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Lipid Production by Scenedesmus Dimorphus: Comparison of Extraction Techniques and Effect of Sodium Nitrate Concentration

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LIPID PRODUCTION BY *SCENEDESMUS DIMORPHUS:* COMPARISON OF EXTRACTION TECHNIQUES AND EFFECT OF SODIUM NITRATE CONCENTRATION

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LIPID PRODUCTION BY *SCENEDESMUS DIMORPHUS* COMPARISON: OF EXTRACTION TECHNIQUES AND EFFECT OF SODIUM NITRATE CONCENTRATION

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ABSTRACT

Exhaustion of fossil fuels, negative effects of excess carbon dioxide in the atmosphere and high gas prices have all made it very crucial to find alternative and renewable sources of energy. Scientists are studying biological and physiological potential of microalgae as an alternative source for biofuel production. Microalgae have shown to be more economical among other crops including corn, sunflower and so on. They are also able to reduce industrial carbon dioxide by consuming it through the growth process and can be grown almost everywhere. Microalgae also have high growth rate which means higher production in a shorter time. They need less land compare to other crop for the same amount of lipid or oil. Among more than 100 species of microalgae, one with great lipid potential is *Scenedesmus dimorphus*.

In this study we developed and evaluated five different permeabilization methods to determine the best lipid extraction method to produce more lipids from *S. dimorphus* biomass. The methods which were used in this research include wet-pellet, freeze-dry, sonication, grinding and freeze-thaw. Our results show that the most promising method is

the grinding method using mortar and pestle, followed by solvent extraction in hexane/isopropanol (3:2, v/v). Different types of batch experiments were performed to determine growth parameters such as growth rate (μ), yield ($Y_{x/s}$) and lipid content (Y). The effect of sodium nitrate concentration on growth rate and lipid content was also studied using a fed-batch experiment with constant concentration of sodium nitrate. This could be resulted that the maximum content of lipid occurred in the medium with 0.00 g $NaNO_3$/L (flask 4). Data shows that flask 3 (0.07 g $NaNO_3$/L) has the highest growth rate among the flasks (μ=0.47 day $^{-1}$). The results are helpful to better understand the effect of nutrients on lipid content.

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CHAPTER I

INTRODUCTION

The exhaustion of fossil fuels, the uncertainties in the oil markets, and global warming as a result of increasing CO_2 from burning fossil-based fuels, are among the major challenges of the world in the current century. One of the ways that scientists have tried to address these issue is to introduce new and renewable sources of energy that can substitute for traditional fossil fuels used for transportation. These renewable alternative energy sources might include plants, algae and animals fat. Specifically, the fat (also called "lipids") from these sources can be reacted with alcohols to produce biodiesels which can then be used as an alternative fuel. The ever growing demand for energy, especially in the transportation industry, makes an even stronger case towards developing biodiesel. Among different sources of biofuels, biodiesel production from algae with its high growth rate and high lipid content has shown to be very attractive.

In the United State alone more than 50% of the oil is imported each year. Considering the average cost of imported oil, which is currently above $110 per barrel, the United States spent over $460 billion on imported foreign oil in 2011, and

consequently the investment of these funds in green energy like biofuels instead of imported oil, could have significant benefits (Jon 2012). In order to end this ever increasing cost and combat global warming, examination of biofuels as an alternative energy source is even more justified. Many scientists are now studying photosynthetic organisms such as plants and algae that can convert sunlight and CO_2 into biomass for such purpose.

Based on an estimate from the United States Department of Energy, if algae-based fuels replace all the petroleum fuel in this country, it would only need 15,000 square miles which is less than 0.42% of the U.S soil (Hartman 2008) and less than 1⁄7 the area of corn harvested in the United States in 2000 (Dyer 2008). Algae can produce up to 300 times more oil per acre than other crops such as corn, rapeseed, palm, and soybean.

Microalgae are good sources for biofuel production for many reasons. They have high photosynthetic biomass rates; can be grown in many different environments including saline water or even in the deserts; have high productivity and higher lipid content compared to other crops (up to 80% of dry weight) (Huang 2010); and can be used to control and reduce carbon dioxide from industrial sources by consuming it through its growth process. Also some species of microalgae have fast growth. The faster they grow, the more lipids can be produced which also makes them potentially economically justifiable. Among different sources for biofuel production, algae have one of the highest lipid contents.

There are almost 300,000 species of algae in the world. They are photosynthetic and consume CO_2 in the presence of sunlight to produce their own nutrition. Most of the

current researches in biofuel are focused on algae due to the high growth rate and high lipid content with the goal of replacing traditional oil crops for biofuel production (Table 1.1).

Table 1.1. Comparison of algae with other oil-producing crops (Riesing 2009).

Crops	**Oil yield (gallon/acre)per year**
Corn	18
Soybeans	48
Safflower	83
Sunflower	102
Rapeseed	127
Oil Palm	635
Microalgae	5000-15000

There are many species of microalgae in the world that potentially can be used as biofuel. The chemical compositions of a number of different microalgae strains are shown in Table 1.2. Among different characteristics of these microalgae species, lipid content is the most important since the lipids are ultimately converted to the biodiesel. As is shown in this table, *Scenedesmus dimorphus* have the highest lipid content (16-40% dw) among the reported species, and is considered a strong candidate for biofuel production. This is a green and unicellular microalgae species and can be found in

colonies of 1, 2, 4 or 8 cells (Figure 1.1). It has been shown that it can reach maximum concentration of 1.3 gdw/L in 17 days of culture (Ying Shen 2009).

Table 1.2. Chemical composition of various microalgae species on dry matter basis (%)(Becker 1994).

Strain	**Protein**	**Carbohydrate**	**Lipid Content (% dw)**	**Nucleic Acid**
Scenedesmus obliquus	50-56	10-17	12-14	3-6
Scenedesmus quadricauda	47	-	1.9	-
Scenedesmus dimorphus	8-18	21-52	16-40	-
Chlamydomonas rheinhardii	48	17	21	-
Chlorella vulgaris	51-58	12-17	14-22	4-5
Chlorella pyrenoidosa	57	2	2	-
Spirogyra sp.	6-20	33-64	11-21	-
Dunaliella bioculata	49	4	8	-
Dunaliella salina	57	32	6	-
Euglena gracilis	39-61	14-18	14-20	-
Prymnesium parvum	28-45	25-33	22-38	1-2
Tetraselmis maculata	52	15	3	-
Porphyridium cruentum	28-39	40-57	9-14	-
Spirulina platensis	46-63	8-14	4-9	2-5
Spirulina maxima	60-71	13-16	6-7	3-4.5
Synechoccus sp.	63	15	11	5
Anabaena cylindrica	43-56	25-30	4-7	-

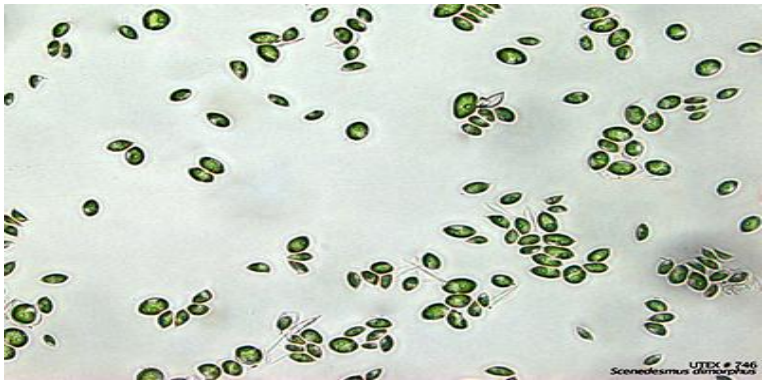


Figure 1.1. *Scenedesmus dimorphus* image (Hegewald 1982).

Biofuel industrial development is challenged by the high costs and high energy usage in the growth, harvesting, and oil extraction processes. In the process of developing biofuels from microalgae, extracting hydrocarbons from dried biomass is a critical step. The techniques used for lipid extraction and quantification at the lab-scale and for research/analytical purposes, are very different from those used purely for extraction/recovery at the industrial-scale. Lipid quantification generally requires two distinct steps: biomass permeabilization, and lipid extraction. Biomass permeabilization involves breaking the cell walls to expose the lipids for removal. There are different permeabilization methods for microalgae, such as use of sonication, a bead-beater, freeze-drying, and grinding. The extraction methods can be divided into three categories: physical (cold press), chemical (extraction with solvents) and combination of both processes (Lee 2010). Among these, solvent extraction is still the main method of choice, because it is simple and relatively inexpensive compared to other methods. One of the purposes of this research is to determine the best analytical technique for quantification

of lipids from the microalgae *Scenedesmus dimorphus*, using commonly available equipment that is convenient for laboratory use.

Methods for increasing the lipid content in microalgae are important for improving the economic potential of the biofuel industry. Enhancement of lipid content by nutrient limitation is one such method. Nitrogen sources, such as sodium nitrate, are necessary for biomass production, and have a significant impact on the amount of biomass produced. When some of these key nutrient concentrations are limited, algal growth rate declines, but in some species, active biosynthesis of fatty acids is maintained when adequate light and carbon dioxide are provided. Nutrient starvation is one of the most common lipid induction techniques and sodium nitrate is one of the single important nutrient affecting lipids in algae. It has been tested on several green microalgae, such as *S. dimorphus*, and all of them showed a significant rise in lipid content.

The main objectives of this research are:

1) To investigate the effect of several lab-scale methods on lipid recovery from *S. dimorphus*. In this work, we compared various methods for permeabilizing the biomass prior to extraction, including freeze-drying, sonication, and freeze-thawing the wet pellet; and grinding of the dry pellet using mortar and pestle. Following permeabilization, hexane/isopropanol (3:2, v/v) was used as a solvent to extract lipids from the biomass. Two phases resulted; a top layer contains the solvent and lipids, and the bottom liquid layer containing the residual biomass. The quantity of lipids obtained was determined gravimetrically following solvent

evaporation. Results were analyzed for precision, effectiveness, and convenience.

