Study of Growth Kinetics of High Lipid Microalgae in Local Environment

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Certification of Thesis Work

We, the undersigned, certify that **Anika Ferdous,** candidate for the degree of **Master of Science in Engineering (Chemical),** has presented her thesis on the subject **"Study of Growth Kinetics of High Lipid Microalgae in Local Environment".** The thesis is acceptable in form and content. The student demonstrated a satisfactory knowledge of the field covered by this thesis in an oral examination held on **August 31, 2020.**

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Candidate's Declaration

It is hereby declared that this thesis or any part of it has not been submitted elsewhere for the award of any degree or diploma.

_____

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Abstract

Clean energy supply has always been a challenge confronting humanity. Wind energy, geothermal energy, solar energy and biofuel are among the most important options proposed during the last century. Biofuel has many unique capabilities that drew attention more than other sources. There are several criteria that make microalgae a potential candidate for biodiesel production. For example- they can be cultivated on non-arable lands using non- potable water, all the year round. Moreover, several species have the ability to accumulate high amount of lipid which can further be increased by modifying culture conditions with the biotechnology techniques. Microalgae are unicellular, photosynthetic microorganisms, they have minimal nutrient requirements and are being used as source materials for a variety of products such as protein rich nutritional supplements, pharmaceutical chemicals and pigments (used in food and cosmetics). The strain *Chlorella vulgaris*, has shown a great potential as a source of oil and also in helping in wastewater treatment. Challenges in the process of producing biodiesel from microalgae include effective techniques to grow microalgae and harvest the grown microalgae, extraction of the algal oil and its trans-esterification to biodiesel. The goal of the present study is to study the behavior of the selected microalgae in the local environment. Microorganisms behave differently in different environments. The culture media which worked in one environment might not produce satisfactory result in another environment. Hence, it is important to find a suitable culture media in the local environment. This study has been crucial to develop an economical process to produce biodiesel from microalgae. The production of microalgae *Chlorella vulgaris* in four different media was investigated in batch culture. The results were analyzed to find the most suitable media. The best response was recorded for modified CH media. In this media, an exponential phase with duration of 15 days was recorded, where the concentration of the biomass at the end of the period was 2.84 g/L. Moreover, the culture in this media was recorded to have the highest specific growth rate (0.136 d^{-1}), lowest doubling time (53.28 hours) and maximum volumetric biomass yield (0.106 $gL^{-1}d^{-1}$). The study also showed that Bold's Basal medium which is the most frequently used medium for culturing *Chlorella vulgaris* did not respond accordingly in our local environment.

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Chapter 1 Introduction

Fossil fuel are depleting. These non-renewable fuel sources have become an inevitable part of human civilaztion. However the prosperity these fuel sources have brought for our civilization has costed us our environment. [2]. On the other hand, today a lot of carbon dioxide is released into the atmosphere due to human activities and carbon dioxide is the main culprit for global warming [3]. According to a report by Global Carbon Project found that carbon dioxide emissions from burning fossil fuels were likely to have increased by 2.7 percent in 2018, after a 1.6 percent increase in 2017 [4]. A way of recycling the carbon and reusing the waste for something beneficial has also become a top priority as a way of fighting global warming. Microalgae have the capacity to fixate inorganic CO_2 into organic matters. A desirable characteristic of many species of microalgae is the capability of producing and accumulating lipids suitable for biodiesel. Some algal species can accumulate polysaccharides as starch for bioethanol purpose. Moreover, microalgal biomass represents an increasingly attractive strategy in the sector of wastewater treatment. Microalgae have the capacity for intensive nutrient removal from wastewater [3].

Microalgae are unicellular, photosynthetic microorganisms, they have minimal nutrient requirements and are already being used as source materials for a variety of products such as protein rich nutritional supplements, pharmaceutical chemicals and pigments (used in food and cosmetics). They also grow extremely fast, in comparison to plants [2].

However, it is essential to modify the system for making it economically feasible. For example, harvesting and de-watering processes for the microalgal culture system are time and energy consuming [5].

Optimization of this biological process is achieved by determining the optimal growth conditions for microalgal biomass. A cost-effective and efficient method is the usefulness of kinetic growth models. With such models “in silico” experiments can be performed to assess the behavior of the microalgal biomass. For development of kinetic models, it is necessary to generate experimental data and validate it against model calibration. Such experiments that aim at obtaining suitable data regarding microalgal growth, often require proxy measurements, *e.g.* chlorophyll content and lipid content. In general the

analysis protocol for these measurements are very time consuming and require good technical practice. Instead of these, measurement of optical density and dry cell weight offer low cost alternatives and are rather easy to perform [6].

1.1 **Background of the Study**

Reduction of CO_2 emissions is expedient but even a 30% reduction (as agreed by some international legislations), is still not enough to stabilize the CO_2 levels into a "safe zone". This requires the development of alternative biofuels, of which biodiesel and bioethanol are the ones with great market potential at the moment. Biodiesel is a popular alternative to petroleum based diesel; it can be used in regular diesel engines. It is eco-friendly, non-toxic, and biodegradable. Moreover, when burned, due to its low sulfur content, it produces fewer emissions than its petroleum counterpart. It can be prepared from renewable sources like edible and non- edible vegetable oils, animal fats and even waste cooking oils. The most common concern with such first generation biofuels is that as production capacities increase, so does their competition with agriculture for arable lands used for food production. Currently, approximately 8% of plant-based oil production is used as biodiesel and this has already contributed to an increase in price of oil crops over the last few years. Second generation biofuels as lignocellulosic and microalgal biofuel systems, have the potential to overcome many of the limitations [7-9]. Some strains of microalgae are reported to produce 15-300 times more oil per area, for biodiesel production, than traditional oil crops. Microalgae have a shorter average harvesting cycle of around 10 days, traditional oil crops are only harvested once or twice a year. The cost of biodiesel could be reduced if one considers non-edible source such as microalgae oil [8, 9]. Different studies have shown *Chlorella* to have promise as a source for oil in the production of biodiesel [10-13].

Despite having numerous pros, the application of microalgae for fuel production on an industrial scale is now offline, as the process is not commercially viable.
However, there is a major drawback and even possibly an obstruction to the microalgal culture systems on industrial scale, and that is- high harvesting cost. Since the harvested microalgae culture is dilute in nature, the dewatering process bears a huge operational cost, which consequently makes the microalgal system less economically feasible.

A way to optimize the biological process is determining the optimal growth conditions for microalgal biomass. Using kinetic growth models can be a cost-effective and efficient method to determine the optimum period for harvesting. For development of kinetic models, it is necessary to generate experimental data. Hence comes the necessity to study growth kinetics of a microalgal system. In general, the analysis protocol for these measurements are very time consuming and require sophisticated technical practice. Instead of these, measurement of optical density and dry cell weight offer low cost alternatives and are rather easy to perform.

1.2 Significance of the Study

Currently the whole world is moving towards renewable energy. Various countries is Europe like Germany, England etc. are shutting down their coal fired power plants. The USA is also reducing it's dependency on fossil based fuels and gravitating towards renewable options. China has declared to go carbon neutral in terms of energy by 2060. It's high time for Bangladesh to join the clean energy club and contribute to fighting climate change. Bangladesh has great potential to become a pioneer in the field of green energy. Microalgae can grow in industrial effluent water. Later these microalgae can be used to produce biodiesel. Hence adopting the process of producing microalgae for biofuel production can offer a two fold advantage- removal of heavy metals from industrial effluent and mitigating the energy crisis. This study is done keeping this big picture in mind. This study will allow us to understand how the selected strain behave in our local environment. This understanding can be later used to develop mass scale production of microalgae to produce biodiesel.

1.3 Research Objectives

The main objective of the study was to study how a microalgae with high lipid content behave in the local environment in a laboratory scale. The specific aims under the main objective to be covered are listed below:

- To select a suitable microalgae strain and environment, i.e. temperature and light flux for controlled environment culture
- To experimentally investigate the effect of nutrient media on the growth of the selected strain

- To analyse the experimental data to determine key parameters
    - ❖ Duration of Exponential Phase
    - ❖ Cell Concentration at the End of the Exponential Phase
    - ❖ Overall Specific Growth Constant
    - ❖ Doubling Time
    - ❖ Biomass Productivity
- To identify a suitable medium for growing the selected the strain in local ebvironment of Bangladesh.

1.4 Thesis Overview

The thesis consists of five chapters. Chapter 1 contains brief introduction to the research topic. Chapter 2 contains a more detailed literature review relevant to the research such as basic information of microalgae, reproduction process of algae in batch culture system, factors affecting the growth of microalgae, determining the growth of biomass and developing kinetic models. This chapter also contains a brief description on situation of microalgae culture in Bangladesh. In chapter 3, materials and methods are listed along with descriptions of the parameters monitored. In chapter 4, results obtained are shown, analyzed and discussed. The final chapter (chapter 5) includes the conclusions that can be drawn from the results obtained as well as recommendations for future work.

Chapter 2 Literature Review

2.1 Microalgae

Algae, one of the most robust organisms on earth, are a large group of primitive, mostly aquatic, photosynthetic chlorophyll-bearing plants, lacking specialized tissues and organs namely roots, stems, leaves, flowers, etc. . Algae can be broadly categorized into two groups: microalgae and macro algae. Microalgae are very small, their sizes can range from a few micrometers to a few hundreds of micrometers while macroalgae can be up to six order of magnitude larger than microalgae. Algae are usually found in damp places or water bodies. Like plants, most algae require primarily three components to grow: sunlight, carbon-dioxide, and water [14]. Algae contains proteins, carbohydrates, fats (lipids) and nucleic acids. Though the percentages vary with the type of algae, there are algae types that contain as much as 70% lipids (oil).It is this fatty acid/lipid (oil) that can be extracted and converted into biodiesel [15, 16].

Microalgae is a third generation biofuel source with several advantages over terrestrial crops owing to its high potential yield of biofuels and relatively fast growth rates [17]. CO_2 can be captured and used in large scale cultivation of algae for biofuel production [18]. Figure 1 illustrates CO_2 mitigation (carbon neutrality) using microalgae as an energy resource.

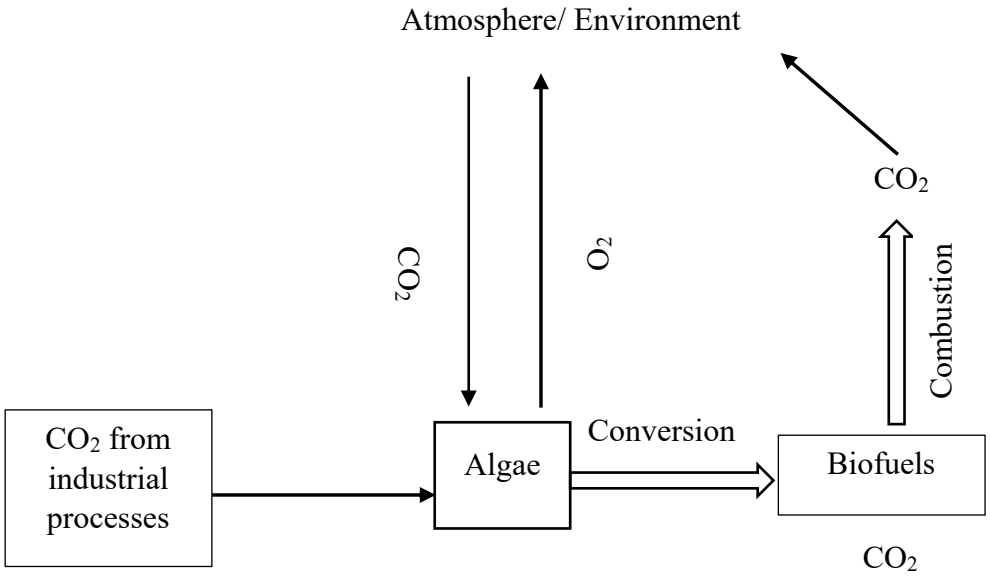

Figure 1: CO_2 mitigation using algae [18]

Microalgae have many different species with widely varying compositions. Microalgae live as single cells or colonies without any specialization. The small size of microalgae makes subsequent harvesting more complicated [19]. Biologists have categorized microalgae according to their pigmentation, life cycle and basic cellular structure; the important categories are: diatoms (Bacillariophyceae), green algae (Chlorophyceae), blue-green algae (Cyanophyceae), and golden algae (Chrysophyceae) [20].

Microalgae can be classified based on their pigmentation, growth conditions, cell wall structure and flagellation. There are six phyla of algae: cyanobacteria, green algae, red algae, diatomaceae, Eustigmatophytes and Prymnesiophyceae. A brief description of each phylum of algae is given below in Table 1 [21, 22]

Table 1 Classification and description of microalgae phyla [21,22]

Algal Phylum	Description
Green algae or Blue-green algae	Algal cells have green chloroplast that contains chlorophyll a and b. These cells have mitochondria. Some species have flagella.
Red algae or Rhodophyceae	Cells have chloroplast with chlorophyll a and d, and phycobillins. They have double cell wall, but do not have centrioles and flagella.
Cyanobacteria	Class of prokaryotic cells that contain chloroplast with no chlorophyll. These are bacteria; but they are assimilated to algae due to their growth through photosynthesis process similar to microalgae. Some strains can grow in soil, marine or fresh water.
Eustigmatophytes	Class of eukaryotic algae which contain yellow-green chloroplast. They include strains growing in marine, freshwater or solid medium such as soil. Algae in this class have chloroplast containing chlorophyll a.
Prymnesiophytes or Prymnesiophyceae	Class of algae in chlorophyll a-c phyletic line. Some strains of this group have one or two flagella. For example, Pavolva strain has smooth flagella of equal length.
Diatomaceae	Class of Bacillariophyceae, Diatomaceae cells have chloroplast carrying chlorophyll a and c. They have hard wall due to the presence of silica. Most of these cells can be found in fresh or salted sea. Majority of diatom species live in cold water.

2.1.1 Microalgae Strains

Many microalgal strains have been reported to have the ability to contain high amount of lipids. Allard and Templier (2000) extracted lipids from a variety of marine and freshwater algae and reported that the lipid contain varied from 1-26% [23]. Miao and

Wu (2004) reported a heterotrophic growth of *Chlorella protothecoides* is capable of as high as 55% of lipid growth, which can be later converted to biodiesel [24]. In spite of low growth rate, *Botryococcus braunii* strain has received vast attention because of its high lipid content (up to 64%) and high hydrocarbon production ability [25, 26].

The properties of microalgae- based biodiesel depend on the strain of microalgae from which it was produced. For instance, *Botryococcus braunii* contains terpenoid hydrocarbons while *Chlorella prothecoides* contains glycerol lipids.

Finding algae strains to grow is not as difficult as cultivating a specific strains of algae for biodiesel production. A class of biomolecules synthesized by many species of microalgae is the neutral lipids, or triacylglycerol (TAGs) and their content in microalgae varies from organism to organism. Some microalgae can produce more than 60% of their dry cell weight in the form of lipids under certain conditions [27, 28]. Table 2 enlists different algal strains with their lipid contents [17].

Table 2 Lipid content of some microalgae [17]

Species	Lipid (% dry matter)
Botryococcus braunii	25 - 75
Chlorella sp.	28 -32
Chlorella vulgaris	22.1- 56.6
Chlorella sorokiniana	19.3
Chlorella protothecoides	23/55
Chlorella minutissima	57
Dunaliella bioculata	8
Dunaliella salina	14–20
Neochloris oleoabundans	35–65
Spirulina maxima	4–9

In recent years, different microalgae strains have been receiving high attention because of their less complex structure, fast growth rate, and high lipid contents. Khan et al. (2009) and Rodolfi et al (2009) studied a variety of microalgae.

Khan and Rodolfi cultured these strains in 250 ml flask at 25^{o} C using air as source of CO_2 and day light fluorescent lamp for two weeks culture period. Table 3 shows some

of the microalgae species along with their lipid content, biomass productivity and lipid productivity.

Table 3 Lipid content and productivity of different phyla of microalgae [21, 29]

Algae phylum	Strains	Biomass productivity (g/L/day)	Lipid content, % biomass	Lipid productivity, mg/L/day	Habitat
Diatoms	*Chaetoceros muelleri*	0.07	33.6	21.8	Marine
	Chaetoceros calcitrans	0.04	39.8	17.6	
	P. Tricomutum	0.24	18.4	44.8	
	Skeletonoma costatum	0.08	21.0	17.4	
	Skeletonoma sp.	0.09	31.8	27.3	
	Thalassiosira pseudonana	0.08	20.6	17.4	
Green algae	*Chlorella sp. F&M-M48*	0.23	18.7	42.1	Fresh water
	Chlorella sorokiniana	0.23	19.3	44.7	
	Chlorella vulgaris CCAP-11b	0.17	19.2	32.6	
	Chlorella vulgaris F&M-M33	0.20	18.4	36.9	
Prymnesiophytes	*Pavlova salina* CS 49	0.16	30.9	49.4	Marine
	Iscochrysis sp. M177	0.17	22.4	37.7	
	Isochrysis sp. M37	0.14	27.4	37.8	
	Pavlova lutheri CS182	0.14	35.5	50.2	
Red algae	*Porphyridium cruentum*	0.37	9.5	34.8	Marine
Eustigm-atophytes	*Nannochlorosis sp.*	0.19	28.4	53	Fresh water
	Monodous subterraneus UTEX	0.19	16.1	30.4	

During recent decades, many scientific organizations around the world are working on microalgae culture. Some of the major microalgae culture collections are listed below [30-32]

- Fresh Water Culture Collection at the University of Coimbra in Portugal, which includes more than 4000 strains representing 1000 species.
- SAG: Experimentelle Phykologie und Sammlung von Algenkulturen; Culture collection at the University of Gottingen in Germany, which includes 2213 strains representing 1273 species.

- UTEX: Culture Collection of Algae at the University of Texas at Austin, USA, which includes 2300 strains.
- Culture Collection at the National Institute for Environmental Studies (NIES) in Ibraki, Japan, which includes 2150 strains representing 700 species.
- CSIRO: The Australian National Algae Culture Collection, with more than 1000 different strains.
- CCAP: Culture Collection of Algae and Protozoa is located within the Scottish Association for Marine Science campus on the Dunstaffinage peninsula near Oban on the scenic west coast of Scotland. This is the most diverse collection of its kind in the world, with approximately 3000 strains of marine and freshwater algae, protista and seaweeds.
- CPCC: Canadian Phycological Culture Centre for Algae, Cyanobacteria and Lemma.
- NMCA: The Provasoli- Guillard National Center for Marine Algae and Microbiota, Maine.
- PCC: Pasteur Culture Collection of Cyanobacteria, Pasteur Institute, Paris, France.

2.1.2 Chlorella Microalgae

Chlorella vulgaris is a green alga. It is widely distributed in freshwater, marine and terrestrial environments and has high photosynthetic ability. It has the capability for rapid growth under autotrophic, mixotrophic and heterotrophic condition [33]. All of these characteristics have made it one of the first microalgae considered for large-scale cultivation and commercial production [34].

C. vulgaris is 2 to 10 μm in diameter. The cells are spherical, sub-spherical or ellipsoid in shape without flagella [35, 36]. It appears as single cells or is able to for, colonies up to a maximum of 64 cells. *C. vulgaris* has a single, cup-shaped chloroplast with or without the presence of pyrenoids (storing starch grains). As a non-motile microalga, *C. vulgaris* reproduces through asexual cell division, by division of the mother cell into 2-32 autospores or daughter cells [37, 38]. Figure 2 shows an image of *Chlorella vulgaris,* which is used in the study mentioned in this thesis.

Microalgae are mainly made up of proteins, lipids, carbohydrates, pigments, minerals and vitamins. However, the composition varies based on species and culture condition such as ight intensity, temperature, pH, salinity and medium nutrients [39, 40]. Table 4 shows the composition of various biochemical components present in *Chlorella vulgaris* [37].

Table 4 Composition of biochemical components in *C. vulgaris* [37]

Biochemical components	% dry weight
Protein	43-58
Lipid	5-58
Carbohydrate	12-55
Pigments	1-2

C. vulgaris contains vitamin A, B, C and E, and also is rich in calcium, potassium, magnesium and zinc.

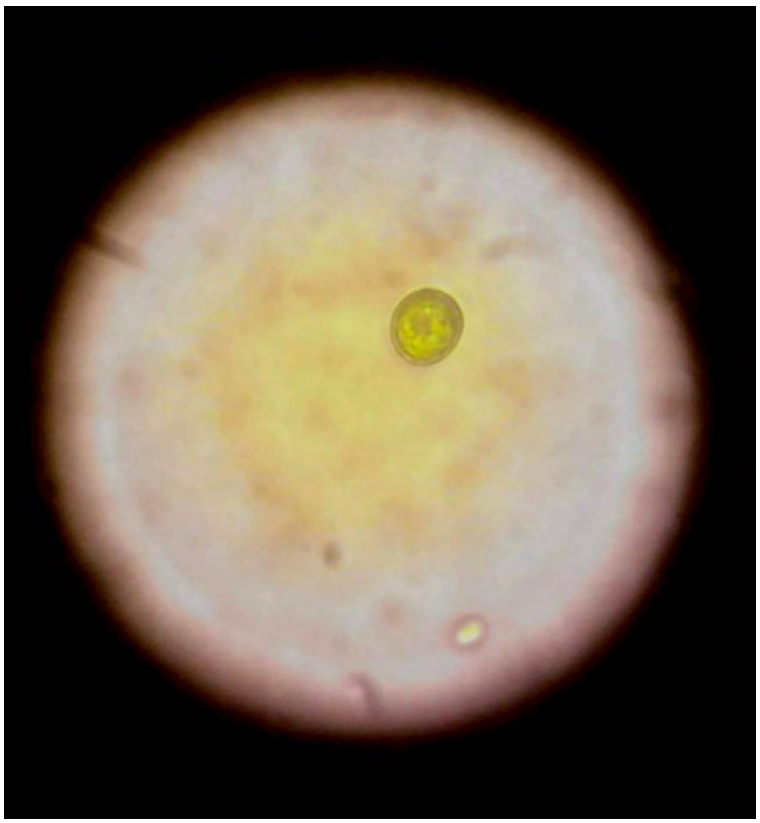

Figure 2: Microscopic image of *Chlorella vulgaris*

2.2 Current Uses of Microalgae

There are multiple areas in where microalgae are used, the specifics will depend on the needs. The whole algal biomass can be used as a source of protein or valuable chemicals (pigments, enzymes) can be extracted. Several studies have shown the potential of microalgae as a therapeutical agent. Preparations containing *Spirulina* have shown to help wound cicatrization, *Scenedesmus*- containing preparations have been tested and shown effects on the treatment of certain skin diseases as eczema. Moreover, some algal compounds have shown inhibitory effects on HIV virus [16, 41]. Microalgae are already used as a source of nutritional supplements, as an additive for cosmetics, in the treatment of wastewater and as a potential source of oil for biofuels [11, 16]. Figure 3 shows use of microalgae in various fields.

