ISOLATION AND CHARACTERIZATION OF MICROALGAE FOR BIOFUEL PRODUCTION

BY

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This thesis is dedicated to my beloved parents and fiancée

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ABSTRACT

Full Name : [Muhammad Arif Ibrahim]

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The world is confronted with depleting fossil fuel resources at a time as energy demand is exploding. The frequent use of fossil fuels is now widely considered as unsustainable. Biofuel derived from microalgae is a sustainable way for fuel production. Algae is carbon neutral and can be produced intensively on relatively small areas of marginal land.

In the past decades, wide-scale screening of naturally occurring algal strains has been characterized all over the world. However, very few investigations related to algae-based biofuel production have been carried out in Saudi Arabia so far; especially studies related to high yielding oleic strains. This thesis is focussed on the isolation of potential microalgae strains for biofuel production from Cornish of Al Khobar and Jubail. Three strains (S1, S2, and S3) were isolated and characterized as *Chlorella kessleri, Chlorella vulgaris, and Nanochloropsis oculata*, respectively. The isolated strains were analyzed for growth kinetics, nitrate and phosphate removal and biofuel productivity at 30°C and 35°C. S2 showed highest optical density and dry weight while S3 showed the lowest value at both temperatures. All the isolated strains showed greater than 90 % nitrate and

phosphate removal at both temperatures. Lipid content of 19%, 27%, and 14% was found in S1, S2, and S3, respectively. On the other hand, fatty acid profiling through GC-FID showed 18%, 22% and 16% concentration of palmitic and 6%, 4% and 5% steric acid in S1, S2, and S3, respectively.

ملخص الرسالة

الاسم الكامل: محمد عارف إبراهيم

عنوان الرسالة: عزل وتوصيف الطحالب لإنتاج الوقود الحيوي

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يواجه العالم تحديا مع استنفاد موارد الوقود الأحفوري في زمن يزيد فيه الطلب للطاقة ، ويرجع ذلك إلى زيادة في اعداد السكان وزيادة التصنيع. أن الاستخدام المتكرر لأنواع الوقود الأحفوري على نطاق واسع يعتبر غير متجدد. أن الوقود الأحيائي المستمدة من الطحالب يتم بطريقة مستدامة لإنتاج الوقود من الطحالب ، حيث تنتج هذه الطحالب بكثافة في مناطق صغيرة نسبيا من الأراضي المستخدمة. لقد تم خلال العقود الماضية إيجاد سلالات طحالب متعددة في جميع أنحاء العالم، ولكن هناك دراسات قليله في المملكة العربية السعودية يتم فيها تعيين سلالات من الطحالب التي يمكن استخدامها في انتاج الوقود الحيوي. حيث يتم فيها تعيين السلالات التي لها القابلية على انتاج مركب الاوليك والذي يستخدم في انتاج الوقود الحيوي.

أن هذه الاطروحة تركز على عزل سلالات الطحالب المجهرية لإنتاج الوقود الأحيائي، حيث تم عزل سلالات التي تحتوي مركب الاوليك بنسب عالية من مناطقتين (كورنيش الخبر وجبيل) في المنطقة الشرقية. لقد تم عزل ثلاثة سلالات (S1، S2 ، S3) حيث تم تصنيفها *Chlorella kessleri, Chlorella vulgaris, and Nanochloropsis oculata*.

وجرى تحليل السلالات المعزولة بدراسه النمو ، وإزالة والنترات الفوسفات وإنتاجية الوقود الأحيائي في 30 درجة مئوية و 35 درجة مئوية. وأظهرت النتائج أن S2 اعطى أعلى كثافة بصرية والوزن الجاف ، بينما أظهر S3 أدنى قيمة في كل درجات الحرارة. لقد أظهرت جميع السلالات المعزولة لها القابلية على إزالة 90% من النترات والفوسفات في جميع درجات الحرارة على حد سواء. لقد وجد أيضا ان محتوى الدهون هو 19% و 27% و 14% في S1، S2، S3على التوالي. كما من ناحية أخرى وباستخدام GC-FID ، ان نسبة الأحماض الدهنية هي 18 %، ونسبة 22% وتركيز 16% من حمض بالميتيك وحامض الاستيرك 6%، 4%،5% في S1 أو S2 أو S3، على التوالي.

CHAPTER 1

INTRODUCTION

Microalgae are the microscopic algae that are found in various types of environment including; marine, freshwater and harsh environment [73]. They are a diverse organism with a number of species ranging from 200,000 to several million species. They [74].

1.1 Background

The world is currently depending on the basic sources of energy e.g. petroleum, natural gas, coal, hydro-electrical and nuclear. The increased population has boost up industrialization which need more energy. The continuous use of fossil fuels is now widely considered as unsustainable due to depletion of these sources as well as the release of greenhouse gasses that cause the increase of global warming. It has been estimated that if the current CO_2 emission remains unchanged between 2060-2090, its concentration will reach up to 850-1130 ppm [1] which is double than the mandatory CO_2 level set by IPCC, 2007. Concurrently, it is expected that fossil fuel reserves will be depleted by the year 2100 at the current rate of global oil consumption [2]. Renewable and environmentally-sustainable alternative sources of fuel are thus an immediate priority, not only to replace fossil fuels but also to overcome fuel demand and supply. Particularly, fuel substitutes for the transportation sector are tremendously required, as it is the sector with the highest oil demand [108].

The idea of utilizing algae as a source of fuel is old but currently it got much attention due to increasing price of petroleum and more importantly, the emerging issue of climate change and global warming associated with burning fossil fuels. Microalgae can provide several different types of renewable biofuels which include methane, biodiesel (methyl esters) and biohydrogen [11, 12, and 13]. Biofuel is the sustainable and renewable energy source which consists of three types; first generation biofuel, second generation biofuel and third generation biofuel. Scientists defined the first generation biofuel as a by-product of sugar crops. It can also be obtained from vegetable oil including oil seed crop and biogas of raw materials [3, 4]. Generally, it is assumed that first generation fuel is ethanol based and come out from food crops [3]. Second generation biofuel is cellulose based produced by non-food crops material like wood, leaves, straw etc. [5]. While third generation biofuel is produced by different microorganisms and algae [6].

A biofuel can be produced at commercial scale with following characteristics e.g. economically cost effective, utilizing the non-agricultural land, minimal discharge of greenhouse gasses or less water use [7]. A judicious use of microalgae could meet all these conditions. So, it can be used as a renewable biomass source. In the last decades, various investigations have been performed for replacing fossil fuels with biofuel to minimize the emission of greenhouse gasses [8, 9, and 10]. Microalgae found to be the best source of renewable, biodegradable and environment-friendly biodiesel capable of fulfilling the global requirement of transport fuels [8].

1.2 Microalgae

Microalgae are considered one of the oldest form of living organisms which are photosynthetic and oleaginous i.e. they directly produce and accumulate storage lipid in quantities averaging 26-85% of dry weight [14]. Table 1.1 shows the lipid production by different microalgae strains to their respective biomass.

Table 1.1: Lipid to biomass percentage of various microalgae strains

Strains	Lipid to Biomass %	References
Botryococcus braunii	36.9	15
Chlorella	38	16
Chlorella vulgaris	56	17
Chricystis minor	22.9–26	18
Scenedesmus Dimorphus	30	19
Microcystis	40.1	20
Chlorella kessleri	1-10	109

Microalgae grow fast with high lipid content and will produce the same quantity of biodiesel using 23% agricultural land as compared to palm plantations that will utilize 61% of the land [21]. Isolation of indigenous microalgae strains with high oil content will be a good alternative. Considering the phenotypic aspects of marine microalgae have further advantages, as these do not compete for land or fresh water for the biomass production. In the present study, we have focused on the isolation of indigenous microalgae strains for biofuel production from Arabian Gulf kingdom of Saudi Arabia.

1.3 Objectives

The major objectives of the project are the following

1- Isolation of indigenous microalgae strains from the Arabian Gulf.

2- Morphological identification of the isolated strains.

3- Check the growth performance of the isolates by

  - Varying the nutrient (NO_3 and PO_4)
  - Varying the temperature (30˚C and 35˚C)

4- Biofuel analysis of the strains by gas chromatography

1.4 Motivation and Justification

Use of unsustainable petroleum sourced fuels increased the accumulation of carbon dioxide in the environment thus add in global warming. International Energy Agency [IEA] estimated that if we go with current CO_2 emission rate then at the end of this century, the CO_2 concentration will fall in the range of 850-1130 ppm [1]. While the level of CO_2 concentration set by IPCC meeting in 2007 [22] will be half at that time. Other than climate change, it has also been estimated that this century will deplete the fossil fuel as well. Renewable and environmentally-sustainable alternative sources of fuel are thus an immediate priority, not only to replace fossil fuels but also to overcome fuel demand and supply.

Microalgae are one of the most promising feedstocks for bioenergy because they convert solar energy into chemical energy by carbon dioxide fixation [23]. These photosynthetic microorganisms grow fast and produce carbohydrate and lipids in large quantities, which later processed into bioethanol and biodiesel respectively. Moreover, microalgae produce a wide range of different valuable compounds like Omega 3, pigments (carotenoids, phycobiliproteins) vitamins (e.g. tocopherols), polyunsaturated fatty acids (PUFAs) and polysaccharides [110]. Due to the production of vital compounds, microalgae are also used as a human nutrition, animal feedstock, fish feeds and feed for rotifers.

Comparing with other terrestrial crops, microalgae is preferable due to

1- Fastest growth rate
2- No need of fertile agriculture land
3- Higher biomass production
4- Grown in diverse habitat e.g. freshwater and waste water
5- No need for extra nutrients of growth
6- No pest or herb threat as for agricultural crops
7- Biomass production by direct fixation of environmental CO_2
8- Residual algal biomass can be used as feed or fertilizer [25-28].

Table 1.2 shows the biodiesel productivity, land use and oil yield of different crops and microalgae.

Table 1.2: Productivity and oil yield of various crops and microalgae

Plant Source	**Biodiesel Productivity (kg/ha/year)**	**Land Use (m^2 Year/ kg)**	**Oil Yield (L / ha/year)**
Corn/Maize (*Zea mays* L.)	152	66	172
Soybean (*Glycine max* L.)	562	18	636
Jatropha (*Jatropha curcas* L.)	656	15	741
Canola (*Brassica napus* L.)	862	12	974
Sunflower (*Helianthus annuus* L.)	946	11	1070
Palm (*Elaeis guineensis*)	4747	2	5366
Microalgae (low oil content)	51,927	0.2	58700
Microalgae (medium oil content)	86,515	0.1	97800
Microalgae (high oil content)	121,104	0.1	136900

It is clear that microalgae biodiesel productivity is thousand times higher than other crops. In the past decades, worldwide screening of naturally occurring algal strains has been done. However, in Saudi Arabia, very few investigations could be available at present relating to the algae-based biofuel production, especially relating to high yielding oleic strains. The current study was conducted to isolate and characterize indigenous microalgae strains for enhanced biofuel production.

CHAPTER 2

ISOLATION TECHNIQUES

Several species of microalgae have been screened for their suitability as a biodiesel feedstock based on their fatty acid profile and so far, *Chlorella* and *Scenedesmus obliquus* have been identified as the most promising species [29, 30].

2.1. Isolation

A different method has been used for the isolation of axenic microalgae strains. The first step for isolation of axenic strains is to collect the sample by a standard method based on the requirement of the project. For example, MacLulichch performed an experiment on epilethic assemblages and used chipping, scrapping, and brushing [31]. General Oceanic Niskin bottles can be used for sampling of non-substrate bound organisms. Method used for isolation of benthic species are I) rock chipping 2) brushing and 3) scrapping. After sampling from selected site, filtration and inoculation are done in order to obtain axenic microalgae strain. Currently, there are various methods used from basic microbiological isolation techniques to modern tools.

2.1.1. Streak Plates

This is an enrichment technique used to isolate strains from a pool of microorganisms. During this, a sterile loop is inserted into the liquid sample by picking green algae growing on filter paper or in the liquid culture. Afterward, this loop is spread over a new agar plate by a specific pattern that all sides of loop touch the agar medium [32].

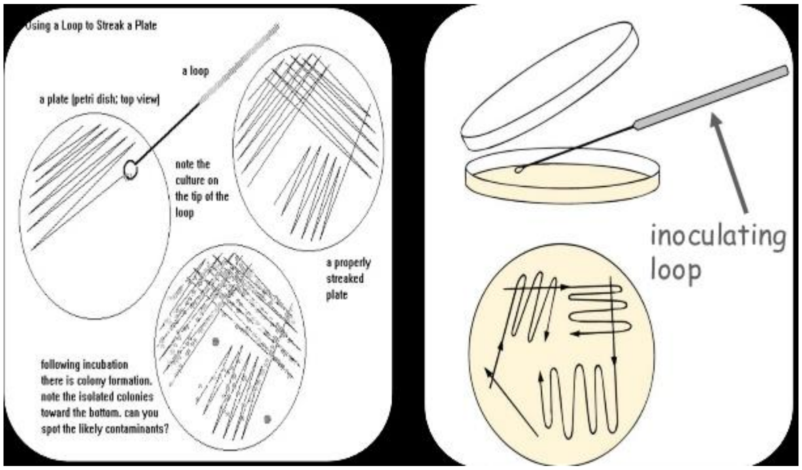


Figure 2.1: General streaking procedure. Image source [33]

The area in the start of streaking line contains more number of cells. But as the streaking line goes further, the number of cells become lesser. This makes the dispersion of cell far enough from each other that single colony can easily be seen. As this developed from a single cell, this can be a pure colony. But if it still contains some organisms, several streaking makes it pure and isolated strain. But before considering it pure, investigate the individual cell under a microscope. This then can be identified on the base of size/shape and color. After phenotypic identification, shift the pure colony in new clean agar plate to make a stock [32, 34]. On the base of sources, we used this method for isolation.

2.1.2. Serial dilution

During this technique, the concentration of microalgae in liquid media is bringing down to the level where it is assumed that higher dilution tubes will contain the pure strain [39]. However, if the higher dilution tubes still contain an impurity, the streaking or another method can be used in parallel to get a pure culture.