2) To measure the kinetics of sodium nitrate effect on algae growth and lipid content and yield. In this research the fed-batch experiment was performed using sodium nitrate as the limiting nutrient in four different flasks with four different sodium nitrate concentrations. The samples were taken from each flask during the experiment and the lipid content and growth rate were measured to determine the effects of the sodium nitrate concentration.

This thesis provides the evidence for the selection of reproducible and practical lipid quantification technique that can be used in a general laboratory. The time-course of lipid enhancement by nutrient starvation is important for determining kinetic models of this process and estimation of kinetic parameters. These kinetic models are essential for large-scale bioreactor system modeling and optimization.

CHAPTER II
BACKGROUND

2.1 Methods for Small- scale Lipid Extraction

Hundreds of different strains of microalgae have been discovered and their lipid contents have been measured and reported (Sheehan 1998). Extractions from a variety of microalgae, both fresh water and marine, have yielded lipid contents in the range of 1 to 40% (Becker 1994). The improvement of methods of lipid extraction has resulted in an increase in measurable lipid content.

2.1.1 Permeabilization

Permeabilization is the process of breaking algae cell walls to separate lipid from the rest of the algae biomass. Algal cell walls contain polysaccharides or glycoproteins or both. When the algal cells are not subjected to permeabilization, only about 3.8% of lipids were extracted. This confirms that the cell wall of microalgae has to be broken down to bring a contact between solvent and lipids (Soštarič 2012). It has been shown

that breaking the cell walls by permeabilization minimizes contamination from external sources and preserves the chemical integrity of the substances within the cells (Mercer 2011). Permeabilization targets the micro algal biomass, which ruptures cell walls and releases the oil, and when permeabilization is used along with solvents to recover oil, it is most effective among other methods.

There is no standard protocol for microalgae permeabilization. However, there are general fractionation methodologies for lipid extraction. There are two common ways to break the algal cells: physical and chemical methods (Widjaja 2009). The physical method can be performed on either the solid, or dry biomass, or in the liquid state. In the solid method, the plant tissue is crushed between a mortar and pestle. The liquid method involves the use of biomass in the liquid medium, which can be performed by various methods such as sonication, freeze-drying, and freeze-thawing. The chemical methods are achieved by using organic solvent. An appropriate lipid extraction method is key to increase the measured value of lipid. To disrupt the cell walls using solvent extraction, various techniques such as (a) microwave, (b) bead beating, (c) sonication, (d) lyophilization, (e) grinding with a pestle and mortar have been used. For instance in microwave method, the high frequency waves break or shatter the cells. This method has been suggested for vegetable oil extraction (Cravotto 2008). In bead beating, beads break the cell walls by mechanical forces due to high speed of bead movement (Lee 1998).

A study by Ying Shen (2009) was done on *Scenedesmus dimorphus* and *C-protothecoides*. At the end of culturing, lipid of these algae biomass was extracted by five different methods: (1) direct extraction with a Soxhlet extractor; (2) sonication; (3) French press; (4) bead beater; and (5) wet milling. A soxhlet extractor is a laboratory equipment to extract lipid from a solid material. The french press ruptures the cells by passing them through a narrow tube under high pressure. The bead beater uses beads to disrupt the cell wall to make the lipid accessible. Sonication was performed twice and each time for 2 minutes. French press applied at 1500 psi for 5 runs and bead beater ran with 1 mm glass beads for 2-minute runs and wet milling for 3 times. After disrupting the biomass under different permeabilization methods, ethanol/hexane (1:1, v/v) and hexane were added to each sample separately. For all five methods, the same solvent solution was used as well. The biomass with solvent tubes was then left on the shaker overnight. After centrifugation, the supernatant was separated and then dried in oven at 95^0C for 1.5 hour. The lipid content of both microalgae strains, *S. dimorphus* and *C.protothecoides,* which were recovered using these five different permeabilization methods with ethanol/hexane (1:1, v/v), is shown in Figure 2.1. Base on their results the wet milling followed by hexane extraction had the highest lipid content for *S. dimorphus* and bead beater had the highest lipid content for *C.protothecoides*. The significant differences between these two species in size and shape may account for the difference in their response to permeabilization techniques.

Using wet milling as a permeabilization method has some advantages including high lipid recovery rate, easy operation, low cost process and maintenance. Other methods such as soxhlet, sonication, French-press and bead beater, which were used in this study, are either expensive or yielded low lipid content.

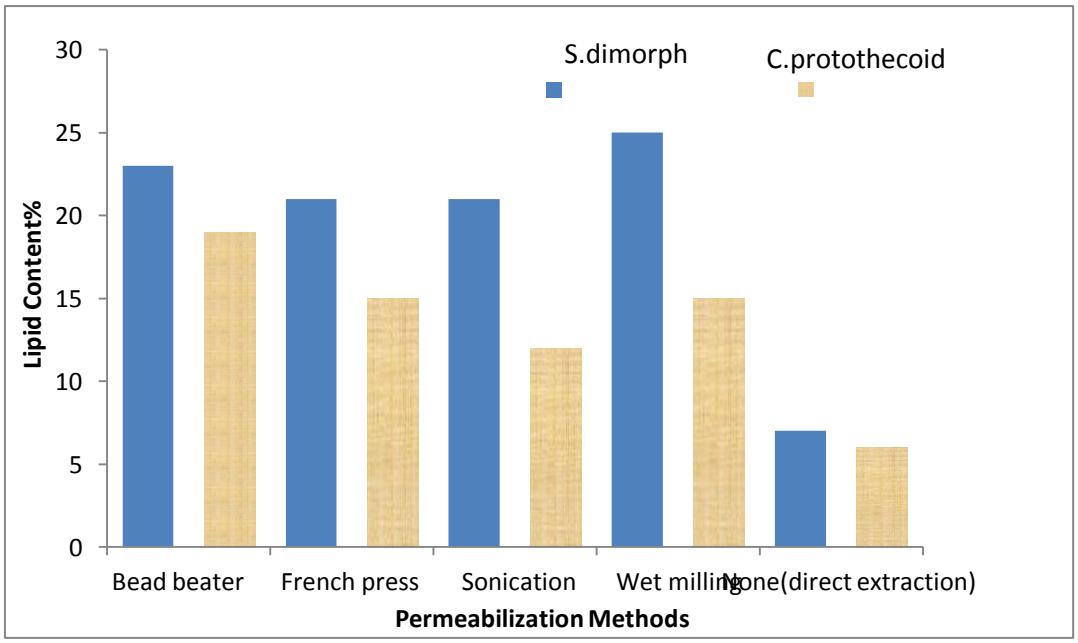


Figure 2.1. Effect of extraction methods on lipid contents of *C.prototheco ides* and *S.dimorphus* (Ying Shen 2009).

Lee et al. (2010) conducted several permeabilization experiments on three different microalgae species; *Butryococcus braunii*, *Chlorella vulgaris* and *Scenedesmus sp*., to identify the most effective method. In their work, five different methods including autoclaving at 125^0C and 1.5 MPa, bead-beating with beads of 0.1 mm diameter at a high speed of 2800 rpm, microwave oven at 100^0C and 2450 MHz, and sonication at 10 KHz were implemented. All experiments were done using 5 minutes of processing, and the solvent system of choice was chloroform/methanol (1:1, v/v). As shown in Figure 2.2, it is clear that without any cell disruption, lipid content is low. Their results showed that

highest lipid content was consistently obtained from each of the three species when using the microwave method. Bead beating was nearly as effective as microwave method. Sonication routinely yielded the lowest recovery, while results with the autoclave were mixed.

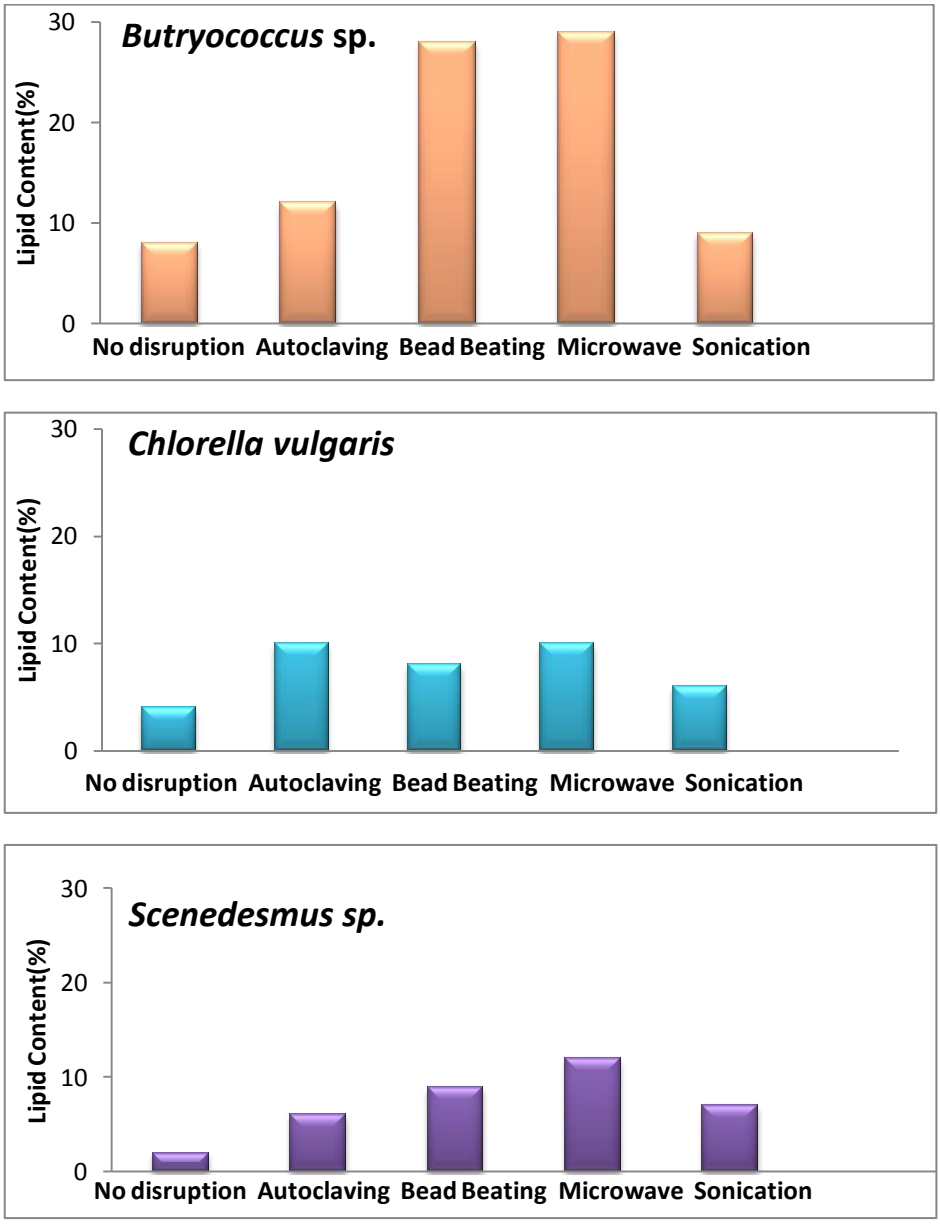


Figure 2.2. Effects of different methods on lipid content on three different microalgae species including *Butryococcus braunii*, *Chlorella vulgaris* and *Scenedesmus sp*. by using solvent solution chloroform/methanol (1:1, v/v) (Lee 2010).