The immense potential of *C. vulgaris* has been revealed in numerous studies. Table 5 summarizes the different applications of biochemical components derived from *C. vulgaris*.

Table 5 Applications of metabolites derived from *Chlorella vulgaris*

Category	Components	Application	Reference
Lipids	Triglycerides, phospholipids, glycolipids	Biodiesel, animal feed, antibiotics	[42, 43]
Proteins	Amino acids	Food supplement, animal feed, pharmaceuticals, bio – fertilizers, food additives, emulsifier	[44, 45]
Carbohydrate	Starch, cellulose, β-1-3-glucans	Food additives, pharmaceuticals, aquaculture feed.	[37, 46]
Pigments	B-carotene, astaxanthin, cantaxanthin, lutein, chlorophyll.	Pharmaceuticals, neutraceuticals, food supplements, dye and paint, cosmetics, aquaculture feed	[37, 47]
Vitamins and minerals	Microelements, macroelements, vitamin A, B, C, E	Antioxidants, aids in metabolism	[37, 48]

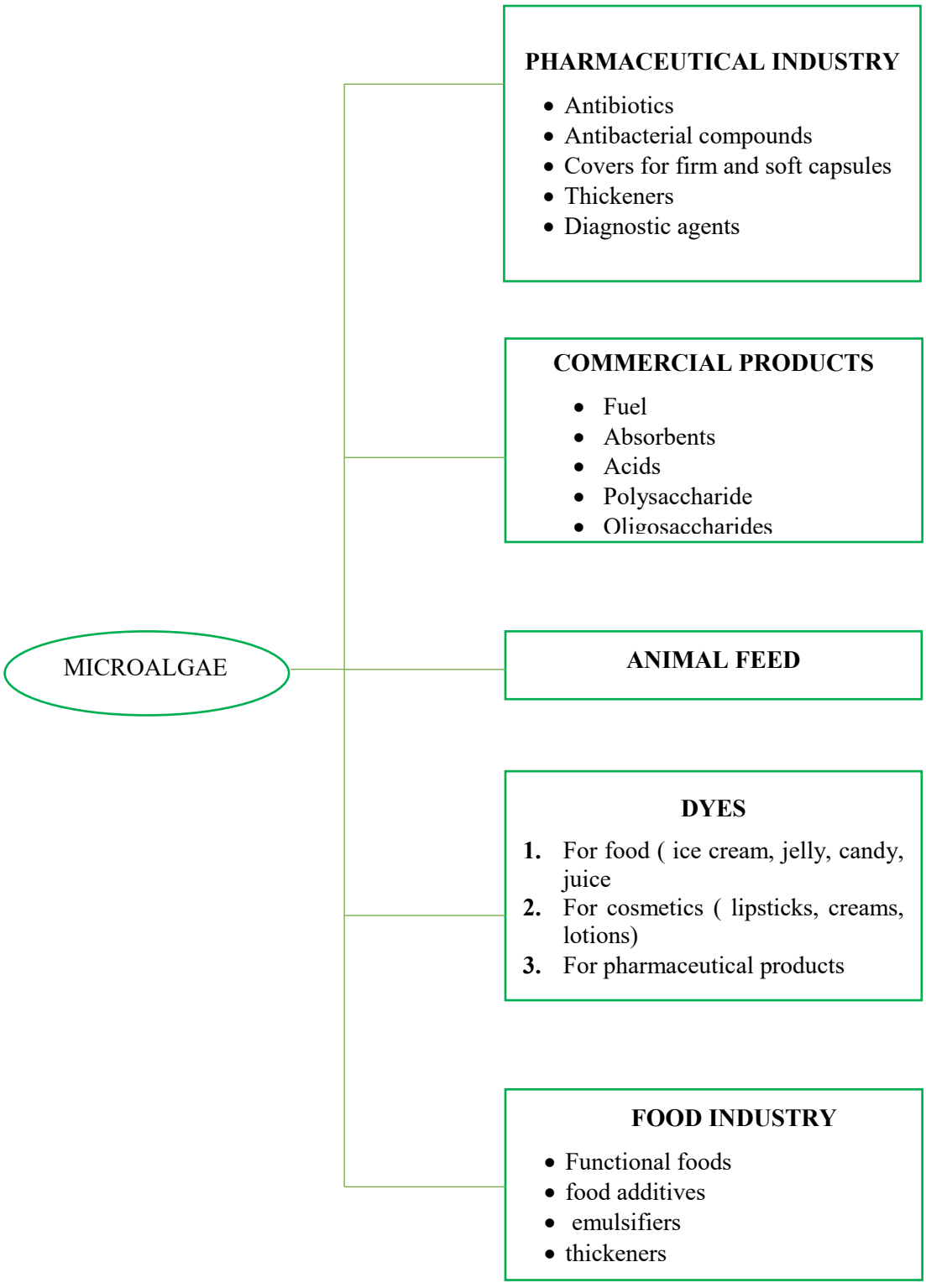

Figure 3 : Various commercial applications of microalgae [1]

2.2.1 Microalgae as potential source of biofuel

Biodiesel is the monoalkyl esters of long-chain fatty acids derived from renewable feedstocks as- vegetable oil or animal fats [49]. Primary advantages of biodiesel are that it is renewable, non-toxic and biodegradable.

Biodiesel is considered 'carbon neutral' as all the carbon dioxide (CO_2) released during consumption had been sequestered from the atmosphere during growing the oil crops[50]. Some advantages of biodiesel compared to many other alternative transportation fuels is that it can be used in existing diesel engines without modification, and can be blended with petroleum diesel in required ratio. Biodiesel performs as well as petroleum diesel, while reducing emissions of particulate matters, carbon monoxide (CO), hydrocarbons and oxides of sulphur (SO_x) [50]. Other environmental benefits of biodiesel are that it is highly biodegradable and it reduces emissions of air toxics and carcinogens.

Algae are very important from an ecological point of view. Algae work as food source for many animals. Their important role as food source is due to the content of minerals, vitamins, and oils and enriched polyunsaturated fatty acids (PUFAs). PUFAs such as α-linolenic eicosapetaenoic and docosaesaenoic acids, belong to the ω-3 group [51, 52].

Microalgae can provide several different types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass [34], biodiesel derived from microalgal oil [53] and photobiologically produced biohydrogen. The idea of using microalgae as a source of fuel is not new, but it is now being taken seriously because of the rising price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning of fossil fuels [54].

The advantages of microalgae over higher plants as a source of transportation biofuels are numerous [21, 29]:

1. Microalgae synthesize and accumulate large quantities of neutral lipids / oil [20-50% dry cell weight (DCW)] and grow at high rates
2. Oil yields per area of microalgae cultures could greatly exceed the yield of best oilseed crops.

3. Microalgae can be cultivated in saline/brackish water/coastal seawater or non-arable land, and do not compete for resources with conventional agriculture.
4. Microalgae tolerate marginal lands (e.g. desert, arid and semi-arid lands) that are not suitable for conventional agriculture.
5. Microalgae utilize nitrogen and phosphorus from a variety of wastewater sources (e.g. agriculture run-off, concentrated animal feed operations, and industrial and municipal wastewaters), providing the additional benefit of wastewater bioremediation.
6. Microalgae sequester CO_2 from flue gases emitted from fossil fuel-fired power plants and other sources, thereby reducing emissions of a major greenhouse gas. 1 kg of algal biomass requires about 1.8 kg of CO_2.
7. Microalgae produce value-added co-products or by-products (e.g. biopolymers, proteins, polysaccharides, pigment, animal feed and fertilizer) and does not need herbicide and pesticide.
8. Microalgae grow in suitable culture vessels (photo bioreactors) throughout the year with higher annual biomass productivity on an area basis.

However, their application for fuel production on an industrial scale due to low efficiency of commercially viable oil production is now offline.

2.2.2 Challenges of using microalgae

Harvesting of microalgae is seen as one of the major challenges of using microalgae for the production of biodiesel. Microalgae that store lipids are generally unicellular, have low densities and are found in suspension, which make the overall separation process difficult. Large scale extraction procedures for microalgal lipids are complex and still in the development stage [55]. Currently research is underway to alleviate these challenges.

Microalgae grown in open pond systems are prone to contamination. Bacterial contamination actively competes for nutrients and oxidize organic matter that could lead to purification of the culture. Control to heterotrophic bacteria may be achieved by increase in pH. Aerobic bacteria generally found in algal ponding systems have an optimum pH of 8.3. Increase in pH beyond this level gives effective inhibition thus preventing competition by influencing nitrogen efficiency [56, 57]. Open systems are

also susceptible to grazers in the form of protozoa and zooplankton. These organisms actively consume microalgae and can devastate algal concentration in relatively short periods of time (2-3 days). Zooplankton can reduce microalgal concentration by up to 90% of the original density in 48 h [58] and *Daphnia* can lower microalgal density by a massive 99% over a few days [57]. Several methods to control these organisms are available including filtration, centrifugation, low dissolved oxygen (DO), application of hormones and increase in free ammonia. These methods however have drawbacks. Moreover, filtration is difficult due to the size of microalgal species such as *Chlorella sp.* making separation technically difficult. Centrifugation is prohibitively expensive at large scale requiring high capital and energy inputs. Photosynthetic activities of microalgae increase the DO as a function of growth.

Increase in free ammonia as a control method may be achieved by pH elevation by volatilization of ammonia. It has been eluded that the toxicity of high pH may actually be due to increased free ammonia levels that are brought by the volatilization of ammonia at high pH [59]. Thus the most appropriate method of controlling zooplankton and bacterial populations is to increase pH to 11 [57].

Other challenges with respect to the use of biodiesel as fuel is that it is susceptible to bacterial oxidation subsequently causing internal corrosion of the storage tanks [60].

2.3 Biofuel Production from *Chlorella vulgaris*

According to Knothe (2008) and Hu et al. (2008), the two major fatty acids present in microalgae-derived biodiesel are C16 and C18 compounds containing palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:2) [61, 62].

Many publications describing research on microalgae have been published. Several researchers have focused on the *Chlorella vulgaris* [24, 63-65], which appears to be a good option for biodiesel production because they are readily available and easily cultured in the laboraotory. Converti et al. [66] attempted to increase the lipid content in microalgae by varying the temperature and nitrogen concentration during the culture of *Nannochloropsis oculta* and *Chlorella vulgaris* and concluded that variation of temperature and nitrogen concentration strongly influenced the lipid content of

microalgae. Although Lee et al. (2010) [67] found that the *Botryococcus sp.* Produced the highest lipid content compared to other species, Griffiths and Harrison (2009) [68] have found that the lipids produced by *Botryococcus braunii* are unsuitable for use in biodiesel production. According to Griffith and Harrison (2010), the hydrocarbons produced by *B. braunii* have a chain length of greater than 30 carbons [68].

Table 6 shows the fatty acid profile of *C. vulgaris*, which had ≥ 40% lipid content when grown under unfavorable growth conditions. Microalgae containing highly saturated and monosaturated fatty acids (14-20 carbons) are suitable for biodiesel production [43, 69].

Table 6 Variation in fatty acid profile of *Chlorella vulgaris* [37]

% of Fatty acids	*C. vulgaris*[a]	*C. vulgaris*[b]	*C. vulgaris*[c]
Palmitic acid (C16 : 0)	13.9	13.09	2.16
Palmitoleic acid (C16: 1)	5.7	0.57	0.28
Stearic acid (C18:0)	3.1	3.03	1.96
Oleic acid (C18: 1n9c)	2.2	11.29	0.48
Linoleic acid (C18:2n6c)	25.3	9.36	3.31
Linolenic acid (C18:3n3)	24.2	3.08	0.97

a = % of Fatty acid content [70]; b= Fatty acid content (weight % dry weight of biomass) in basal medium, miotrophic [43] ; c= Fatty acid content (weight % dry weight biomass) in basal medium, photoheterotrophic [43].

Some properties that make *Chlorella vulgaris* appropriate for use in food industry, aquaculture, cosmetics, pharmaceuticals, waste water treatment and the production of biofuel, are-

- Rapid growth
- Easy and flexible terms of culture
- Resistance against interfering factors
- Require little or no arable land
- Have the potential to attenuate greenhouse effects

C. vulgaris captures CO_2 from the atmosphere at 10-50 times greater efficiency than terrestrial plants [71]. Even though the production of biodiesel from *C. vulgaris* is still

expensive, combining both wastewater treatment plants and fuel production plants could offset the cost. The microalgae consume the nutrients in municipal effluent, mitigating the effect of pollution and aquatic eutrophication and simultaneously producing biofuels and other high-value metabolites [37].

2.4 Algae Growth

Algae cells multiply by cell division (mitosis). During the mitosis process, algal cells are divided into two identical daughter cells. Certain strains of algae such as diatoms undergoes gametes fusion through syngamy followed by mitosis . It is believed that the syngamy process enhances algal cell enlargement in volume. Algal cells capture carbon from CO_2 in an intermediate step and transform it into complex carbohydrate molecules such as lipids, which performs as the raw material in biodiesel production [22].

Algal cells are either photoautotrophic or heterotrophic.

Photoautotrophic cells capture carbon dioxide and photonic energy to convert into sugars (glucose), then lipid as shown in the following reactions .

$$6\ CO_2 + 6\ H_2O + 8\ \text{photons} \rightarrow C_6H_{12}O_6 + 6\ O_2$$

$$C_6H_{12}O_6 \rightarrow \text{TGA (or lipid)}$$

Heterotrophic cells, on the other hand, use other organic compounds as source of carbon and do not undergo photosynthesis.

In photoautotrophic algae, the photosynthetic reaction takes place within the chloroplast. CO_2, photons and water are key elements in algae growth process (photosynthetic reaction). It is important to note that algal cells can tolerate CO_2 up to certain concentration. The level of tolerance depends mostly on the algal strains. Kurano and Miyachi (2004) studied the impact of CO_2 fixation in algal culture and showed that algal culture of certain Chlorella strains were inhibited by CO_2 if its concentration exceeded 5%. However, certain Chlorella strains can reach high biomass productivity with CO_2 concentration exceeding 10% [72]. Khan et al. (2009) showed that *Chlorella kesslerican* reach high biomass productivity of 87 mg per liter per day with supplied CO_2 exceeding 10% [29].

2.5 Reproduction and Growth in a Batch System

Vegetative, asexual, reproduction through cell division is common for most of the microalgal species. Other asexual reproduction occurs through fragmentation and production of spores. Sexual reproduction occurs for species in a life-time but is not a universal feature [33]. In general, small microorganisms grow faster than big ones.

A culture growing in a closed system where nothing is added or taken away is called a batch culture. An inoculum of cells is added and the batch culture is kept in an environment, which favors growth. The growth is divided into three phases; the lag, the log and the stationary phase.
The *lag* phase varies in time depending on the growth conditions. If an exponentially growing culture is transferred to a similar environment the exponential growth starts without delay. If the cell sample is transferred from a stationary phase to a new fresh medium an adaptation occurs, *i.e. a lag phase*. If cells are coming from a rich medium to a poor medium the cells need to produce enzyme for production of essential metabolites missing, hence a long *lag phase* occurs.

Cells growing by division in a *log phase*, synonymously *exponential phase*, are often in their healthiest state where one cell divides into two new cells which each divide into two new cells and so on. If the number of cells is plotted in a *semi logarithmic* way a straight light will be seen. Exponential growth cannot grow indefinitely in a batch culture and only occurs in the *log phase.*

The amount of absorbed energy most often depends on the amount of cells in a cell suspension rather than photon flux density [73]. If a stream of photons with low flux density passes through a cell suspension with low cell concentration some photons slip through. As the cell concentration increases there could be a point where all the photons are captured. The increase in cell density will be exponential until this limiting point and hereafter the biomass production occurs at a linear rate as a function of added light.

Either log phase or the linear phase will last until any other limiting factor occurs. Finally, the nutrients start to deplete in a batch system as no new medium is added. Either the essential nutrient is depleted or waste and toxic products accumulate and limit growth.

As a result the exponential phase starts to decline and the *stationary phase* starts. At the end a *death phase* can occur as nutrients are depleted or toxic substances are released as there are too many cells in the solution. The quality of the harvested cells in a batch culture varies with time and phase and is thus less predictable compared to continuous systems.

2.6 Factors Affecting Algal Growth

Photosynthetic growth of algae requires light, carbon di oxide, sugar and inorganic salts. Growth medium provides the nutrients and inorganic salts that ensure the unhindered algal growth. The physical and chemical conditions that must be assured to get satisfactory algal yield are described below:

Light:

Light helps algae to convert carbon di oxide into carbohydrate via photosynthesis. Microalgae needs a light/dark regime for productive photosynthesis, it needs light for a photochemical phase where ATP and NADPH are produced. The dark phase is necessary for biochemical phases where essential molecules for growth are synthesized [74].

Researches have shown that, both the growth rate and cell density of microalgae increase proportionally with light period duration, even at different densities [75]. Further research on this topic has revealed that a 12:12h (light: dark) photoperiod resulted in a higher productivity and maximum cell density compared to a 14:10h (light: dark) photoperiod [76].

The intensity of light depends on the depth and density of the algal culture. At higher depth and cell concentrations, the light intensity should be increased high enough so that in can penetrate through the culture. Light may be natural or supplied by fluorescent tubes. Generally microalgae use light of wavelengths from 400 to 700 nm for photosynthesis [77].

Mixing:

Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification

(e.g. in outdoor cultures) and to improve gas exchange between the culture medium and the air [77].

The rate at which atmospheric CO2 can diffuse into an algal culture will definitely affect the growth rate. Scientists from UK conducted an experiment to investigate the effect of CO2 aeration on microalgae biomass production. The experiment that was done on two different algal strains, showed that combination of blue light and 15% CO2 induced higher rate of liquid accumulation in both the strains [78].

Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, Erlenmeyer's), aerating (bags, tanks), or using paddle wheels and jet pumps (ponds). However, it should be noted that not all algal species can tolerate vigorous mixing [79].

Temperature:

Growth temperature affects biomass production and growth rate of microalgae. This is because both photosynthesis (oxygen production or carbon dioxide fixation) and respiration process in microalgae are temperature- dependent [80]. The optimum temperature used for culture varies among microalgal species, ranging from 15 to 26^{o} C [81]. Lower temperatures (below 16^{o} C limit cell growth and higher temperature accelerate the metabolic rate of microalgae, but temperatures above 35^{o} C inhibit growth. The optimal growth temperature for *C. vulgaris* is in the range of 25-30^{o} C, demonstrating the highest biomass production [66, 82]. Its growth rate decreased when the temperature was more than 30^{o} C and at 38^{o}C the cells dies. However, it is a noteworthy observation that the lipid production of *C. vulgaris* increased to 14.7% when the growth temperature decreased from 30 to 25^{o}C, and the content of oleic acid in *C. vulgaris* increased up to 34% when it was culture at 38^{o}C, making it a potential for nutraceutical production.

Nutrient medium:

Media composition has a robust effect on both the growth rate and final concentration of microalgae. Two major nutrients are nitrogen and phosphorus, which both play a role in controlling growth rates and lipid production. Microalgae have different nutrient requirements not only by composition but also by concentration of the nutrients supplied.

Micoralgal growth media are therefore composed of macronutrients, generally consisting of a nitrogen source, phosphate and a metal chelator. Iron is generally supplied in as a micronutrient. Other essential nutrients are carbon, hydrogen, oxygen, sulfur, calcium, magnesium, sodium, potassium, and chlorine. Nutrients needed in minute quantities include iron, boron, manganese, copper, molybdenum, vanadium, cobalt, nickel, silicon, and selenium [83].

Nitrogen plays a vital role in cell growth and lipid production [84], composing 1-10% of total dry matter in microalgae [85]. Nitrogen can be supplied as a nutrient to microalgae in different forms such as ammonium, nitrate, yeast, urea and peptone and each of these will have a different effect on growth [86]. *C. vulgaris* prefers ammonium over nitrate, depleting it first when both of the compounds were present in the media. However, in mixotrophic culture urea was depleted faster than both nitrate and ammonium [84]. This indicates that under different culture conditions, *C. vulgaris* has different preferences for the nitrogen source and mixotrophic cultivation is a feasible approach for lipid production [84].

It must be noted that nutrient requirement must be maintained in an optimal range to ensure maximum growth. Too little nutrient can reduce the growth rate and too much of it can be toxic and inhibiting [87].

Table 7 summarizes the optimal cultivation conditions for the same species. Table 8 contains several nutrient media along with their components, used widely for *Chlorella vulgaris* culture.