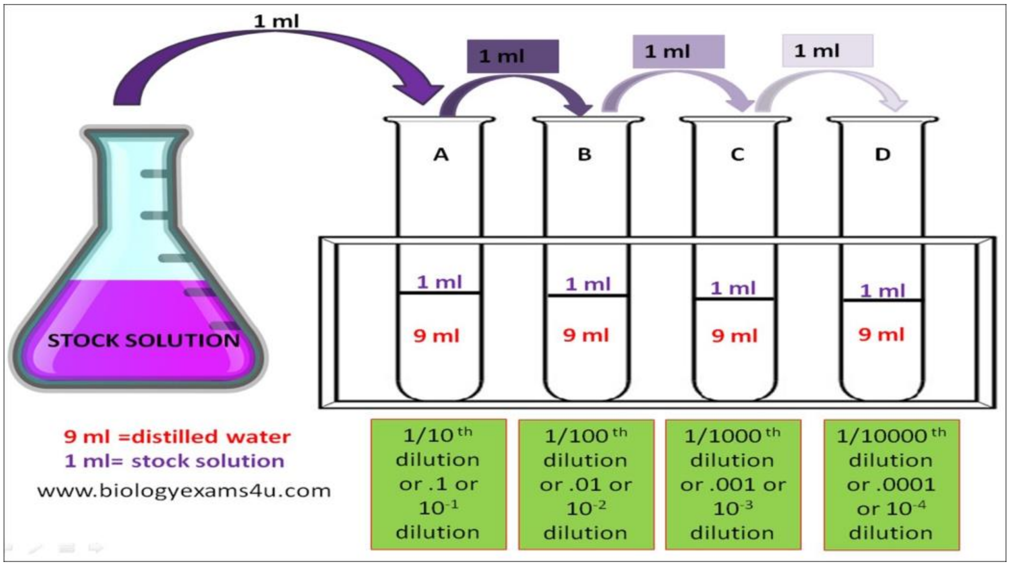


Figure 2.2: Serial Dilution procedure. Image source [35]

During this method, a series of test tubes (up to 10) are prepared to contain a certain amount of liquid media. From the inoculum, 1 ml is taken and pour into the first series tube (Tube 1). After mixing the first tube, take one ml from Tube 1 and pour it into Tube 2 and mix it. This process is repeated to the last series tube where the dilution reaches to 10^{-10}. From the last tube, agar plate media is inoculated through streaking for a better result. As shown in figure 2.2. Higher the dilution more is the purification. After 2-4 weeks, agar plate needs to check under the microscope.

2.1.3. Micromanipulation

During this method, capillary tubes are first manipulated by heating and pulling on both ends. The narrow ends should be of twice the diameter of the cell to be micromanipulate. Silicone tube is attached to the thick side of the micropipette to suck from the culture media. This process is operated under the microscope [34].

The cells obtained by sucking are shifted to new sterile medium to wash. They again transfer to another medium by mouth pipetting. At the end, the pipetting cells are transferred to tissue culture well, petri dish or media tube as shown in figure 2.4. However, as the number to suck the cell increases, the risk to damage the cell also increases.

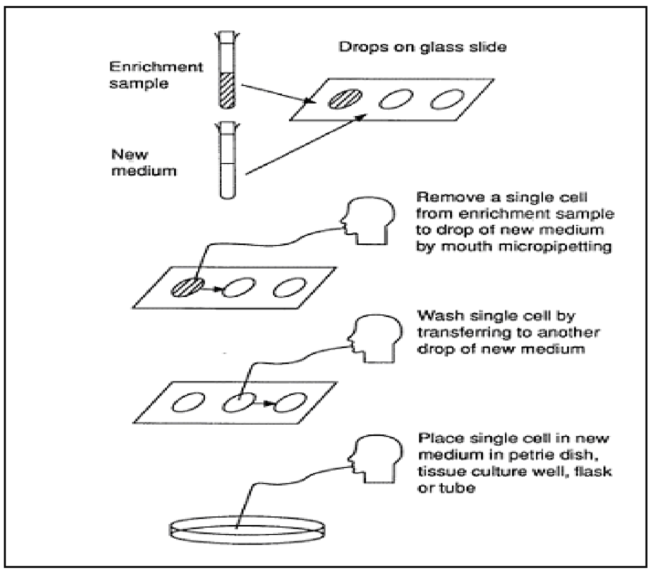


Figure 2.3: Micromanipulation of a sample with capillary micropipette to obtain uni algal culture]

2.2. Growth Kinetics

Many biomass measurement methods are developed and used currently. Microalgal cell concentration in a medium is quantified as cell number or cell mass. Flow cytometry, hemocytometry, optical density measurement, dielectric permittivity and dry weight are techniques being used for quantification of cell number or cell mass.

2.2.1. Optical Density and Dry Weight

Among the most frequently used techniques, optical density is widely used for estimation of biomass [123]. OD is measured through spectrophotometer at a specific wavelength. During the measurement, light pass through the sample and fraction of it is transmitted which is determined by Beer-Lambert Law of Absorbance [124]. This law, actually, correlates cell concentration with the attenuation of light. A linear relationship is necessary between cell number and optical density. Various wavelength is used for measurement, however, the suitable is the one which is absorbed by chlorophyll of the cell. Chlorophyll a content absorbs the light of 680 nm wavelength and assessment the cell number [125]. However, this light not only relates to cell number but also to cell morphology. Cell size and shape are also related to specific growth rate so for phototropic study, an independent calibration curve is plotted [126].

However, this method fails if there is any insoluble particulate matter as this also hinder the light and transmit it [127]. Although this is non-destructive and rapid but sensitivity is limited as in some cases up to 10^7 cells per ml [128]. So the measurement of dry weight is preferable and accurate for quantification of biomass.

2.2.2. Factors Affecting Biomass

The growth of microalgae is influenced by various physical (light and temperature) and chemical factors (nutrients, salinity, and pH) [40].

If the light intensity is sharp the growth rate of microalgae enhanced. Over the saturation point, growth and biofuel productivity is reduced through reactive oxygen species [41]. Similarly, temperature variation has a significant role for biofuel productivity. Microalgae optimum biofuel yield is achieved at optimum temperature. Above optimum temperature, microalgae growth is reduced due to cell death as well as a reduction in biofuel production. However, low temperature compensates the biomass loss caused by respiration during night period [42]. Maximum biofuel production is obtained at a high temperature conceding the optimum range during the day and low temperature at night. Each factors affecting the growth kinetics are discussed separately here.

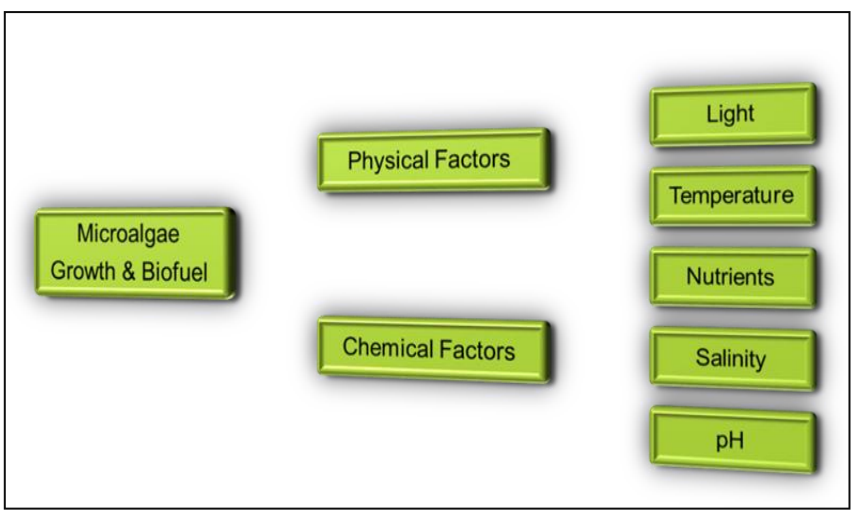


Figure 2.4: Flow chart of various factors affecting microalgae growth. Image source [43]

2.2.2.1. Temperature

Temperature indirectly changes the growth of microalgae if a change in intensity of light occurs [47]. Temperature below 4°C completely inhibits the photosynthesis of microalgae. As temperature increases from 4 to 11°C, growth substantially shows a positive response. But after 10°C, growth shows a linear relation with temperature [48]. Actually, temperature affects the reaction rate of intercellular enzyme which influences algal photosynthesis, respiration intensity and growth rate as well [49].

Butterwick and his colleague studied growth behavior of 21 freshwater microalgae with a large range of temperature from 2-35°C. Only four showed a decent growth rate of 0.4/day at the 5°C, while the remaining 17 showed very less growth rate with no significant effect. However, at 25°C all strains grew well [50]. But this range of temperature doesn't mean the optimum range to work best. The optimum range shows a narrow line of temperature from 20-30°C [51]. Generally, in the optimal range, increase in temperature cause the increase in biomass. But above the optimum level cause the reduction in biomass and in the severe case leads to death as well. Therefore, high biomass accumulation can be achieved by increasing the temperature to optimal in the morning (to enhance productivity during the day) and decreasing the temperature at night (to avoid biomass loss) [52].

2.2.2.2. Nutrients

Nutrients supply to microalgae cultures include macronutrients i.e. nitrogen, phosphorus, carbon, and sulfur and micronutrients i.e. iron, zinc, copper, and cobalt respectively. The nutrient limitation may cause morphological and physiological changes of microalgae cells, and therefore decrease the growth rates and biomass production. The most important nutrient for microalgae growth and development are nitrogen and phosphorus. Their ratio especially limits the yield of microalgae [53].

Nitrogen involves the formation of many important compounds like nucleic acid, protein, and photosynthetic pigments. Microalgae uptake nitrogen in the form of ammonia, nitrate, and nitrite. However, these sources can affect the growth of microalgae: like various ammoniacal form NH_4–N, NH_3–N and NH can be a useful nutrient element for microalgae, according to research, the NH_4–N is easier for microalgae to absorb than NH_3– N [54].

Nitrogen limitation cause the reduction in protein synthesis [64, 65], enhancement of lipid accumulation [55, 56] and triglycerides as well [57, 58]. This nitrogen reduction phase triggers the carbon fixation to carbohydrate compounds [61]. The other negative impacts are decrease in oxygen evolution, carbon dioxide fixation, and chlorophyll content and tissue production [62]. Phycobilisomes and light harvesting antennae of photosystem II can destroy during nitrogen limitation as well [63]. So, for higher lipid productivity, an appropriate amount of nitrogen is needed to be applied without limiting the other factors.

Phosphorus: Although, N: P ratio is very important for the growth of microalgae but it has been found that phosphorus is the primary limiting factors for microalgae growth and development [64]. Comparative to nitrogen, it is needed in very less amount due to less bioavailability [21], so applied in higher quantity than required. It constitutes about 1% of the microalgae dry weight [65]. Phosphorus limitation causes the reduction of light utilization due to a lower generation of the substrate in C3 cycle, hence limit the carbon fixation. It also causes accumulation of lipid as well. As more than double content of lipid has been found (23-53%) by limiting the phosphorus from 2 mg/L to 0.1 mg/L [66] in *Scenedesmus sp*.

Similar to nitrogen, reduced phosphorus causes the accumulation of carbohydrate content and reduce the chlorophyll a and protein amount [67]. Similarly, it also causes the destruction of Phycobilisomes as well as respiration rate [63]. The atomic ratio of N: P in microalgae cell is 16: 1, lower ratio make nitrogen limiting nutrient while higher ratio makes phosphorus limiting factor [68]. As every strain has a specific atomic ratio of the cell, so the requirement for nitrogen and phosphorus vary from strain to strain [69].

2.3. Lipid Measurement

Lipid measurement consists of two steps, lipid extraction, and its measurement or quantification. Various chemical and mechanical methods are used to extract lipids like folch, Bligh, and dyer, bead beating, sonication, microwave, and electroporation. However, in this study chemomechanical method (direct esterification) was used for lipid extraction on the basis of resource availability.

2.4. Nitrate and Phosphate Removal

Nutrient and toxic metals found higher than the prescribed level in wastewater, which need to be removed from the water before discharge and reuse. As the conventional method of treatment (chemical and physical) are not eco-friendly. Microalgae proves to be an alternative for efficient removal of nitrogen, phosphorus, and toxic metals from a wide variety of wastewaters [93, 94] including agricultural [95, 96], industrial [97, 98] and municipality wastewater [99, 100]. These investigations portray that microalgae can remove a substantial amount of pollutants. Hence controlled microalgae cultivation has a great potential for biological treatment of wastewater.

2.4.1. Potential of Species for NP Removal

Currently, microalgae have gained much attention to producing at commercial level due to its environmentally friendly approach. A large number of investigations carried out for strain selection and optimum cultivation system [101]. After cultivation, it's harvesting at commercial scale is also another challenging issue. Macroalgae being multicellular can easily harvest and consider more suitable for nutrient removal than microalgae. However, due to high production of lipids and other important biomolecules, makes microalgae more attractive for multi-purpose tasks.

Nutrient removal by microalgae relies on factors including the potential of different species in diverse environmental conditions i.e. temperature, pH, light intensity, inoculation density, nutrient concentration, N/P ratio and other factors including the type of species, the source of wastewater and its type. Among all these, nutrient concentration including NP ratio, the source of wastewater and type of species are the key contributors for removal.

Three types of nitrogen found in wastewater e.g. nitrate, nitrite and ammonium while phosphorus existed in phosphate form. Li Xin *et al.*, 2010 [84] conducted a comprehensive study to elaborate the removal of nitrate and phosphorus of various forms and their interaction. They showed that phosphorus removal was quicker than nitrogen forms of nutrient by *Scenedesmus* LX1 sp. Among the nitrogen forms, nitrate and urea removal were more as compared to ammonia as shown in the below figure. Similarly, with phosphorus as a nutrient, ammonia removal was also low than others forms. However, this study contradicts too many investigations. However, the initial concentration does matter as mentioned in Table 3 if the concentration is low it is completely utilized. However, as the concentration is higher, the removal percentage goes up and down respectively in both genus *Scenedesmus* and *Chlorella* sp. [84].

Table 2.1: Nitrate and phosphate removal by various microalgae strains

Species	Time days	Nitrogen In. cons. Mg/l	Removal rate mg/l/day	Phosphorus In. cons. Mg/l	Removal rate mg/l/day	References
Scenedesmus quadricauda	3	70 18	10-12.6 4.9-5.7	16	3.7	84
Scenedesmus dimorphus	3	70 18	10-12.6 4.9-5.7	16	3.7	84
Scenedesmus obliquus	7	8.7	1.24	1.7	0.24	102
Scenedesmus sp	12	15	1.03-1.2	1.3	0.1	66
Scenedesmus rubescene	6	25.2	4.15	1.74	0.6	103
Chlorella vulgaris	10	13.2-21.2 41.8-92.8 > 129	1.3-2.1 2-3.1 ~3	7.7	0.7	75
Chlorella vulgaris	4	8.7	2.17	1.7	0.42	102
Chlorella sp	14	82 116	5.5 7.37	212	13.7	99
Chlorella vulgaris	3	25.2	8.3	1.74	0.56	103
Chlorella reinhardtii	4	25.2	6.2	1.74	0.42	103

The integrative study showed that N/P ratio of 5:1-8:1 caused the efficient removal of both nitrogen and phosphorus by Scenedesmus sp. However, N/P ratio of 5:1-20:1 is also convenient for nitrogen removal, but higher than this can cause a reduction in nitrogen removal due to phosphorus deficiency [66]. It is evident from table 3, where N/P ratio is higher than 20:1 even than the nitrogen removal rate (1.5 mg/l/day) is higher because of lower initial concentrations [102,104]. After the induction growth phase, most of the pollutants are taken up at the start of log or exponential phase. So removal rate always observes higher at the initial log phase, but at the end of this phase, the rate declines significantly.

