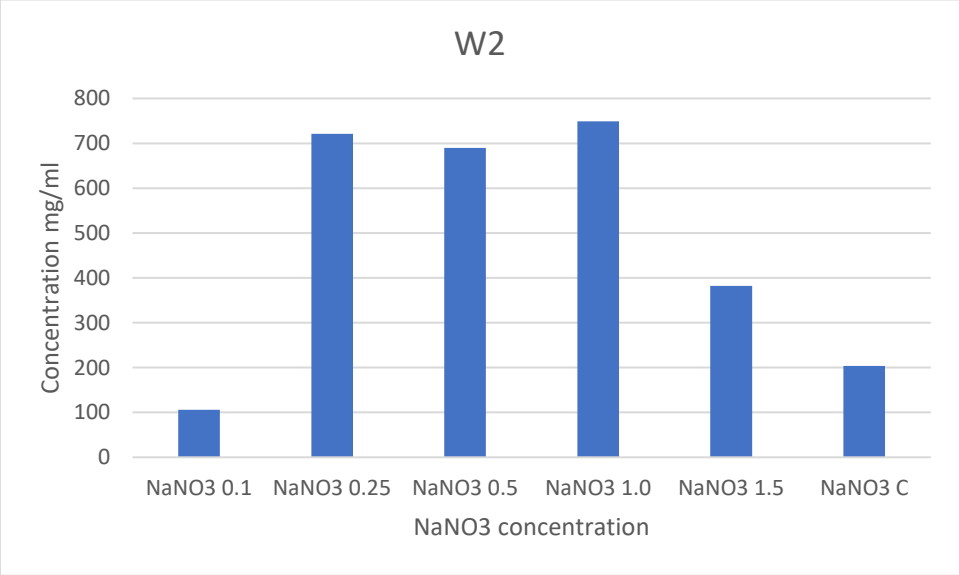


Fig 15 B:

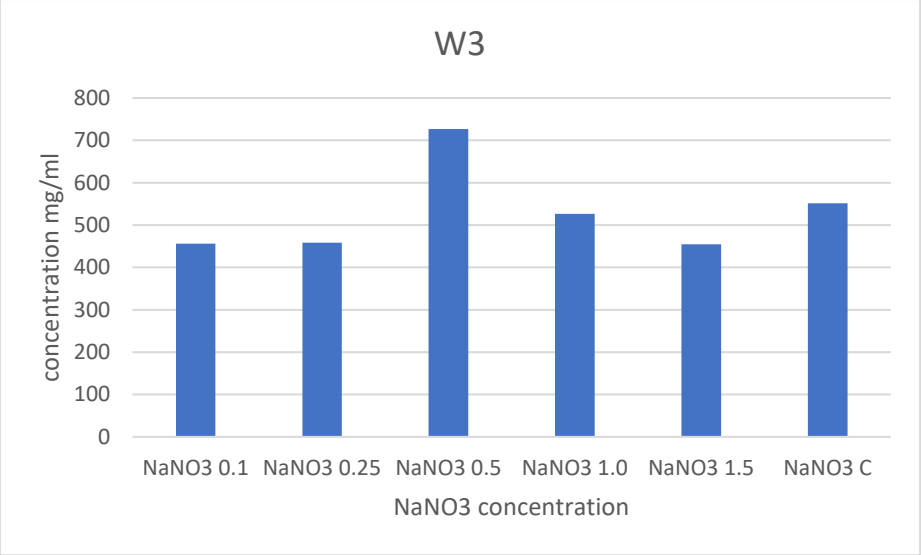


Fig 15 C:

The lipid accumulation in the *Chlorella sp.1*. culture after 1 week of incubation was maximum when the $NaNO_3$ concentration of the growth medium was 1.5 g/l. After 2 weeks of incubation, the maximum lipid accumulation was seen at 1.0 g/l $NaNO_3$ concentration. After 3 weeks of incubation, the maximum lipid accumulation was seen at 0.5 g/l $NaNO_3$ concentration.