Table 7 Optimal cultivation conditions for *Chlorella vulgaris*

Parameters	Preferences	References
Nitrogen source	Ammonium (autotrophic)	[88]
	Urea (mixotrophic)	[84]
Carbon source	1.2 g/L of $NaHCO_3$	[89]
	8% CO_2	[90]
	10% of CO_2 with 24h light provision	[48]
	1% w/v glucose (mixotrophic)	[91]
Light intensity and quality	1000 to 2000 lux/m^2	[92]
	Yellow, red and white light (achieves log phase faster)	[93]
Temperature	30^oC (biomass production)	[66, 82]
	25^oC (lipid production)	[66]
Culture condition	Aeration 200 ml/min (lipid production)	[91]

Table 8 Different nutrient media and their composition

Name of the medium	Name of compounds used	Reference
Bold's Basal (BB)	$NaNO_3$, $MgSO_4.7H_2O$, NaCl, K_2HPO_4, KH_2PO_4, $CaCl_2.2H_2O$, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$, MoO_3, $CuSO_4.5H_2O$, $Co(NO_3)_2.6H_2O$, H_3BO_3, EDTA, KOH, $FeSO_4.7H_2O$, H_2SO_4	[94]
MLA	$MgSO_4.7H_2O$, $NaNO_3$, K_2HPO_4, H_3BO_3, H_2SeO_3, Biotin, Vitamin B_{12}, Thiamin-HCl, Na_2EDTA, $FeCl_3$, $NaHCO_3$, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, $CaCl_2.2H_2O$	[95]
Blue Green (BG)	$NaNO_3$, $K_2HPO_4.3H_2O$, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, Citric acid, Ferric ammonium citrate, EDTA, Na_2CO_3, H_3BO_3, $MnCl_2.4H_2O$, $ZnSO_4.7H_2O$, $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $Co(NO_3)_2.6H_2O$	[96]

2.7 Determining the Growth of Biomass

In view of microalgal system optimization it is essential to have insight in the kinetics related to the microalgal growth. Microbial growth can be measured as an increase of biomass in the algal culture or it can also be measured with a surrogate parameter which is proportional to cell amount [97]. Measuring an increase in biomass or a related surrogate parameter in general gives insight in the microalgal growth rate [98].

There are different methods of estimating biomass and growth where optical density (OD), dry weight (DW) and cell count are commonly used methods. Dry weight and cell counting are time consuming methods whilst OD measurements are faster. For unicellular microorganisms, optical density is proportional to cell count and dry weight, within certain limits. However, Madigan & Martinko explain that work has to be made beforehand. A standard curve has to be made to couple direct measurements, cell count or dry weight, to indirect measurements as OD [3].

The proceedings of the Eutrophication Biosimulation Assessment Workshop stated that algae crops are measured by a variety of techniques including cell counts, absorbance, gravimetric, carbon-14, fluorescence, and volumetric. Each technique has certain advantages and disadvantages over the others. Counts have the major advantage of being determinant at concentrations far below those which are measurable gravimetrically. Fluorescence is an excellent technique for measuring algal crops which does not involve destruction of the samples. More clumping of algae can interfere with this technique. Absorbance measurements are also virtually useless when clumping of algae has occurred. The radio carbon techniques as set forth in "Provisional Algal Assay Procedure (PAAP)" seems needlessly complex, delicate and subject to error in the hands of inexperienced personnel [2].

2.7.1 Optical Density (OD)

To follow growth of microorganisms optical density, or turbidity, is of great importance. Absorbance (or scattering) of light through a cell suspension can be directly coupled to biomass, furthermore coupled to DW or cell count on the same sample [99]. The method

assumes that the cells are unicellular and that the scattered light is linear to the number of cells.

However, there are insecurities by OD-measurements for microalgae. Pigments in the cells affect the optical properties by absorbing and harvesting of light at certain wavelengths [43]. Harrison et al. mean that it affects the correlation between OD and DW. Even though a standard curve has been made there can be errors in estimating biomass as pigment content varies depending on growth cycle phase and environmental circumstances. Harrison et al. have several advices to minimize the errors. One of them is to use wavelength outside the absorbance range of the light harvesting pigments, i.e., use wavelength with reported absorbance minimums. Two suggested wavelengths are 550 nm and 750 nm.

2.7.2 Direct Microscopic Count in a Counting Chamber

Cell count can be either viable count, where only live cells are of interest, or it can be a total count where all cells are counted, including dead cells [22]. There are two ways of performing a direct microscopic count. The samples can be dried on slides and counted, or a counting chamber can be used if cells are in a suspension. In the counting chamber a grid is engraved on the surface of the counting chamber. All squares have the same area (known) and by multiplying with a conversion factor number of cell per milliliter is obtained. Drawbacks are that cell count include both dead and living cells, small cells are hard to distinguish, and precision is hard to obtain. Dilutions could be necessary to get an acceptable number of cells to count.

2.7.3 Dry Weight

Dry Weight (DW) is a method for estimating biomass and is important to know for many aspects. The DW is measured by taking a sample with known volume, drying it and expresses the obtained cell weight as a matter of suspension volume, i.e., the biomass suspension. There are two main estimations, the dry weight (DW) and ash free dry weight (AFDW). The difference is that AFDW subtracts the weight of the salts after the sample has been ashed.

The filters must be conditioned and pre-weighed to be able to subtract the filter weights from the sample weights. The samples should be dried in an oven and then weighed together with the filter of known weight.

2.7.4 Growth Kinetics

To estimate growth rates, one must have a series of measurements, at different times, that will permit the calculation of the rate of change in biomass concentration. Cell number should be counted either through a direct method, as through light microscopy with a hemacytometer, or indirectly through biomass concentration (as dry weight) or optical density, as long as this measurements correlate linearly with the number of cells [100-102].

Under a typical homogenous batch regime (in a closed system), microalgae will pass through the following growth phases illustrated in Figure 4.

A. Adaptation (lag phase)

B. Exponential growth phase (log phase)

C. Stationary phase

D. Logarithmic death phase

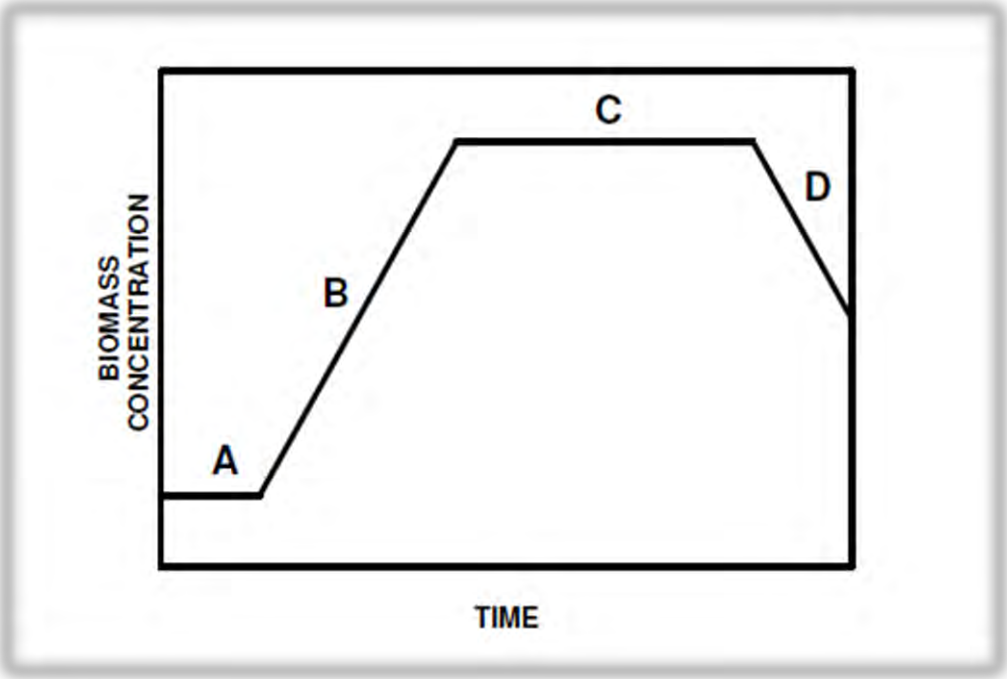

Figure 4: Microalgae batch growth profile [97]

The individual phases, shown in Figure 4, are not always clearly defined; their length or slope might change according to the culture conditions. During **lag phase (phase A)** the microalgae cells are adapting to the new environment conditions, at the end of lag phase, the cells are well adjusted and then start to multiply rapidly, this is **exponential phase (phase B)** , in this phase, the number of living cells, doubles regularly with time [101, 103].

During this period, equation (2.1) and (2.2) describe cell growth.

$$\frac{dn}{dt} = \mu n \text{ or } \frac{1}{n}\frac{dn}{dt} = \mu \qquad (2.1)$$

$$n = n_o \text{ at } t = t_o \qquad (2.2)$$

Where n and n_o are the number of cell (or cell concentration) at different time t and t_o , where t_o is the initial time and μ is the specific growth rate.

From integration of equation (2.1) the following is obtained:

$$\ln\frac{n}{n_o} = \mu\,(t - t_o)$$

$$\mu = \frac{\ln {}^{n}/_{n_o}}{t - t_o} \qquad (2.3)$$

From equation (2.3) it can be deduced that the time period for cell population to double (t_d) is given by:

$$t_d = \frac{\ln 2}{\mu} \qquad (2.4)$$

During the exponential phase t_d or μ can be used to characterize the state of **the cell population**.

In an open system, media with fresh nutrients is added in at the same rate that medium is withdrawn from the culture; this keeps growth parameters and cell concentration at a constant level. The following mass balance can be applied to any component of the system:

$$rate\ of\ addition\ to\ system - rate\ of\ removal\ from\ system + rate\ of\ production\ within\ system = 0 \qquad (2.5)$$

The above can be reformulated as:

$$f x_o - f x + V r_x = 0 \qquad (2.6)$$

Where f is the volumetric flow rate, x_o is the cell or biomass concentration in the feed stream, x is the cell concentration inside the system, V is the culture volume and r_x is the

rate of cell formation. It is assumed that the growth rate is much higher that cell death rate and cell death is neglected, if μ denotes the specific growth rate ($^{r_x}/_{r}$), then it can be substituted in equation (2.6) to obtain the following equation 2.7: [103]

$$Dx_o = (D - \mu)x \qquad (2.7)$$

The ratio of the volumetric flow rate, at which fresh medium is added to the culture, is referred to as the dilution rate d, so that:

$$D = {}^{f}/_{V} \qquad (2.8)$$

When the liquid feed stream to a continuous culture consists only of nutrients, so that$x_o = 0$, meaning the algal cell concentration is constant in the system, the following equations (2.9) and (2.10) are fulfilled [101].

$$\frac{dx}{dt} = 0 \qquad (2.9)$$

$$\mu = D \qquad (2.10)$$

2.7.5 Kinetic Model of Algae

An important aspect of designing a Photo Bio-reactor (PBR) for culturing microalgae in large scale, is the kinetic modeling of photosynthetic cell growth. Kinetic models or growth kinetics of algal cells are generally expressed by a rate of cell growth. A kinetic model can be affected by different parameters such as medium composition, environmental and/or growth conditions [104]. Lee (2001) described growth kinetic as a result of several complex networks of biochemical/chemical reactions and transport phenomena involving numerous phases and multiple component systems [105].

Monod model is a classic one, which is based on the specific growth rate (μ_{max}) of cell grown in any reactors. Equation (2.11) gives the empirical expression of the Monod equation or specific growth rate (inverse of time) [105].

$$\mu = \mu_{max} * \frac{C_s}{K_s + C_s} \qquad (2.11)$$

Where C_s, K_s and μ_{max} represent the concentration of the limiting nutrient, the saturation constant and the maximum growth rate respectively.

However, growth limiting nutrients in culture medium have to be determined in order to use Monod model (Equation 2.11). If growth limiting nutrients are not determined, then there is a need to develop a kinetic model specific to reactor, culture performed and strain in use.

Obviously, each growth phase has a different expression of growth rate; however, the number of cells or cells concentration in the **lag** and **stationary** phases are approximately the same resulting in negligible growth rates. Thus, the focus for a kinetic model is more in the **exponential phase** where cells multiply rapidly.

Huesemann et al. (2009) studied biomass productivities in algae. They calculated the maximum specific growth rate (μ_{max} in$\frac{1}{s}$) in the exponential growth phase of algae batch culture using Equation 2.12.

$$\mu_{max} = \frac{1}{\Delta t} * \ln \frac{C_f}{C_i} \qquad (2.12)$$

Where Δt (s), C_f and C_i (gmol/L) represent respectively the length period of incubation time (exponential growth), the final and initial biomass concentration over Δt time period. Equation 2.12 is applied in exponential or growth phase only; therefore, the incubation period corresponds to the duration of exponential phase.

Huesemann et al. (2009) also demonstrated that photosynthesis reaction can be a rate limiting catalyst in algae growth caused by illumination (light intensity) and carbon fixation. They demonstrated that photosynthetic oxygen evolution rate termed by P was a function of light intensity as shown in an empirical expression in Equation 2.13 [106].

$$P = P_{max} * \tanh\left(\frac{a+I}{P_{max}}\right) R_{dark} \qquad (2.13)$$

Where, P_{max} , I, R_{dark} and "a " represent respectively the photosynthetic oxygen evolution's maximum rate, the light intensity, the rate of oxygen uptake in the absence of light. The initial slope in Huesemann model indicates the photonic energy efficiency in photosynthetic activity. It indicates the minimum number of photons require to produce one molecule of oxygen during the photosynthesis reaction.

Another kinetic model correlating growth rate to light intensity was developed by Chojnacka and Marquez-Rocha (2004). They conducted a study of photoautotrophic microalgae cultures which involved light as the source of photonic energy for photosynthesis reaction. Photosynthetic activity can be limited by light and CO_2. Light was a growth limiting catalyst in Chojnacka and Marques-Rocha (2004) experiment. They considered light as physical substrate. Therefore, Monod model was applied in the absence of photo-inhibition to determine specific growth rate as shown in Equation 2.14.

$$\mu = \mu_{max} * \frac{I_o}{K_{I_o} + I_o} \qquad (2.14)$$

Where μ_{max} , I_o and K_{Io} are respectively the maximum growth rate, the incident light intensity and light saturation constant. The constant K_{Io} is expressed in the units of light intensity I_o. It can be seen that equation 2.14 and 2.11 are exactly the same, except that light intensity I_o was substituted by substrate concentration C_s. If photo-inhibition is observed during photosynthetic activity, then Monod model in Equation 2.14 is replaced by Haldanc model in Equation 2.15 [107].

$$\mu = \mu_{max} * \frac{I_o}{K_{Io}+\frac{I_o^2}{K_I}+I_o} \qquad (2.15)$$

Where K_I represents the inhibition constant for incident light intensity. The constants K_{Io} and K_I have the same units as I_o. All the other parameters in Equation 2.15 were defined early in this section.

2.7.6 Maximum Specific Growth Rate

The knowledge of microalgal growth rate is essential for several reasons. Some are-
(A) To make a selection of the microalgae with highest growth rates for the valorization of biomass and / or nutrient recuperation
(B) To control the efficiency of the wastewater treatment and removal of nutrients
It should be noted that, the growth rate depends on metabolism and availability of nutrients, on the operating conditions provided by the system under operation, and on the produced biomass for the effective nutrient removal [108]. In Table 9 maximum specific growth rates of different microlagal species are summarized with their residing environment and prevailing temperature. Most of the data obtained are in the temperature range between 15-30 ^oC for both marine and freshwater microalgae. Minor differences between the μ_{max} for freshwater and marine algae, respectively 1.55± 0.82 d^{-1} and 1.19± 0.46 d^{-1} in this temperature range could be observed.

Researchers describe how exponential growth can be estimated with optical density or cell count by making a plot where the growth is plotted on a logarithmic scale and time arithmetically, hence a *semi logarithmic* plot is obtained. The linear correlation indicates that the cells are growing in their exponential phase and the specific growth rate can be read in the graph. The maximum specific growth rate in a batch culture is calculated by the slope of the straight line of the semilogarithmic plot, using the following equation (2.16). There are fast growing species, where doubling time can be as low as 3.5 hours.

$$\ln(X) = \ln(X_o) + \mu_{max} t \quad (2.16)$$

Where, μ_{max}= Maximum specific growth rate (h^{-1})

X and X_o = Relative measurements of the biomass concentration (*e.g.* OD)

t = Time

Table 9 Maximum specific growth rates of different microalgal species in different temperature [109]

Algae	**Value (d^{-1})**	**Temperature (°C)**
Chlorella vulgaris	2.1	20
	1.92	25
	1.3	30
	1.55	25
Chlorella pyrenoidosa	1.68	37.7
	2	38.7
	2.15	39.6
	1.36	19

2.7.7 Determination of Specific Growth Constant (μ)

After the growth curves have been plotted, the next step is to calculate the specific growth rate constant (μ) of the exponential growth phase. Since the curve of the exponential growth phase is constantly increasing it is difficult to find a "straight" line portion to measure. Figure 5 represents a typical microorganism growth curve. One solution is to calculate Log10 of each of the average daily cells/fields estimates and plot these lag values against time. The exponential part of the curve will now be a straight line. The resulting graph will look like the one in Figure 6.

Now, the linear portion of the graph is identified and represented as the exponential growth phase.

From this line, any two points are chosen which lie which lie within the exponential growth phase. The first of these points represents the Log10 of the cell number represents the Log10 of the cell number/field at time zero (t_0) i.e. (Log10 N_o) and the second number represents the Log10 of cell number/field at time 'one' (t_1), i.e. (Log10 N_1). If the points which correspond to the exponential growth phase are scattered, it may be necessary to fit a straight line through the points by performing a 'linear regression'. From the straight line the Log10 values for the t_o and t_1 in days can be calculated.

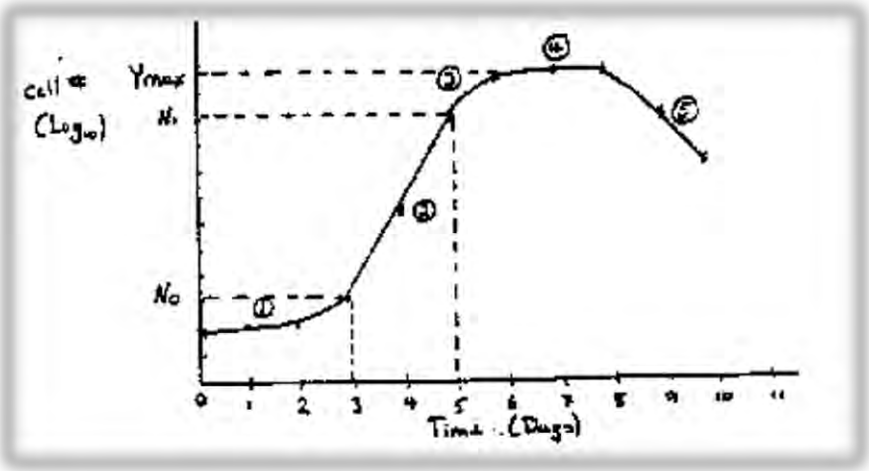

Figure 5: Growth curve for a typical algal batch culture [110]

Where, point 1 indicates – lag or induction phase

Point 2 indicates – exponential phase

Point 3 indicates- phase of declining relative growth

Point 4 indicates- stationary phase

Point 5 indicates- death phase

Once the values of (Log10 N_o) and (Log10 N_1) have been determined, the specific growth constant (μ) is then calculated using the following formula:

$$\mu = \frac{Log10\ N_1 - Log10\ N_o}{t_1 - t_o} \qquad (2.17)$$

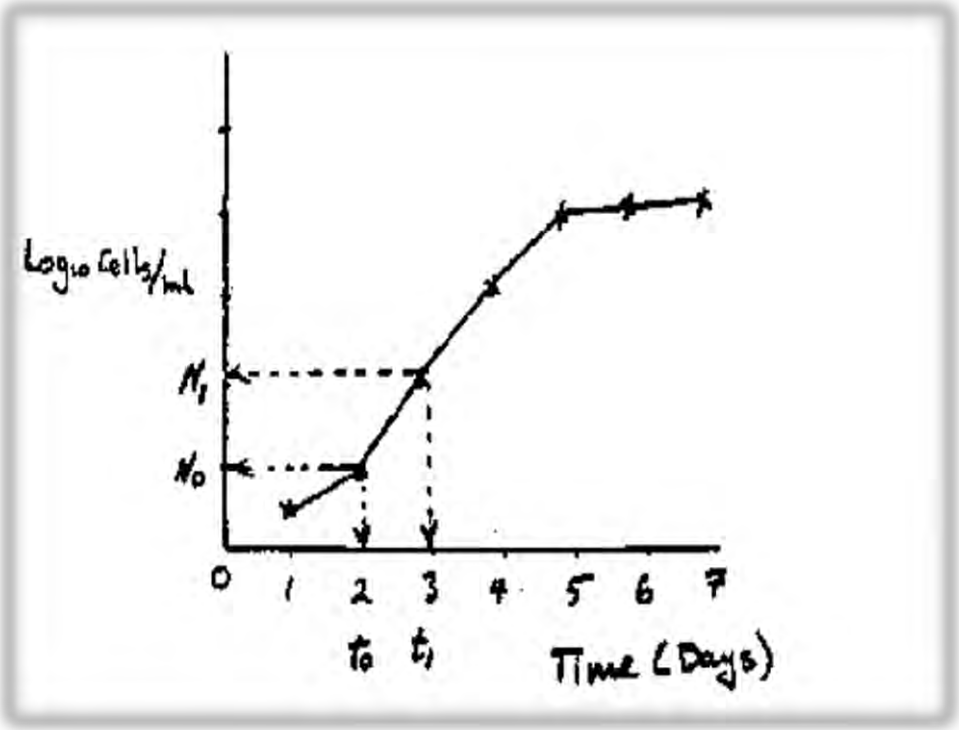

Figure 6: Example of estimation of data points [110]

2.7.8 Determination of Doubling Time, G

While the specific growth constant represents a measure of the ability of the organism to grow under a given set of environmental conditions, doubling times are more easily understood or meaningful. The doubling time is simply the time (in hours) required for the cells to divide. A doubling time value means slow growth, while a small doubling time value means rapid growth. Doubling time (G) can be calculated using the following formula:

$$G = \frac{Log\ 10_2}{\mu} \times 24$$

$$\text{or,}\quad G = \frac{0.301}{\mu} \times 24 \qquad (2.18)$$

2.7.9 Estimation of "Maximum Yield" (Y_{max})

In any closed system, such as the culture flask, exponential growth cannot be maintained indefinitely. Factors such as depletion of nutrients, shading, and production of toxins, waste products or simply overcrowding will lead to a decrease in the rate of growth of the population of algae in the culture by about day 6. At some point a maximum yield (Y_{max}) will be attained. This is the time in the growth of the culture when the number of cells dying is equal to the number of cells being produces, therefore the total cell number

does not change. The maximum yield may directly be estimated from the original growth curve, Log10 (cell number) vs. time in days. This Y_{max} is actually the "carrying capacity" of that culture (environment). That is it is the number of individuals that can be supported by the resources available in that environment. This is one of the most important concepts in ecology.