When the only simple culturing method is used, the removal rate is less as compared with the use of higher CO_2 concentration. Like [105] *Scenedesmus obliquus* showed higher nitrogen and phosphorus removal rate as compared to *Scenedesmus sp* [102, 104] under 15% CO_2 concentrations. Without foreign interference, or the use of any other organisms and biotechnology, the removal rate more than 10 mg/l/day can be achieved by *Scenedesmus dimorphus* [84] by using microalgae membrane photoreactor (mMR). The initial concentration was much higher (TN >80 mg/l and TP 16 mg/l) than all other cited investigations. However, the NP ratio was much lower. The expected higher level of total nitrogen in municipal wastewater is 15-90 mg/l [106], so the use of microalgae membrane photoreactor would be beneficial than another traditional method as it could remove up to 40 mg/l [84].

Similar to *Scenedesmus*, *Chlorella* sp is also dependent and sensitive to the initial concentration of NP, their ratio, and type to remove nitrogen and phosphorus from the municipal wastewater. From the table 3, in batch culture, *Chlorella vulgaris* removal capacity for NP goes down as the initial concentration rise by keeping the NP ratio constant. The removal rate of nitrogen about 2 mg/l/day was observed when the initial concentration was 21 mg/l. however, further increase causes the reduction in removal percentage though the removal rate is high [75]. The highest removal rate of nitrogen 12 mg/l/day and that of phosphorus 4 mg/l/day could be achieved by using mMR by keeping in mind the normal nitrogen and phosphorus quantity in municipal wastewater.

2.4.2. Mechanism of Removal

Microalgae needs nitrogen and phosphorus as nutrients and possess more N and P in their body than plants [38].

2.4.2.1. Nitrate Removal

Nitrates, nitrite, nitric acid, ammonium, and ammonia and nitrogen gas are the chemical forms of nitrogen commonly used by microalgae to produce organic nitrogen. To know the capacity of nutrient removal, it is necessary to understand uptake mechanism of nutrients by microalgae. Simple diffusion and transport mechanism are used by eukaryotic algae for nitrogen uptake [111]. It starts as the translocation of inorganic nitrogen does across the plasma membrane and follows by the oxidized nitrogen reduction and the addition of ammonium into an amino acid. Reduction occurs by reductase enzyme which converts nitrate into nitrite and ultimately to ammonium [112]. Therefore, before the organic conversion of nitrogen forms all are converted into ammonium.

Ammonium is the preferred form of inorganic nitrogen uptake by microalgae. Studies revealed algae could not consume the nitrate until the complete uptake of ammonium [101]. However, uptake is limited by the type of species and amount of nutrient in the wastewater. But its uptake can be enhanced by optimizing the Glutamate Synthetase enzyme. Similarly, NR and NiR can also increase the uptake and utilization of NO_3 and NO_2^{-1} respectively [107]. The tolerance level of ammonium nitrogen of microalgae varies from species to species and range from 25-1000 μ mol NH_4^+-N L^{-1} [107]. As the excess amount leads to a reduction in activity of microalgae [104], approaches can be used that enhance the uptake and removal of waste nutrient. Glutamine synthetase enzyme increases the removal as it enhances the uptake of ammonium up to 70% at cell level [104]. Nitrogen removal from wastewater is not only done by cell metabolism but also by ammonium stripping as the increased pH and temperature cause volatilization [66].

2.4.2.2. Phosphate Removal

Similar to nitrogen, phosphorus removal done by two ways. First, cell metabolism in which P is uptake preferably by H_2PO_4 and HPO_4^{-2} and used in phosphorylation to produce ADP (adenosine diphosphate) by the use of ATP (adenosine triphosphate) [105]. This energy comes from electron transport chain or from light. Therefore, phosphates transferred across the membrane actively. Inorganic phosphorus is utilized by microalgae but some varieties can also consume organic ester as their food [102].

CHAPTER 3

METHODOLOGY

Table 3.1: Approaches utilized for achieving objectives

Objective No	Approach to achieving the objective
1	**Sampling for the isolation of indigenous microalgae strains from diverse sites:** Samples were taken from Corniche of Al-Khobar and Jubail sites in Arabian Gulf in order to isolate different microalgae strains. **Isolation of pure microalgae strains culture:** Microalgae strains were isolated by streaking and single-cell isolation methods to obtain a pure culture.
2	**Identification of microalgae strains** Isolates were identified morphologically through a light microscope.
4	**Analysis of growth kinetics** The selected isolates were tested at two temperatures (30˚C and 35˚C) and three Nitrate and Phosphate levels N1= 61.5 mg/L N; 1.45 mg/L P N2= 123 mg/L N; 2.9 mg/L P N3= 246 mg/L N; 5.8 mg/L P **Lipids Analysis through GC** Lipid from selected strains was extracted by direct esterification method and was analyzed GC.

3.1. Microalgae Sampling

Before sampling site selection, sampling method and type of sample container were already defined.

3.1.1. Site Selection

Coast of Al-Khobar and Jubail industrial city were selected for sampling.

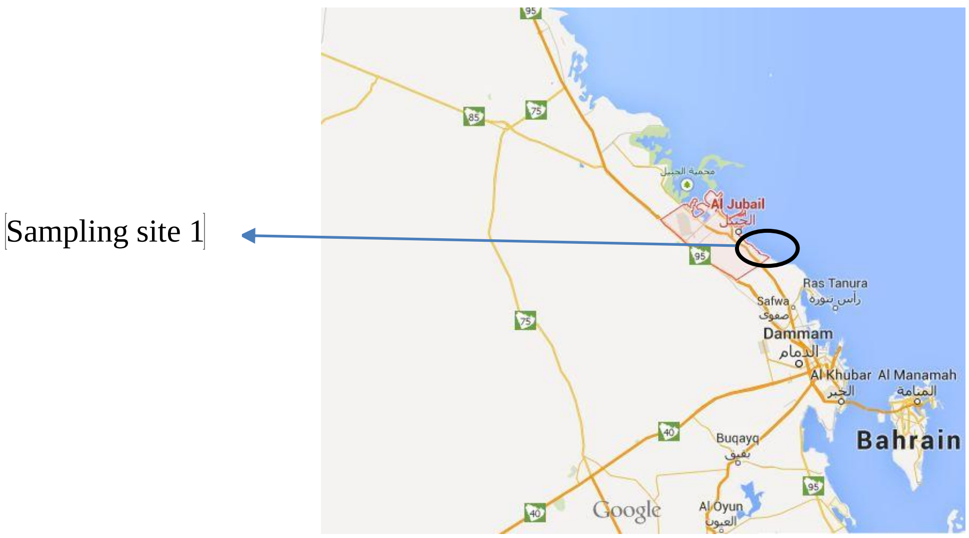
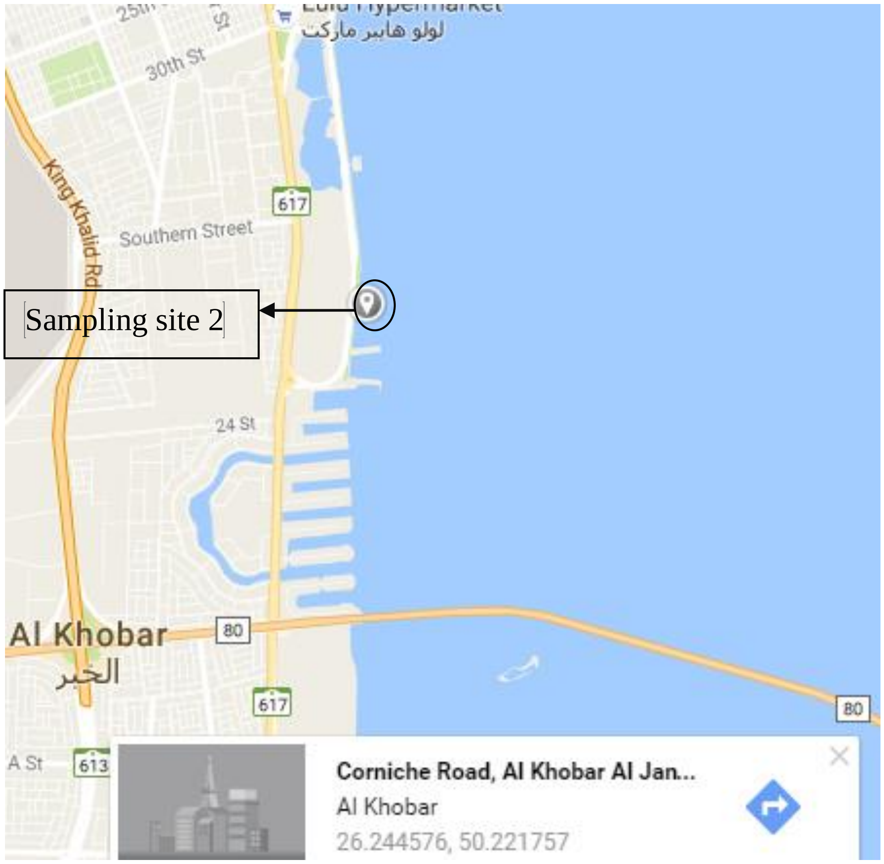


Figure 3.1: Google image for the Sampling site. Image source Google



3.1.2. Sampling tool and Container

One sampling net, twenty-one 50 ml test tubes, and marker for labeling was kept during the visit.

3.1.3. Sampling Method

Two visits were conducted at the coast of Jubail and Al-Khobar, however, the same method was adopted for sample collection.

1- Samples were collected randomly from Jubail Industrial City Coast. Total 14 samples were collected in 50 ml test tubes, through sampling net against the flow of water and remaining from the shore of the coast by scraping the soil surface. The collected samples were soon shifted to the lab for further study.

2- Coast of Al-Khobar was visited to bring samples through sampling net. Seven samples were collected nearly 200 meters away from the outlet of wastewater treatment plant.

3.1.4. Sample Labelling

Samples were labeled immediately after the sampling. A1-A7 labeled for water samples, while B1-B7 were the soil samples of Jubail coast. C1-C7 were the water samples collected from Al-Khobar coastal area near the wastewater treatment plant.

Cleaning and sterile techniques: Prior to the culturing of microalgae, all new glass wares were soaked overnight in 10% of 1 M hydrochloric acid to remove any residual chemicals. The glassware and other culturing equipment were washed with Decon-90, a phosphate-free detergent, and then rinsed 6 times with tap water and 6 times with de-ionized or distilled water [113]. Sterile conditions were employed by steam autoclaving to all culturing and sub-culturing equipment's, while inoculation, sampling, and the medium transfer were carried out in front of a Bunsen burner flame in a laminar flow hood with the bench top surface cleaned with 70% ethanol to prevent any bacterial contamination.

3.2. Media Preparation

SN medium was used for the experiment. For one liter of medium, 750 ml seawater was taken from the sampling sites and kept in storage vessel until further use. Seawater was filtered through double Whatman No. 1 filter paper to remove any sand and suspended particles [114] and mix with 250 ml distilled water. This mixed water and prepared stock solution was then autoclaved at 121°C (4 hours for every 1L of seawater) and left to cool at room temperature before the addition of nutrients and culture inoculation. This sterilization is done to destroy all organisms in the medium even the heat bearing bacteria [115]. The table below shows stock solution with their concentration for preparing one liter of the SN media.

Table 3.2: SN media recipe

Nutrients	Amount of 1L
$NaNO_3$ (300.0g/L.dH_2O)	2.5ml
K_2HPO_4 (anhydrous) (6.1 g/L dH_2O)	2.6ml
$Na_2EDTA.2H_2O$ (1.0 g/L dH_2O)	5.6 ml
Na_2CO_3 (4.0 g/L dH_2O)	2.6 ml
Vitamin B_{12} (1.0 mg/L dH_2O)	1.0 ml
Cyano Trace Metal Solution	1.0 ml
Cyano trace metal solution/ L	
Citric Acid.H_2O	6.25 g
Ferric Ammonium Citrate	6.0 g
$MnCl_2.4H_2O$	1.4 g
$Na_2MoO_4.2H_2O$	0.39 g
Co $(NO_3)_2 \cdot 6H_2O$	0.025 g
$ZnSO_4.7H_2O$	0.222 g

For preparing stock solution, 150 g of $NaNO_3$ was dissolved in 500 ml of distilled water. Similarly, all other stocks were prepared in 500 ml of distilled water in 1 L Erlenmeyer flasks. Three liters liquid inoculation media was prepared by adding 7.5 ml of $NaNO_3$, 7.8 ml of K_2HPO_4, 7.8 ml of Na_2CO_3, 16.8 ml of $Na_2EDTA \cdot 2H_2O$, 3 ml of Vitamin B_{12} and 3ml of Cyano Trace Metal Solution in 3L of already autoclaved seawater [121].

3.3. Media Inoculation

Before inoculation, 30 Whatman No. 1 filter papers and all flask were autoclaved at 121˚C for 4 hours [115]. The liquid samples were filtered through these autoclaved paper and transfer into a flask containing 100 ml liquid media (Samples A1-A7 and C1-C7). Soil samples were directly transferred into the liquid media flasks (samples B1-B7). All the marked Sample flasks were incubated in a growth chamber at 32˚C for 12:12 light: the dark period for three weeks.