Table 10: Effect of pH on *Chlorella sp. 2*

Effect of pH on *Chlorella sp 2.*			
	Concentration mg/ml		
pH	W1	W2	W3
pH 5	20.88	259.71	576.76
pH 6	21.18	451.47	420
pH 7	47.35	370	551.47
pH 8	64.12	551.47	478.82
pH 9	35.88	341.18	311.47

Fig 16: Effect of pH on lipid accumulation in *Chlorella sp.2*. over 3 weeks

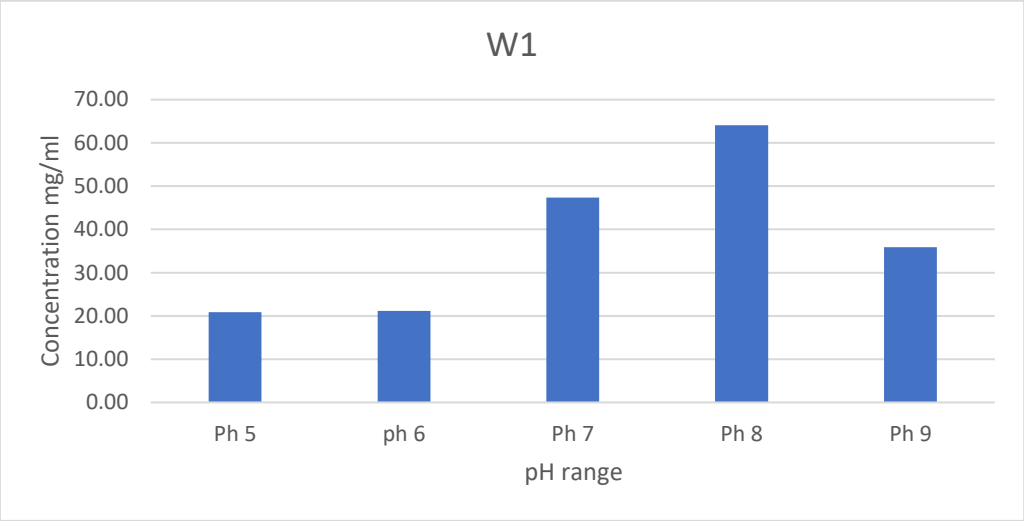


Fig 16 A:

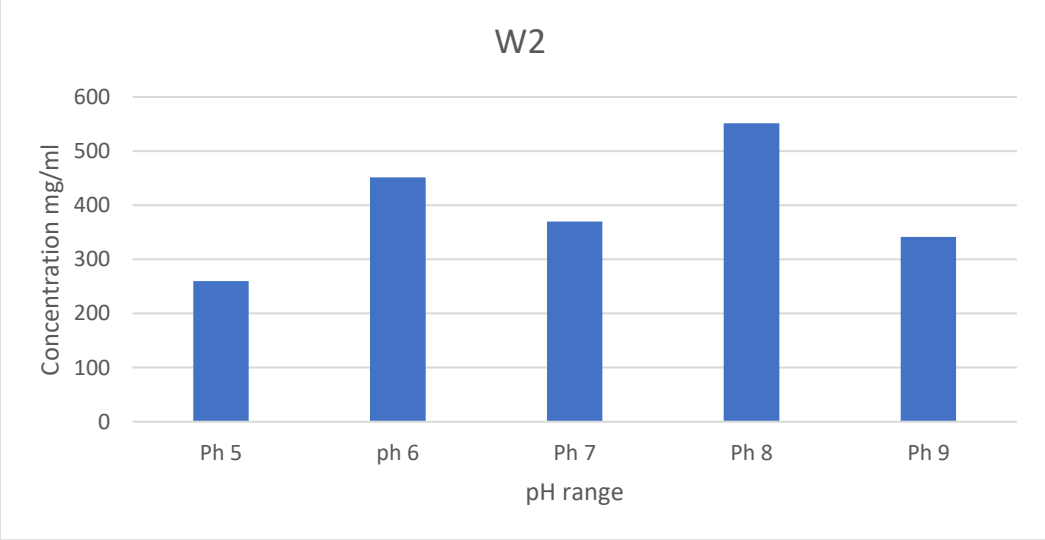


Fig 16 B:

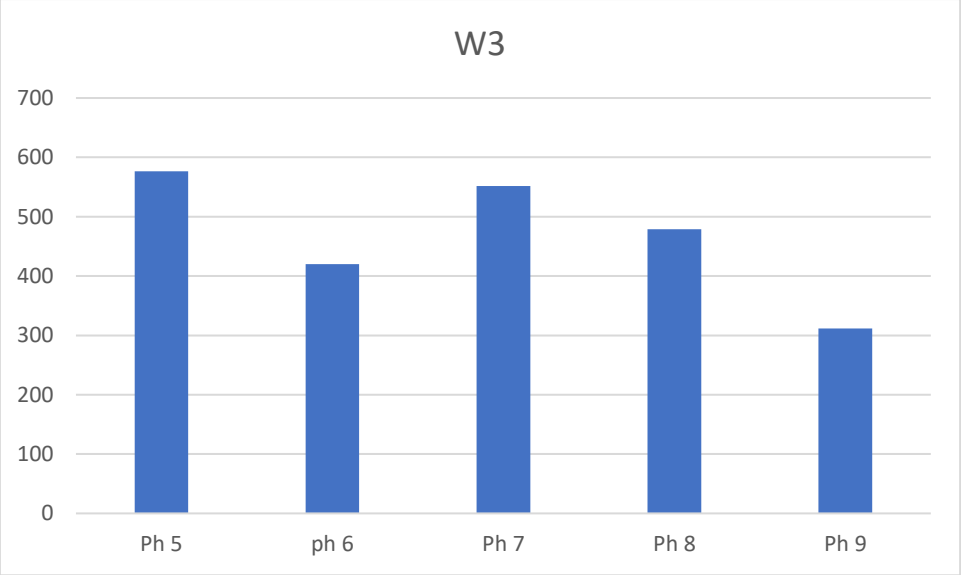


Fig 16 C:

The lipid accumulation in the *Chlorella sp* 2. culture after 1 week of incubation was maximum when the pH of the growth medium was 8. After 2 weeks of incubation, the maximum lipid accumulation was seen at pH 8. After 3 weeks of incubation, the maximum lipid accumulation was seen at pH 5.

Table 11: Effect of $NaNO_3$ on Chlorella sp. 2

Effect of $NaNO_3$ on *Chlorella sp. 2*			
NaNO3 CONC	W1	W2	W3
0.1	7.65	41.76	156.76
0.25	37.65	37.06	228.24
0.5	103.53	44.12	356.47
1.0	28.82	123.53	112.65
1.5	35.59	197.06	299.41
C	25	52.06	194.41

Fig 17: Effect of $NaNO_3$ on lipid accumulation in Chlorella sp.2 over 3 weeks

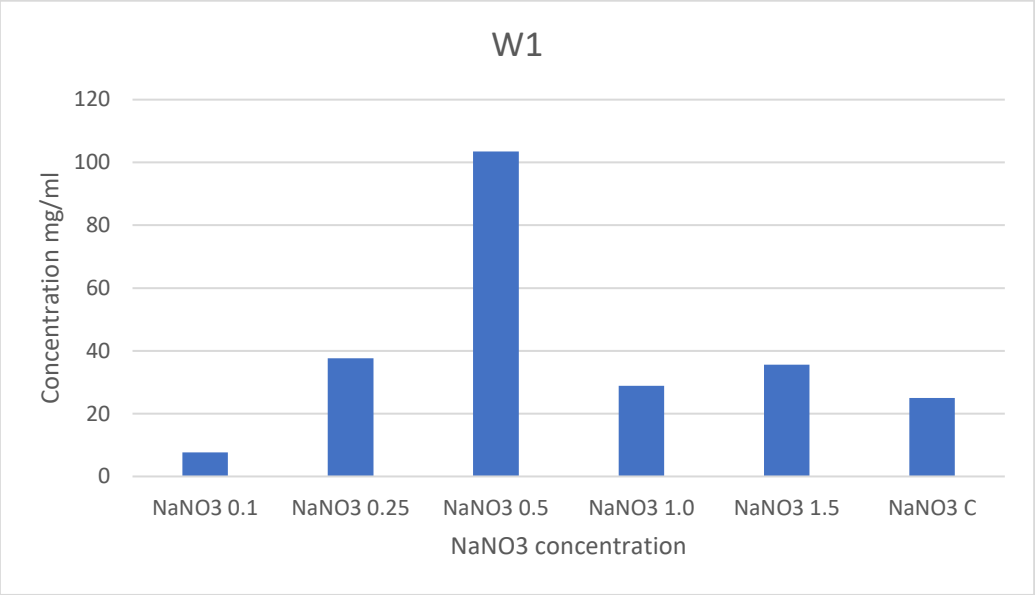


Fig 17 A:

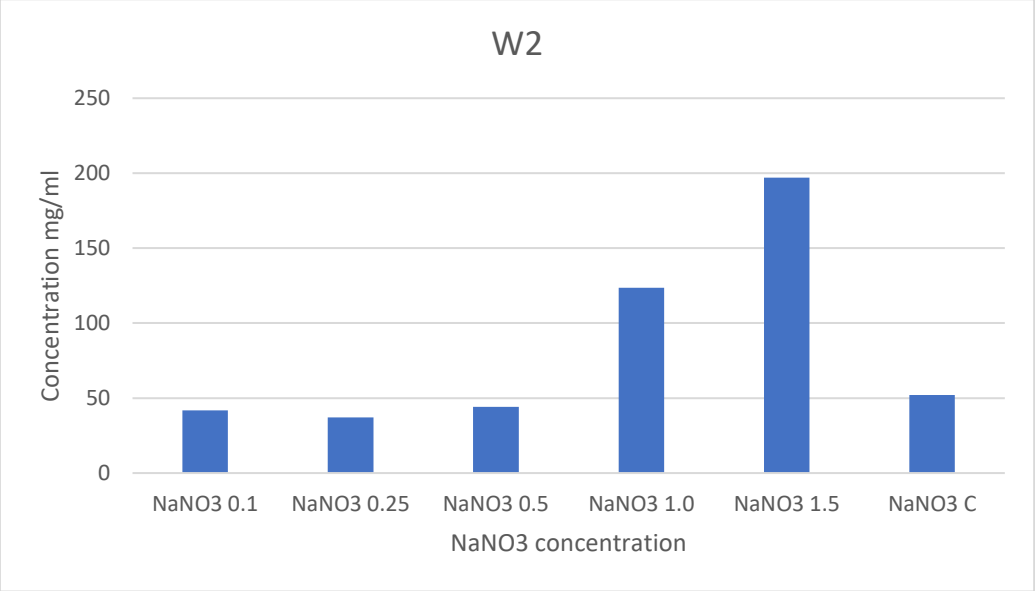


Fig 17 B:

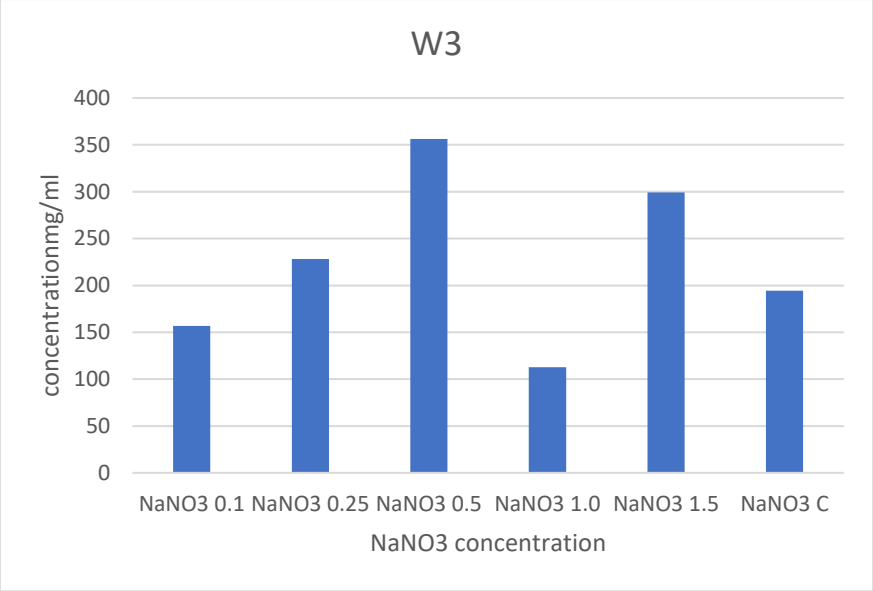


Fig 17 C:

The lipid accumulation in the *Chlorella sp.2.* culture after 1 week of incubation was maximum when the $NaNO_3$ concentration of the growth medium was 0.5 g/l. After 2 weeks of incubation, the maximum lipid accumulation was seen at 1.5 g/l $NaNO_3$ concentration. After 3 weeks of incubation, the maximum lipid accumulation was seen at 0.5 g/l $NaNO_3$ concentration.