2.8 Importance of Studying Growth Kinetics

Kinetic study of the algae biomass is an important parameter for determining its suitability for commercial or large scale mass production for the extraction of oil and other useful compounds. The algal cultivation and harvesting/processing systems for high value bio-products seem to be profitable for producers due to high cost of the final product, but the same is not true when biofuel production (biogas, bioethanol and biodiesel) is considered [3, 111]. Algal cultivation and harvesting/processing are both energy- and cost-intensive [9], especially for algal biofuel production, existing cultivation and processing methods are not economical or sustainable [4, 5]. For algal biomass production, suitable growth medium with nutrients is essential [9]. Current methods utilize large quantities of nutrients which results in overall imbalance of energy and environmental benefits. Considering tight profit margin of the algae feedstock for biofuel production, algal growth optimization with minimum nutrients is warranted. Algae essentially require light (energy), carbon source (CO_2 for autotrophic metabolism), growth medium (water) and nutrients (nitrogen and phosphorous) for reproduction. Some algae species can utilize waste organic sources such as municipal and industrial wastewaters as carbon source through heterotrophic metabolism. Among the requirements for algal growth, nutrients are the most cost-involving as their production involves natural resource and energy utilization. Optimizing the nutrients required for algal growth can mitigate their production costs and significantly improve the downstream process economics.

2.9 Microalgae cultivation: Bangladesh Perspective

Microalgae, with a rich diversity of over 50,000 species, exist all over the world. In case of Bangladesh, no updated data is available as we do not have a national microalgae collection and culture center. However, a total of 200 marine algal taxa (seaweeds) have

been reported so far, by several Bangladeshi researchers [112]. In Bangladesh, a large number of algal species were reported to occur in freshwater, brackish water, and marine habitats. However, the potential of algal biomass production for biofuel has not been properly addressed.

In Bangladesh Council of Scientific and Industrial Research (BCSIR), a team of five researchers studied the suitable culture media for a local strain of *Spirulina* [113]. In Biological Research Division, BCSIR, Dhaka, *Spirulina* was cultured at pilot scale for over 12 years. Bangladesh medium (Bd_1) was developed in the laboratory for commercial production of *Spirulina* in Bangladesh [114].

Another group of researchers in University of Dhaka, conducted a study to optimize growth conditions of *Chlorella vulgaris* and *Anabaena variabilis* for use as biofuel feedstock [115]. The study was conducted in modified Chu-10D medium to determine their potential as feedstock for biofuel production. The study also showed effects of aeration, and addition of supplements to the media.

Chapter 3 Materials and Methods

The project was to select an algal strain which has high lipid content and is suitable for growing in the local environment and also finding a growth media that will provide the maximum biomass production. The study was conducted using four nutrient media in Erlenmeyer flasks and the growth was observed by OD_{678}- measurements, dry cell weight measurement, microscopic imaging and visual change in color.

3.1 Instruments, Chemicals and Reagents

3.1.1 Materials / Instruments

In this study several media were prepared to study the growth kinetics of microalgae. Different reagents were used to prepare these media which are listed in a following table. Also several parameters had to be studied to conduct the study. Instruments that were used for the study are also listed below. The Erlenmeyer flasks were used for preparing algal culture in lab scale. An electric balance was used to conduct the gravimetric measurements, and measuring the dry algal biomass, while graduate cylinders were used for measuring liquid volumes. A spectrophotometer was used to measure the optical density of the solution, a microscope was used to observe the cell density, and a TDS meter was used to measure the total dissolved solid of the algal solution. An electric oven was used for drying algal samples and glasswares containing excess moisture. All the materials and instruments used in this experiment are tabulated below along with their uses, are tabulated below in Table 10.

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Table 10 Materials/ Instruments used in this work and their functions

Materials/ Instruments	Functions/Comments
HACH Spectrophotometer, DR-6000	Measure the Optical Density (OD) of algal solution
Nikon YS-100 Student Microscope	Observing the cell density
HANNA TDS meter	Measuring the value of total dissolved solid
VIBRA Balance	Gravimetric measurement, dry algal biomass, nutrients etc.
National Oven	Dry algal samples and glassware containing excess moisture
250 ml Erlenmeyer flasks	Propagating the culture for lab scale study
Graduate Cylinders	Liquid Volume Measurement
Pipette	Transferring sample
Pipette filler	

3.1.2 Reagents

Reagents used in this work and their functions are listed below in Table 11. However, the salts that are used as nutrients are not included in this table, they are shown in table 12.

Table 11 List of reagents used in this work

Reagents	Use/Comments
Distilled water	Prepare nutrient solution
Ethanol (Research grade)	Sterilize glass rods and pipettes before sample collection

3.2 Experimental Procedures

3.2.1 Algal Strain Selection

In Bangladesh, the only strain that is cultured in large scale is *Spirulina maxima* and it is produced solely for food purpose. As suggested by literature, it is not suitable for biodiesel production due to its very low lipid content.

Literature also suggested that, even though *Botriococcus braunii* has very high lipid content, the quality of the lipid is not suitable for producing biodiesel. Hence, *Chlorella vulgaris*, the next best option in terms of lipid content, was selected for the present study.

If the strain is collected from an open source, it may contain a symbiotic culture, which has the probability to contain toxic strains as well. Hence, it will be difficult to identify the suitable strain for studying the growth kinetics. After extensive literature review, it could be found that *Chlorella sp.*is appropriate for biodiesel production due to its high oil content, fast growing and resistance to contamination. The strain used for this study was *Chlorella vulgaris*, which was collected from Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia.

3.2.2 Growth Media

In this work the growth kinetics of *Chlorella vulgaris* were studied in four different nutrient media namely- CH medium, Modified CH medium, Bold's Basal (BB) medium and Modified BB medium.

BB medium is a universally used medium for growing freshwater microalgae, whereas, CH is a locally developed medium. CH stands for *Chlorella*. CH medium was solely developed to grow *Chlorella sp.* by BCSIR.

The analytical grade of the following chemicals were used to prepare growth media for Chlorella vulgaris: $MgSO_4.7H_2O$, $NaNO_3$, K_2HPO_4, KH_2PO_4, H_3BO_3, H_2SeO_3, $NaHCO_3$, $CaCl_2.2H_2O$, KNO_3, $FeSO_4.7H_2O$, $CaSO_4.2H_2O$, NaCl, EDTA, KOH, H_2SO_4, , Na_2EDTA, $FeCl_3$, $MnCl_2.4H_2O$, $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $CoCl_2.6H_2O$, $Na_2MoO_4.2H_2O$, $MnCl_2.4H_2O$, MoO_3, $Co(NO_3)_2.6H_2O$. Laboratory grade ultra-pure water was used for solution preparation and dilution.

Table 12 Composition of Nutrient Media- CH, Modified CH, BB, and Modified BB

Name of the chemicals	Concentration (g/L)			
	CH	**Modified CH**	**BB**	**Modified BB**
$MgSO_4.7H_2O$	10.42×10^{-3}	10.42×10^{-3}	7.5	7.5
$NaNO_3$	-	-	25	25
K_2HPO_4	40.42×10^{-3}	40.42×10^{-3}	7.5	7.5
KH_2PO_4	-	-	17.5	17.5
H_3BO_3	-	-	11.42	11.42
$CaCl_2.2H_2O$	-	-	2.5	2.5
KNO_3	1.0833	1.0833	-	-
$FeSO_4.7H_2O$	5×10^{-3}	5×10^{-3}	4.98	4.98
$CaSO_4.2H_2O$	2×10^{-3}	2×10^{-3}	-	-
NaCl	-	-	-	-
EDTA	-	-	50	50
KOH	-	-	31	31
H_2SO_4	-	-	1 ml/L	1 ml/L
Biotin	-	50×10^{-9}	-	50×10^{-9}
Vitamin B_{12}	-	50×10^{-9}	-	50×10^{-9}
Thiamin HCl	-	0.1×10^{-3}	-	0.1×10^{-3}
$MnCl_2.4H_2O$	-	-	1.44	1.44
$CuSO_4.5H_2O$	-	-	1.57	1.57
$ZnSO_4.7H_2O$	-	-	8.82	8.82
MoO_3	-	-	0.71	0.71
$Co(NO_3)_2.6H_2O$	-	-	0.49	0.49

3.2.3 Addition of Vitamin

Three different vitamins were added to regular CH and BB medium. The physical and chemical properties of vitamins may change and distort if autoclaved, therefore the vitamins were added after the media was autoclaved. The added vitamins were Biotin, Vitamin B_{12} and Thiamin HCl.

Table 12 shows the composition of the nutrient media used for growing *Chlorella vulgaris*.

3.3 Microalgae Culture

The microalgae was cultured in 250 ml Erlenmeyer flasks. Different batches of the experiments for each medium were conducted in series. The flasks were first filled with 200 ml nutrient medium and then autoclaved for 20 minutes. After cooling down to the room temperature, each flask was inoculated with 5 ml of algae stock solution. The cultures were mixed by means of sterile air in a laminar airflow chamber. The flasks were kept in a plant-growth room having a controlled temperature of 25±2° C, and lit with 1200 lux/m^2 fluorescent light with a photoperiod of 8 hours a day. Li Figure 7 shows the setup of the experiment in a culture room with controlled environment.

The E-flasks had cotton balls to seal the culture and also to ensure transmission of gas.

Figure 7: Experimental Setup for Growth Kinetics Study

3.4 Growth Kinetics Study

The parameters observed for conducting the study of growth kinetics of algae are- absorbance/ optical density and dry cell weight of the biomass. After substantial growth period a portion of the algae biomass was separated and analyzed for measurement of optical density and microscopic imaging. The study was continued for 30 days and samples were collected on day 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30. Then the solution of biomass was dried to find the mass of biomass present in the specific solution. The same methodology was adopted for rest of the algae biomass for various growth periods. Spectroscopic analysis was supported by visual change of color in the algal biomass solution and the microscopic imaging, and was validated with dry cell mass measurement in this study.

3.4.1 Absorbance/ Optical density

HACH Spectrophotometer, DR-6000 was used to measure the optical density of the microalgae cultures. Optical density is defined as the absorption of visible radiation. According to literature, the optimal wavelength to be used for studying absorbance of *Chlorella vulgaris* is within the range of 670-750 nm. For this study, the optimal wavelength used was 678 nm, which was determined based on the UV spectrum. Optical Density of the samples were measured at a regular time interval for 30 days. Figure 8 shows the spectrophotometer model used in this experiment.

Figure 8: HACH Spectrophotometer, DR-6000

3.4.2 Microscopic Imaging

For this step, **Nikon YS-100 Student Microscope** was used to observe the increase in cell density. The pictures of cells were taken using a mobile camera to present a pictorial demonstration of the cell growth. Figure 9 shows the setup of microscopic imaginf using Nikon YS-100 Student Microscope**.**

Figure 9: Nikon YS-100 Student Microscope

3.4.3 Dry Cell Weight

For this step, 10 ml of cultivated sample was collected from each media and initially the Total Dissolved Solid (TDS) value was measured for each sample. Then the samples were transferred to clean beakers, which were bone dried earlier. Before transferring the samples, the weight of the dry, empty beakers were recorded. The beakers were then put inside a desiccator to be heated around 80^{o}C overnight. This removed any moisture content from the sample and later the weight of the dried sample along with the beaker was calculated. Assuming all the dissolved solids left with moisture, leaving behind only the suspended solids, which were dried *Chlorella vulgaris,* Dry Cell Weight (DCW) was calculated from the above data, using the following equation –

$$\boldsymbol{DCW = (weight\ of\ beaker + solid) - Weight\ of\ dry\ beaker - TDS}$$

Figure 10 shows the balance and the TDS meter that were used in this experiment to determine Dry Cell Weight (DCW).

(A)

(B)

Figure 10: (A) VIBRA Balance for measuring dry biomass

(B) HANNA TDS meter for measuring TDS value

Figure 11 demonstrates the method and work process of this experiment.

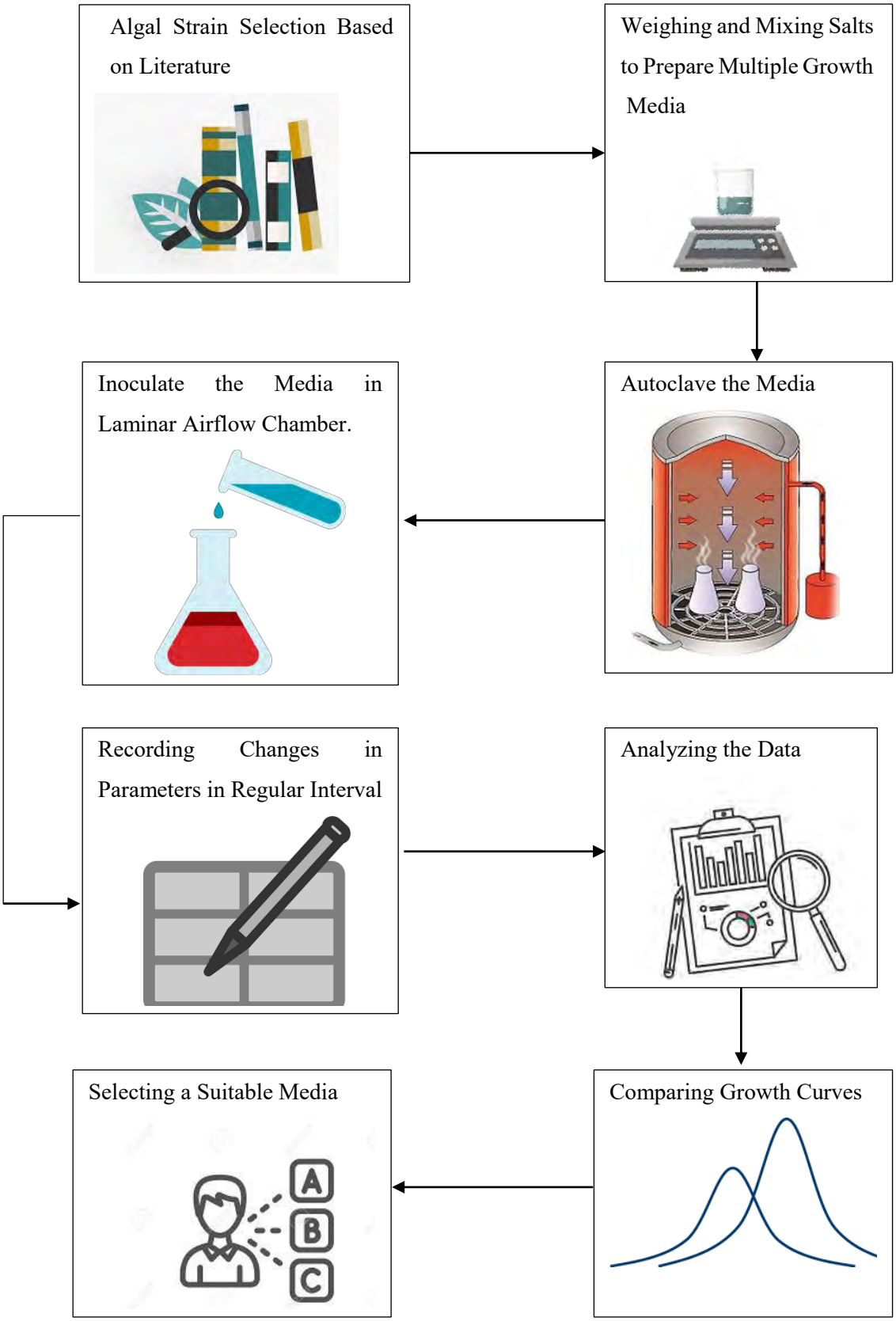


Figure 11 : Steps of conducting growth kinetics study

Chapter 4 Experimental Study of Growth Kinetics and Analysis

4.1 Change in Color of Broth and Microscopic Imaging

Images were captured to show change in the color of the culture broth during the study period. The images in Table 13 show how the color of the biomass culture changes over time.

Microscopic images were taken as well to demonstrate the change in density of microalgal colonies. The microscopic images to demonstrate the change in cell density are tabulated in Table 14.

The growth kinetics analysis using spectrophotometric and gravimetric data was carried out to explain this visual change in color.

4.2 Specific Growth Rate and Growth Curve Using Dry Cell Weight

The dry cell weight measured over time was used to calculate specific growth rate constants. First the cell weight obtained by drying 10 ml of sample was converted to dry biomass concentration (C_t) and then it was used to calculate specific growth rate constant for a period of 30 days for four media.

The formula used for the calculation is –

$$C_t = \frac{mass\ of\ algal\ dry\ biomass\ (g)}{medium\ volume\ (L)}$$

Where, C_t = Concentration of algal biomass at a given time (g/L)

$$\mu = \frac{\ln \frac{C_{t2}}{C_{t1}}}{t_2 - t_1}$$

Where, μ = Specific growth rate (d^{-1})

t_1, t_2= Two successive days of recording the value

C_{t1}, C_{t2}= dry biomass concentration in two days (g/L)

The change in biomass concentration recorded over the 30 day period was used to produce growth curves of *Chlorella vulgaris* in four different media, namely- CH, Modified CH, BB and Modified BB.

Table 13 Growth of *Chlorella vulgaris* in CH, modified CH, BB and modified BB media

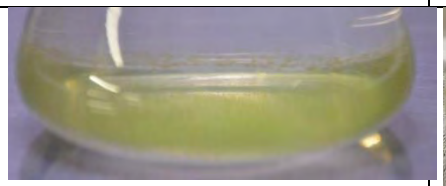
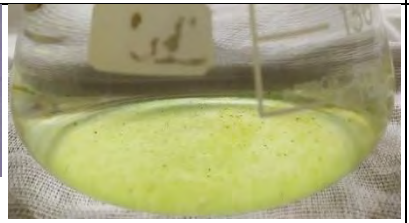
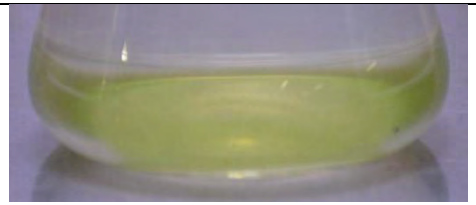
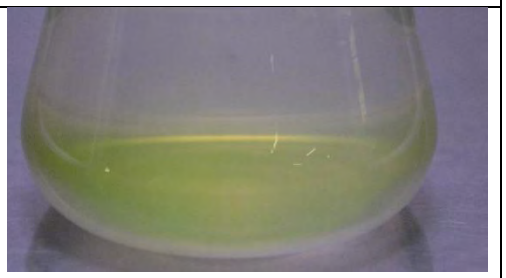
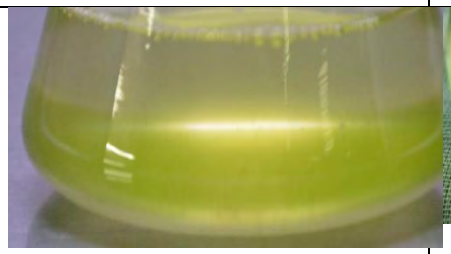
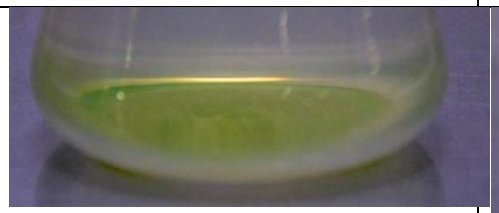
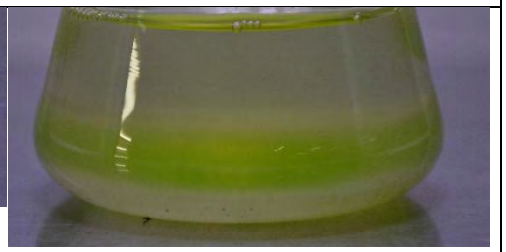
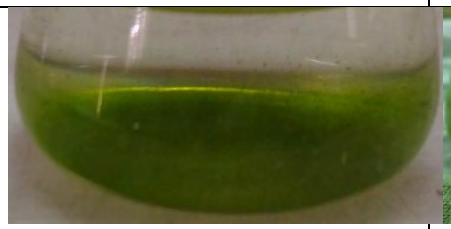
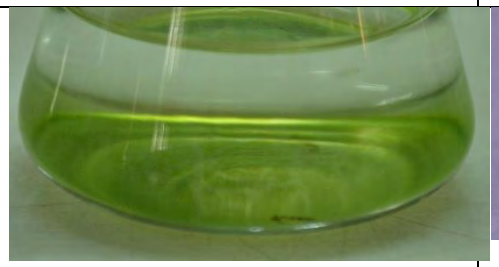
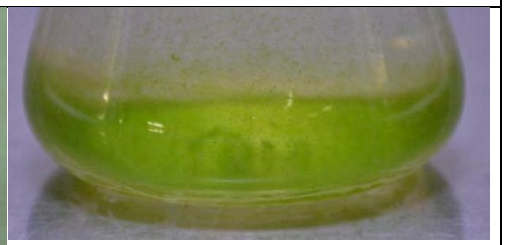
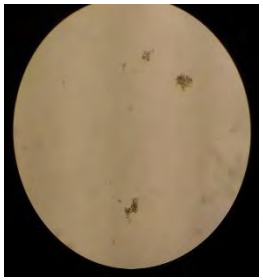
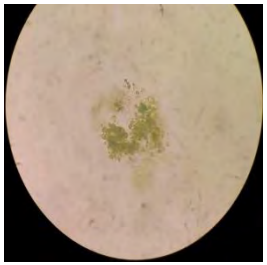
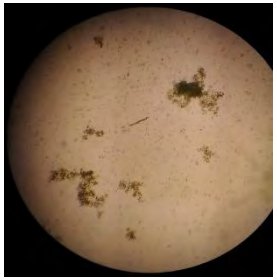
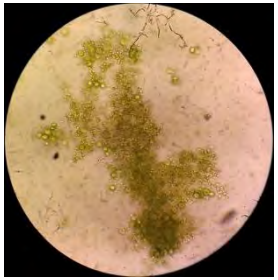
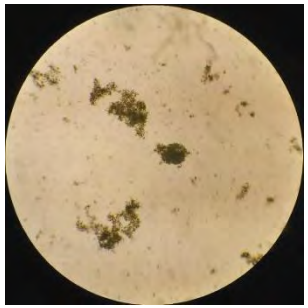
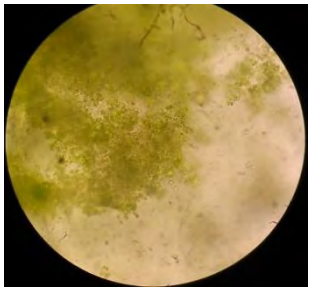
Time	Algae growth in CH medium	Algae growth in modified CH medium	Algae growth in BB medium	Algae growth in modified BB medium
Day 10				
Day 20				
Day 27				

Table 14 Growth of colonies of *Chlorella vulgaris* in modified CH medium (Microscopic image)

Day	10 X image	40 X image
Day 7		
Day- 14		
Day- 21		

4.2.1 Growth Kinetics and Growth Curve for CH medium

Table15 contains calculated values of specific growth rate of *Chlorella vulgaris* from day 0 to day 30 with 3 days interval for CH medium.