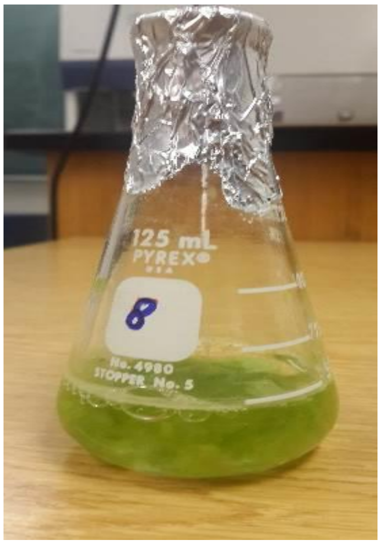


Figure 3.2: Microalgae growing sample during the experiment

3.4. Microalgae Isolation

After 4-week incubation, agar media was prepared by the same method as liquid media by adding 1% agar in the sea water before autoclaving. Streak plate method was used for isolation and purification of strains. A sterile loop was introduced into liquid sample showing clear growth of green algae on filter paper and in the liquid culture. Afterward, this loop was spread over a new agar plate by a specific pattern that all sides of loop touch the agar culture [34]

After two weeks of culturing, the single isolated slant was picked and streak again on a new agar plate. Before culturing, the colony was observed under the compound light microscope by preparing the glass slide to check the axenic nature of the colony. Streaking was performed seven times in order to get axenic strains [34]. The individual colony was differentiated on the basis of size, shape, and color. All agar plates were incubated in the growth chamber. After obtaining an axenic culture, these slants were characterized on the basis of their morphological characteristics by taking images under a compound light microscope.

Figure 3.3: Third Streak Agar plate during the incubation

3.5. Identification

3.5.1. Morphological Identification

The isolated slants were identified by taking images at 40x, 60x and 100x under a microscope. Glass slides were prepared by transferring the isolated strains, fixed by glycerin and analyzed under a microscope. To identify the isolated and axenic strain, following manuals were used.

1) Algal Identification Lab guide by Agriculture and Agri-food Canada [117]

2) Microalgae Identification for Aquaculture by Barry H Rosen Florida Aquaculture [118]

3) Easy Identification of most common freshwater algae by Sanet *et al* 2006 [119]

4) Freshwater algae of North America Ecology and classification John D. Wehr and Robert G Sheath [120]

3.5.2. Genetic Identification

The isolates (S1-S3) were characterized by 18S rRNA analysis.

3.6. Growth Analysis

In order to study the effect of nutrients and temperature on the growth of isolated strains, the experiment was performed in three replicates. For growth, optical density and dry weight were measured.

3.6.1. Optical Density

OD was measured by spectrophotometer after every two days' interval. The samples were centrifuged, the supernatant discarded and the biomass pellets were mixed with autoclaved seawater. Then 2 ml of sample was taken and measured the OD. In order to get same initial OD, same dry weight (0.018g/1000 ml) was used in all treatments.

3.6.2. Dry Weight

Dry weight was measured by the following procedure. First, combust the GF/C filter paper for 24 hours at 50˚C in the oven then weighed it and added 10.0 ml sample culture through the separating funnel. A filter containing wet biomass washed with 10.0 ml distilled water to remove the salts. The filter was removed then and put in the oven at 50˚C for 24 hours to desiccate and evaporate moisture content. Dry filter then weighed again, the difference between weight of filter paper and biomass+ filter was the dry weight of the sample [129]

DW= (weight of biomass+ filter paper) - weight of dry filter

Treatments:

The samples were incubated at two temperatures [T1= 30˚C and T2 35˚C]. The treatment layout was as below, where:

- T1=30˚C, T2=35˚C
- S1= Strain 1, S2= Strain 2, S3=Strain 3,

- N1= 61.5 mg/L NO_3 Nitrogen, 1.45 mg/L PO_4^{3-}Phosphorus
- N2= 123 mg/L NO_3 Nitrogen, 2.90 mg/L PO_4^{3-}Phosphorus
- N3= 246 mg/L NO3 Nitrogen, 5.8 mg/L PO_4^{3-}Phosphorus

T1			**T2**		
N1	**N2**	**N3**	**N1**	**N2**	**N3**
S1	S1	S1	S1	S1	S1
S2	S2	S2	S2	S2	S2
S3	S3	S3	S3	S3	S3

All treatments were incubated for 24 days. Optical density and dry weight were measured after every two days' interval.

3.7. Phosphorus and Nitrogen Measurement

Nitrate and Phosphate were measured by using total nitrate and total phosphate kit of Hach Company.

3.7.1. Phosphorus Measurement

For phosphorus removal measurement, PO_4^{3-} measured in the culture media after every two days' interval. DR 6000 Spectrophotometer and DRB200 Reactor and Test 'N Tube TM Vials of Hach was used for the measurement of PO_4^{3-}. The method used was molybdovanadate with acid persulfate digestion. The procedure was followed by adopting the Molybdovanadate Method with Acid Persulfate Digestion prescribed by Hach Method 10127 [121]. The following procedure was performed.

First, the DRB200 turned on and heated up to 150°C then DR 6000 was turned on and 542 P Total HR TNT method was selected. Then 5.0 ml of deionized water was added to a Total Phosphorus Test 'N Tube Vial and to prepare a blank labeled as B. Then 5.0 ml of sample added into another Total Phosphorus Test 'N Tube Vial to prepare the sample and labeled with sample code. One potassium persulfate powder pillow added into each sample and blank vials and put in the DRB200 reactor for 30 minutes. After 30-minute heating at 150°C, the vials removed from the reactor and allowed to cool. Then 2.0 ml of 1.54 N Sodium Hydroxide added to each vial, capped and mixed. An addition of 0.5 ml molybdovanadate reagent was also done to each vial before mixing again. Then vials were kept at the normal temperature for seven-minute. The blank was put in the 16-mm cell holder of DR6000. The display of the instrument was 0.0 mg/L PO_4^{3-} when reading was made ZERO. After the blank, labeled sample placed in the 16-mm cell holder and reading of the instrument was noted [121].

3.7.2. Nitrogen Measurement

Nitrogen measured in the form of NO_3^-. Hach Nitrate measuring kit was used to measure the nitrate. Similar to phosphate DR6000 spectrophotometer, DR200 reactor and Test N' Tube^{TM} Vials (Hach) was used during measurement. The procedure was followed by adopting the standard Chromotropic Acid method prescribed by Hach Method 10020 [122]. Among the stored program on DR6000 instrument, "344 N, Nitrate HR, TNT" selected and1.0 ml of sample added in the bottle of NitraVer X Reagent A. After adding reagent, sample mixed ten times and inserted into the 16mm cell holder for Reading. An instrument made ZERO by pressing zero buttons. Zero vial was removed and one powder pillow of NitraVer X Reagent B added. After capping, this vial was mixed ten times and kept for 5 minutes for a reaction. After reaction time, vial inserted in cell holder for nitrate reading. The display was noted for each sample in mg/L NO_3^- [122].

3.8. Lipid Measurement

For lipid measurement first its extraction is needed from biomass. Then its quantification was performed on GC-FID

3.8.1. Extraction

Direct esterification method was used for extraction of methyl ester from microalgae biomass. The algal biomass harvested at 3000 rpm for 5 minutes. From the collected wet biomass dry weight was measured by the same method as mentioned above. First acid hydrolysis was done with 1 ml of 1 M H_2SO_4 then heated at 90°C on a heating block for 30 minutes. Then base hydrolysis by 1 ml of 5M NaOH and heated at 90°C for 30 minutes and cooled it at room temperature. The hydrolyzed sample centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and 1 ml deionized water was mixed with a pellet.

The pellet was centrifuged at 3000 rpm for 10 minutes again and the supernatant collected. All collected supernatant was mixed with 3 ml of 0.5 M H_2SO_4. Centrifugation at 3000 rpm. The addition of 5 ml Hexane to the precipitate and heating at 90^0C for 15 minutes. Again centrifugation and collection of hexane phase and evaporation of the solvent under a fume hood. The extracted lipid residue was esterified into FAMEs by 5% H_2SO_4 with Methanol and heating [130].

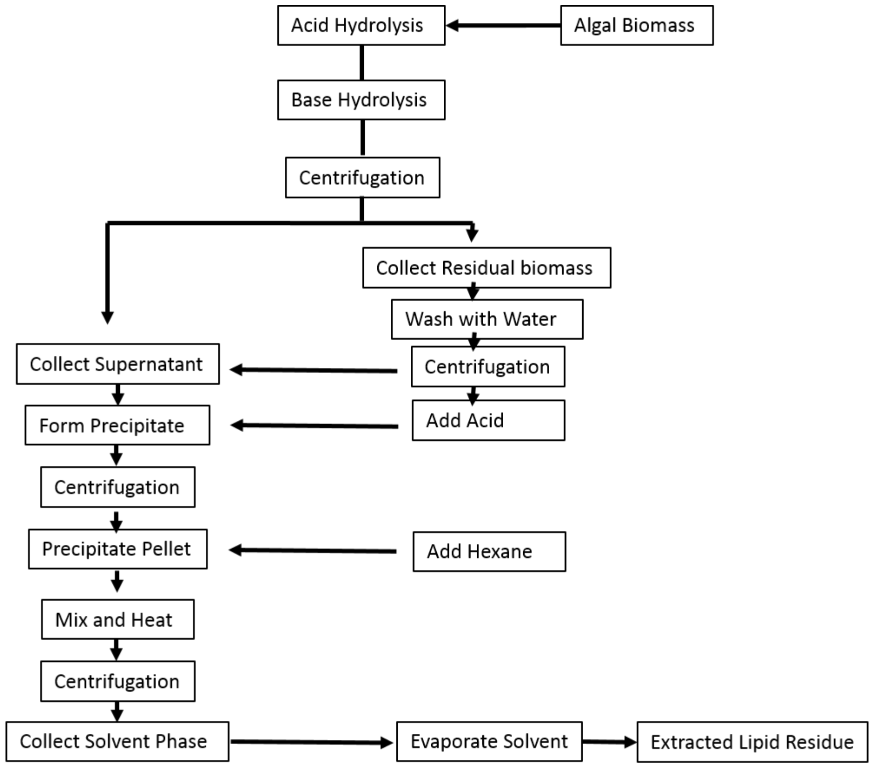


Figure 3.4: Flow chart for the Adopted Extraction procedure during the experiment

3.8.2. Quantification

For quantification of lipid content, GC-FID was used. Supleco^{TM} 37-Component FAME Mix (Catalog No. 18919-1AMP) standard was used. Standard was prepared by dilution in 10mg/ml of methylene chloride as prescribed by the method [131]. After running the standard, Blank sample was run and checked the obtained peaks. After that, all 18 samples were run in triplicate and peaks were integrated with the standard run.

CHAPTER 4

RESULTS

4.1. Sampling

After two weeks' incubation at temperature 30˚C and 35˚C in SN medium, 11 microalgae slants were grown among 21 samples (table 4.1). Growth was observed on the filter paper as well as the medium. This may be due to instant change of media from sea water to SN Media. Although SN media is sea based but the composition of nutrient in the sea and SN never be the same. Among the samples of Khobar Cornish (C1-C7), only one sample (C5) showed pure growth while the remaining have fungal contamination therefore discarded. However, all samples collected from Jubail (A1-A7), exhibited growth except (A1) (table 4.1).

Table 4.1: Samples result after incubation of three weeks

Sample number	Sample ID	Sample location	Microalgae growth
1	A1	Jubail	NO
2	A2	Jubail	YES
3	A3	Jubail	YES
4	A4	Jubail	YES
5	A5	Jubail	YES
6	A6	Jubail	YES
7	A7	Jubail	YES
8	B1	Jubail	NO
9	B2	Jubail	YES
10	B3	Jubail	YES
11	B4	Jubail	YES

12	B5	Jubail	YES
13	B6	Jubail	NO
14	B7	Jubail	NO
15	C1	Al-Khobar	NO
16	C2	Al-Khobar	NO
17	C3	Al-Khobar	NO
18	C4	Al-Khobar	NO
19	C5	Al-Khobar	YES
20	C6	Al-Khobar	NO
21	C7	Al-Khobar	NO

4.2. Isolation

At the end of five subsequent streaking, six slants (A3, A5, B2, B3, B5, and C5) were isolated and found axenic as shown in table 4.2. Unlike initial culturing, agar plates showed quick growth even after seven days, this is due to acclimatization of the organisms with the media. After third streaking, most of the slants showed uniformity, but A1 and A8 could not found uniform under microscope investigation because of symbiotic relation with some other organisms. So, at the end of consecutive four months streaking, six slants were found uniform and axenic.

Out of 21 samples, 6 slants were isolated. But three were considered for identification and characterization A3, B5 and C5, one from each sampling site.

Table 4.2: Result of Subsequent 7 Streaking

Sample number	Sample ID	Result Streak 1	Result Streak 2	Result Streak3	Result Streak4	Result Streak 5
Jubail	**A2**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Jubail	**A3**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Jubail	A4	Yes	No			
Jubail	**A5**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Jubail	A6	Yes	Yes	No		
Jubail	A7	Yes	No			
Jubail	B1	Yes	Yes	Yes	No	
Jubail	B2	Yes	No			
Jubail	**B3**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Jubail	**B5**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Al-Khobar	**C5**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**

Possibility of various strains occurred at different sampling site:

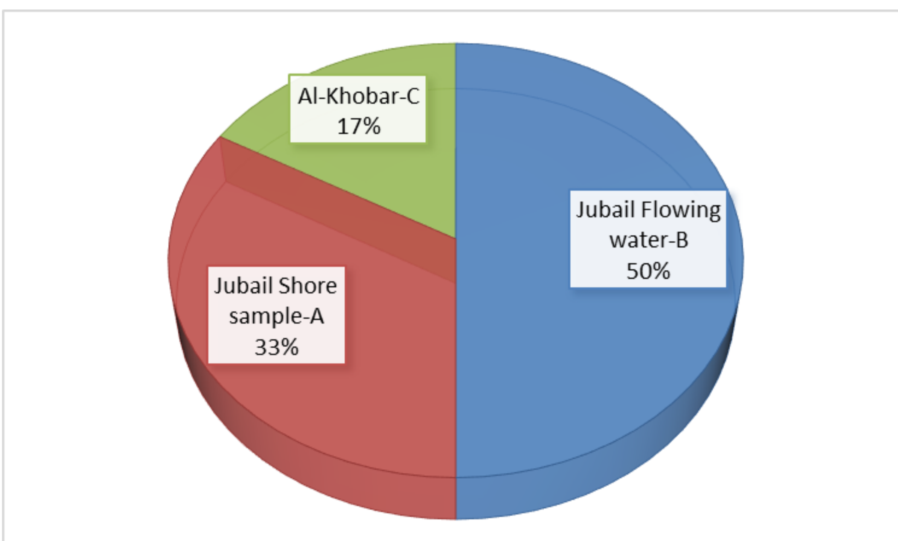


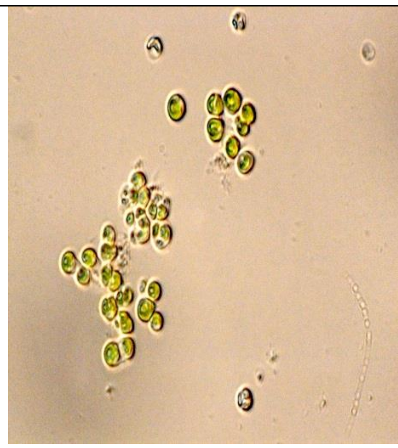
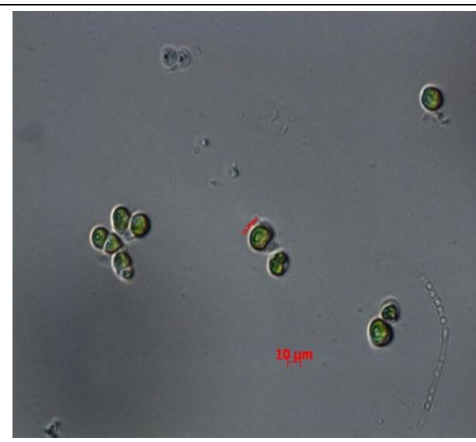
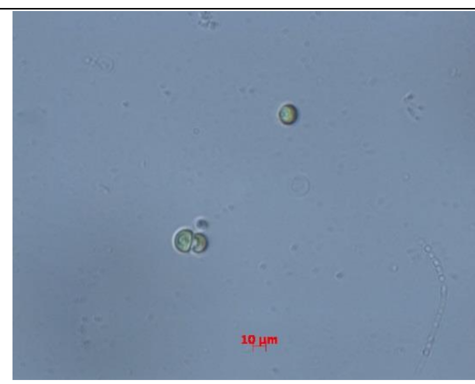
Figure 4.1: Percentage of isolated strains at various sampling sites