The same study (Lee 2010) also showed that the bead beating method resulted in higher lipid content from *Butryococcus braunii* than sonication, french press and lyophilization methods. Overall, the microwave method was shown to be the most effective among all the permeabilization methods in this study. Microwaving was also suggested to be advantageous in that it is simple and inexpensive.

In another study, Sostaric et al. (2012) studied the effect of different permeabilization methods on lipid content in the microalgae C*hlorella vulgaris*. The permeabilization methods that were used in this study were either single or combinations of: grinding in a mortar, microwave oven, and sonication. All of these methods were applied on lyophilized biomass by drying them for 24 hours at 70^0C and Lipids were then extracted using chloroform/methanol (1:2, v/v) solvent solution. The microwave method was done at 1000 watt for 2.5 minutes and sonication was performed in ultrasound bath at 10 KHz for 5 minutes. In their method, after adding solvent, all the mixtures were sonicated for another 30 minutes. The results (Table 2.1) show that when the cells are subjected to permeabilization, larger amounts of lipid can be recovered. Also they suggested that the combination of permeabilization methods results in higher lipid content.

Table 2.1. Lipid content (%) using different permeabilization methods on *chlorella vulgaris* microalgae (Sostaric 2012).

Permeabilization Methods	**Lipid Content (%)**
Without cell disruption	3.80
Grinding in a mortar	8.51
Grinding + microwave	9.59
Grinding + microwave + sonication	9.82

2.1.2 Solvent extraction

The choice of solvent for lipid extraction is very important for microalgae since the extraction efficiency of solvent depend on type of organism and cell wall permeability. Different combinations of solvents have been proposed to extract lipids from microalgae. For instance, hexane/ isopropanol have been used for tissue (Hara 1978); DMSO/petroleum ether for yeast (Park 2007); hexane/ethanol for microalgae (Cartens 1996); and hexane/isopropanol for microalgae (Nagle 1990). The hexane system has shown to be an attractive system in solvent extraction processes because hexane and alcohol will separate into two separate phases when water is added, which leads to more efficient separation process. Methanol and chloroform are toxic thus selecting ethanol or isopropanol seems reasonable as well. Other than using hexane/ethanol in solvent extraction systems, solvent systems such as 1-Butanol or ethanol have also been shown to be applicable (Grima 1994).

Samori et al. (2010) studied lipid extraction from freeze-dried samples of *Botryococcus braunii* microalgae in four different solvent systems (n-hexane and chloroform/methanol (2:1, v/v); DBU (1, 8-diazabicyclo-undec-7-ene); DBU/ethanol; and DBU/octanol at 60^0C for 4 hours. They also investigated the effects of different solvents using wet biomass. They demonstrated that DBU/octanol system had the highest lipid recovery than the other solvent systems on both dried and liquid samples.

In a study by Mulbry et al. (2009) on green algae *Rhizoclonium hieroglyphicum*, optimization of lipid extraction process was applied. In their experiment, dried biomass

was ground in a Wiley Mill to produce 3 mm particles which then were stored in sealed plastic bags at 20-25^{0}C. To extract lipid from dried biomass, they used two different methods: accelerated solvent extraction (ASE) and conventional extraction method. In ASE method, solvents tested included chloroform/methanol (2:1, v/v), isopropanol/hexane (2:1, v/v) and hexane. In the other method, lipid content was calculated by using a modified Folch method (Folch 1957) and chloroform/methanol (2:1, v/v) was used as a solvent solution. The results yielded significantly higher lipid contents when using ASE method with chloroform/methanol and isopropanol/hexane. The results showed that the manual method yielded 46-55% of total lipid but the ASE extractions with chloroform/methanol yielded 78-81% of total lipid and with isopropanol/hexane 78-84% of lipid and ASE with hexane was even higher compared to those other solvents and up to 87-92% lipid extraction.

Lee et al. (1998) studied a rapid method for extraction of lipid from the green algae *Botryococcus braunii*. This microalgae was grown in a photo-bioreactor and after 2 weeks of incubation, the algal cells were harvested by centrifugation at 8000 g for 10 minutes. The permeabilization of algal cells was then performed by: direct extraction using solvent only, sonication, homogenization, French press, bead beater and lyophilization, and five different solvent solutions widely used for lipid extraction including chloroform/methanol (2:1, v/v), hexane/isopropanol (3:2, v/v), dichloroethane/methanol (1:1, v/v), dichloroethane/ethanol (1:1, v/v) and acetone/dichloromethane (1:1, v/v).

The effect of solvent systems on lipid extraction is shown in Figure 2.3. It shows that the most effective solvent system was chloroform/ methanol which has lipid content of 28.6% (dw).

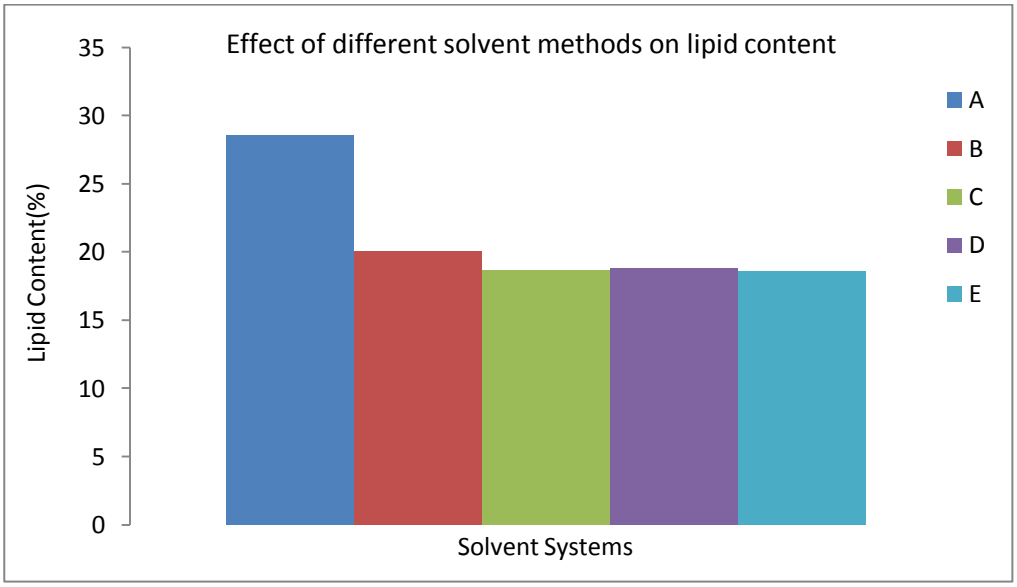


Figure 2.3. Effects of different solvent systems on lipid extraction from *Botryococcus braunii*.A, chloroform/methanol (2:1, v/v); B, hexane/isopropanol (3:2, v/v); C, dichloroethane/methanol (1:1, v/v); D, dichloroethane/ethanol (1:1, v/v); E, acetone/dichloroforme (1:1, v/v) (Lee 1998).

Long et al. (2011) studied the effect of different solvent systems for efficient lipid extraction from *Nannochloropsis* microalgae. The dried samples of *Nannochloropsis* were grounded to fine powder then solvent extraction methods were used to determine lipid content. The solvent extraction methods were based on Bligh and Dyer protocol (Bligh 1959), Folch et al. method (1957) and a mixture of hexane/isopropanol as described earlier (Hara 1978). For Bligh and Dyer and Folch protocols, a mixture of chloroform/methanol solvent with a ratio of (1:2, v/v) and (2:1, v/v), respectively, were used. In their experiments, dried algae was mixed (approximately 5 g mass) with 100 mL

of solvent and left over night in solvent. It appears that the Folch method (27.1%) resulted in slightly better results than the Bligh and Dyer method (24.8%) and hexane/isopropanol (17.5%). In this study the chloroform/ methanol method had the highest lipid content for the dried microalgae at room temperature.

Since the choice of solvent system is very important for lipid extraction from algae and it also varies between algae strains, in this research, hexane and isopropanol were chosen to be used as the solvent of choice. Hexane is the most popular chemical for solvent extraction which is relatively safe (can be toxic of inhaled), cheap, largely flammable and a volatile non polar solvent. It is an organic solvent and good alternative for hydrocarbons extraction (Gouveia 2009). Hexane isomers are un-reactive; they are used as inert solvents in organic reactions because of their non polarity (Wikipedia 2012). Alcohols are also very good solvents for most lipids and one of its most popular and common one is isopropanol which is colorless and flammable with a strong odor with molecular formula of C_3H_7OH. It dissolves in a wide range of non-polar compounds. It is non toxic and evaporates quickly (Wikipedia 2012). Isopropanol was used in addition to hexane to dissolve lipids or oils. In general, the hexane/isopropanol is a good and low-toxicity solvent.