Table 15 Specific growth rate calculation for CH medium using dry cell weight

Mass of dry biomass (g)	Concentration of dry biomass, C_t (g/L)	Time,t (days)	$\frac{c_{t2}}{C_{t1}}$	$t_2 - t_1$	$\ln \frac{c_{t2}}{c_{t1}}$	Specific growth rate, μ (d^{-1})
0.0056	0.056	0	0.56	0	0	0
0.00561	0.05614	3	1.002499	3	0.002496	0.000832
0.00568	0.056842	6	1.012497	3	0.01242	0.00414
0.00578	0.057801	9	1.016881	3	0.01674	0.00558
0.00592	0.05915	12	1.023338	3	0.02307	0.00769
0.00605	0.060501	15	1.022847	3	0.02259	0.00753
0.00613	0.061324	18	1.013592	3	0.0135	0.0045
0.00617	0.061735	21	1.006712	3	0.00669	0.00223
0.0062	0.062049	24	1.005083	3	0.00507	0.00169
0.00622	0.06223	27	1.002914	3	0.00291	0.00097
0.00623	0.06231	30	1.001285	3	0.001284	0.000642

The values of cell concentration were put against time to generate the growth curve, from which lag phase, exponential phase, phase of declining relative growth and stationary phase have been detected. Growth curve for CH medium is shown is Figure 12.

Figure 13 shows the change in specific growth rate values with time, for CH medium. It can be seen there is a gradual rise to reach a plateau briefly and then the specific growth rate values decrease gradually.

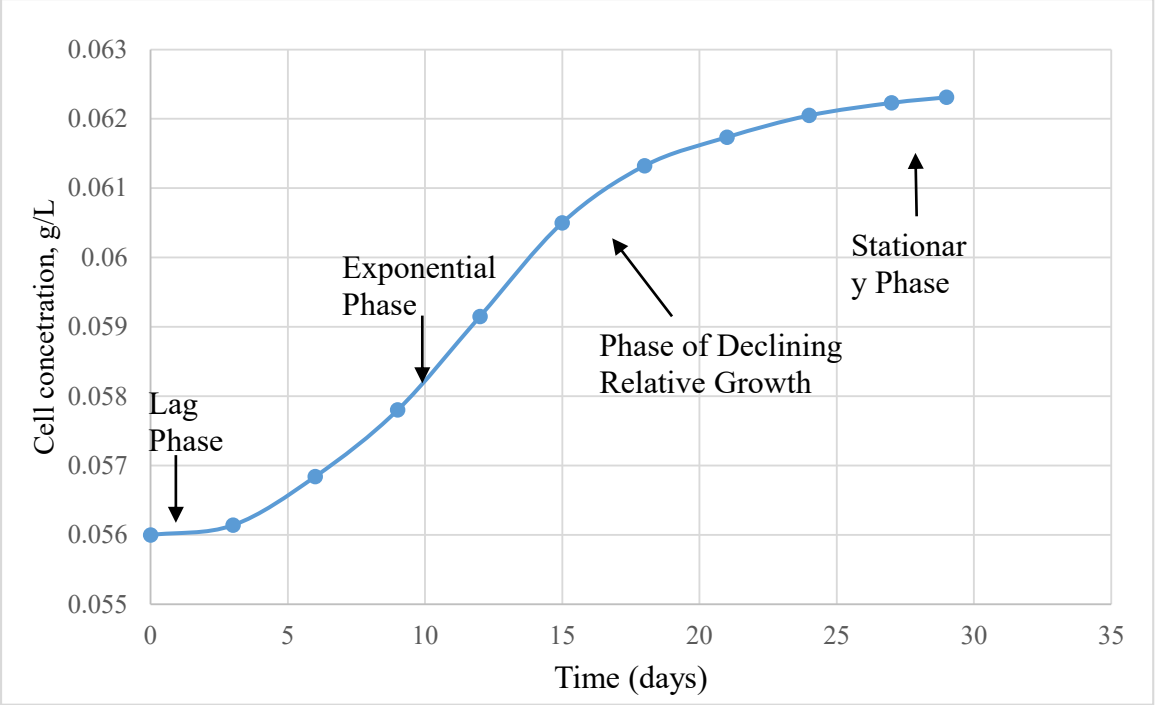


Figure 12 **:** Growth curve for CH medium using cell concentration

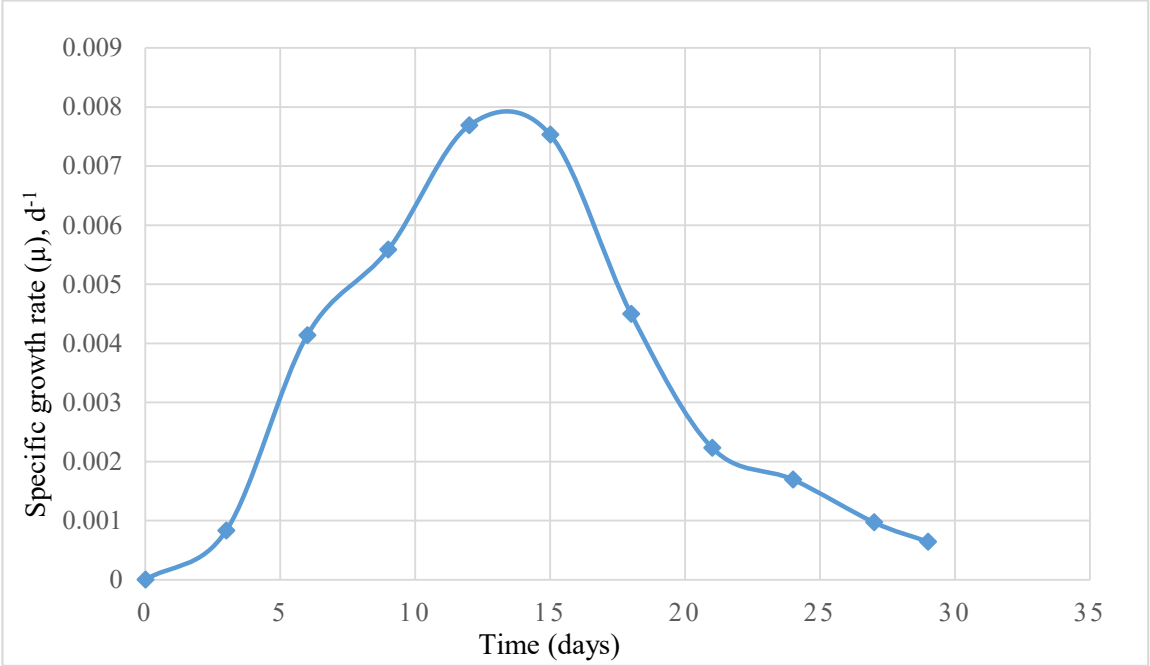


Figure 13: Change in value of specific growth rate with time in CH medium

4.2.2 Growth Kinetics and Growth Curve for Modified CH medium

Table16 contains the variation in values of specific growth rate of *Chlorella vulgaris* from day 0 to day 30 with 3 days interval for modified CH medium.

Table 16 Specific growth rate calculation for modified CH medium using dry cell weight

Mass of dry biomass (g)	Concentration of dry biomass, C_t (g/L)	Time,t (days)	$\frac{c_{t2}}{C_{t1}}$	$t_2 - t_1$	$\ln \frac{C_{t2}}{c_{t1}}$	Specific growth rate, μ (d^{-1})
0.00756	0.0756	0	0.756	0	0	0
0.00791	0.0791	3	1.046296	3	0.045257	0.015086
0.00911	0.0911	6	1.151707	3	0.141245	0.047082
0.0271	0.271	9	2.974753	3	1.090161	0.063387
0.07401	0.7401	12	2.730996	3	1.004666	0.100349
0.11085	1.1085	15	1.497771	3	0.403978	0.146592
0.16905	1.6905	18	1.525034	3	0.422017	0.155041
0.28395	2.8395	21	1.679681	3	0.518604	0.158679
0.29397	2.9397	24	1.035288	3	0.03468	0.08156
0.29412	2.9412	27	1.00051	3	0.00051	0.012753

The values of cell concentration were put against time to generate the growth curve, from which lag phase, exponential phase, phase of declining relative growth and stationary phase have been detected. Growth curve for modified CH medium is shown is Figure 14.

Figure 15 shows the change in specific growth rate values with time, for modified CH medium. It can be seen there is a gradual rise to reach a plateau briefly and then the specific growth rate values decrease gradually.

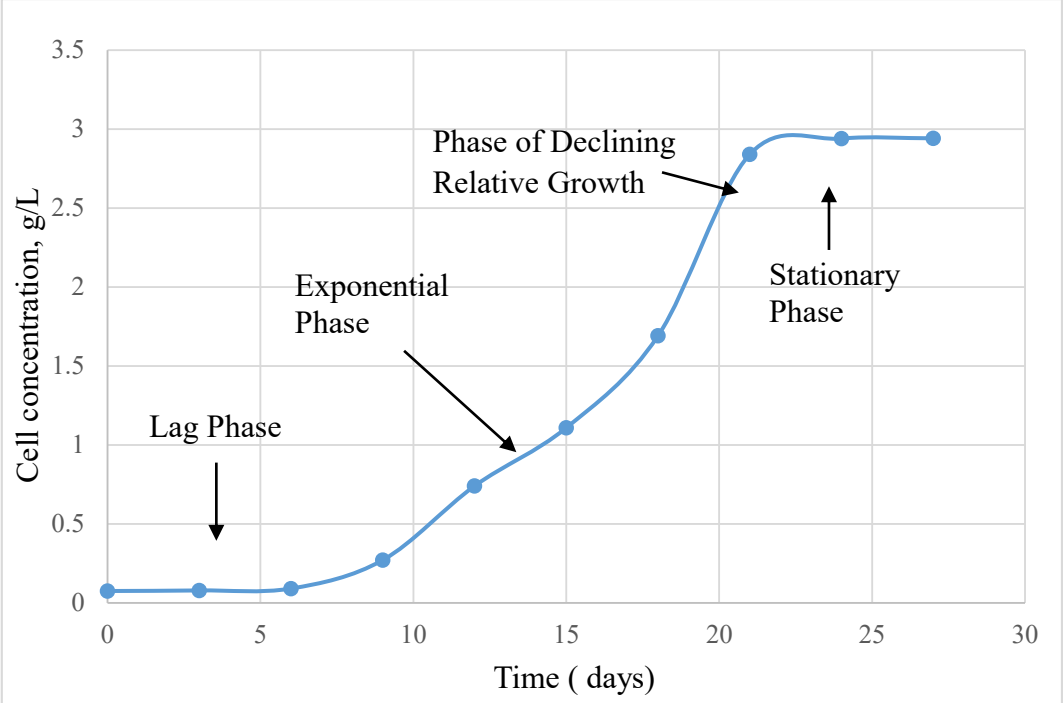


Figure 14: Growth curve for modified CH medium using cell concentration

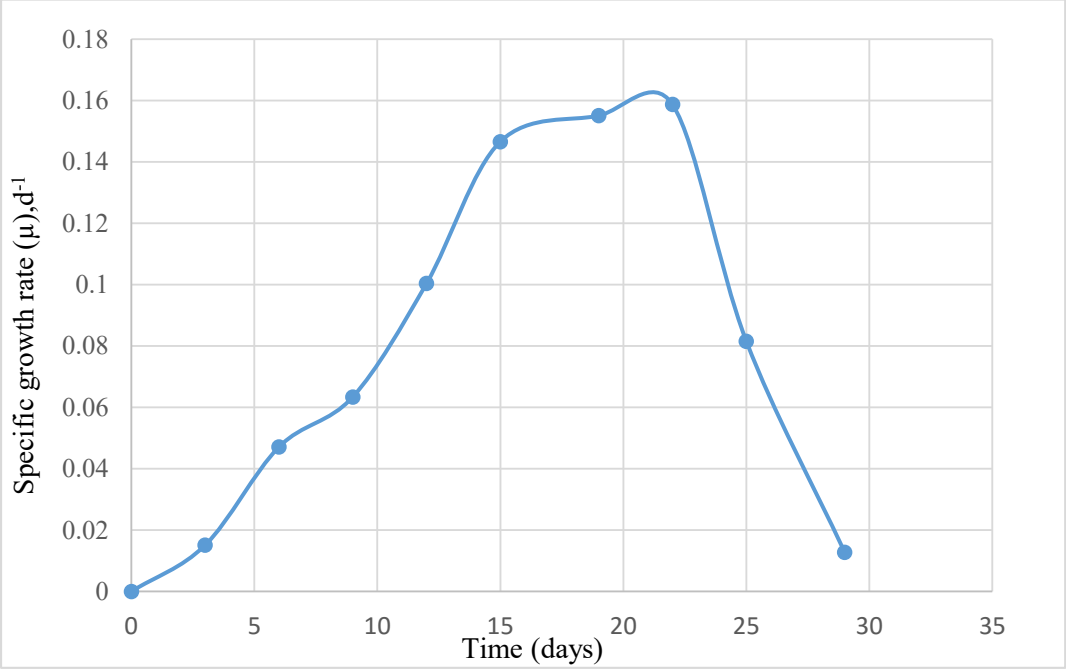


Figure 15: Change in value of specific growth rate with time in modified CH medium

4.2.3 Growth Kinetics and Growth Curve for BB medium

Table17 contains the variation in values of specific growth rate of *Chlorella vulgaris* from day 0 to day 27 with 3 days interval for BB medium.

Table 17 Specific growth rate calculation for BB medium using dry cell weight

Mass of dry biomass (g)	Concentration of dry biomass, C_t (g/L)	Time,t (days)	$\frac{c_{t2}}{C_{t1}}$	$t_2 - t_1$	$\ln \frac{c_{t2}}{c_{t1}}$	Specific growth rate, μ (d^{-1})
0.0058	0.0580	0	0.058	0	0	0
0.00582	0.0582	3	1.003448	3	0.003442	0.001147
0.00587	0.0587	6	1.008591	3	0.008554	0.002851
0.00598	0.0598	9	1.018739	3	0.018566	0.006189
0.00608	0.0608	12	1.016722	3	0.016584	0.005528
0.00615	0.0615	15	1.011513	3	0.011447	0.003816
0.00618	0.0618	18	1.004878	4	0.004866	0.001622
0.00621	0.0621	21	1.004854	3	0.004843	0.001614
0.006235	0.06235	24	1.004026	3	0.004018	0.001339
0.006246	0.06246	27	1.001826	3	0.001824	0.000608

The values of cell concentration were put against time to generate the growth curve, from which lag phase, exponential phase, phase of declining relative growth and stationary phase have been detected. Growth curve for BB medium is shown is Figure 16.

Figure 17 shows the change in specific growth rate values with time, for BB medium. It can be seen there is a gradual rise to reach a plateau briefly and then the specific growth rate values decrease gradually.

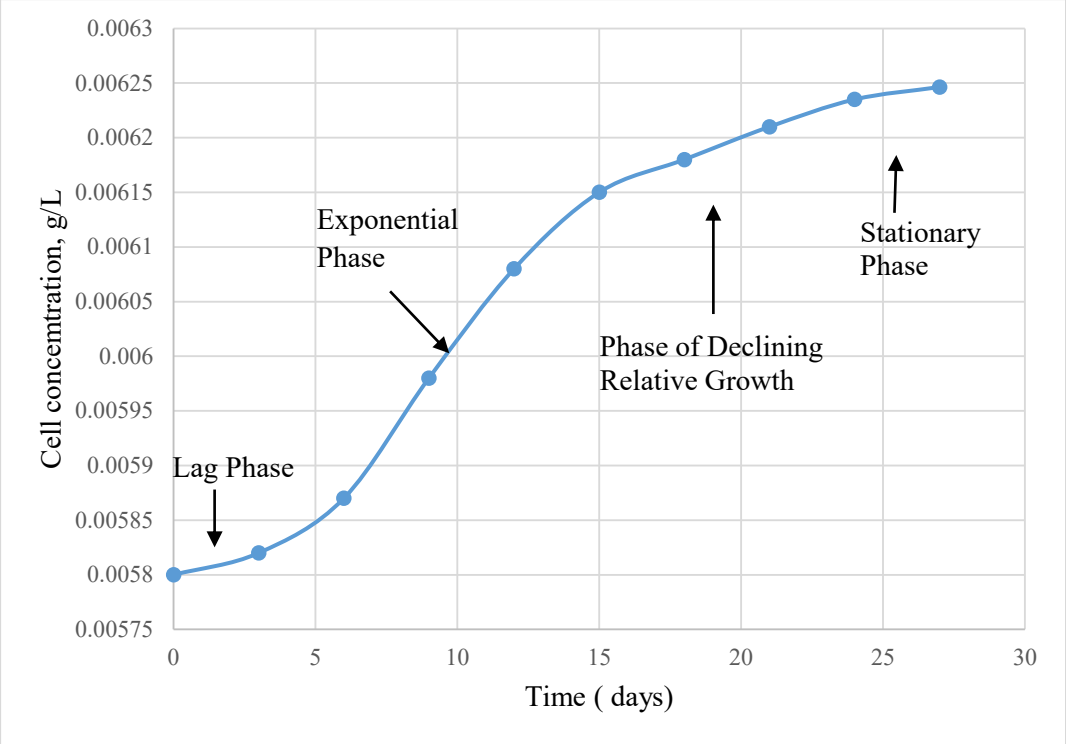


Figure 16: Growth curve for BB medium using cell concentration

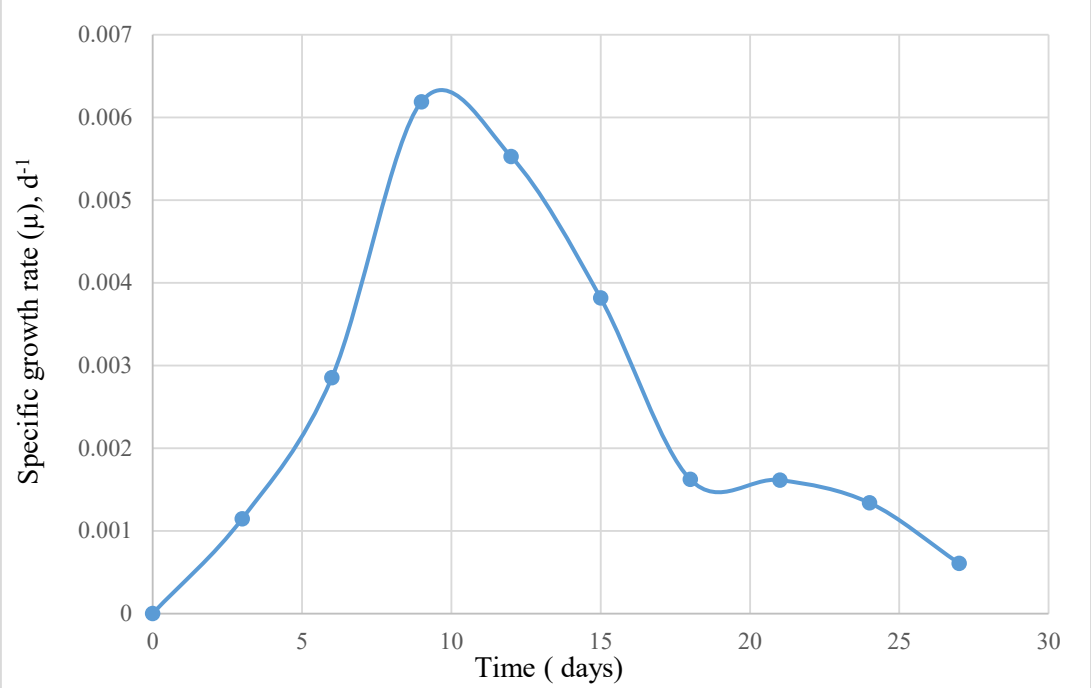


Figure 17: Change in value of specific growth rate with time in BB medium

4.2.4 Growth Kinetics and Growth Curve for Modified BB medium

Table18 contains the variation in values of specific growth rate of *Chlorella vulgaris* from day 0 to day 27 with 3 days interval for modified BB medium.