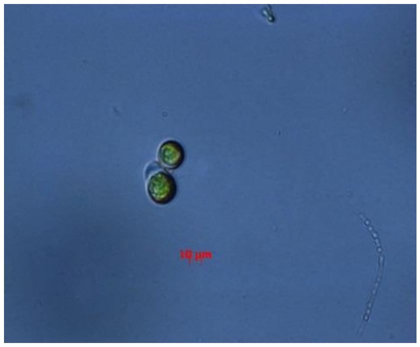
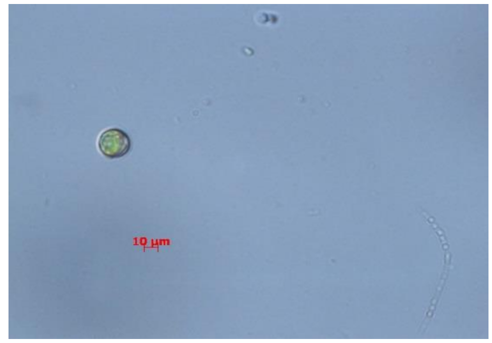
4.3. Identification

4.3.1. Morphological Identification

Isolated strains were characterized by morphological identification through Optical Microscope images taken at 40x, 60x and 100x.The microscopic description of all three isolates strains is mentioned in table 4.3.

Table 4.3: Representative sample for identifying the pure strains

ID	Colonial	Cellular
A3=S1		
B5=S2		

C5=S3		

Strain 2 is a small spherical green cell, remain solitary and is non-flagellated. Its chloroplasts are thin and cup-shaped just like *Chlorella vulgaris*. The cell wall is generally thin and smooth. The only method of reproduction is asexual by means of 4 or 8 (rarely 16 or more) auto spores which are formed internally through cell division.

4.3.2. Genetic Identification

The strain S1 were identified as *Chlorella kessleri*, S2 as *Chlorella vulgaris* and S3 as *Nannochloropsis oculata*.

4.4. Growth Analysis

Growth kinetics were measured by taking optical density and dry weight and drawing their relationship as well.

4.4.1. Optical Density

4.4.1.1. OD at T1

Trends of optical density were observed in the three strains at three nutrient levels at 30^{0}C as shown in figure 4.2

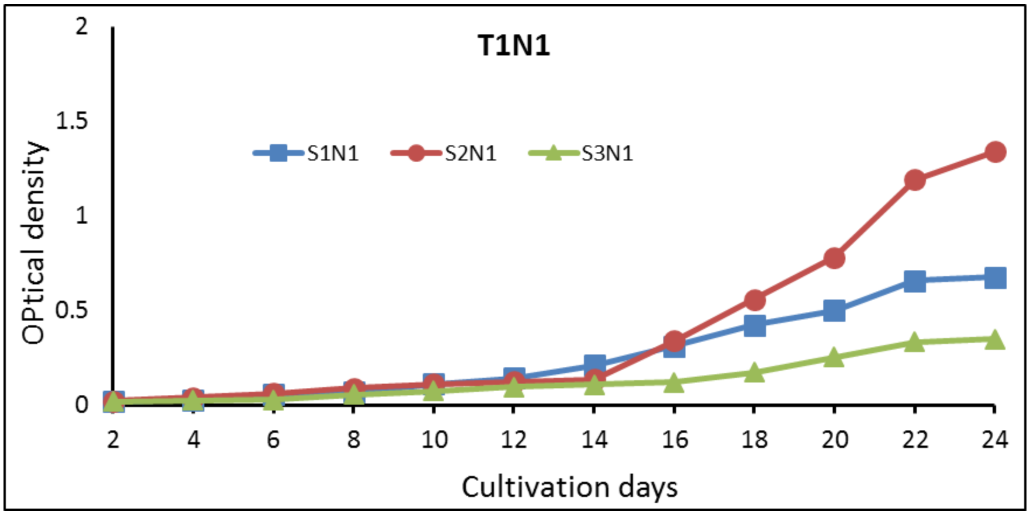


Figure 4.2: Effect of N1 level on optical density of three isolated strains at T1

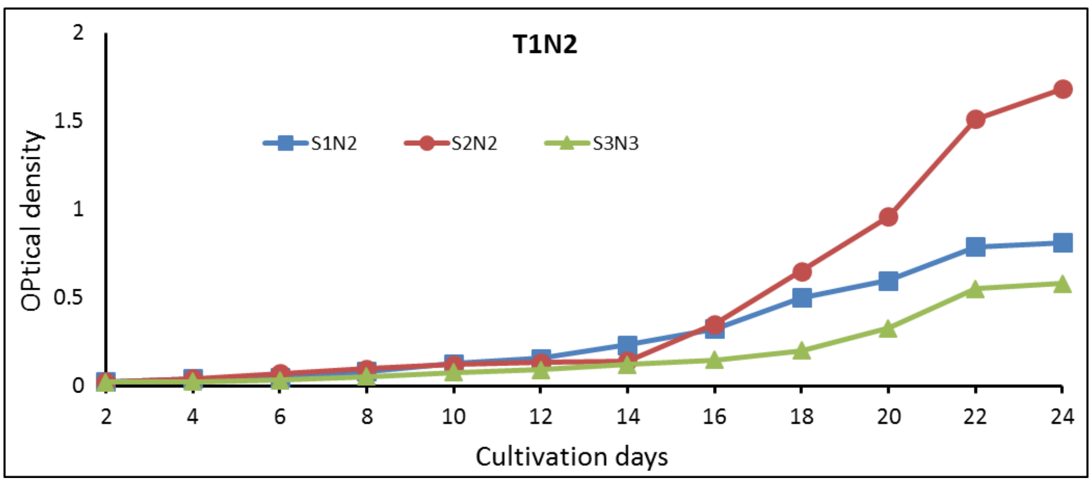


Figure 4.3: Effect of N2 level on optical density of three isolated strains at T1

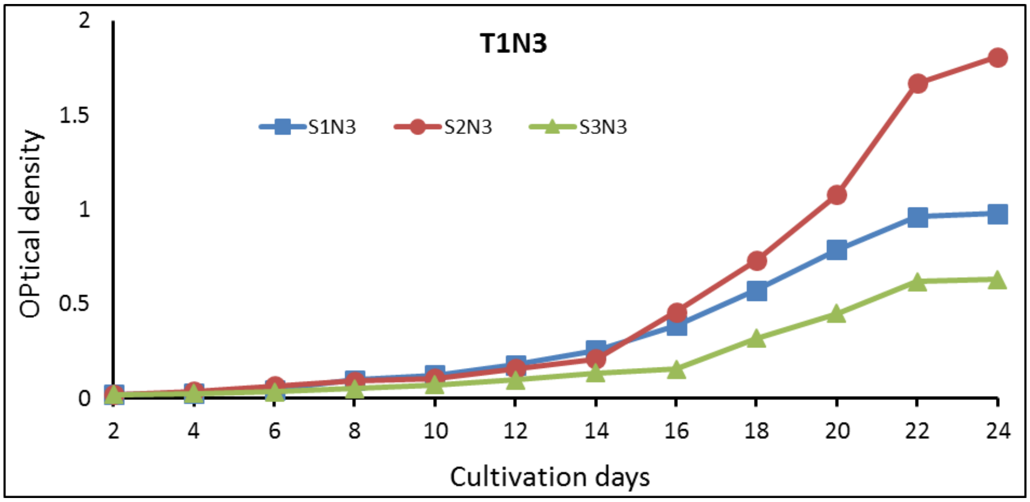


Figure 4.4: Effect of N3 level on optical density of three isolated strains at T1

S1=Strain A3: S2=Strain B5: S3=Strain C5: N1= 61.5 mg/L NO_3-N, 1.45 mg/L PO_4-P: N2=123 mg/L NO_3-N, 2.9 mg/L PO_4-P: N3= 246mg/L NO_3, 5.8 mg/L PO_4-P

Among all the three strains, Strains 2 showed the highest value of optical density (1.81) followed by S1 (0.981) while S3 showed lowest one (0.63). While among the nitrate and phosphate levels, N3 showed the highest optical density of 1.81 while N1 showed the lowest value of 0.35.

4.4.1.2. OD at T2

Effect of Nitrate and phosphate levels on optical density of three strains at temperature T2

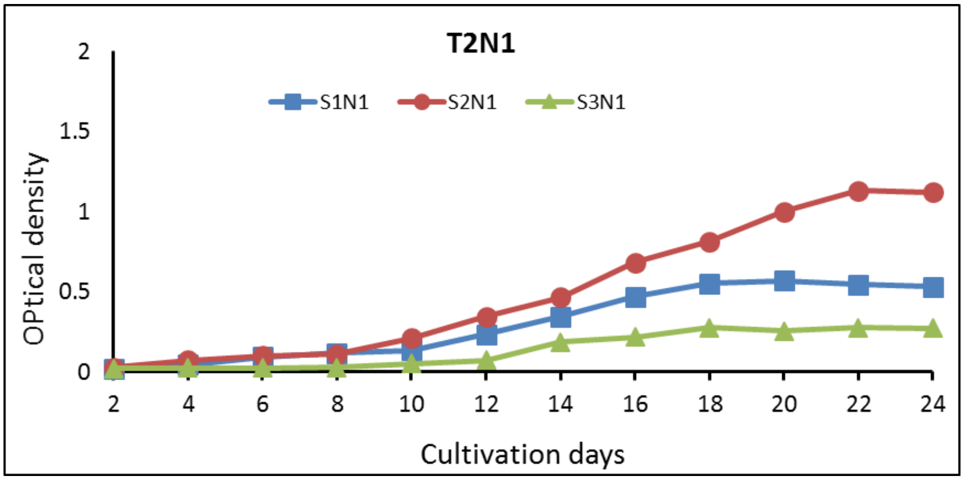


Figure 4.5: Effect of N1 level on optical density of three isolated strains at T2

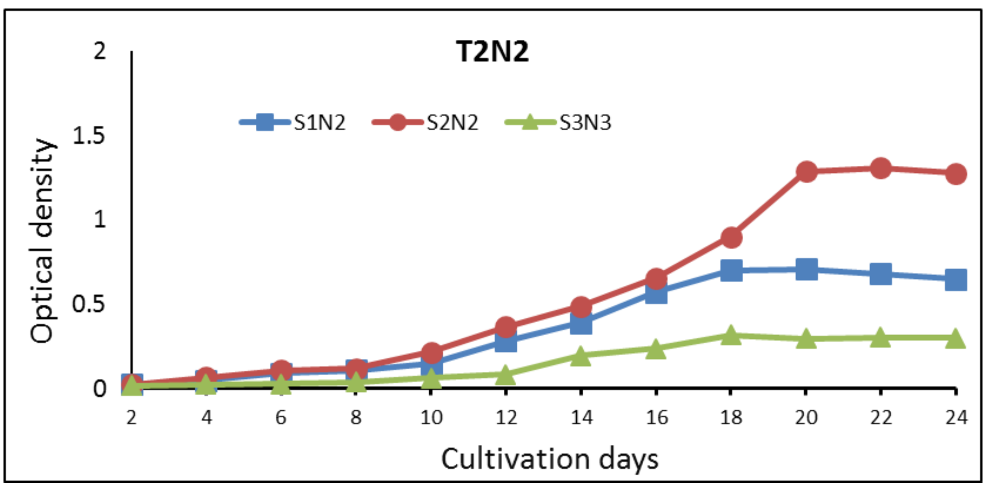


Figure 4.6: Effect N2 level on optical density of three isolated strains at T2

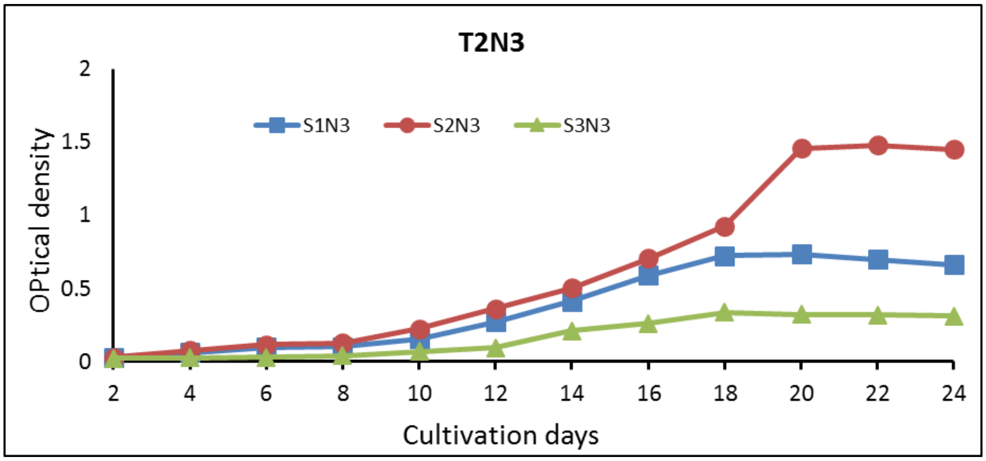


Figure 4.7: Effect of N3 level on optical density of three isolated strains at T2

S1=Strain A3: S2=Strain B5: S3=Strain C5: N1= 61.5 mg/L NO_3-N, 1.45 mg/L PO_4-P: N2=123 mg/L NO_3-N, 2.9 mg/L PO_4-P: N3= 246mg/L NO_3, 5.8 mg/L PO_4-P

At 35˚C, the same trend was observed for the optical density. S1 showed the least value (0.273) while S2 showed the highest value (1.45). The Nitrate and Phosphate level again showed the same trend of higher growth at higher nitrate and phosphate level, the strains growth increased. The overall, optical density of three strains became low by increasing the temperature from 30˚C to 35˚C.