2.1.3 Lipid Induction by Nitrogen Limitation

Lack of nutrients such as nitrogen limitation is an efficient way to increase lipid content as has been reported during past decades by many other researchers. Generally lipid accumulation happens under nutrient limitation when light and carbon dioxide are available. Microalgae remove nitrogen from medium by up taking it into algal biomass

(Aslan 2006). One of the most widely studied genuses of microalgae for nitrogen uptake is *Scenedesmus* (Jing Shi 2007). The concentration of nitrogen is seemed to be a fundamental factor and has a direct influence on algal growth and lipid accumulation. Each species of microalgae produces different ratios of lipids. These tiny organisms are able to manipulate their metabolism through changes of the chemical composition of the medium (Behrens 1996). For example, with low nitrogen and iron-supplemented medium, *Chlorella vulgaris* has lipid contents of 40% and 56.6%, respectively (Illman 2000).

Ying Shen et al. (2009) also studied the effect of nitrogen on lipid content of *S. dimorphus* and *C.prototheco*ides microalgae. They chose these two strains because of their well-studied characteristics and identified them as good candidates (Renaud 1994; Xiong 2008). The algae were inoculated into sterilized MB medium; KNO_3 was replaced by urea, nitrate and glycine as different sources of nitrogen. The system used in this experiment was a fed-batch system. The effects of nitrogen source on these microalgae are shown in Table 2.2 and 2.3. As shown in Table 2.2, the maximum biomass and lipid content achieved by *S. dimorphus* in 17 days were in high nitrate and urea concentrations, and low glycine. The lipid yields in urea were at least 38% higher than in nitrate medium and 48% higher than glycine medium. The effects of nitrogen sources on *C.prototheco*ides were shown in Table 2.3. It shows that in contradiction to *S. dimorphus* strain, *C.prototheco*ides produced more lipids in lower concentration of nitrogen and it is also concluded that nitrate was the best source of nitrogen for lipid production. Maximum lipid content in nitrate media was 103% higher than urea media and 38% higher than yeast extract media.

Table 2.2. Effects of nitrogen on biomass dry weight (DW) and LY of *S. dimorphus*

Nitrogen source	Nitrogen concentration	Biomass (DW) (g/L)	Lipid yield*(g/L)
Nitrate	Low (A-1)	0.9 ±0.1	0.21 ±0.01
	Medium (A-2)	1.0 ±0.1	0.22 ±0.01
	High (A-3)	1.2 ±0.1	0.29 ±0.01
	Low (A-4)	1.0 ±0.1	0.32 ±0.01
Urea	Medium (A-5)	1.1 ±0.1	0.31 ±0.01
	High (A-6)	1.3 ±0.1	0.40 ±0.01
	Low (A-7)	1.2 ±0.1	0.27 ±0.01
Glycine	Medium (A-8)	NS	NS
	High (A-9)	NS	NS

Note: *lipids were obtained by bead-beater disruption followed by hexane extraction; NS-algae did not survive.

Table 2.3. Effects of nitrogen on biomass DW and LY of *C. Protothecoides*

Nitrogen source	Nitrogen concentration	Biomass (DW) (g/L)	Lipid yield*(g/L)
	Low (H-1)	11.7 ±0.5	5.89 ±0.03
Nitrate	Medium (H-2)	11.2 ±0.4	4.57 ±0.02
	High (H-3)	12.8 ±1.2	4.46 ±0.02
	Low (H-4)	11.8 ±0.6	2.90 ±0.01
Urea	Medium (H-5)	2.5 ±0.4	0.60 ±0.01
	High (H-6)	6.9 ±0.3	1.66 ±0.01
	Low (H-7)	12.2 ±0.2	4.27 ±0.02
Yeast extract	Medium (H-8)	14.2 ±0.4	3.56 ±0.02
	High (H-9)	8.3 ±0.5	1.07 ±0.01

Note: *lipids were obtained by bead-beater disruption followed by hexane extraction.

Mandal et al. (2009) evaluated the relationships between critical parameters such as temperature, pH, nitrogen and phosphate lipid content. Different concentrations of nitrate (0.005-0.1 g/L) were used to study the effect of nitrate limitations on lipid accumulation on *S.obliquus* microalgae. Nitrate limitation was implemented by substituting KNO_3 in the medium with KCl with the same molar concentration. The maximum lipid production of 43% was achieved as a result of nitrogen deficiency when the cultures reached their stationary phase. Their results are in agreement with the report of Shifrin and Chisholm (1981) in which lipid content of up to 58.3% (dw) of *S.obliquus* was achieved.

Xin et al. (2010) also studied the effects of different nitrogen concentrations on the growth, nutrient uptake and lipid content of microalgae *Scenedesmus.sp*. To culture *Scenedesmus.sp,* the modified BGII medium was used in their experiments. The nitrate was used as the nitrogen source. In their experiments, five samples with initial concentrations of nitrate of 2.5 mg/L, 5 mg/L, 10 mg/L, 15 mg/L and 25 mg/L were prepared. The results showed that the maximum algal density increased with an increase in initial nitrogen concentration. The lipid content of dry weight was calculated and it was shown that, at the lowest concentration of nitrogen (2.5 mg/L), the lipid content was almost 30% (highest) and at the highest concentration of nitrogen (25 mg/L), the lipid content was 25%. Their studies showed that at lower initial concentration of nitrogen, *Scenedesmus. sp.* contained high lipid content per algal biomass. This may be due to exhaustion of nutrient earlier at low cell density when the initial nitrogen concentration was low, so *Scenedesmus.sp.* started to accumulate lipids under good source of light and CO_2 (Courchesne 2009).

In another study by Xu et al. (2001), effects of different nitrogen sources and concentration on growth rate and total lipid content on *Ellipsoidion sp*. microalgae was investigated. The culture was grown in batch system and lipid was extracted with chloroform/methanol (2:1, v/v). In this experiment, sodium nitrate, ammonium chloride and urea were used as the nitrogen sources. The growth rate and lipid content of *Ellipsoidion sp* are shown in Table 2.4.

Table 2.4. Effect of nitrogen on the specific growth rate and total lipid content of *Ellipsoidion sp*. microalgae (Xu 2001).

N source	**N-free**	**NaNO3**	**NH4Cl**	**urea**
Specific growth rate (day^{-1})	0.17±0.01	0.29±0.01	0.31±0.01	0.28±0.02
Total lipid content (%)	7.99±0.21	27.6±0.35	33.3±0.39	21.5±0.51

The culture had a low growth rate and lipid content in nitrogen-free medium and high growth rate and lipid content in ammonium medium. The possible reason of the slow growth in nitrogen-free medium might be that the nitrogen is still not exhausted in algal cells. This showed that urea was not as effective as other nitrogen sources. In general, the algae grew well in ammonium medium, but the growth rate was limited to nitrogen concentrations less than 0.64 mmol/L and so the maximum lipid content (33.5 % of dry weight) was obtained in the media containing 2.56 mmol/L NH_4Cl.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell Line and Media preparation

Two types of biomass were used in the experiments: a dry vegetable plant powder(EARTH SOURCE Greens & More, from Solgar Company, NJ) and algal sample of *Scenedesmus dimorphus* (UTEX 746, obtained from University of Texas, TX).

The vegetable plant powder consisted of 22% Soy Lecithin powder, 11% blue-green *spirulina* and the remains was made of various grasses, some chlorella powder, various grains and variety of other vegetables, fruit and plant materials. The dry vegetable powder contains 11 wt% lipids according to the manufacturer.

Bold-Basal medium with 3-fold nitrogen and vitamins (3N-BBM+V), was used as the growth media for *S. dimorphus* (Table 3.1). Some experiments were done without adding vitamins to the media (3N-BBM) as described later.

Table 3.1. Stock solution, P-IV metal and vitamins ingredients for preparation of 1 liter media (Source: http.//www.utex.org).

#	Component	Amount	Stock Solution Concentration	Final Concentration
Stock Solution				
1	NaNO3	30 mL/L	12.5 g/500mL dH2O	0.294 M
2	CaCl2·2H2O	10 mL/L	1 g/400mL dH2O	0.017 M
3	MgSO4·7H2O	10 mL/L	3 g/400mL dH2O	0.0225 M
4	K2HPO4	10 mL/L	3 g/400mL dH2O	0.0431 M
5	KH2PO4	10 mL/L	7 g/400mL dH2O	0.1287 M
6	NaCl	10 mL/L	1 g/400mL dH2O	0.0427 M
7	P-IV Metal Solution	6 mL/L		
8	Vitamins	1 mL/L		
P-IV Trace Metal Solution				
1	Na2EDTA·2H2O	0.75 g/L		2 mM
2	FeCl3·6H2O	0.097 g/L		0.36 mM
3	MnCl2·4H2O	0.041 g/L		0.21 mM
4	ZnCl2	0.005 g/L		0.037 mM
5	CoCl2·6H2O	0.002 g/L		0.0084 mM
6	Na2MoO4·2H2O	0.004 g/L		0.017 mM
Vitamins				
1	H(Biotin)		0.0016g/40mL 50mM HEPES solution	
2	B1(Thiamine)		0.0442g/40mL 50mM HEPES solution	
3	B12(Cyanocobalamin)		0.0063g/40mL 50mM HEPES solution	

All the components in the stock solution and P-IV metal were obtained from Sigma-Aldrich. The following amounts of stock solutions were used in preparing 1 L of growth media: 30 mL of $NaNO_3$; 10 mL of NaCl, $MgSO_4$, K_2HPO_4, KH_2PO_4 and $CaCl_2$ solutions each; 6 mL of trace metal solution and 1 mL of each vitamin (H(biotin), B_1 (thiamine) and B_{12} (cyanocobalamin)). Vitamins were added to the growth media through a 10 micron Watman sterilized filter. Stock and P-IV solutions were kept at room temperature and vitamins were stored at +4°C prior to the experiments. For media preparation all of the components were completely solublized in the DI water to the total volume of 1 L.

The flasks or bottles containing media (without vitamins added) were covered with foam stopper and covered loosely with aluminum foil and autoclaved for 30 minutes at 121^0C, 150 psi. After autoclaving, the flasks were kept under sterile hood to cool and then the vitamins were added if necessary.