Table 18 Specific growth rate calculation for modified BB medium using dry cell weight

Mass of dry biomass (g)	Concentration of dry biomass, C_t (g/L)	Time,t (days)	$\frac{c_{t2}}{C_{t1}}$	$t_2 - t_1$	$\ln \frac{C_{t2}}{c_{t1}}$	Specific growth rate, μ (d^{-1})
0.00652	0.0652	0	0.652	0	0	0
0.01797	0.1797	3	1.087772	3	0.084132	0.028044
0.01928	0.1928	6	1.072899	3	0.070365	0.023455
0.04167	0.4167	9	2.161307	3	0.770713	0.256904
0.08786	0.8786	12	2.108471	3	0.745963	0.248654
0.09078	0.9078	15	2.178546	3	0.778658	0.259553
0.17104	1.7104	18	1.884115	3	0.633458	0.158365
0.18894	1.8894	21	1.104654	3	0.099532	0.033177
0.190002	1.90002	24	1.005621	3	0.005605	0.001868
0.19594	1.9594	27	1.031252	3	0.030774	0.007693

The values of cell concentration were put against time to generate the growth curve, from which lag phase, exponential phase, phase of declining relative growth and stationary phase have been detected. Growth curve for modified BB medium is shown is Figure 18.

Figure 19 shows the change in specific growth rate values with time, for modified BB medium. It can be seen there is a gradual rise to reach a plateau briefly and then the specific growth rate values decrease gradually.

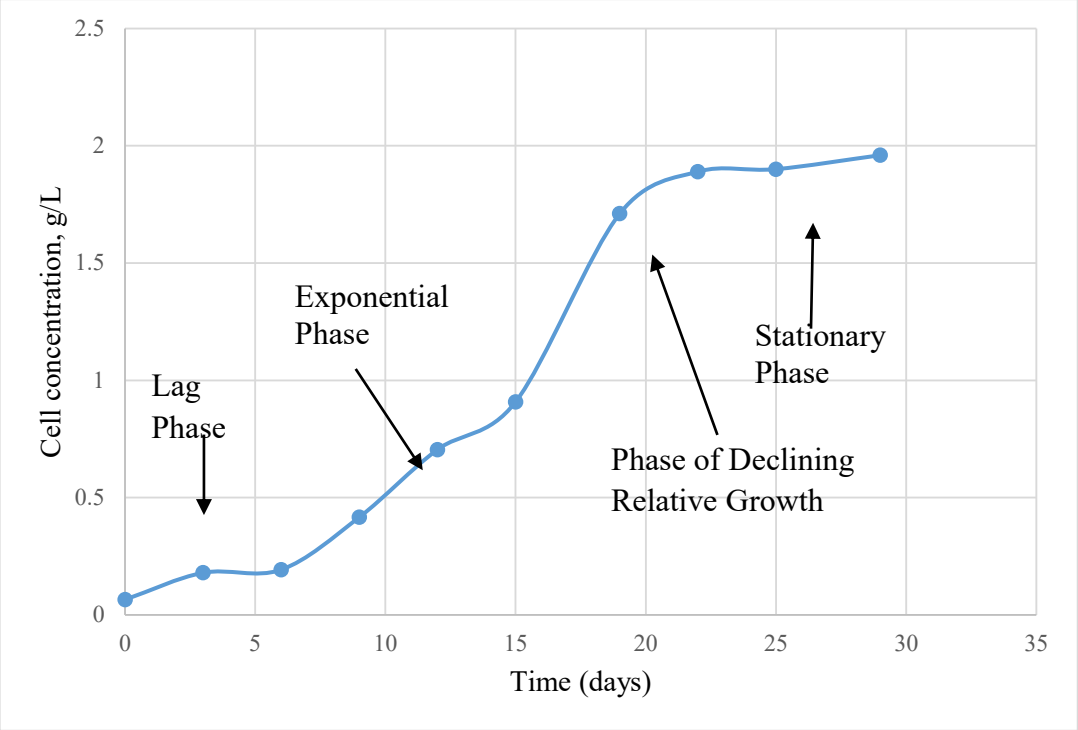


Figure 18: Growth curve for modified BB medium using cell concentration

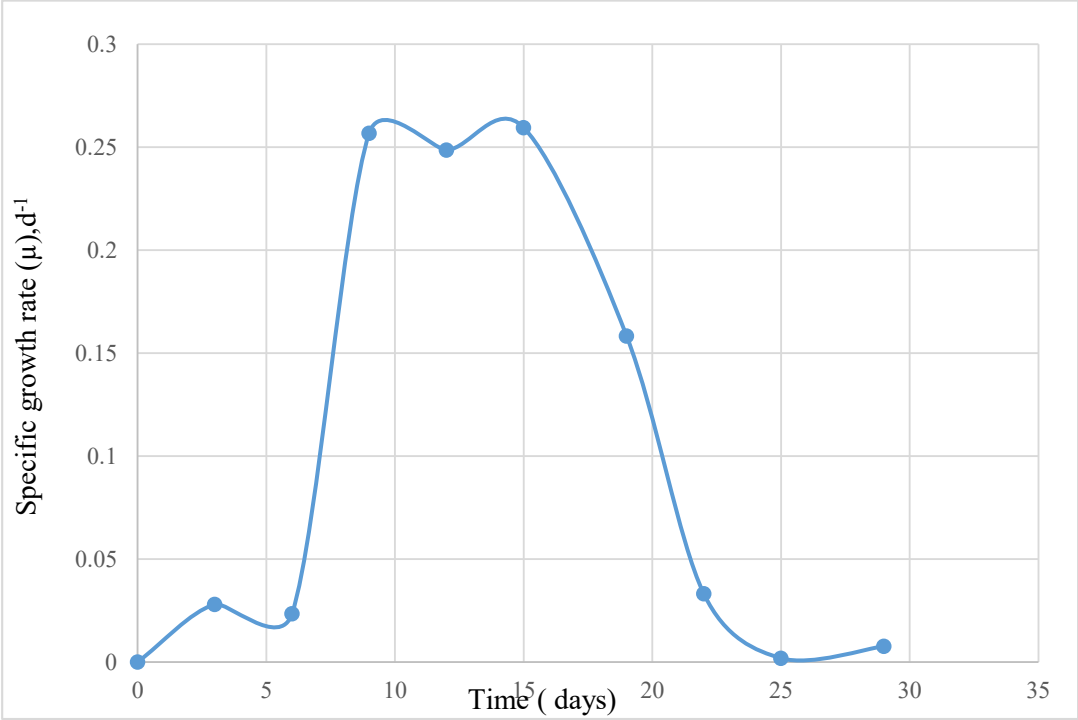


Figure 19: Change in value of specific growth rate with time in modified BB medium

4.3 Specific Growth Rate and Growth Curve Using Optical Density at 678 nm

Optical Density of a sample is a lesser time consuming way of monitoring the growth. Following procedures mentioned above, specific growth rate constants were calculated and growth curves were generated using Optical Density of samples measured at 678 nm. The same calculation procedure was carried out for CH, Modified CH, BB and Modified BB medium.

4.3.1 Growth Kinetics and Growth Curve for CH medium

Table19 contains the variation in values of specific growth rate of *Chlorella vulgaris* from day 0 to day 27 with 3 days interval for CH medium.

Table 19 Specific growth rate calculation for CH medium using optical density

Optical density, OD_{678}	Time,t (days)	$\frac{OD_{t2}}{OD_{t1}}$	$t_2 - t_1$	$\ln \frac{C_{t2}}{C_{t1}}$	Specific growth rate, μ (d^{-1})
0.00015	0	0	0	0	0
0.000154	3	1.026069	3	0.025735	0.008578
0.000157	6	1.021057	3	0.020838	0.006946
0.000602	9	3.827682	3	1.342259	0.44742
0.001224	12	2.034243	3	0.710124	0.236708
0.001803	15	1.472101	3	0.386691	0.128897
0.002158	18	1.197353	4	0.180113	0.060038
0.002336	21	1.082412	3	0.079192	0.026397
0.00247	24	1.057103	3	0.055532	0.018511
0.002558	27	1.036012	3	0.035379	0.011793

The values of optical density were put against time to generate the growth curve, from which lag phase, exponential phase, phase of declining relative growth and stationary phase have been detected. Growth curve for CH medium is shown is Figure 20.

Figure 21 shows the change in specific growth rate values with time, for CH medium. It can be seen there is a spike to reach a peak value and then the specific growth rate values decrease gradually.

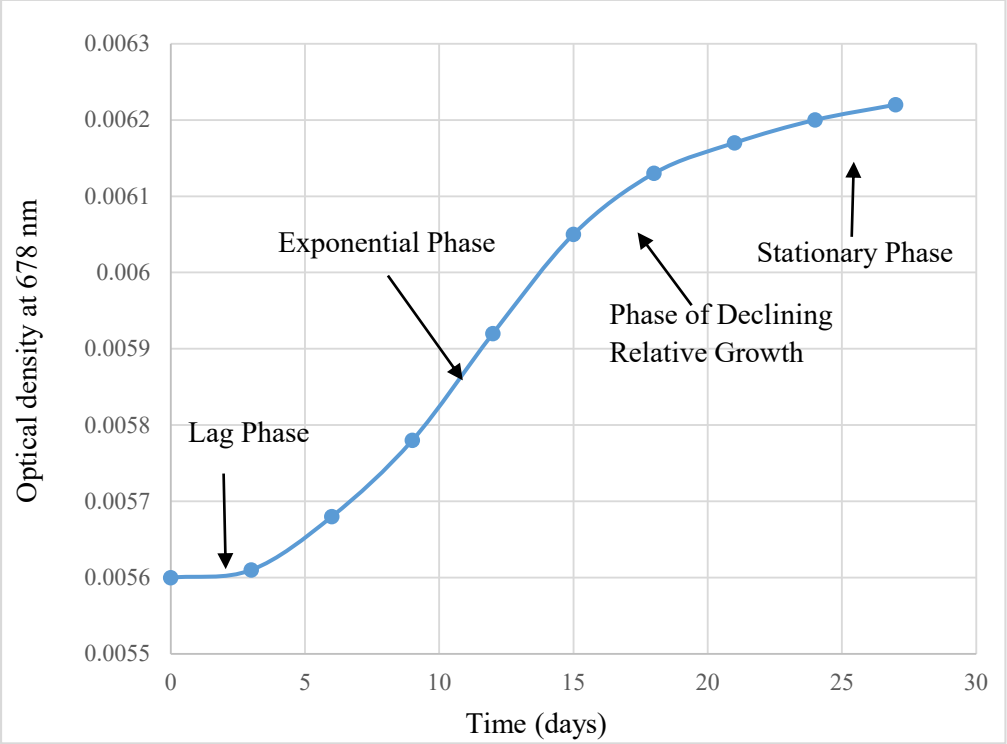


Figure 20: Growth curve for CH medium using optical density

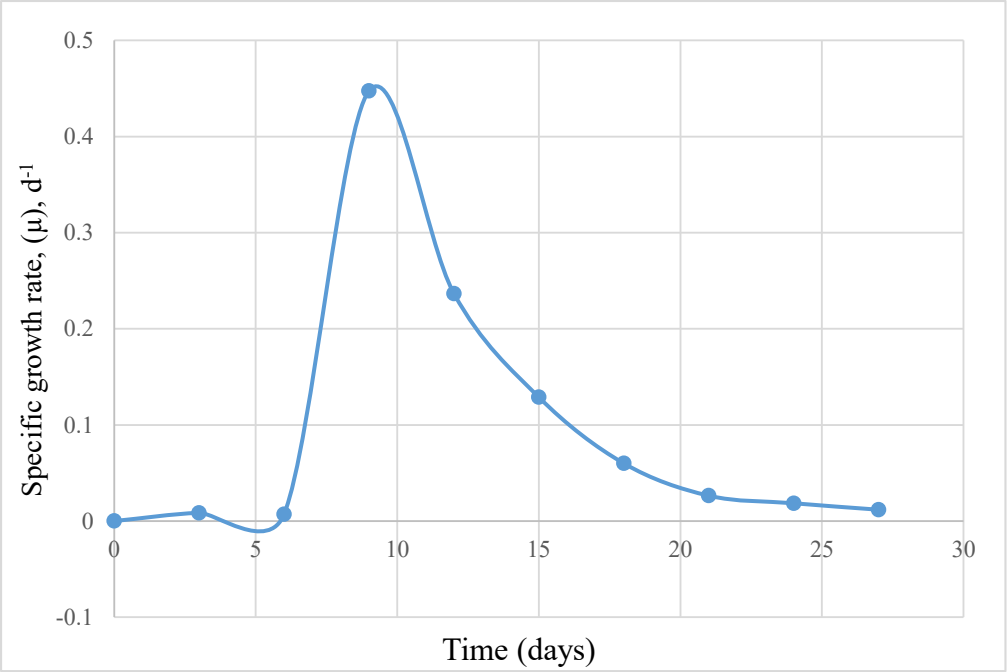


Figure 21: Change in value of specific growth rate with time in CH medium

4.3.2 Growth Kinetics and Growth Curve for Modifed CH medium

Table 20 contains the variation in values of specific growth rate of *Chlorella vulgaris* from day 0 to day 27 with 3 days interval for modified CH medium.

Table 20 Specific growth rate calculation for modified CH medium using optical density

Optical density, OD_{650}	Time,t (days)	$\frac{OD_{t2}}{OD_{t1}}$	$t_2 - t_1$	$\ln \frac{OD_{t2}}{OD_{t1}}$	Specific growth rate, μ (d^{-1})
0.015	0	0	0	0	0
0.016	3	1.066667	3	0.064539	0.021513
0.032	6	2	3	0.693147	0.231049
0.065	9	2.03125	3	0.708651	0.236217
0.126	12	1.938462	3	0.661895	0.220632
0.178	15	1.412698	3	0.345502	0.115167
0.2258	18	1.268539	4	0.237866	0.079289
0.2403	21	1.064216	3	0.062238	0.020746
0.2513	24	1.045776	3	0.044759	0.01492
0.258	27	1.026661	3	0.026312	0.008771

The values of optical density were put against time to generate the growth curve, from which lag phase, exponential phase, phase of declining relative growth and stationary phase have been detected. Growth curve for modified CH medium is shown is Figure 22.

Figure 23 shows the change in specific growth rate values with time, for modified CH medium. It can be seen there is a gradual rise to reach a plateau and then the specific growth rate values decrease gradually.

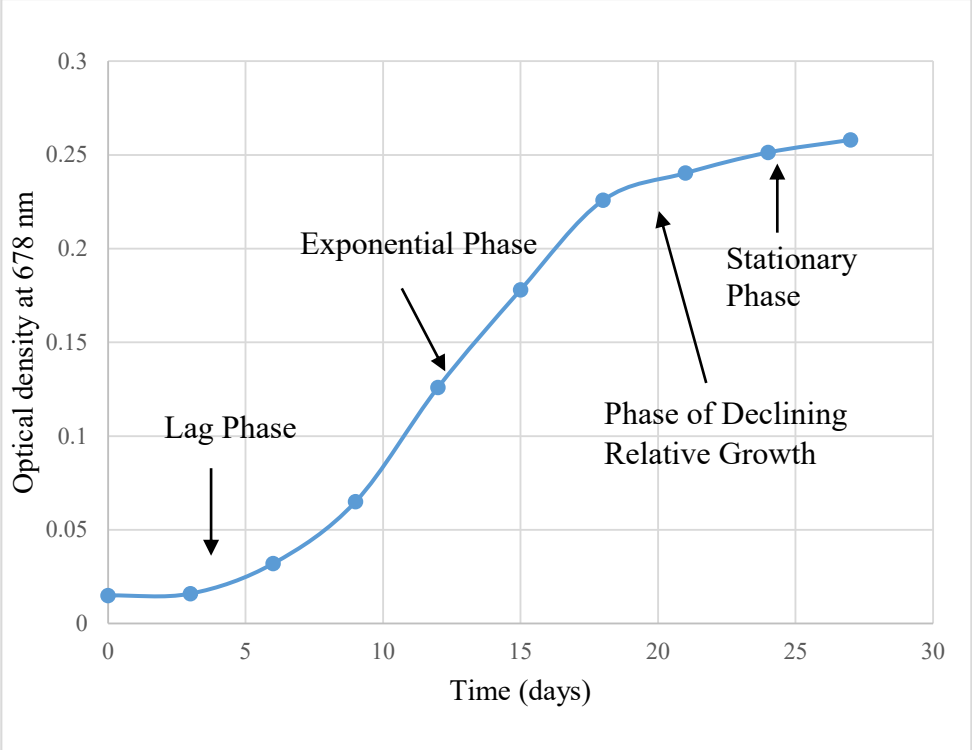


Figure 22: Growth curve for modified CH medium using optical density

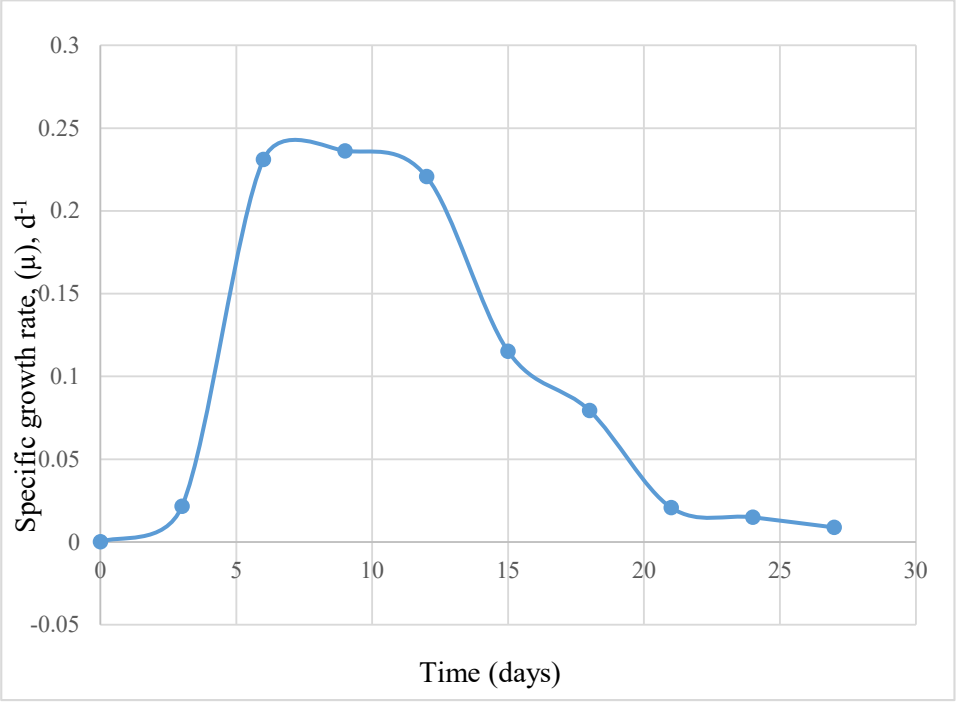


Figure 23: Change in value of specific growth rate with time in modified CH medium

4.3.3 Growth Kinetics and Growth Curve for BB medium

Table 21 contains the variation in values of specific growth rate of *Chlorella vulgaris* from day 0 to day 27 with 3 days interval for BB medium.

Table 21 Specific growth rate calculation for BB medium using optical density

Optical density, OD_{650}	Time,t (days)	$\frac{OD_{t2}}{OD_{t1}}$	$t_2 - t_1$	$\ln \frac{OD_{t2}}{OD_{t1}}$	Specific growth rate, μ (d^{-1})
0.009	0	0	0	0	0
0.011	3	1.222222	3	0.200671	0.06689
0.013	6	1.181818	3	0.167054	0.055685
0.017	9	1.307692	3	0.268264	0.089421
0.033	12	1.941176	3	0.663294	0.221098
0.057	15	1.727273	3	0.546544	0.182181
0.066	18	1.157895	3	0.146603	0.048868
0.07	21	1.060606	3	0.058841	0.019614
0.073	24	1.042857	3	0.041964	0.013988
0.076	27	1.041096	3	0.040274	0.013425

The values of optical density were put against time to generate the growth curve, from which lag phase, exponential phase, phase of declining relative growth have been detected. After the phase of declining relative growth, another upword growth is seen in the curve. This is an anomaly. Growth curve for BB medium is shown is Figure 24.

Figure 25 shows the change in specific growth rate values with time, for BB medium. It can be seen there is a rise to reach a peak value and then the specific growth rate values decrease gradually. However, there is no platue, rather two spikes can be observed.