4.4.2. Dry Weight

Effect of two temperatures and three nutrient levels on strains S1, S2 and S3 are given below by the graphs. Among three nutrient treatments and two temperature levels, N3 and T1 showed the highest gain in biomass as compared to other treatments.

4.4.2.1. DW at T1

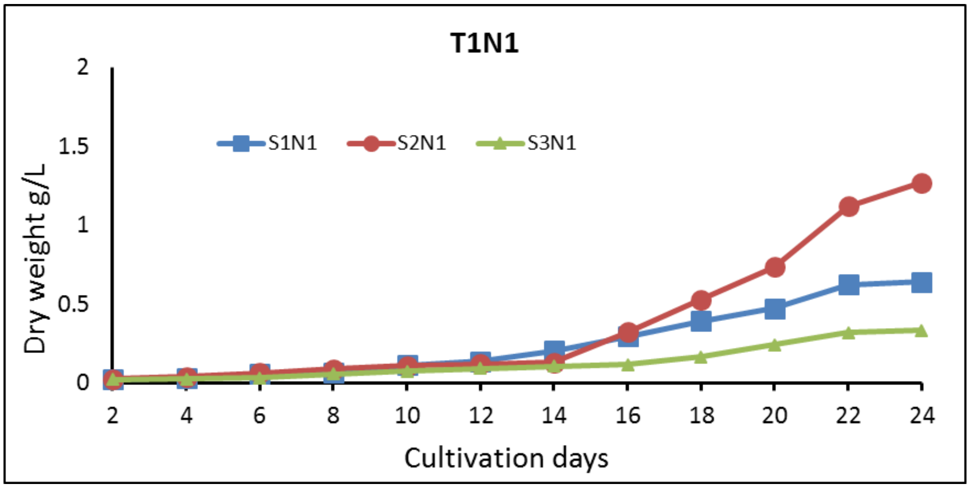


Figure 4.8: Effect of N1 level on dry weight of three isolated strains at T1

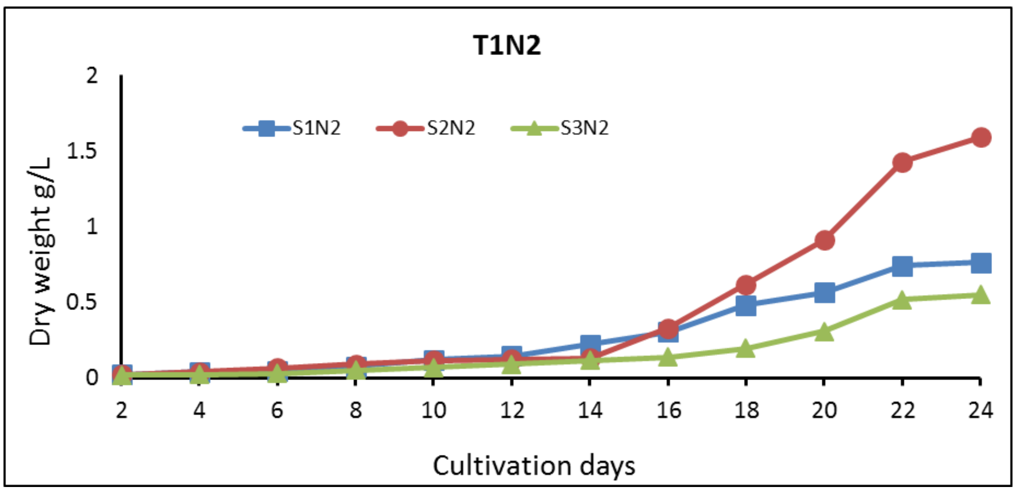


Figure 4.9: Effect of N2 level on dry weight of three isolated strains at T1

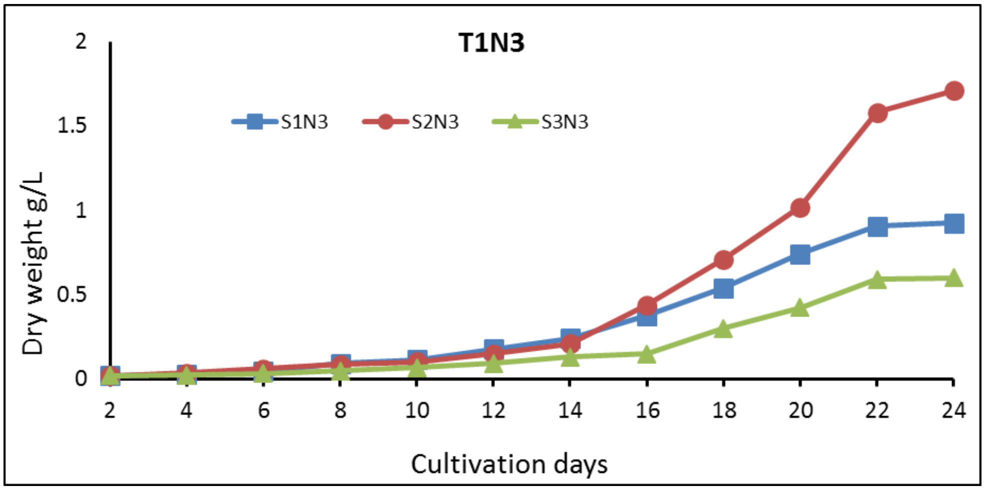


Figure 4.10: Effect of N3 level on dry weight of three isolated strains at T1

S1=Strain A3: S2=Strain B5: S3=Strain C5: N1= 61.5 mg/L NO_3-N, 1.45 mg/L PO_4-P: N2=123 mg/L NO_3-N, 2.9 mg/L PO_4-P: N3= 246 mg/L NO_3, 5.8 mg/L PO_4-P

The graphs figure 4.8, 4.9 and 4.10 depict that as the cultivation days increase the dry weight increase. In the start of the cultivation, the gain in dry weight is less because of initiation phase of all strains. But as the initiation phase end and exponential phase starts the increase bump up quickly and stick to the highest level. All studied strains showed the start of exponential phase after 16 days of cultivation as shown in the figures 4.8, 4.9 and 4.10 at N1, N2, and N3 nutrient levels. However, after 22nd days of cultivation, the exponential phase transfers into declining phase which is evident from the figures 4.8, 4.9 and 4.10.

This decline in growth after 22nd days of cultivation may be due to the limitation of nutrient in the medium. However, all three strains (S1, S2, and S3) showed highest dry weight at 24th day of incubation.

As the nutrient increases from Nitrate=61.5-246 mg/L & Phosphate 1.45-5.8 mg /L, the dry weight increases as well. This increase is due to more availability of nitrogen and phosphorus which are an integral part of the protein and ATP. But at N3, the strains S1, S2 and S3 showed highest dry weight (0.6, 1.71 and 0.92 mg/L) than N2 and N1 as shown in figure 20. Dry weights of S1, S2 and S3 at N1 are 0.64, 1.27 and 0.33 mg/L while at N2 are 0.76, 1.59 and 0.55 mg/L respectively.

4.4.2.2. DW at T2

As the temperature increased from 30 to 35˚C, the dry weight of all treatments decreased. The similar result was also observed by [72] which can be seen by the graph (figure 21-23).

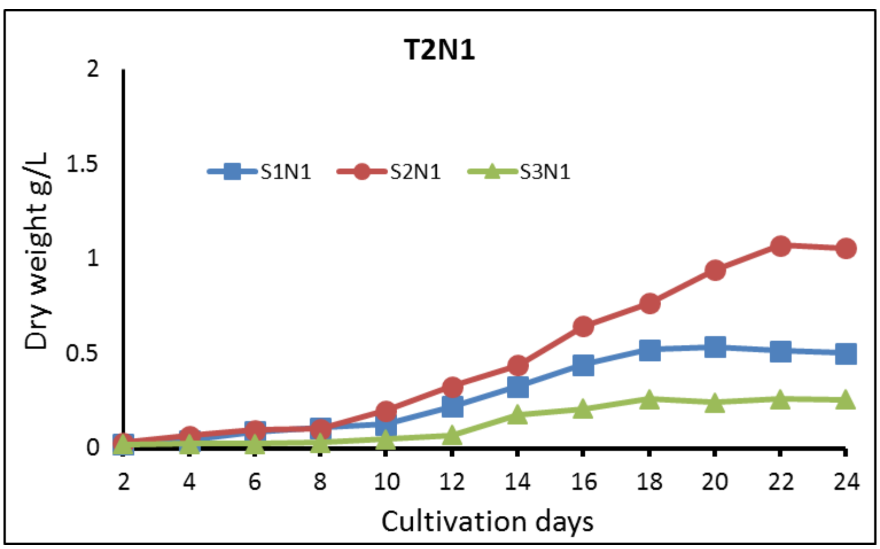


Figure 4.11: Effect of N1 level on dry weight of three isolated strains at T2

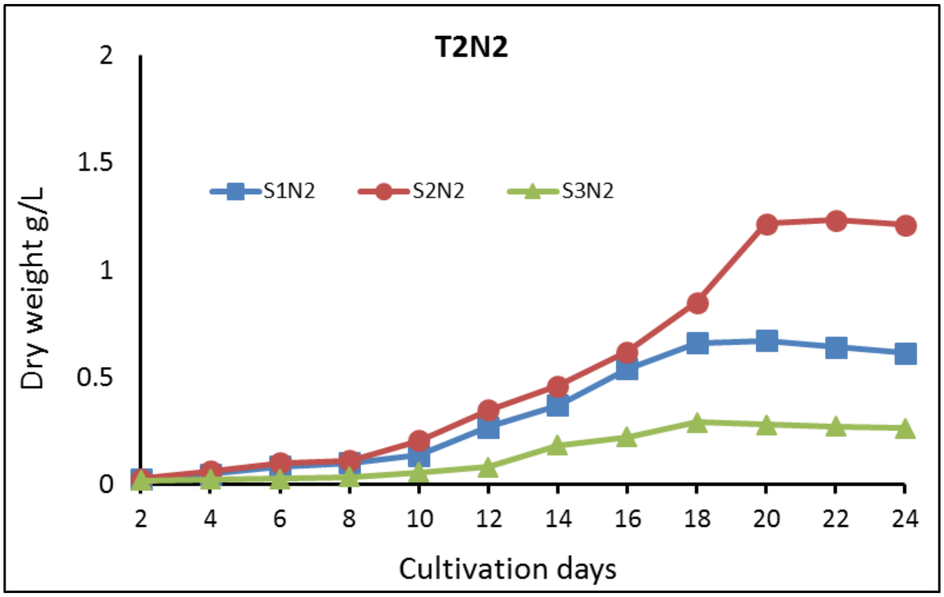


Figure 4.12: Effect of N2 level on dry weight of three isolated strains at T2

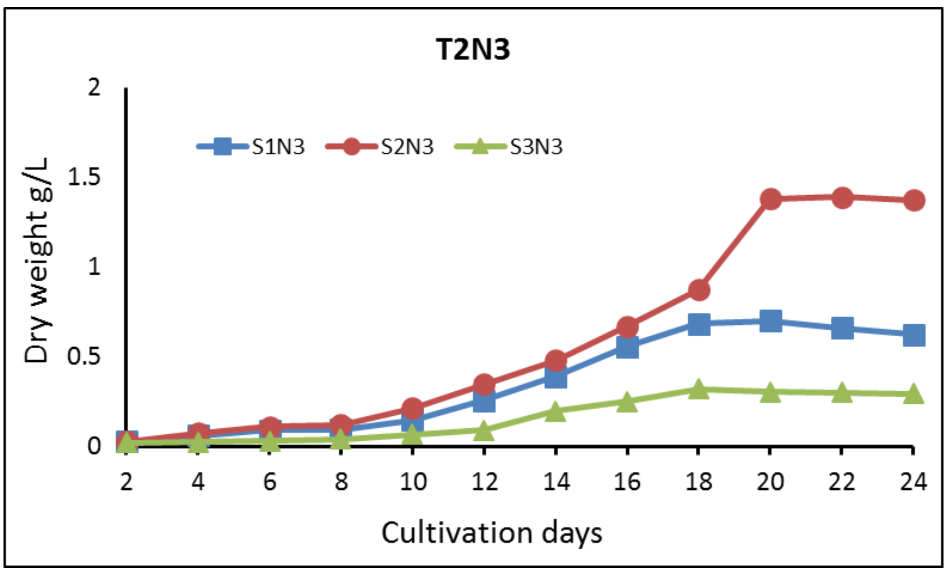


Figure 4.13: Effect of N3 on dry weight of three isolated strains at T2

S1=Strain A3: S2=Strain B5: S3=Strain C5: N1= 61.5 mg/L NO_3-N, 1.45 mg/L PO_4-P: N2=123 mg/L NO_3-N, 2.9 mg/L PO_4-P: N3= 246mg/L NO_3, 5.8 mg/L PO_4-P

Regarding exponential phase, when the temperature increases from 30 to 35˚C, the initiation phase shifted after 12 days of incubation. Similar to the results at 30˚C, the highest gain in dry weight was observed at N3 when 35˚C temperature applied. Strain S2 showed the higher dry weight of 1.38 mg/L at 20th day of cultivation, while the lowest dry weight 0.32 mg/L was seen by S3 at 18th day of cultivation.

The growth declined after 22nd days of incubation may be due to nutrient limitation or completion of the growth cycle. This trend was also followed by the treatments T2N1 and T2N2. However, dry weight at T2N1 and T2N2 were lower than of at T2N3. The lowest dry weight of all three strains was observed at N1 when 270 mg/L nitrate and 4 mg/L phosphate was applied. This because as the nitrate increases, microalgae growth increase.

4.5. Phosphorus and Nitrogen Removal

Total nitrogen and phosphorus were measured after every two days' interval. However, the data is shown for after four days' interval.

4.5.1. Phosphorus Removal

Three phosphorus levels (1.45, 2.9 and 5.8 mg Phosphorus/L) were analyzed to check the removal capacity of three strains S1, S2, and S3. Phosphate was applied in the form of K_2HPO4.