3.2 General Method for Culturing Algae

Algal cells were maintained in their growth phase by cultivation in a 1 L sterile batch reactor called the seed jar. The seed jar was used as inoculums source for experiments in this research. The seed jar was started by taking the seed cells from agar and inoculating them in 1 L of 3N-BBM or 3N-BBM+V. The 5% CO_2 in air was sparged into the bottle at a flow rate of 0.1 LPM continuously and the solution was stirred using a stir bar at room temperature under a sterile illuminated hood.

In 10 to 14 days the seed jar was ready to be used for inoculation into the experimental flasks or bottles. A cell suspension volume from the seed jar, equal to

approximately 5% of the final volume of the experimental flask, was added to each experimental flask, and 3N-BBM or 3N-BBM+V media added to the experimental flask to make up the remainder of the working volume. The gas mixture, at a flow rate of 0.85 LPM (5% carbon dioxide in air v/v), was supplied to each flask through 0.2 μm sterilized filter and 200 μL pipette tip and the excess gas was vented out through 0.2 μm sterilized filter through a 3/16 inch ID stainless steel tube (Figures 3.1,2). All the experiments had a same gas flow rate. Ho et al. (Ho 2010) have shown that growth rate in *S.obliquus* may increase with increasing CO_2 level up to 20%. But there are contradicting studies which have shown the growth rate in *S.obliquus* is maximized by 6% CO_2 level (Morais 2007) then the CO_2 level should be monitored closely. Since algae are sensitive to pH changes, excess of CO_2 can form carbonic acid with water and drops the pH levels of the culture media. For instance, growth rate of *S.acutus* was reduced 50% at pH 5 and even growth stopped at pH 4.8 (Nalewajko 1997) and the flasks were agitated continuously in a shaking water bath at 150 rpm. Agitation is necessary for *Scenedesmus dimorphus* to prevent sediment formation. The temperature in the water bath was maintained at 30±1°C. Seven 14-watt lights with intensity of 515-550 ft-candles were provided by 24" fluorescent bulbs in bath water with 12h:12h light and dark periods.

Each flask was equipped with a sampling system, comprised of a Tygon silicon tube with 3/16 inch internal diameter (ID), which was clamped when not in used to avoid bacterial contamination of the growth media. Nutrients were injected to the system using a similar tube equipped with a sterilized filter.

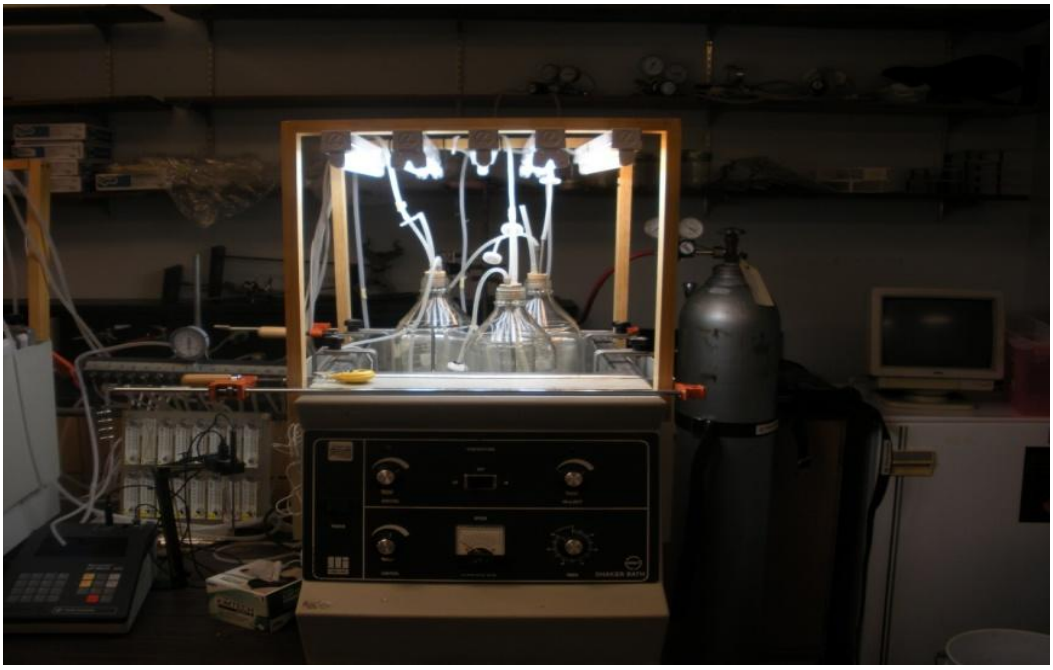


Figure 3.1. Culturing *Scenedesmus dimorphus* in water bath shaker system.

At the end of the growth process the cell suspension was centrifuged in 50 mL centrifuge tubes at 2000 rpm for 60 minutes. After centrifugation, the supernatant was removed and the pellets from each centrifuge tube were combined into a glass centrifuge tube. DI water was used to transfer all the remaining biomass to the new glass tube. The pellets in the new glass tube were centrifuged for another 30 minutes at 2000 rpm. The top layer (supernatant) was discarded and the wet pellet was used in various permeabilization and lipid extraction procedures as described later.

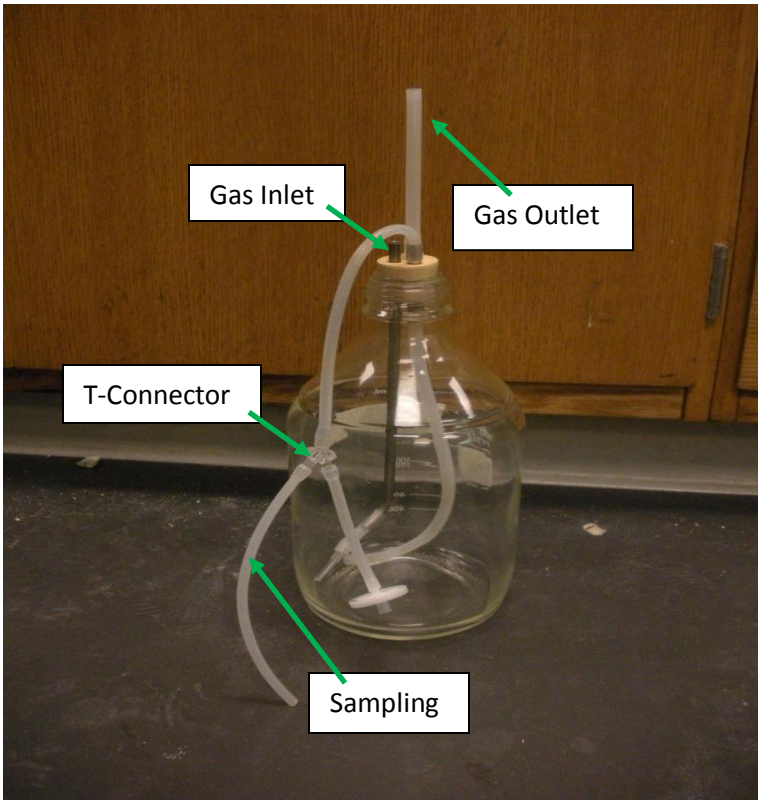


Figure 3.2. Bubbling/Sampling for a cell culturing system.

3.3 Solvent Extraction

To extract lipid from *Scenedesmus dimorphus* microalgae, a two-stage hexane/isopropanol (3:2, v/v) (Acros Co. NJ) solvent extraction method was employed. After centrifugation and separation of water from biomass, the biomass (either dried or wet pellets) was transferred to a pre-weighed glass tube. For all extractions 5 mL of hexane/isopropanol (3:2, v/v) was added to each wet pellet sample. The samples were then placed on a shaker platform at 200 rpm for 3 hours at room temperature to extract lipid from biomass. After 3 hour of extraction, the supernatant containing solvent and lipids was pipetted to a new pre-weighed glass tube by glass pipette. The second solvent

extraction stage was performed using another 5 mL of hexane/isopropanol (3:2, v/v) solvent added to the residual biomass for another 3 hours on a shaker platform at 200 rpm at room temperature, and the new lipid plus solvent layer was added to the first extract tube. The glass tubes were placed under the hood at room temperature with connections to a vacuum manifold and aspirator system (Figure 3.3), to expedite the solvent evaporation process. The solvent was evaporated after approximately 10-12 hours. The solvent and residual biomass tubes were weighed to obtain the masses of the lipid and dry biomass.

In a number of experiments, after adding the second 5 mL of solvent, the samples were placed on a shaker platform at 200 rpm for 20 hours for extraction process, which is called the "2nd long stage" process.

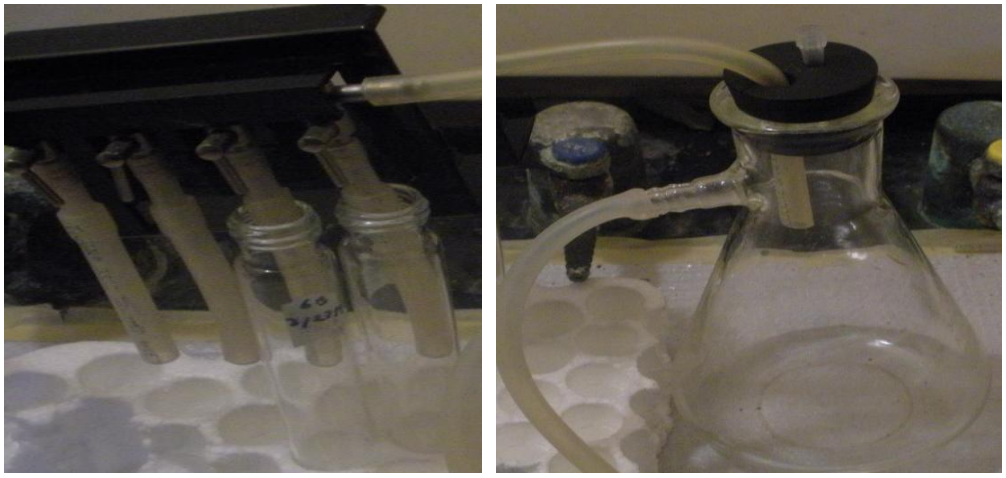


Figure 3.3. Vacuum and aspirator systems.