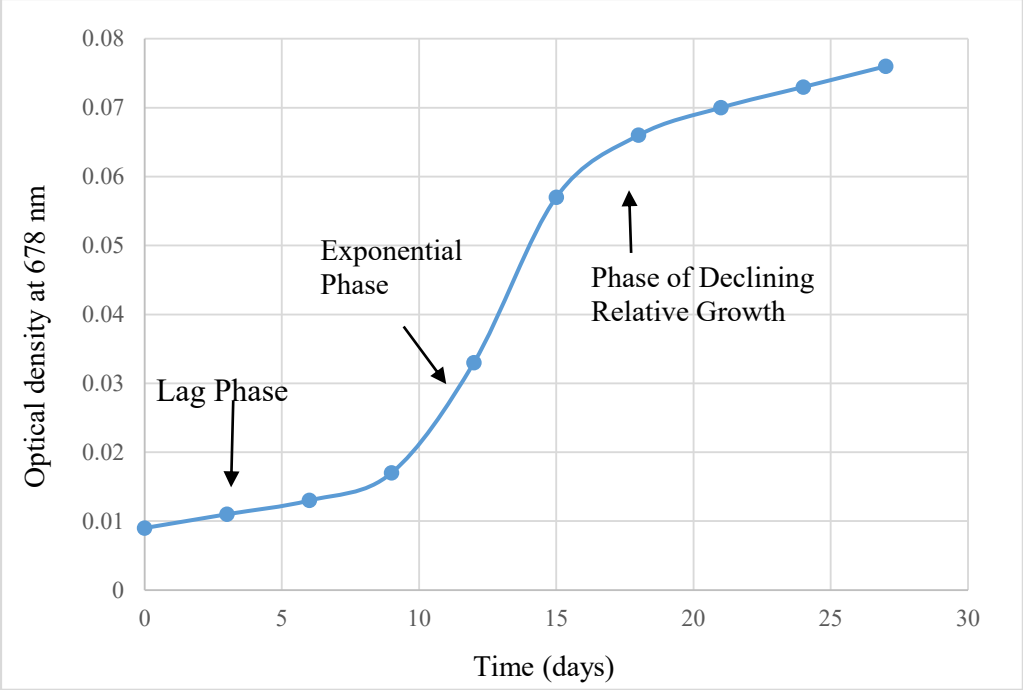


Figure 24: Growth curve for BB medium using optical density

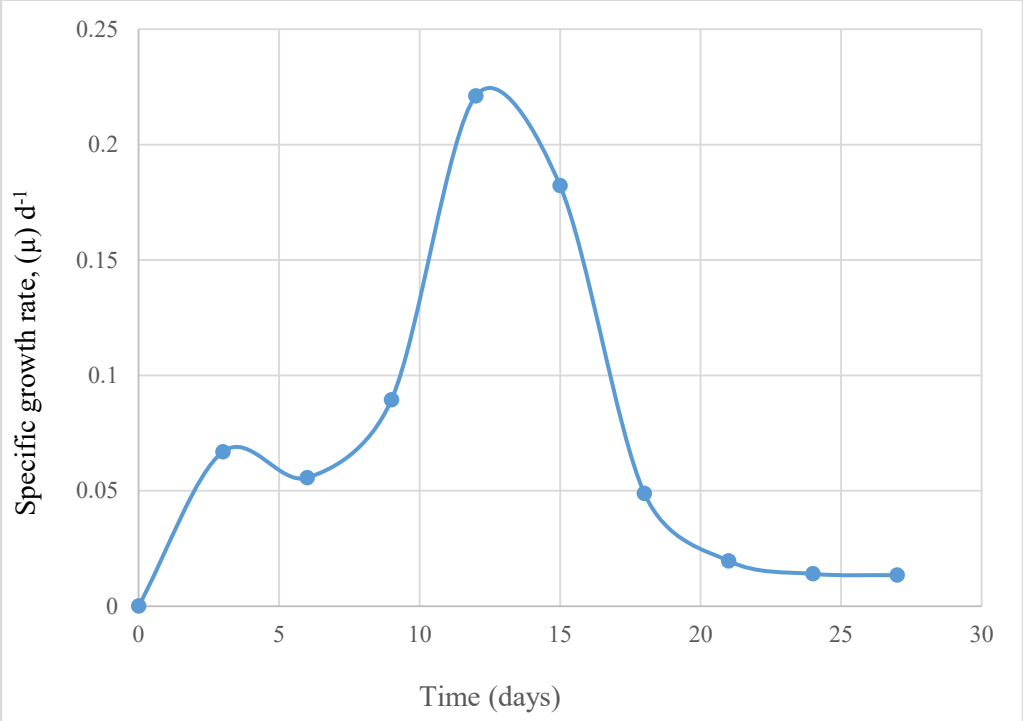


Figure 25: Change in value of specific growth rate with time in BB medium

4.3.4 Growth Kinetics and Growth Curve for Modified BB medium

Table 22 contains the variation in values of specific growth rate of *Chlorella vulgaris* from day 0 to day 27 with 3 days interval for modified BB medium.

Table 22 Specific growth rate calculation for modified BB medium using optical density

Optical density, OD_{650}	Time,t (days)	$\frac{OD_{t2}}{OD_{t1}}$	$t_2 - t_1$	$\ln \frac{OD_{t2}}{OD_{t1}}$	Specific growth rate, μ (d^{-1})
0.008	0	0	0	0	0
0.0085	3	1.0625	3	0.060625	0.020208
0.0092	6	1.082353	3	0.079137	0.026379
0.022	9	2.391304	3	0.871839	0.290613
0.048	12	2.181818	3	0.780159	0.260053
0.065	15	1.354167	3	0.303186	0.101062
0.071	18	1.092308	3	0.088293	0.029431
0.078	21	1.098592	3	0.094029	0.031343
0.081	24	1.038462	3	0.03774	0.01258
0.084	27	1.037037	3	0.036368	0.012123

The values of optical density were put against time to generate the growth curve, from which lag phase, exponential phase, and phase of declining relative growth have been detected. Growth curve for modified BB medium is shown is Figure 26.

Figure 27 shows the change in specific growth rate values with time, for modified BB medium. It can be seen there is a rise to reach a peak value and then the specific growth rate values decrease gradually.

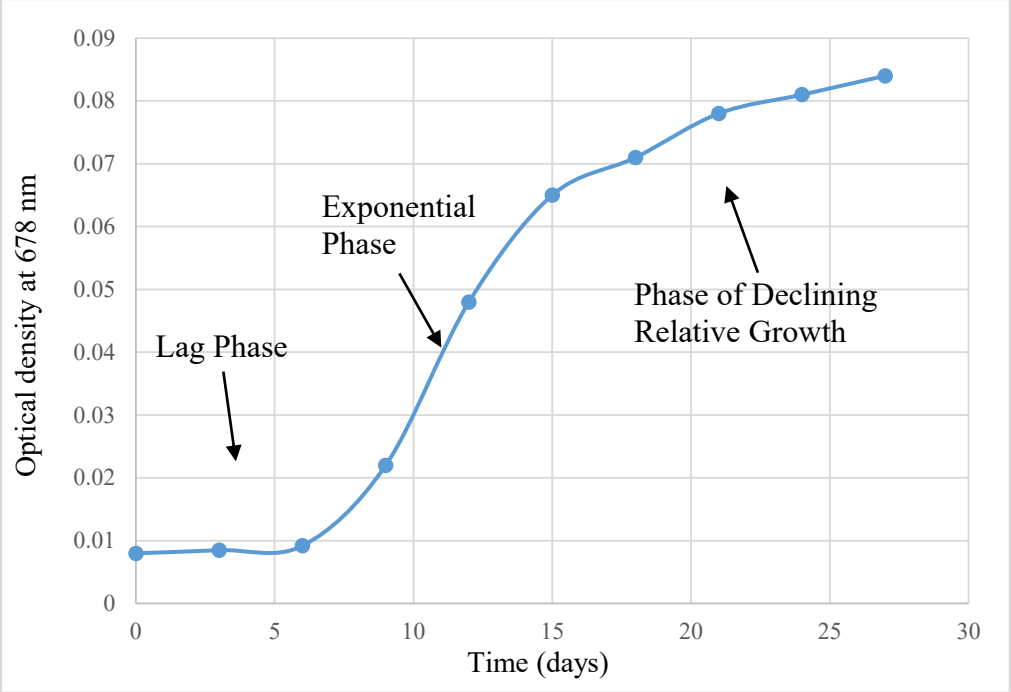


Figure 26: Growth curve for BB medium using optical density

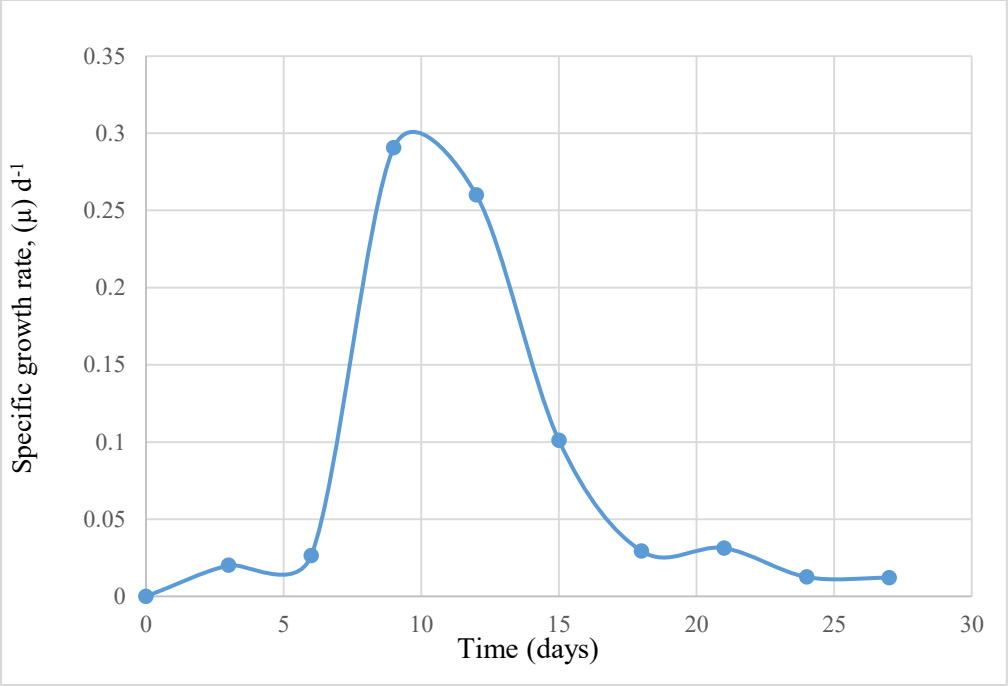


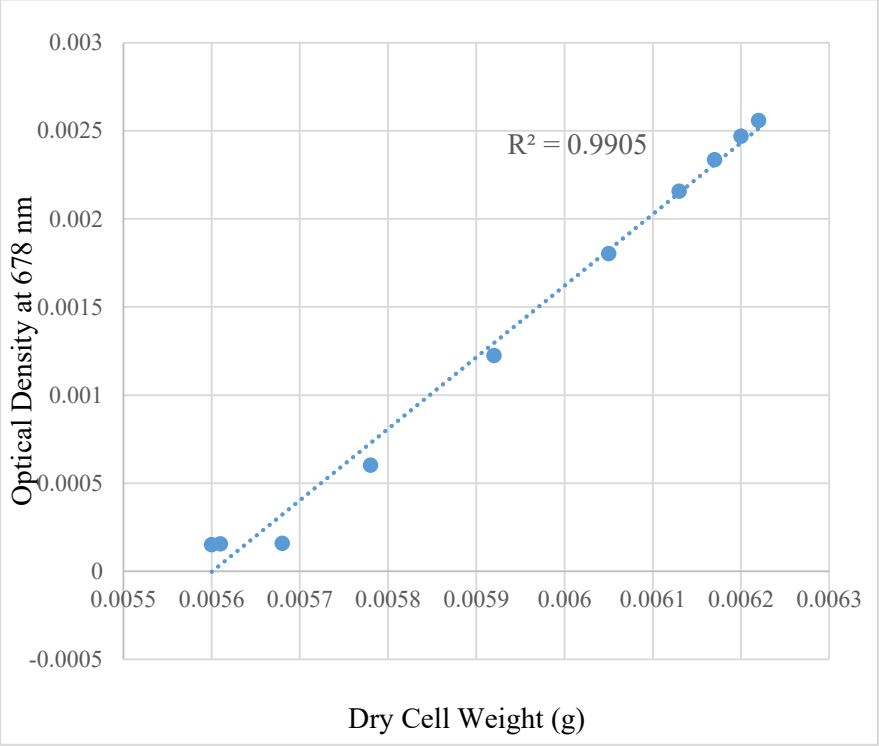
Figure 27: Change in value of specific growth rate with time in modified BB medium

4.4 Direct Determination of Dry Cell Weight from Optical Density

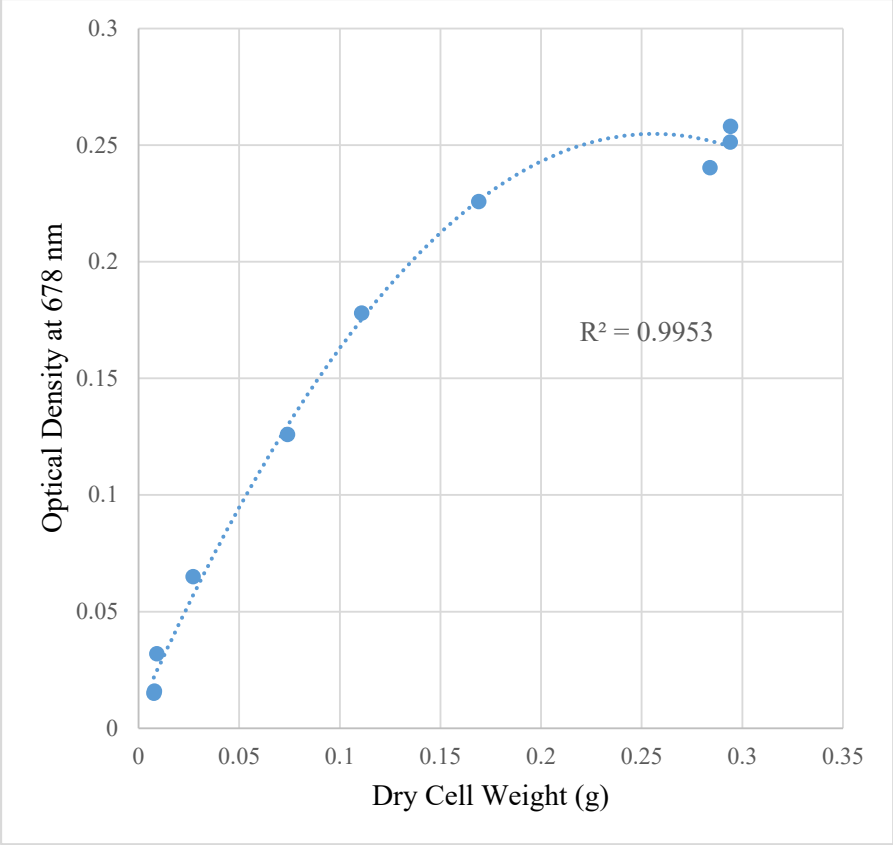
Absorbance also known as Optical Density is used as a convenient indirect measurement of biomass concentration in microbial cell suspensions. Absorbance of light by a suspension can be related directly to cell density or dry cell weight using a suitable standard curve.

A calibration curve was prepared to correlate the optical density or absorbance of the culture *Chlorella vulgaris* with dry cell weight (DCW). Figure 28 shows the correlation of dry cell weight (biomass concentration) with the culture's absorbance at 678 nm for the four media used in this study. 678 nm was chosen due to a preliminary study showing 678 nm to be the wavelength of maximum absorbance for a culture of the specific strain of *Chlorella.*

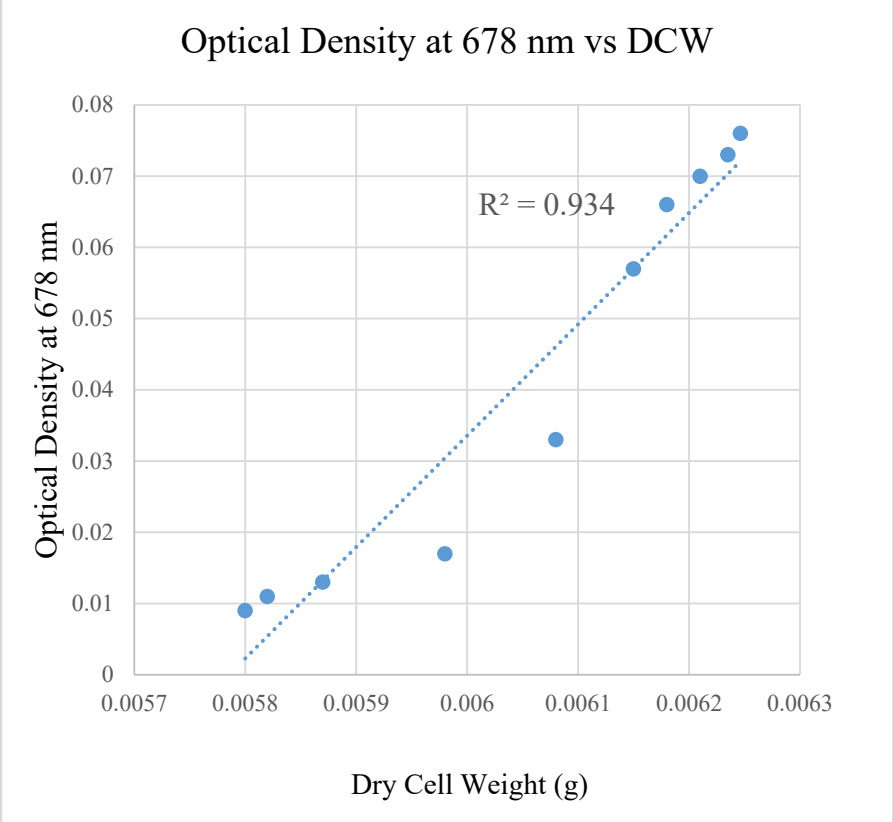
Even though the calibration curves show linear trendline for CH and BB media, it shows a parabolic trendline for modified CH and modified BB.



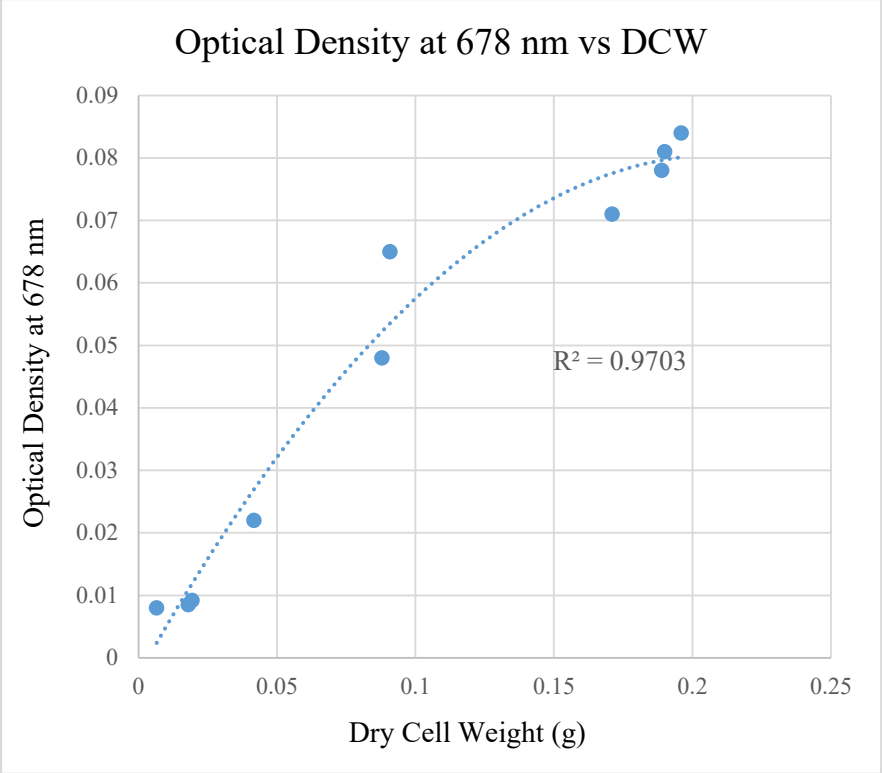
(a) Calibration curve for CH medium



(b) Calibration curve for modified CH medium



(c) Calibration curve for Bold's Basal (BB) Medium



(d) Calibration Curve for modified Bold's Basal (BB) medium

Figure 28: Calibration Curve for (a) CH medium (b) Modified CH medium (c) BB medium (d) Modified BB medium

4.5 Comparison of Exponential Phase for Various Media

The main purpose of culturing microalgae *Chlorella vulgaris* is to harvest the cells for further use. The cells reach their optimum condition of harvesting at the end of exponential phase. When exponential phase ends, the nutrients start to decline and the cell concentration declines as well. Hence the best period of harvesting the cells is immediately after the exponential phase.

Table 23 and Figure 29 show the duration of exponential phase and the cell concentration at the end of exponential phase for each of the four media used in the study.

Table 23 Properties of Exponential Phase for Four Media

Name of the media	Duration of exponential phase (days)	Concentration at the end of exponential phase (g/L)
CH	13	0.060501
Modified CH	15	2.8395
BB	16	0.0618
Modified BB	14	1.7104

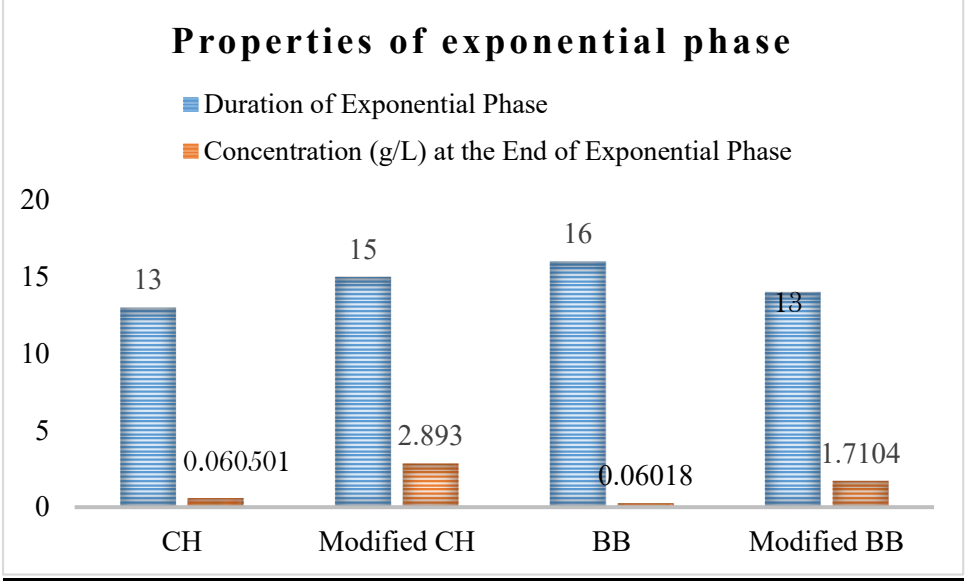


Figure 29: Properties of Exponential Phase

4.6 Specific Growth Rate (Overall), Doubling Time and Volumetric Biomass Yield

To draw a comparison among the four culture media used, three decisive parameters, namely- Overall Specific Growth Rate, Doubling Time and Volumetric Biomass Yield were calculated.

These parameters for each medium were calculated using values of the biomass concentration at the beginning and end of the study period.

The technique has been described earlier in the literature review section.