At 30˚C

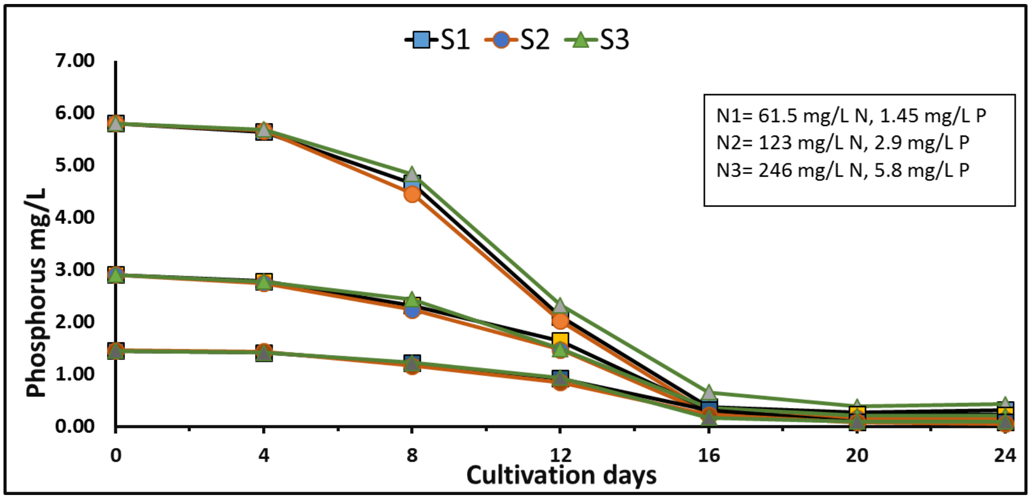


Figure 4.14: Phosphorus removal by S1, S2, and S3 at T1

Over 90 % of the applied phosphate removed by the three strains. Strains (S1, S2 & S3) at three nutrient levels showed about >95 % removal of phosphate.

At 35°C

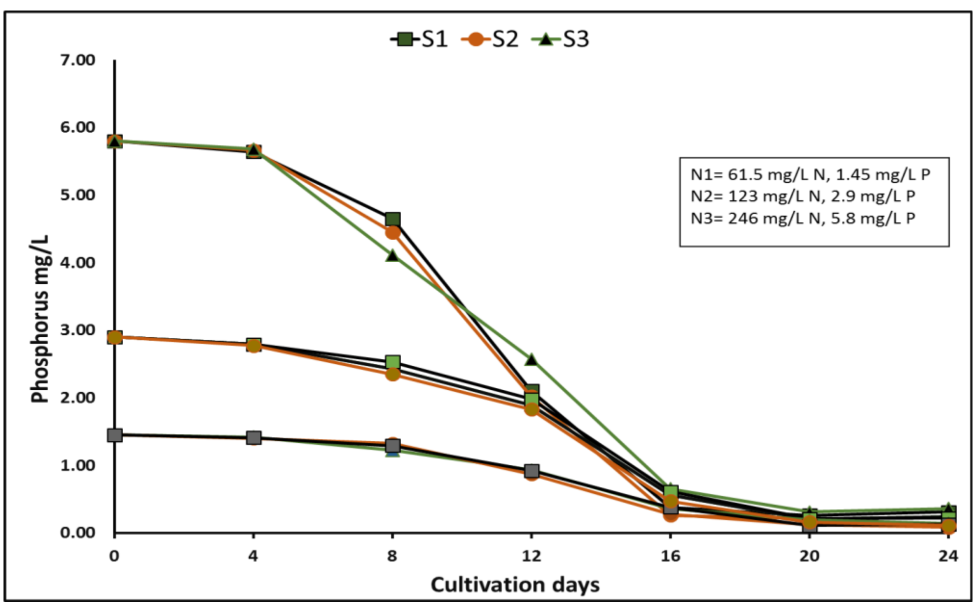


Figure 4.15: Phosphorus removal by S1, S2, and S3 at T2

At T2, the same results were obtained as for T1. More than 90% total phosphate was removed by three strains (S1, S2 & S3). About 80% of the nitrate was removed in the first 12 days of incubation. This small variation is may be due to fewer microalgae cells at 35^{0}C. S2 strains showed >94 % phosphate removal at the end of the experiment. Highest removal by S2 was found at the N1 level of 96%. Similarly, S1showed removal of 95% at 2.9 mg/L nutrient level. Interestingly, S3 showed a different behavior, after 12 days it showed very less removal (87.5%) of phosphate at 5.8 mg/L nutrient level than another nutrient level. At 1.45 and 2.9 mg/L it showed 92 and 96% removal.

4.5.2. Nitrogen Removal

At 30°C

The same outcome was observed with nitrate removal as for phosphate removal.

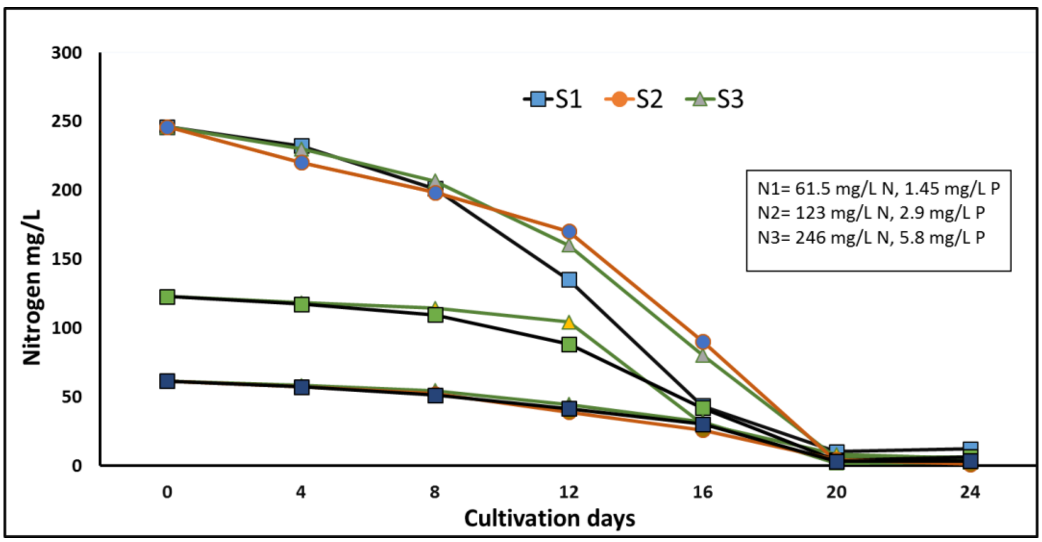


Figure 4.16: Nitrogen removal by S1, S2, and S3 at T1

Total nitrate was removed from the medium by strains (S1, S2 & S3) as shown in figure 4.17.

At 35°C

Very interestingly, the result of total nitrate at T2 was just similar to T1. At the end of the experiment, all nitrate and phosphate were removed efficiently from the media. The removal was more than 99%.

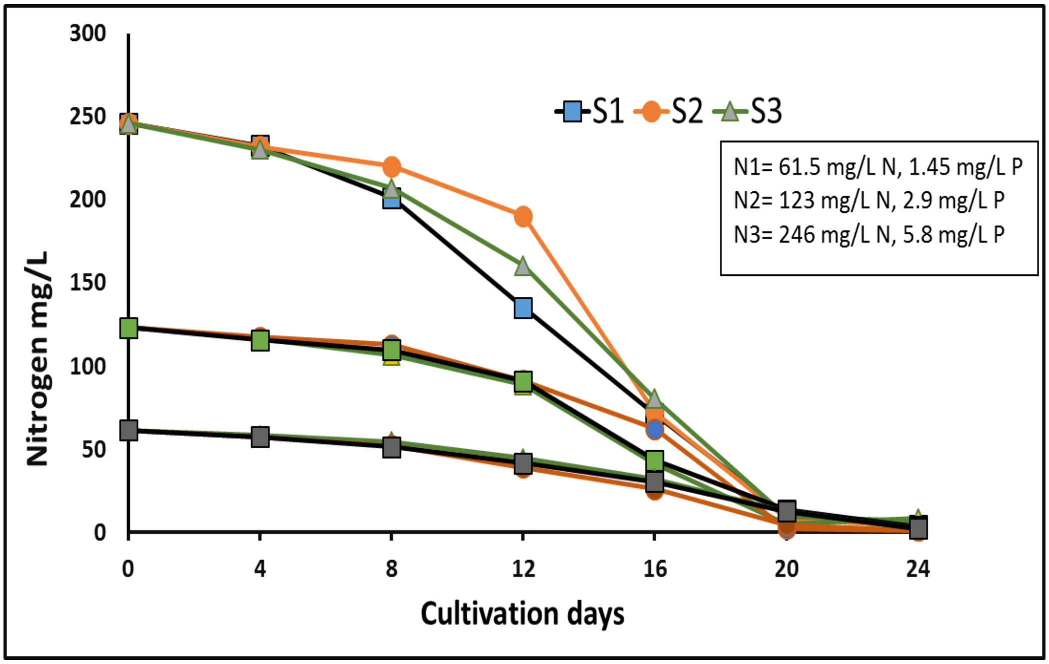


Figure 4.17: Nitrogen removal by S1, S2, and S3 at T2

The relationship between cultivation temperature and nutrient removal was rarely studied. Similarly, in our study, no significant difference of nutrient removal was observed at 30 and 35^{0}C.

4.6. Oil Content

Lipid productivity was measured and quantify by using GC. All the treatments were run and checked the peak time to qualitatively identify the FAMEs isolated and extracted. The hp-88 column was used during the analysis on Agilent 7890a GC-FID. Helium was used as a carrier gas including with Air. The oven temperature was held at 100^0C for 5 minutes then ramped to 240^0C with 10^0C per minute increasing the rate and held for 10 minutes. Injection volume was 1 μl. Supleco analytical FAME standard was run for the correlation with samples.

4.6.1. Extraction of Lipid

Percentage lipid content was measured by dividing the weight of recovered lipid by the dry weight. For this same volume of culture was taken for dry weight and lipid content. The formula for the lipid content measurement is

$$\text{Lipid content \%} = weight\ of\ lipid\ content\ (g)/Dry\ weight\ (g)$$

From the table, it is obvious the highest lipid content was observed from *Chlorella vulgaris* (~28%) while the least from *Chlorella kessleri* (~15). However, Nannochloropsis oculata gave sufficient amount of lipid. The average lipid yields of *chlorella vulgaris*, *Chlorella kessleri* and *Nannochloropsis oculata* were 27.7%, 19.9% and 14.6% respectively. The lipid productivity was measured by the equation

$\nu = CL/t$

Where CL is a concentration of lipid at the end of incubation while t is the duration of the run.

Table 4.4: Lipid content of S1, S2 and S3 strains at T1

Treatment	Dry weight g/L	Lipid content %	Lipid productivity $g/l^{-1}day^{-1}$
S1N1	0.641	19.7	0.82
S1N2	0.764	19.8	0.82
S1N3	0.924	19.7	0.82
S2N1	1.27	27.3	1.13
S2N2	1.59	27.3	1.13
S2N3	1.71	27.2	1.13
S3N1	0.331	14.1	0.58
S3N2	0.55	14.1	0.58
S3N3	0.6	14.1	0.58

At T2:

At 35^0C, again the *Chlorella vulgaris* gave highest lipid content (20%) while *Nannochloropsis oculata* gave lowest (14%). By comparing the results at 30 and 35^0C, no marked variation was found.

Table 4.5: Lipid content of S1, S2 and S3 strains at T2

Treatment	Dry weight g/L	Lipid content %	Lipid productivity $g/l^{-1}day^{-1}$
S1N1	0.641	19.6	0.82
S1N2	0.764	19.7	0.82
S1N3	0.924	19.7	0.82
S2N1	1.27	27.2	1.13
S2N2	1.59	27.3	1.13
S2N3	1.71	27.2	1.13
S3N1	0.331	14.1	0.58
S3N2	0.55	14.1	0.58
S3N3	0.6	14	0.58

4.6.2. Quantification of Lipid

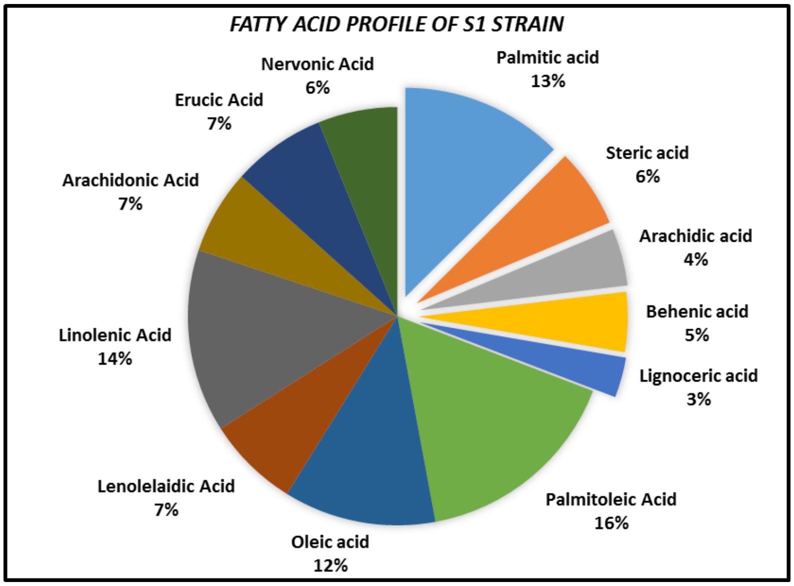
All the samples were run on GC-FID for quantification as per prescribed and mentioned method. Strains S1, S2, and S3 showed similar result at all nutrient levels.

4.6.2.1. Fatty Acid Profile of S1

Table 4.6: Fatty acid profile of S1 strain

Fatty Acid	Name	FAME %	FAME % DW
C-16	Palmitic acid	12.5	2.5
C-18	Steric acid	6.1	1.2
C-20	Arachidic acid	4.4	0.9
C-21	Behenic acid	4.6	0.9
C-23	Lignoceric acid	3.1	0.6
Total Saturated		**30.7**	**6.1**
C16-1	Palmitoleic Acid	16.2	3.2
C18:1	Oleic acid	11.7	2.3
C18-2	Lenolelaidic Acid	7.1	1.4
C18-3	Linolenic Acid	14.1	2.7
C20-4	Arachidonic Acid	6.5	1.3
C22-1	Erucic Acid	7.2	1.4
C24-1	Nervonic Acid	6.1	1.2
Total Unsaturated		**69**	**13.6**
Total FAME		**100%**	**19.7%**

The S1 strains constitute of 31% saturated and 69% unsaturated fatty acid by dry weight. Palmitoleic Acid was the compound in the largest amount of 16% of the fatty acids. However, Palmitic acid (12% of the FAs) and Linolenic Acid (14% of FAs) were also in a higher amount. As the palmitic and steric acid are favored for biofuel, both these constitute about 60 % of the saturated FAs.