3.4 Biomass Permeabilization Methods

After algae preparation and harvesting, the biomass pellets were ready to be used in different permeabilization methods prior to solvent extraction, as described here:

Wet Pellet**.** The wet biomass pellets were combined in a pre-weighed glass tube. In this method, the biomass was neither permeabilized nor dried before solvent extraction. The lipids were then extracted using the two-stage solvent process (or 2nd long stage).

In a different approach to this method, the solvent and were separated lipid from biomass by using paper filter (0.65µm).

Freeze-dry**.** In this method, the wet biomass pellets were resuspended in glass tubes and left in -80°C freezer overnight. On the next day the frozen biomass pellets were lyophilized (Labconco Co.) for about 15-18 hours. Then the two-stage solvent extraction was performed as described in section 3.3. The combined solvents and the extracted biomass were left under the hood at room temperature using the vacuum system (Figure 3.3) to dry and were weighed to obtain the masses of the lipid and dry biomass.

One issue with this method was that the biomass stuck to the sides of the glass tube and made it difficult to resuspend it in solvent. Even though some of the biomass was scraped back into the solvent, but it was still hard to measure dry biomass weight accurately.

Sonication**.** Solvent (hexane/isopropanol (3:2, v/v)) was added to the wet biomass pellet in the glass tubes. Sonication was performed at 40% of maximum amplitude for 1 minute, and then another 5 mL of hexane/isopropanol (3:2, v/v) solvent was added to the glass-

tube and placed on the shaker at 200 rpm for 3 hours. The supernatant was transferred to a new pre-weighed glass tube and placed under the hood for solvent evaporation. Another 5 mL of the solvent was then added to the pellet and the glass tube was placed on a shaker platform for 3 hours at 170 rpm. The top layer was then transferred to the glass tube containing the first extracted lipid and placed back under the hood to evaporate the solvent. The combined solvents and the extracted biomass were left under the hood at room temperature to dry and weighed to obtain the mass of the lipid and dry biomass. The issue with this method is stickiness of the biomass to the upper sides of glass tubes during sonication and that made it difficult to extract lipid and measure dry biomass.

Grinding**.** The wet biomass pellets were kept in an oven at 45-50^{0}C to dry completely and until a constant weight was measured. Pellets were usually dry completely after two days. The dried pellets were ground to a fine powder using a mortar and pestle. The fine powder was transferred to a new pre-weighed glass tube. Lipids were extracted using the two-stage solvent extraction process as described in section 3.3.

In another approach to prepare the wet pellet by using grinding method, the algae suspension was transferred into 50 mL centrifuge tubes and centrifuge for 1.5 hours at 2000 rpm. The supernatant was discarded and the pellets were rinsed with DI water, vortexed and transferred to 1.5 mL pre-weighed micro-centrifuge tubes. They were centrifuged at 14000 rpm for 20 minutes. Then wet pellets were left in the oven at 45-50^{0}C to dry. The pellets were completely dried after less than 20 hours and the rest of process was the same as described before.

Freeze-thaw. The wet biomass pellets were resuspended in a few milliliters DI water and were left at -20^{0}C freezer overnight. The samples were then brought back to room temperature and kept for about 4 hours, and then were placed back at -20^{0}C for another night. The samples were thawed again and then centrifuged at 2000 rpm for 30 minutes. The supernatant was removed and 5 mL hexane/isopropanol (3:2, v/v) solution was added to the wet pellet and placed on a shaker platform at 200 rpm for 3 hours. Then centrifugation was performed for another 30 minutes at 2000 rpm to separate the lipid plus solvent from the biomass and water. The top layer was then transferred to a new pre-weighed glass tube and left under the hood for solvent removal using aspirator device. The combined solvents and the extracted biomass were left under the hood at room temperature to be dried and weighed to obtain the mass of the lipid and dry biomass.

3.5 Growth experiments

Samples were taken every 24 hours to measure the absorbance at 600 nm (Spectronic Genesys 5). Absorbance is linear with biomass concentration only when $A_{600}<1$, so samples with absorbance greater than 1.0 were diluted with 3N-BBM, and the adjusted absorbance reported.

Nitrogen was measured using the Vernier Nitrate Ion Selective Electrode (NISE) (Vernier Software & Technology) (Figure 3.4). The nitrate concentration was measured for some of our experiments. Before each nitrate measurement, the probe was calibrated using two point calibration from 1 ppm to 100 ppm nitrate. If the measured nitrate concentration was higher than 100 mg NO_3^-/L; it was diluted with DI water and re-measured.

The volume of samples taken during each experiment to measure the absorbance, nitrogen concentration and lipid content, varied by each experiment. In each nitrogen measurement, 2-3 mL sample volumes were used. Before nitrate measurement, 2-3 mL of sample volumes were vortexed for a few seconds and then nitrogen was measured by the probe.

Figure 3.4. Vernier Nitrate Ion-Selective Electrode.
(http://engineering.vernier.com/general/sensors/no_3-bta/).

3.5.1 Batch Growth Experiments

In batch experiments 2 L bottles were inoculated with *S. dimorphus* in 3N-BBM at a working volume of 1500 mL. After the inoculation, samples were taken daily to measure absorbance at 600 nm to monitor the growth rate. The purpose of these experiments was to determine the growth rate and lipid content of the algae during exponential and early stationary phases.

A typical growth curve for batch culture is shown in Figure 3.5. The growth curve has four phases: 1) lag phase, 2) growth phase or exponential phase,3) stationary phase and 4) death phase. The lag phase occurs right after inoculation as the cells adjust themselves to the new environment. The growth phase or exponential phase happens when the culture has plenty of nutrients and the growth rate is independent of the nutrient concentration. Cells start the transition to stationary phase when either the nutrient is depleted or toxic by-product has accumulated. During the stationary phase the specific growth rate is zero but metabolic function such as lipid production is still active. The death phase happens when all metabolic functions have stopped (Shuler 2002).

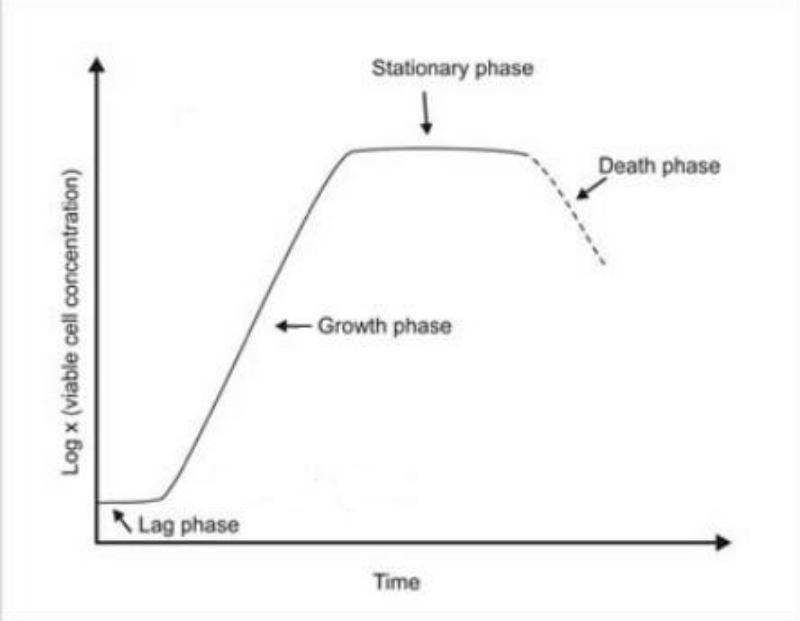


Figure 3.5. Typical growth curve.

The model for algae growth in batch culture, during exponential phase, is:

$$\mu = \frac{1}{X}\frac{dx}{dt} \quad (1)$$

where μ is the specific growth rate, X is the biomass concentration and t is time (Shuler 2002). Then, rearranging and taking the integral of Equation 1 with respect to biomass concentration and time, the concentration can be related to the growth rate as follows:

$$ln\,X = ln\,X_0 + \mu t - \mu(t_0) \quad (2)$$

where X_0 is biomass concentration at time 0 (just after inoculation), X is biomass concentration at time t. A plot of natural log of biomass concentration versus time yields the growth rate from the slope of the linear region.

To determine the lipid content (Y), following equation was used

$$Y = L/m_0 \times 100\% \quad (3)$$

where L is mass of lipid recovered(g) and m_0 is total biomass dry weight(g)

Biomass recovery (R) was obtained by:

$$R = m_0/m_i \quad (4)$$

where m_i is initial amount of dry biomass and m_0 is total biomass dry weight (g) after lipid extraction which is the total of the weight of lipid-free dry biomass (m_f) and the lipid.

To determine the lipid concentration (C_L) in g/L, the following equation was used:

$$C_L = Y \times C_M \quad (5)$$

where C_M is biomass concentration (g/L). To calculate the biomass concentration, following equation was used:

$$C_M = m_0/V_s \quad (6)$$

where V_s is the volume of the original cell suspension sample.

Samples were taken for lipid extraction during exponential and early stationary phases. The volume of samples taken, varied in batch experiments, from 100-200 mL, different from each experiment to another. The grinding method with 2-stage solvent extraction was used for lipid extraction (see section 3.4).

To calculate the yield coefficient, experimental data of biomass and sodium nitrate concentration as functions of time were used. To obtain the yield coefficient in units of g/g, biomass concentration should be converted from absorbance units to g/L, using:

$$\text{Biomass(g/L)} = 0.50\text{*absorbance} \quad (7)$$

based on data presented in Figure IV.4.

Using the slope of the graph of biomass versus sodium nitrate concentration ($\frac{\Delta x}{\Delta s}$), the following equation can be used to calculate the yield coefficient:

$$Y_{\frac{X}{S}} = -\frac{\Delta X}{\Delta S} \quad (8)$$

3.5.2 Fed-batch experiments

This experiment was done to determine effect of nitrogen concentration on growth rate and lipid content. The algal cells were grown in BBM with specific nitrogen concentration, in four 2 L flasks. The initial volume of liquid in each flask was 1500 mL. All flasks were kept under the same growth conditions as mentioned in Section 3.2. Bottles were inoculated at the same time. The initial concentration of sodium nitrate in each flask was as follows: 0.75 g $NaNO_3$/L, 0.14 g $NaNO_3$/L, 0.07 g $NaNO_3$/L and 0.0 g $NaNO_3$/L in the first to fourth flasks, respectively. The required volumes of sodium nitrate added to each bottle are shown in Table 3.2. BBM media (without nitrogen) was then added to bring the total liquid volume in each flask to 1.5 L. The flasks were operated as fed-batch, to keep sodium nitrate concentration constant during the experiment. To monitor and maintain the sodium nitrate concentration constant during this experiment, samples were taken every two days.