The formula used for calculation of overall specific growth rate is-

$$\mu_{overall} = \frac{Log10\ N_{end} - Log10\ N_o}{t_{end} - t_o}$$

Where, $T_{end} - T_0$ = Duration of study period (days)

N_{end} = Biomass concentration at the end of study period (g/L)

N_0 = Biomass concentration at the beginning of study period (g/L)

The formula used for Doubling Time is-

$$G = \frac{Log\ 10_2}{\mu_{overall}} \times 24$$

Or $$G = \frac{0.301}{\mu_{overall}} \times 24$$

Where, $\mu_{overall}$ = overall specific growth rate

The formula used for calculating the volumetric biomass productivity is

$$P_{Biomass}\ (g\ L^{-1}d^{-1}) = (X_2 - X_1).(t_2 - t_1)^{-1}$$

Where, X_1 and X_2 are dry cell weight concentrations of Chlorella vulgaris in gL^{-1} on days t_1 (start point of cultivation) and t_2 (endpoint of cultivation) respectively.

Table 24 and Figure 30-32 show a comparative representation of these values.

Table 24 Calculation of Specific Growth Rate, Doubling Time and Volumetric Biomass Yield

	Biomass Concentration at day 0, X_0 (g/L)	Biomass Concentration at day 27, X_{27} (g/L)	Study Period (days)	Overall Specific Growth Rate, μ (d^{-1})	Doubling Time, G (hours)	Volumetric Biomass Yield (g L^{-1} d^{-1})
CH	0.056	0.06223	27	0.00391	1847.57	00002
Modified CH	0.0756	2.9412		0.135597	53.276	0.106
BB	0.0580	0.06246		0.00274	2636.49	0.00017
Modified BB	0.0652	1.9594		0.040754	177.26	0.005

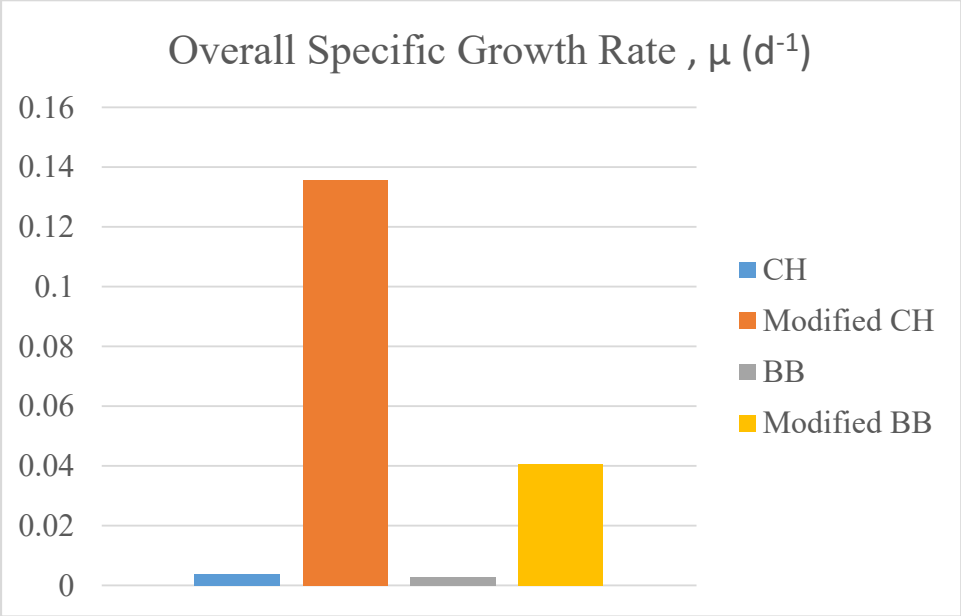


Figure 30: Comparison among Overall Specific Growth Rate of CH, Modified CH, BB and Modified BB

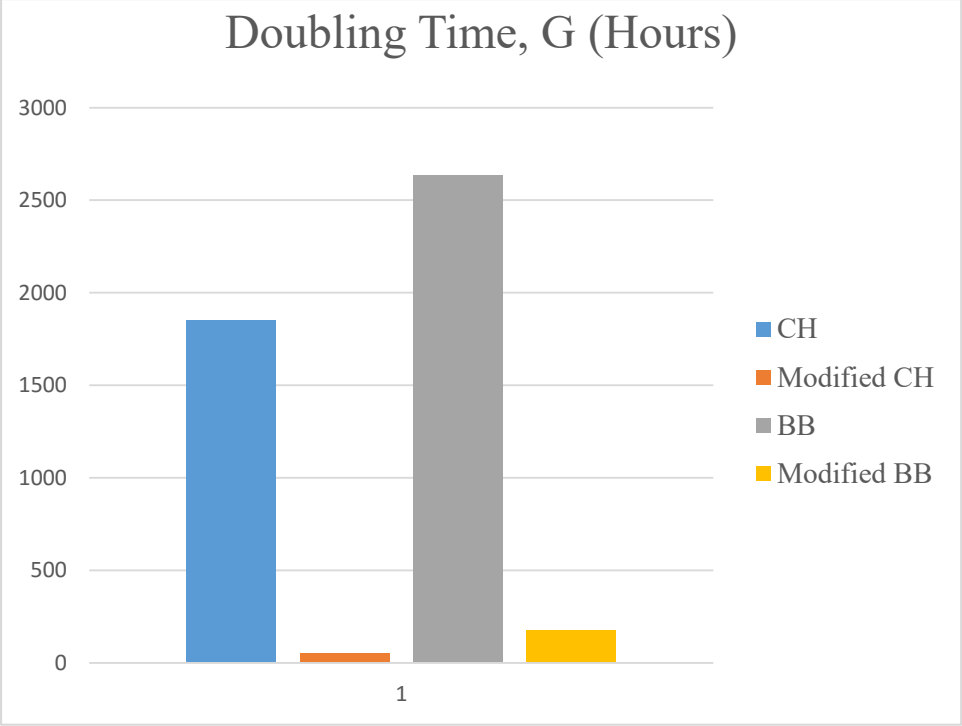


Figure 31: Comparison among Doubling Time of CH, Modified CH, BB and Modified BB

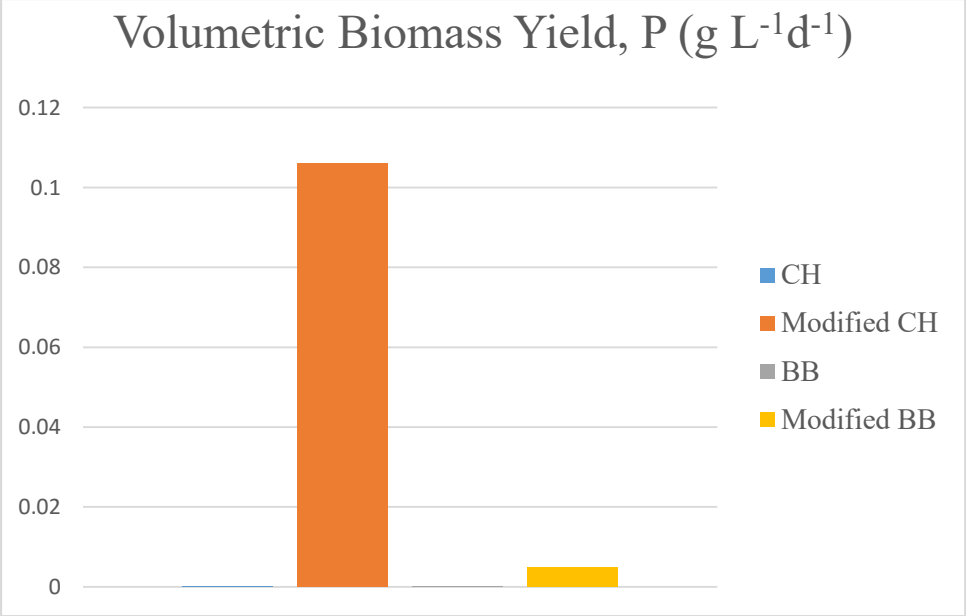


Figure 32: Comparison among Volumetric Biomass Yield of CH, Modified CH, BB and Modified BB

4.7 Selection of Suitable Growth Media

The criteria for selecting the suitable growth media are-

- Short exponential phase with high yield
- High specific growth rate
- Low doubling time and
- High volumetric biomass yield

Considering all the criteria, it can be seen from the above study and consequent analyses, that the Modified CH media is the most suitable media for growing *Chlorella vulgaris,* a high lipid content algae in the local environment.

4.8 General Discussion

In this work a comparative study was done between a locally developed medium - CH and a universally used medium- Bold's Basal (BB) . In this work effect of vitamins on the growth was also studied. The result shows among the four media used, the best result is obtained for modified CH medium, which leads to a conclusion that modified CH is the most suitable one among the four to grow *Chlorella vulgaris* in local environment. However, the obtained result for overall specific growth rate for modified CH medium is 0.134 d^{-1} and the suggested value from literature is 1.92 d^{-1} at 25^{o}C. This discrepancy may occur due to human error or due to the prolonged adaptaibility of the strain with local environment. Whichever reason, further study needs to be conducted to reach a conclusive decision.

This study has proven to be a crucial one for choosing a suitable growth media for *Chlorella vulgaris*, because, Bold's Basal (BB) medium, which is a universally used medium for growing fresh water microalgae, like *Chlorella*, has shown unsatisfactory result in the local environment.

Chlorella vulgaris is a robust strain of microalgae. The strain does not get infected frequently by contaminating bacteria or fungus. This makes the strain a good contender for mass cultivation in the outdoor. However, during the study, it was also observed that the strain is vulnerable to changes in temperature and humidity.

From the study of growth using optical density, it can be seen that the culture did not reach stationary phase for BB and modified BB medium. However, from the growth curves generated from dry cell weight data, a clear stationary phase can be seen for both the media. The reason behind it can be the susceptibility of BB and modified BB medium to contamination. Both the CH and modified CH medium showed less susceptibility to contamination than BB and modified BB medium.

This work also proves that study on biological organisms like microalgae can be unpredictable sometimes. The slightest change in humidity can destroy the whole culture. Hence, while conducting a labscale study, it is very necessary to control every parameter. Contamination can sometimes occur from if any culture nearby gets contaminated. It is absolutely necessary to autoclave everytime and maintain a sterile environment to obtain satisfactory results in labscale.

Chapter 5 Conclusion and Future Work

To tackle the vicious cycle of development and environmental pollution, we need to find out a clean energy source like biodiesel. Microalgae has proven to be a potential candidate for biodiesel production. To make the whole process of biodiesel production from microalgae, economically viable, it is necessary to understand the growth kinetics of microalgae. Prior to going into mass production of a microalgae strain in local environment it is necessary to understand it's behavior. The study conducted in this tesis, shed light on how the microalgae *Chlorella vulgaris*, will behave in our local environment. This study also investigated the effect of various nutrient media on the growth of this strain, in the local environment of Bangladesh.

The results of this study, which was carried out in batch culture, show that composition of the nutrient media and addition of vitamins can affect the growth of the strain. According to the study, the nutreient medium shich showed the shortest exponential phase of 15 days, with highest yield of 2.84 g/L at the end of the exponential phase, is modified CH medium. The highest overall specific growth rate of 0.136 d^{-1}, lowest doubling time of 53 hours were also achieved for this medium. The highest volumetric biomass yield of 0.106 g L^{-1} d^{-1}, was obtained for modified CH media as well. Hence, according to the study, modified CH medium was found to be the most suitable medium for growing *Chloella vulgaris* in the local environment of Bangladesh. This study has proven to be a crucial one for choosing a suitable growth media for *Chlorella vulgaris*, because, Bold's Basal (BB) medium, which is a universally used medium for growing fresh water microalgae, like *Chlorella*, has shown unsatisfactory result in the local environment.

However, the performance of nutrient media could be improved by installing aeration and regular stirring. The effect of incorporating aeration and stirring on the growth of *Chlorella vulgaris* can be conducted as a future study.

Moreover, literature suggests that lipid accumulation in microalgae can be increased by starvation of nutrients. For future study it can be observed as well, whether nutrient media composition play any specific role in lipid accumulation.

Another suggestion based on the literature study is effect of temperature in lipid accumulation. Since the temperature in Bangladesh can vary between 20-35^{o} C, the effect of temperature on lipid accumulation and growth can also be studied. This can help to devise a plan to culture the microalgae all year long.

This study was conducted as a foundation for a bigger work which is the production of biodiesel. Understanding the growth kinetics of microalgae is a crucial part of the whole biodiesel production process. If this part is not optimized, it is not possible to come up with a feasible and economical plan for biodiesel production from microalgae.

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Appendix

Appendix A: Working Procedure of a Spectrophotometer

Spectroscopy is the study of how electromagnetic energy interacts with matter. There are many types of spectroscopy and they are used to detect, identify and quantify data about material samples as gases, liquids and solids. As such, spectroscopy procedure is used to determine both the chemical composition as well as measure the physical properties of matter.

The system focuses electromagnetic energy from the light source onto the material sample. Depending upon the system configuration, light is either reflected off the sample or transmitted through it. After the light is collected from the sample, it its focused onto the entrance slit of a monochromator. The wavelength is set to a specific value for the compound being measured. The wavelength is determined by the maximum absorbance of that compound.

A typical spectrophotometer consists of a light source, a wavelength selector (monochromator), a cuvette containing the sample and a detector.

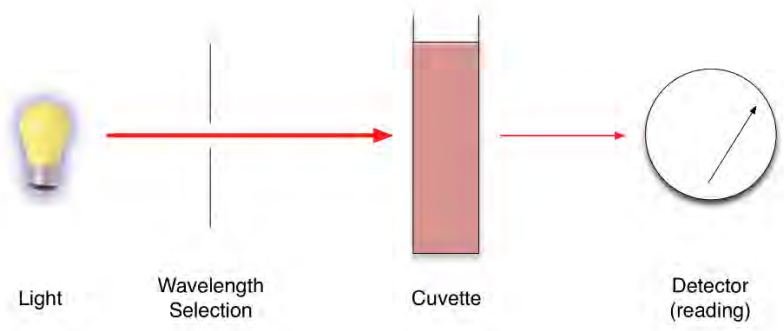

Figure 33 : A typical spectrophotometer

In UV/ Vis/ NIR spectroscopy the ultraviolet (170 nm to 380 nm), visible (380 nm to 780 nm), and near infrared (789 nm to 3300 nm) are used.

Spectrophotometers are designed to transmit light of narrow wavelength ranges. A given compound will not absorb all wavelengths equally- that is why things are of different colors. Since different compounds absorb light at different wavelengths, a

spectrophotometer can be used to distinguish compounds by analyzing the pattern of wavelengths by a given sample. Additionally, the amount of light absorbed is directly proportional to the concentration of absorbing compounds in that sample, so a spectrophotometer can also be used to determine concentrations of compounds in solutions. Finally, because particles in suspension will scatter light (thus preventing it from reaching the light detector), spectrophotometers may also be used to estimate the numbers of cell in suspension.

When studying a compound in solution by spectrophotometry, the sample is put in a sample holder called a cuvette and placed in a spectrophotometer. Light of a particular wavelength passes through the solution inside the cuvette and the amount of light transmitted (transmittance) or absorbed (absorbance) by the solution is measured by a light meter.

Since other compounds in a solution (or the solvent itself) may absorb the same wavelengths as the compound being analyzed, the absorbance of the test solution is compared to a *reference blank*. Ideally, the *reference blank* should contain everything found in the sample solution except for the substance being analyzed or measured.

The amount of light transmitted through a solution is referred to as *transmittance* (T). the transmittance is defined as the ratio of the light energy transmitted through the sample (I) to the energy transmitted through the reference blank (I_O). Since the compound being tested is not present in the reference blank, the transmittance of the reference blank is defined as 100% T.

$$T = \frac{I}{I_o}$$

This number is multiplied by 100 to determine percent transmittance (%T), the percentage of light transmitted by the substance relative to the reference blank.

$$\%\,T = {}^{I}/_{I_o} * 100$$

For most biological applications however, absorbance is measured (A_λ, also referred to as optical density or OD_λ, where λ is the wavelength used for the measurements), the amount of light absorbed by a solution. Absorbance is related logarithmically to transmission thus,

$$A = -logT$$

Optical density (OD), also known as absorbance or turbidity, is used frequently as a rapid and non- destructive measurement of biomass in cultures of bacteria and other unicellular

microorganisms. The amount of light absorbed by a suspension of cells can be related directly to cell mass or cell number.

Selection of wavelength:

The wavelength is set to a specific value for the compound being measured. This wavelength is determined by the maximum absorbance of that compound.

The graph below shows the absorption spectra for Nicotinamide adenine dinucleotide (NAD).

At 260 nm both oxidized (NAD+) and reduced (NADH) absorb the light. However, at 340 nm only the reduced NADH will absorb. This means that in a reaction that is producing NAD, the rate of reaction can be recorded by measuring the appearance of NADH at 340 nm. However, if the measurement is carried out at 260 nm, no or very little change may be seen as the absorbance due to NAD+ would decrease as it gets converted to NADH.

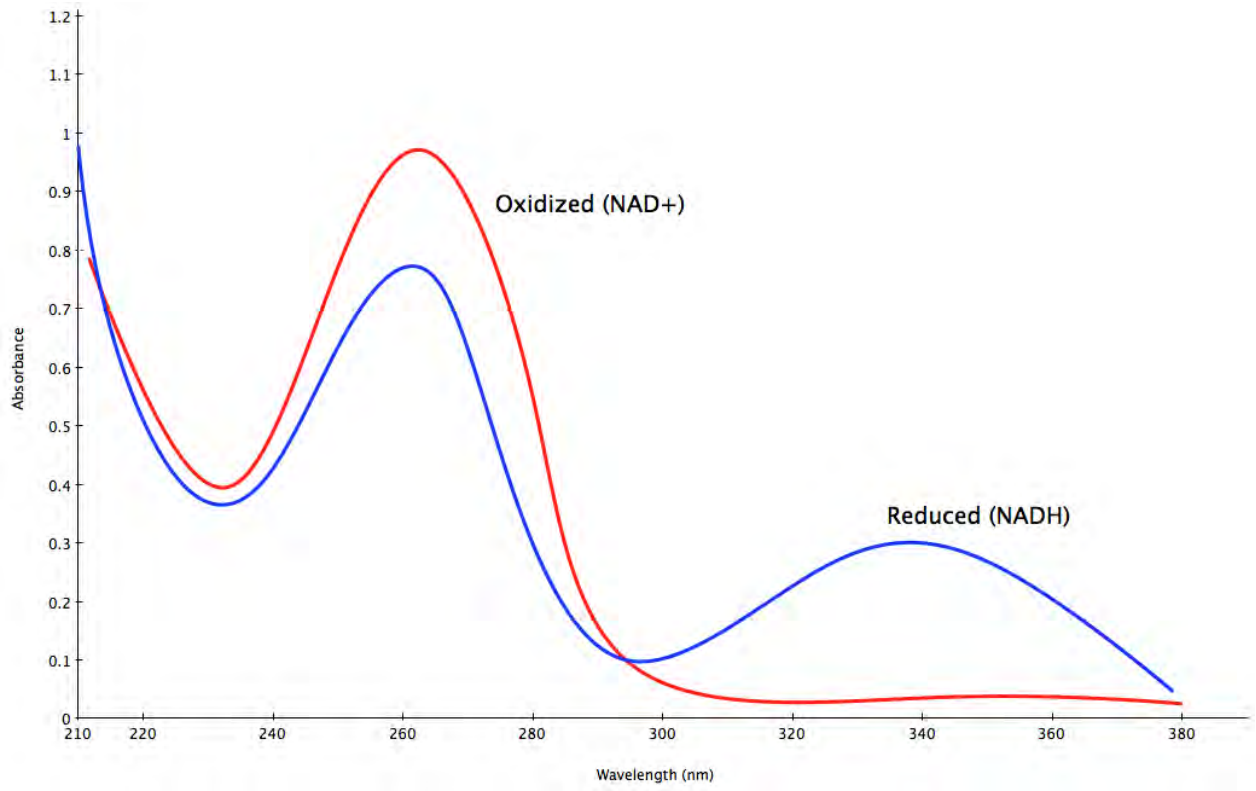

Figure 34: Absorption spectra for Nicotinamide adenine dinucleotide (NAD)

Pigmented samples can bias absorbance, as the color absorbs in a particular region of the light spectrum. This can affect measurement of cell concentration by OD in pigmented cells.

Microalgae have relatively high pigment contents, consisting of mainly chlorophylls and carotenoids. Pigment content can vary from 0.1 to 9.7% of the wet biomass.

Algal culture media are usually clear and there is little consensus in the literature about which wavelength to use for microalgal cultures. Absorbance by pigment is greater in certain parts of the light spectrum. Choosing a wavelength within the maximal absorbance range of the pigment is expected to give the largest signal. The use of wavelengths within the maximum absorbance range of chlorophyll a (400-460 nm and 650-680 nm) has been reported frequently.

Appendix B : List of Publications

Publication 1

Article Title: Algae in a Vat may Power the Future: A Review on Algal Biodiesel Production

Name of the Book: BIOFUELS – Advances and Perspectives

Authors: Farid Ahmad, Anika Ferdous and Mohidus Samad Khan

Publication 2

Article Name: Studying Growth Kinetics of *Chlorella vulgaris*, a Microalgae with High Lipid Content, to Produce Biodiesel in Local Condition

Journal Name: Journal of Natural Science and Sustainable Technology, Volume 11, Number 4. ISSN: 1933-0324. Nova Science Publishers, Inc.

Authors: Anika Ferdous, Farid Ahmed, Md. Shajid Khan, John Liton <unshi, Chapol Kumar Roy, Husna Parvin Nur and Mohidus Samad Khan.

Publication 3

Article Name: Study of Growth Kinetics of High Lipid Content Algae in Local Environment

Name of the Conference: Abstract accepted in **ICChE 2020.**

Authors: Anika Ferdous, Md. Mursalin Rahman Khandaker, Farid Ahmad, John Liton Munshi and Mohidus Samad Khan.