Table no. 12: Effect of pH on NMG

NMG pH concentration	W1	W2
5	89.12	272.65
6	89.71	244.12
7	100.29	260
8	104.71	272.94
9	66.76	213.53

Fig18: Effect of pH on lipid accumulation in NMG over 2 week

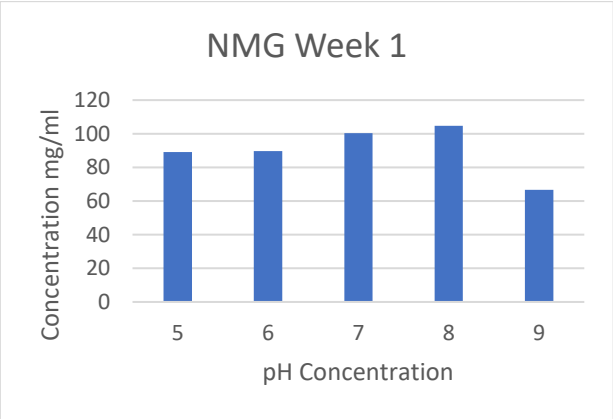


Fig 18 A:

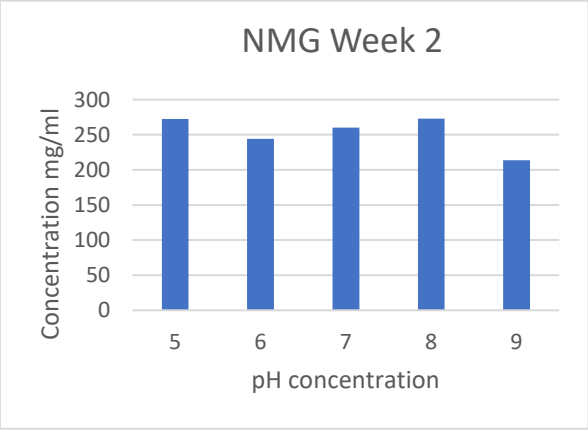


Fig 18 B:

The lipid accumulation in the NMG. culture after 1 week of incubation was maximum when the pH of the growth medium was 8. After 2 weeks of incubation, the maximum lipid accumulation was seen at pH 8.

Table 13: Effect of $NaNO_3$ on NMG

NMG $NaNO_3$ Concentration	W1	W2
0.1	91.76	238.82
0.25	110.00	249.41
0.5	96.47	276.18
1	111.47	265.29
1.5	67.35	241.47
C	73.53	290.88

Fig 19: Effect of $NaNO_3$ on lipid accumulation in NMG over 2 week

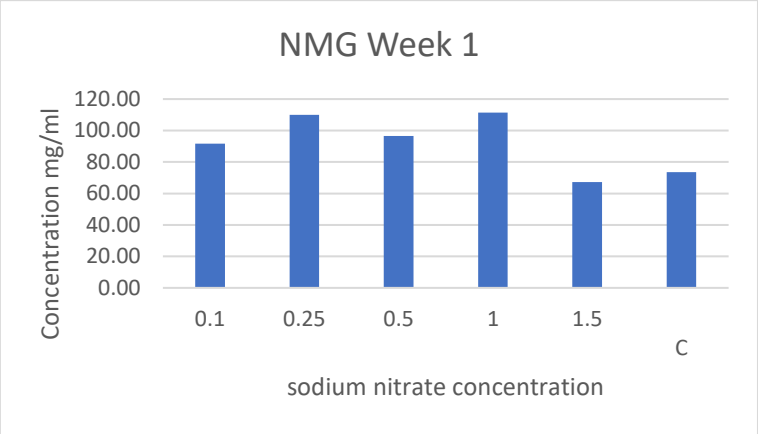


Fig 19 A:

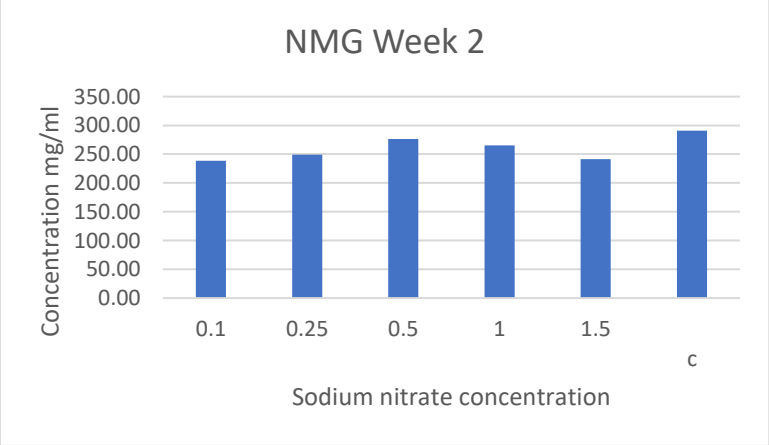


Fig 19 B:

The lipid accumulation in the NMG. culture after 1 week of incubation was maximum when the $NaNO_3$ concentration of the growth medium was 1.0 g/l. After 2 weeks of incubation, the maximum lipid accumulation was seen at 0.5 g/l $NaNO_3$ concentration

Table 14: Effect of pH on SMG

SMG pH concentration	W1	W2
5	88.82	256.76
6	56.76	253.53
7	29.71	265.59
8	84.41	265.29
9	109.71	184.41

Fig 20: Effect of pH on lipid accumulation in SMG over 2 week.

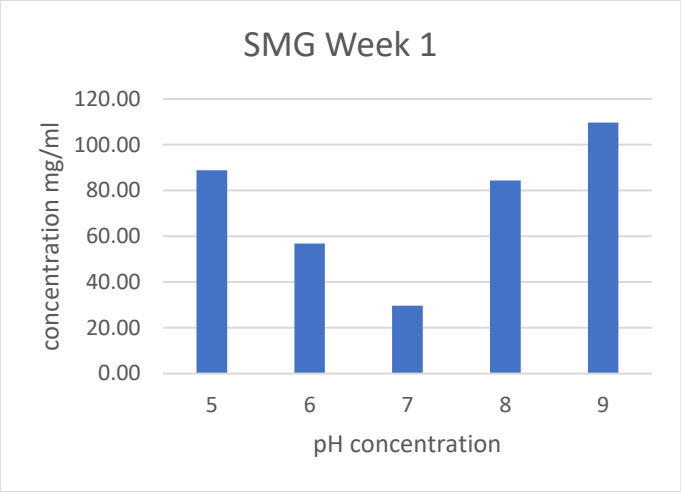


Fig 20 A:

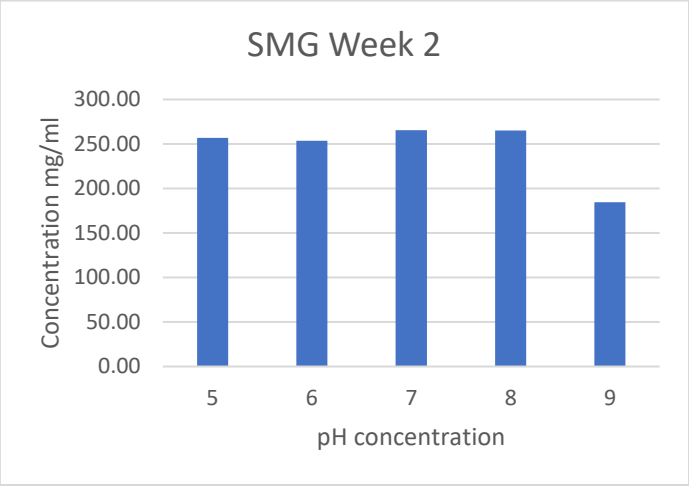


Fig 20 B:

The lipid accumulation in the SMG. culture after 1 week of incubation was maximum when the pH of the growth medium was 9. After 2 weeks of incubation, the maximum lipid accumulation was seen at pH 7.