Table 3.2. Required volume of $NaNO_3$ stock solution added to each 2-L flask (1500 mL working volume) in fed-batch experiment.

Flask Number	**Initial concentration of sodium nitrate (g/L)**	**Required volume of sodium nitrate stock solution (mL)**
1	0.75	45.7
2	0.14	8.31
3	0.07	4.15
4	0.0	0.0

For measuring the lipid content in the fed-batch experiment, 175 mL samples were extracted by grinding method followed by 2-stage solvent extraction. Samples were collected in exponential phase every 2 days and the absorbance at 600 nm was measured. To keep the concentration of sodium nitrate constant in each flask, the concentration of $NaNO_3$, was measured every 2^{nd} day and if there was significant difference to the targeted concentration, a volume of sodium nitrate from stock solution (25 $gNaNO_3/L$) also added to the flask. The volume of solution to be added was determined by first calculating C_{diff}, defined as:

$$C_{diff} = C_T - C_m \quad (9)$$

where C_T is the target concentration of sodium nitrate, C_m is measured sodium nitrate concentration.

The required volume of sodium nitrate from stock solution was then calculated using:

$$V_{Req} = \frac{C_{diff}}{K} \times V_R \quad (10)$$

where V_{Req} is the required volume, V_R is the media volume and K is sodium nitrate concentration of stock solution which is equal to 25 $gNaNO_3/L$. Desired amount of sodium nitrate was filtered and injected to the flask by using a sterile filter and syringe through sampling tube.

3.5.3 Correlation of biomass concentration with absorbance

This experiment was performed by colleague Jacob Schwenk (2012), which is described here briefly. *S. dimorphus* microalgae grown in flasks with 150 mL working volume in 3N-BBM with variation in initial concentration of nutrients of magnesium sulfate and digestate.

In this experiment samples were taken daily and absorbance measured at 600 nm. To measure biomass concentration and lipid content, 75-100 mL of samples were taken on the final day of growth (day 15 for the digestate experiment and day 16 for the factorial design experiment). The biomass concentration were measured for all samples as described in section 3.4 with grinding method and then plotted against absorbance. Details can be found in Jacob Schwenk's thesis (2012) and Appendix-C.

3.6 Data analysis

Data was analyzed by using P-values in the student T-test. Student T-test is statistical hypothesis test in which the test supports the null hypothesis. It is one of the most common statistical tests which would follow a normal distribution if the value of a scaling were known. When the scaling term is unknown and is replaced by an estimate based on the data, the student T-test under certain conditions will apply. The analysis was done assuming unequal variances and one tail. The P-values in the student T-test indicate the significance of each parameter on the results. P-values less than 0.05 indicate a

significant effect of a parameter at the 95% confidence level. In this study, error bars indicate the standard deviation and error bars in growth rates indicate the LINEST function in Microsoft Office Excel. The slopes in all graphs are reported as mean ±stdev. All the calculations in this research were done using Microsoft Office Excel.

In this research it is assumed that all of the nitrogen comes from sodium nitrate ($NaNO_3$) as the nitrogen source and the yield coefficient is $Y_{X/S}$ where S is measured sodium nitrate concentration and X is the biomass concentration. The initial sodium nitrate concentration (S_0) for the experiments was based on the amount of sodium nitrate added to the media, and not measured with the nitrate probe.

CHAPTER IV

RESULTS AND DISCUSSION

Lipid extraction was performed on dry vegetable powder and green microalgae *Scenedesmus dimorphus*. Lipid extraction consists of two steps: permeabilization and solvent extraction. Since different microalgae species have different cell shape, size and wall structures and also algal lipids have different characteristics, the objective of this work was to identify the best methods for permeabilization and lipid extraction from an algae of choice: *Scenedesmus dimorphus*. In this research, the effect of five permeabilization methods, including wet-pellet, freeze-dry, sonication, freeze-thaw and grinding, on lipid recovery from dry vegetable powder and *S. dimorphus* microalgae were investigated. In addition, the effects of sodium nitrate concentration on lipid content were studied.

4.1 Permeabilization and lipid extraction of vegetable powder

The effect of various permeabilization methods on lipid recovery was first investigated using a sample of vegetable powder with known lipid content, as described in Chapter III. The four methods for permeabilization and extraction were chosen to be wet pellet, freeze-dry extraction, grinding and wet pellet by using paper filter, all described in Section 3.4. To determine the lipid extraction and biomass recovery content equations 3 and 4 were used. The lipid contents of the three sets of experiments on dry vegetable powder are summarized in Figure 4.1. The grinding method using mortar and pestle and freeze-dry method were done using dry vegetable powder while the remaining extractions were done on wet vegetable powder. Comparing the lipid contents in this experiment with that of the manufacturer (11 wt %), our experiment shows much higher lipid content (more than 21%). This significant difference between reported lipid content from manufacturer and our measurement could be due to error in manufacturer's report in this batch or might be because of the presence of some residual water in biomass after the drying process or lack of filtering allowed some biomass to end up in lipid fraction.

The lipid contents among different methods were comparing using Student T-test statistical analysis (assuming unequal variances, one tail, unpaired) as shown in Table 4.1. P-values less than 0.05 indicate a significant effect of a parameter on the measured outcome. The student T-test suggests that the difference between grinding method and wet pellet with paper filter ($p=0.00$) is insignificant. Differences among all other samples might not found significant.

Table 4.1. Pair comparison Student T-test for lipid content in vegetable powder under 2 samples assuming unequal variances and one tail.

Comparison in Lipid Content	**p-values**
wet pellet to freeze-dry	0.16
freeze-dry to grinding	0.12
grinding to wet pellet+ paper filter	**0.00**
wet pellet to grinding	0.21
wet pellet to wet pellet+ paper filter	0.48
freeze-dry to wet pellet+ paper filter	0.13

By comparing the student T-test results (Table 4.1) and Figure 4.1, it can be concluded that wet pellet, freeze-dry and grinding methods, all have the same course of action on lipid extraction from a wet vegetable powder and are all better than the wet pellet with filtering method. The last method (wet pellet + paper filter) yields the lowest lipid content measured probably due to the finely grounded vegetable powder which ended up in lipid glass tube or filtering (10 micron Watman sterilized filter), which caused a decrease in total biomass dry weight. It also can be concluded that lack of filtering in other methods might allow some non-lipid biomass to transfer into lipid fraction and causes overestimation of lipid content.

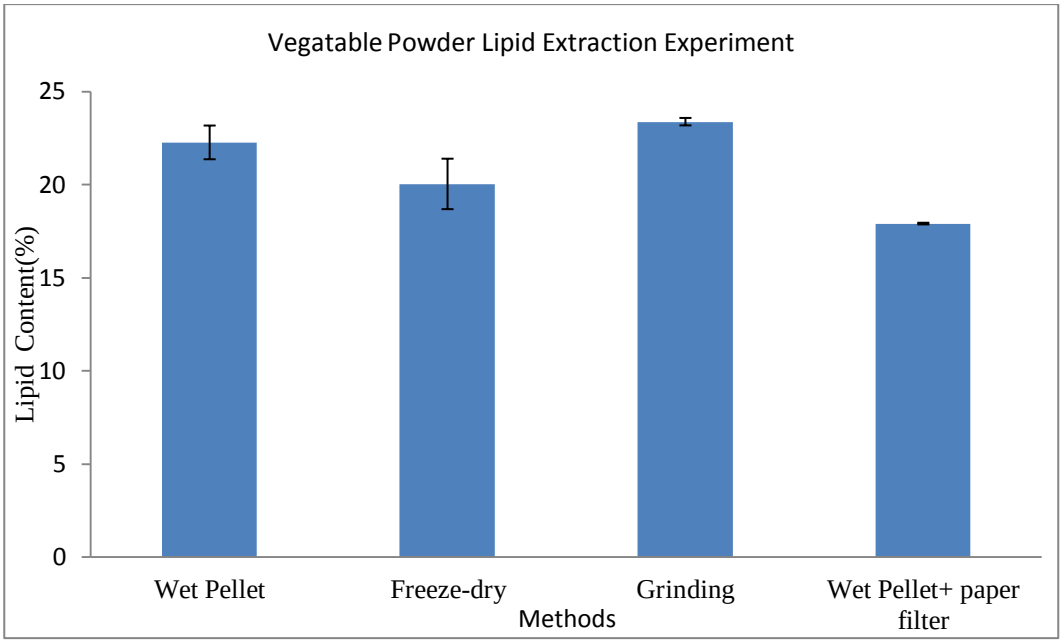


Figure 4.1. Lipid content in different methods on dry/wet vegetable powder (n=2 for wet pellet, freeze-dry and wet pellet+ paper filter methods, n=4 for grinding method).

The p-values in Table 4.1 indicate that three methods including wet pellet, freeze-dry and grinding are the best methods to be used with vegetable powder and results in the most accurate values for lipid content. But as mentioned before, freeze-dry method is not recommended for future use due to practical difficulties. Therefore, wet pellet and grinding methods are considered for further experiments on microalgae *S. dimorphus*. All experiments had a 2-stage solvent extraction, so solvent timing was not a factor.

4.2 Permeabilization and lipid extraction of *Scenedesmus dimorphus*

This experiment was performed on 150 mL of *S. dimorphus*, grown in 3N-BBM+V in 4 Erlenmeyer flasks. The goal of this experiment was to determine which permeabilization method is more efficient by determining the lipid content. Each method was also tested to help determining the shortest extraction times, by allowing the sample to be in contact with the solvent for different time periods. All the experiments were performed on algae during its exponential or stationary phases. Samples from the same seed jar were grown in different and separate growth bottles. Growth bottles were kept in a water bath shaker under the same temperature and lighting conditions to allow the comparison among the batch cultures.