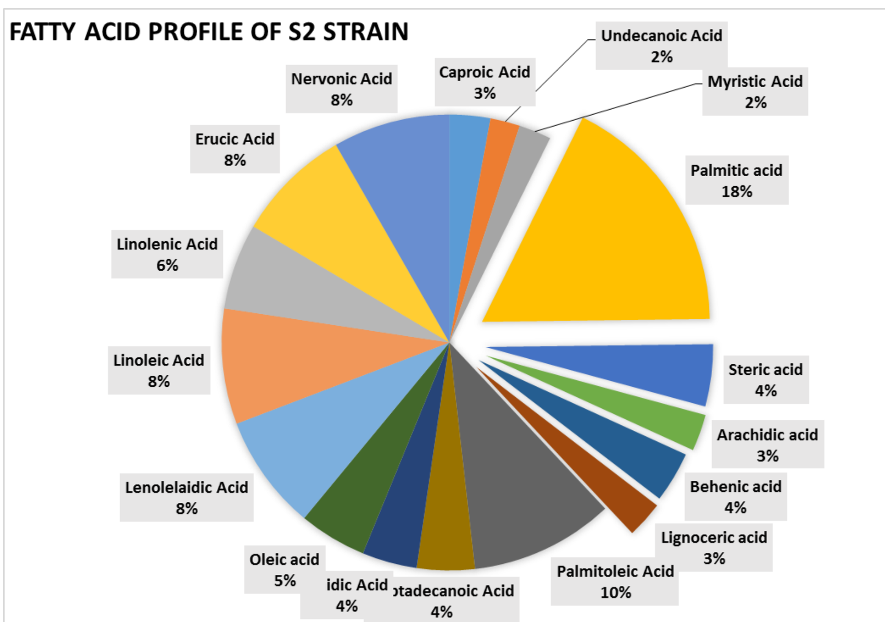
4.6.2.2. Fatty Acid Profile of S2

Table 4.7: Fatty acid profile of S2 strain

Fatty Acid	Name	FAME %	FAME % DW
C6	Caproic Acid	2.9	0.8
C11	Undecanoic Acid	2.1	0.57
C14	Myristic Acid	2.3	0.63
C16	Palmitic acid	17.5	4.8
C18	Steric acid	4.4	1.2
C20	Arachidic acid	2.6	0.7
C23	Lignoceric acid	2.6	0.7
Total Saturated		**34.4**	**9.4**
C16-1	Palmitoleic Acid	10.2	2.7
C17-1	Heptadecanoic Acid	4.1	1.1
C18-1	Elaidic Acid	3.9	1

C18:1	Oleic acid	8.4	2.3
C18-2	Lenolelaidic Acid	8.2	2.2
C18-2	Linoleic Acid	8.2	2.2
C18-3	Linolenic Acid	6.1	1.6
C22-1	Erucic Acid	8.2	2.2
C24-1	Nervonic Acid	8.3	2.3
Total Unsaturated		**65.6**	**17.6**
Total FAME		**100**	**27.3**

S2 strain, showing 34% saturated FAs, have about 22% palmitic acid and steric acid. As its biomass productivity was also high, so it is the most promising line for biofuel production.

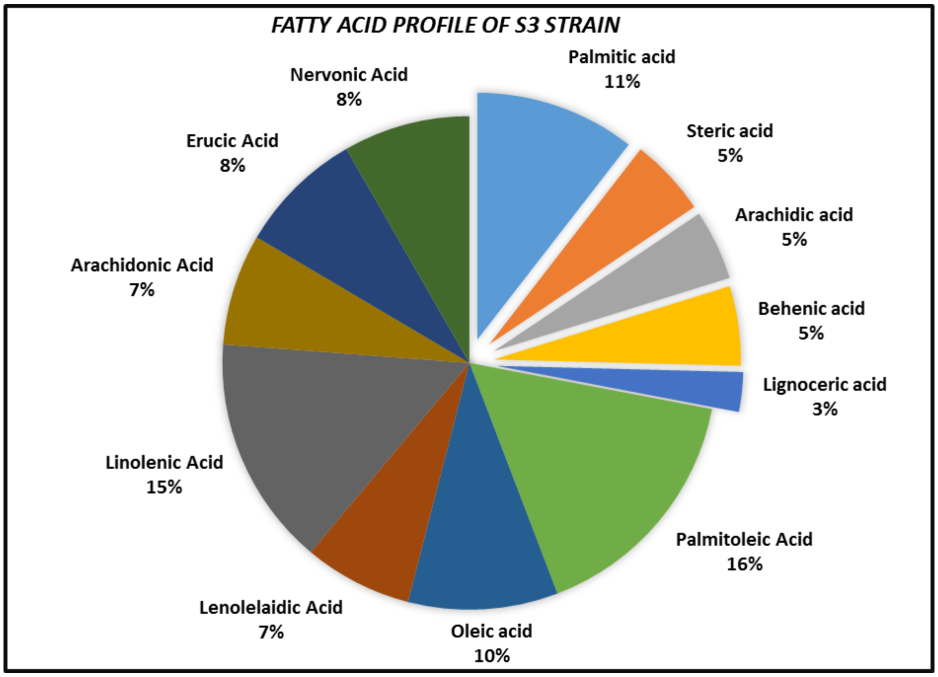


4.6.2.3. Fatty Acid Profile of S3

Table 4.8: Fatty acid profile of S3 strain

Fatty Acid	Name	FAME %	FAME % DW
C-16	Palmitic acid	10.5	1.47
C-18	Steric acid	5.1	0.71
C-20	Arachidic acid	4.6	0.64
C-21	Behenic acid	5.2	0.73
C-23	Lignoceric acid	2.6	0.36
Total Saturated		**28**	**3.92**
C16-1	Palmitoleic Acid	16.2	2.26
C18:1	Oleic acid	9.8	1.37
C18-2	Lenolelaidic Acid	7.1	0.99
C18-3	Linolenic Acid	15.1	2.11
C20-4	Arachidonic Acid	7.3	1.02
C22-1	Erucic Acid	8.2	1.14
C24-1	Nervonic Acid	8.3	1.16
Total Unsaturated		**72**	**10.08**
Total FAME		**100%**	**14%**

In S3 strain, Palmitic acid and steric acid together constitute about 16% of the FAs.



The FA concentration among all the strains varied from 14 to 27% of the dry weight.

Chapter 5

DISCUSSIONS

The size of *Chlorella vulgaris* range from 2-15 µm in diameter (cells are often overlooked because of their small size) [71]. So it was supposed that our slant 2 (B5) resemble the *Chlorella vulgaris*. Similarly, *Nanochloropsis oculata* is found larger in size than *C. vulgaris* but the shape remains same as our strain 3 (C5). Slant 1 (A5) were found to be floating in the medium all the time and under the microscope showed "u" shape chloroplast and more spherical than *Chlorella vulgaris* as shown in table 8 having similar characteristics of *Chlorella kessleri* [70]. On the basis of these morphological characters our isolated strains were identified.

Optical density at T1: All the treatments give maximum optical density at the 30°C. Our study was supported by Fakhry who found maximum optical density at this temperature at day 24 [72].

Optical Density at T2: OD was lower in this study at 35°C then 30°C. This study was supported by *Cho et al.,* which found the maximum cell density of *Nannochloropsis oculata* at 30°C temperature. They varied the salinity of the solution but the optical density was maximum at 30°C than 35°C. However, when the temperature was below 25°C the density reduced [132].

DW at T1: This result is further supported by Bartley et al., 2015 which found the highest cell density at 25th day of cultivation *Nannochloropsis sp* [78]. Similarly, Nigam *et al.,* 2011 found that *chlorella sp* gave 0.315 g/L of biomass production at 24^{th} day of culturing [79].

DW at T2: As the temperature increased from 30 to 35°C, the dry weight of all treatments decreased. The similar result was also observed by some scientists [72].

Observing the result obtained at 30°C and 35°C, dry weight of S1, S2 and S3 was observed higher at 30°C (0.92, 1.71 and 0.6 mg/L respectively). Generally, the temperature effect on microalgae biomass concentration may be species dependent. As no significant difference was observed by varying the temperature from 10-30°C on *Scenedesmum* sp. LX1 under 15 days cultivation [81]. While *Isochrysis galbana* increased linearly with temperature but with a small difference [82]. Similarly, *Chlorella sp* HQ showed significant responses to 12-25°C temperature and a high temperature of 38 °C could not be endured [83]. The similar findings show that *chlorella vulgaris* don't grow well over 30°C as it is considered as heat stress [80].

Removal of P at T1: The removal of 8 mg /L by *chlorella vulgaris* is further supported by another study [75] using batch culture media. Similarly, *chlorella kessleri* was also found to remove the phosphate up to 15 mg/L in batch culture [76]. However, one study showed the very high removal of 42 mg/L phosphate by *chlorella vulgaris* [77]. This removal actually depicts the potential of species for nutrient absorption. If the concentration increases from the optimum level it hinders the growth and hence removal is restricted.

Generally, from various investigations, it is evident that municipal wastewater having total nitrogen up to 90 mg/l and phosphate up to 16 mg/l can be removed by *Chlorella sp* more than 80% [84].

N and P Removal: Result from the current study showed that nitrate and phosphate were almost completely removed within 20-day incubation period for both temperatures, regardless how high the initial concentration (61.5-246 mg/L TN, 1.45-5.8 mg/L TP). It means the initial 246 mg/L nitrate and 5.8 mg/L phosphate did not cause the nutrient uptake inhibition to the studied strains. The initial higher concentration of 178 mg/L was also tolerated by *Chlorella sp.* in one study which supports this study [133].

Fatty Acid Profile: Various compounds are found in microalgae lipids like phospholipid, glycolipid, and glycerides [86, 87]. However, their ratios are species and growing condition dependent. Free fatty acid range about 1-2% of the microalgae lipids [88]. Most of these are linked with glycerol forming acyl glycerol. Generally, mono, di, and tri glycerol are found among which try glycerol are easily converted into biofuel by transesterification method [89]. So for estimation of biofuel production, one has to consider the fatty acid composition rather than only the lipid content [90, 91].

Palmitic acid and Palmitoleic acid are found prominently among all the strains. The highest value of palmitic acid (17.5% of the dry weight) was found in S2 strain, while the lowest were found in S3 (10.5% of the dry weight). However, the S2 strains showed highest amount of saturated fats (38%) while S3 showed 28% saturated fats. As the saturated FAs and MUFAs higher in proportion, the lesser will be the problem in fuel combustion [85]. Moreover, the quantity of palmitic and steric acid is the further indicator of biofuel efficiency, because, after ignition in the engine, these are the first compounds that precipitate [92]. From the table 4.4, 4.5 and 4.6, it is obvious that S2 stain has a higher value of palmitic and steric acid than the S1 and S3. So, the biofuel obtained from S2 is better than S1 and S3.

Conclusion

The following conclusion was drawn from this study.

After sampling from the coast of Jubail and Al-Khobar, six strains were isolated through continuous streaking method. Among these six two were the samples taken from flowing water of Jubail coast while one each from sediment sample of Jubail shore and flowing water from Al-Khobar. Three strains (S1, S2, and S3) identified through their morphological behavior and 18S rRNA analysis.

Two growth parameters OD and DW were measured by varying three nitrates and phosphate and two temperature levels (30 and 35°C). At T1, among the tested nutrient levels, N3 gave higher optical density (1.81) value then followed by N2 and N1 respectively. While S2 gave highest OD of 1.81, followed by S1 0.98 and S3 0.68. A similar trend was observed at T2, where higher OD was observed at N3, followed by N2 and N1. S2 again showed higher OD than the S1 and S3 as like T1. So highest OD was seen from S2 at all nutrient levels.

At 30°C, the trend of dry weight was seen alike optical density. The highest dry weight of 1.71 g/L was observed at N3 and lowest biomass of 0.33 g/L was at N1. N3 nutrient level showed higher biomass accumulation than N2 and N1. The s2 strain showed the high dry weight of 1.27, 1.59 and 1.71 g/L at N1, N2 and N3 respectively. At T2, again the same trend is seen where the high dry weight of 1.37 g/L obtained at N3 then followed by N2 1.21 g/L and N1 1.05 g/L. The highest dry weight of g/L was seen by S2 at N3 nutrient level then g/L at N2 and the lowest at N1 of g/L. S3 produced DW was 0.29 g/L when N3 was applied in the medium and followed by 0.26, 0.25 g/L at N2 and N1 respectively. So, among all the treatments, S2 showed more DW at all nutrient levels.

Nitrate and phosphate uptake was also observed at two mentioned temperatures keeping NP ratio (42:1) constant and changing the initial concentration of nitrate and phosphate (in the form of NaNO3 and K2HPO4). Nitrogen varied from 61-246 mg/L while Phosphorus from 1.45-5.8 mg/L. All the strains showed slow removal from day 1-8, then increase proportionally till the maximum removal. No marked difference found for the removal of nitrate and phosphate at two studied temperatures. A similar trend was also found for S1, S2 and S3 strains individually at both temperatures. The strain S1 showed >94, 98 and 95% nitrate and 94, 96, 94% phosphate removal at N1, N2 and N3 respectively when incubated at 30°C. The s2 strain showed the removal of >94, 98 and 95% nitrate and 92, 95 and 93% phosphate at N1, N2, and N3 respectively. While S3 showed >95, 98 and 97% nitrate and 95, 98, 94% phosphate removal at N1, N2 and N3 respectively at T1.

When S2 was cultivated at 35°C, nitrate removal was 95, 98 and 97% while phosphorus removal was 94, 96 and 92% at N1, N2, and N3 respectively. The strain S1 showed 91, 94 and 90% phosphate and 96, 98 and 97% nitrate removal at N1 N2 and N3 respectively when incubated at 35°C. More than 90% phosphate was removed from the culture media after 16 days while more than 90% nitrate was removed after 20 days.

Lipid content by percent of dry weight showed no variation on two studied temperatures and three nutrient levels (N1, N2, and N3). The strain S2 showed highest lipid content of 27% followed by S1 19% and S3 14% of the dry weight. Highest lipid productivity of 1.13 g/L/day obtained from S2 while the lowest of 0.58 g/L/day from S3 strain. Strain S1 showed 0.82 g/L/day. Quantification of lipid content through GC-FID showed 30.7, 34.4 and 28% saturated fatty acid in S1, S2, and S3 respectively.

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