Table 15: Effect of pH on SMG

SMG $NaNO_3$ Concentration	W1	W2
0.1	85.88	265.29
0.25	72.35	259.41
0.5	45.00	285.88
1	43.53	272.65
1.5	80.59	197.06
c	89.12	256.76

Fig 21: Effect of $NaNO_3$ on lipid accumulation in SMG over 2 week

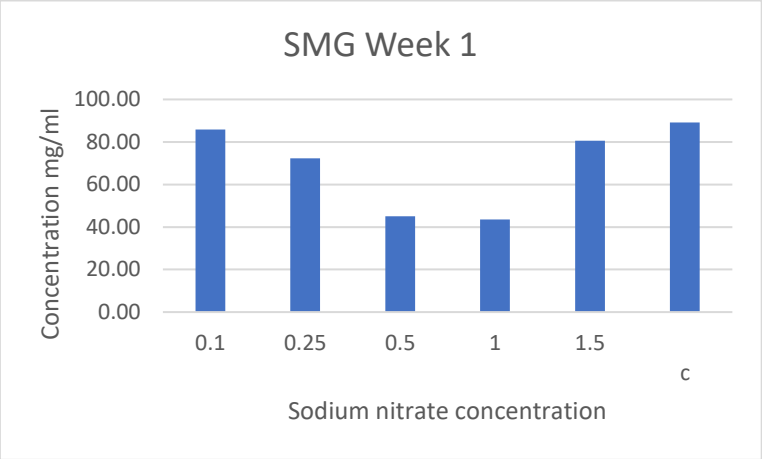


Fig 21 A:

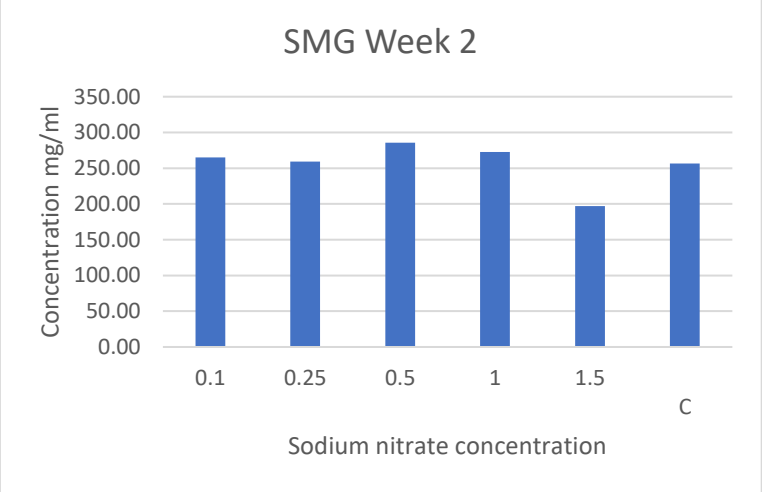


Fig 21 B:

The lipid accumulation in the SMG. culture after 1 week of incubation was maximum when the $NaNO_3$ concentration of the growth medium was 0.1 g/l. After 2 weeks of incubation, the maximum lipid accumulation was seen at 0.5 g/l $NaNO_3$ concentration.

Discussion

In the current study, we have attempted to enhance the lipid production in microalgae isolated from Nanda and Sarzora lake. Those which have high lipid content could be used for biodiesel production. In the tested cultures of this study it was observed that higher lipid production was seen at a sodium nitrate concentration of 0.5 to 1.0 g/l. Also higher lipid production was seen at pH of 6 to 8. Nitrogen deficiency has been identified as the most effective trigger for increasing the lipid content in various microalgal species, such as Botryococcus sp. (Yeesang and Cheirsilp, 2011). Additionally, the pH of the medium significantly affects microalgal growth by influencing nutrient availability, metabolism, and cellular biochemical composition (Richmond, 2000; Bajhaiya et al., 2010). Utilizing $NaNO_3$ as a nitrogen source has been observed to enhance both the growth and lipid synthesis of A. quadricellulare R-56. Similarly, employing sodium nitrate as a nitrogen source has resulted in increased biomass and lipid content in Chlorella vulgaris (Yu Ren and Kong, 2023).

Conclusion

Microalgae are better and cleaner source in the biodiesel production as their growth and maintenance in compassion to others it more cost-effective loss maintenance higher yield is available throughout the year also the lipid production can be enhanced by changing the growth parameter. The lipid production was checked using the SPV calorimetric method which gives an overview of the lipid content also it is a quick and easily method as there is no lipid degradation and hence give better results compared to others

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