The lipid contents for the four cultures are shown in Figure 4.3. For each Erlenmeyer, one of the following procedures were used: wet pellet using 2-stage solvent extraction process; wet pellet using one long solvent extraction process ; freeze-thaw with 1-stage solvent extraction; and sonication with 2-stage solvent extraction (section 3. 4). The maximum and minimum lipid content was 33% and 5.4%, respectively for the experiments. The maximum lipid content resulted from the wet pellet method.

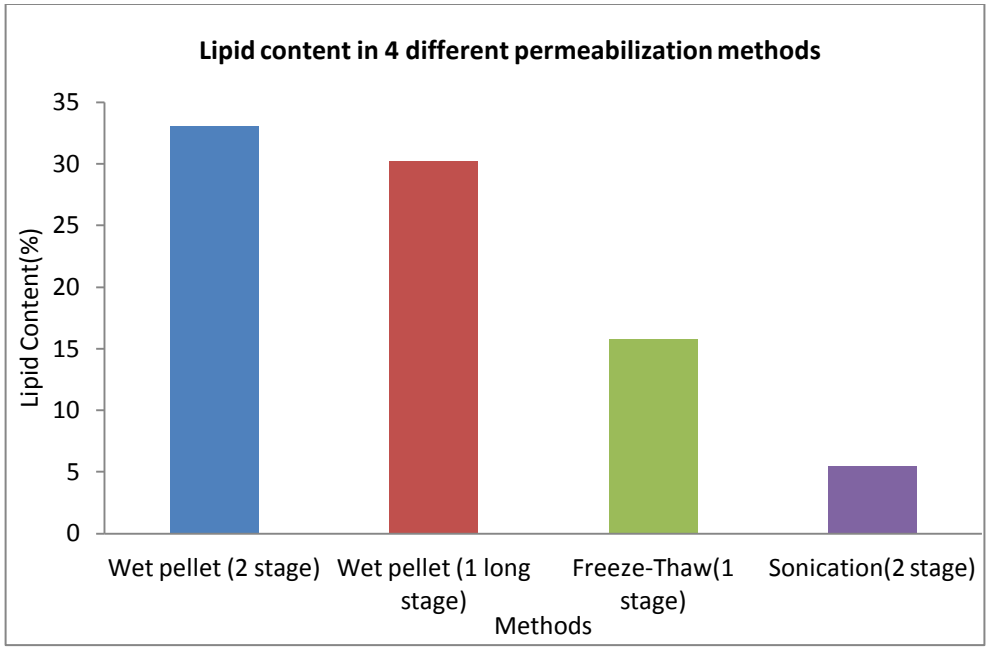


Figure 4.2. Content of lipid extracted from *S. dimorphus* in 3N-BBM+V for different methods (n=1 for all methods).wet pellet performed in 2 stage and one long stage (20 hours)extraction methods.

The results shown in Figure 4.2 indicate that wet pellet permeabilization methods (2-stage and 1-long stage) have the highest lipid content (30-33%). These two lipid extraction methods have nearly the same lipid content but the 2-stage extraction method requires less time and so it was used for further experimentations.

The sonication and freeze-thaw methods were not performed any further due to the formation of an emulsion and stickiness of biomass. They resulted in multiple liquid layers including an emulsion layer which made it very difficult to recover the residual biomass and the lipids from the sample. Therefore, the lipid content was difficult to quantify. In freeze-thaw method there are some difficulties due to the stress on algal cells

(freezing and thawing) which causes the cells to stick to each other or to the sides of glass tubes, and it might be the main reason why this method was inefficient. Stickiness was also one of the problems in freeze-dry and sonication methods. Similar problems were also observed in other experiments with *S. dimorphus*, using the freeze-dry method as well as additional tests with sonication and freeze-thaw (data not shown). Therefore, these three methods are discontinued.

The grinding method used the old-fashioned technique of pestle and mortar. In contrast to other methods, it appears that grinding method using mortar and pestle always results in consistent outcome and it is the least complicated technique among other cell disruption methods. In contrast to the other methods, like sonication and freeze-dry, the samples were dried completely before adding solvent. But the time needed to complete the extraction process using the grinding method is almost two days and some of the biomass is lost in powder form. However in general and in comparison to other cell lysing methods, the grinding method appears to be more efficient.

4.3 Correlation of absorbance to biomass concentration

A set of experiments were performed on *S. dimorphus* in 3N-BBM+V in sixteen Erlenmeyer flasks with 150 mL of working volume. The growth media in each flask has a different composition (Appendix-C, Table C1) as described in section 3.5.3. The goal of this experiment was to determine the correlation between the biomass concentration and absorbance at 600 nm. The biomass concentration was measured for all samples at the end of day 16 of each experiment using the method described in section 3.4. In this

experiment, samples were taken daily and absorbance at 600 nm was measured. The samples with the absorbance values above 1.0 were diluted and an 'adjusted absorbance' was calculated, since previous results in the lab indicated that absorbance is only linear with biomass concentration up to absorbance less than 1.0. The biomass concentration is plotted versus adjusted absorbance in Figure 4.3 and for this experiment the slope was determined to be 0.51±0.05 gdw/L/A_{600}.

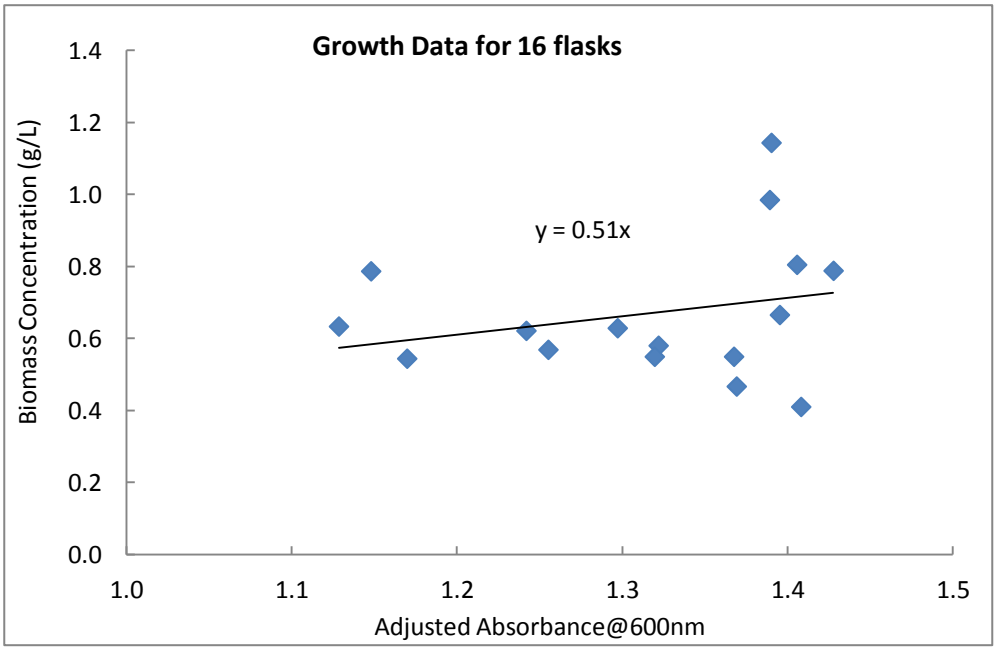


Figure 4.3. Growth data of sixteen flasks on *S.dimorphus* in 3N-BBM+V.

The next experiment was performed on *S. dimorphus* grown in 3N-BB+V media in 40-250 mL Erlenmeyer flasks of 150 mL working volume. This set of experiment consists of 5 different runs (media recipe in Appendix-C, Tables C2-C6).

Figure 4.4 shows the relationship between the biomass concentrations, measured at day 8 of the culture to absorbance at 600 nm of the five different sets of flasks. The slope for this experiment is measured to be 0.50 ± 0.03 gdw/L/A_{600}.

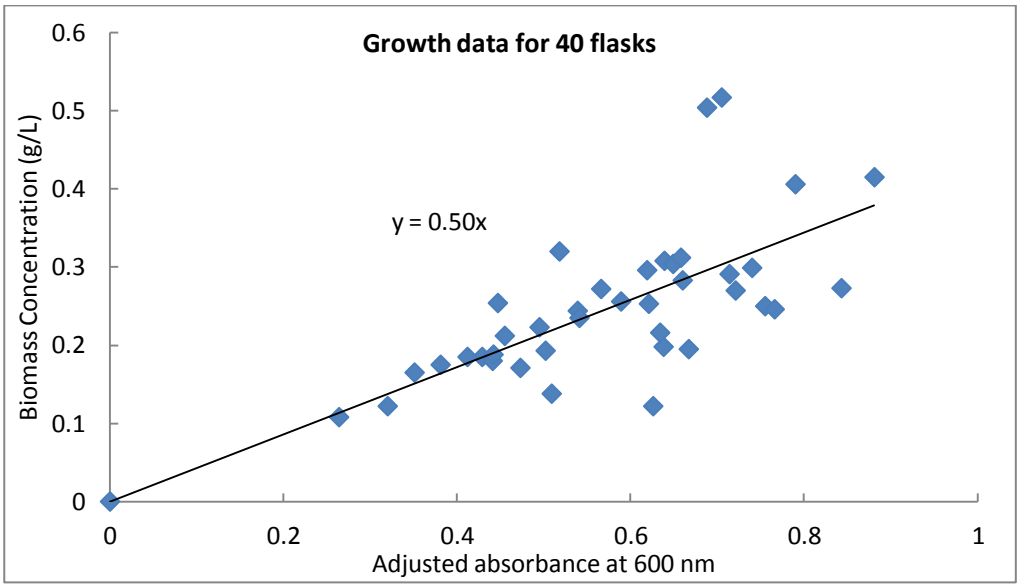


Figure 4.4. Relationship between biomass concentration and adjusted absorbance at 600 nm for *S.dimorphus* in 3N-BBM+V in 40 Erlenmeyer flasks with 150 mL working volume and p value of zero.

The correlation between biomass concentration and absorbance shows a linear relationship between these two parameters. This is an indication that absorbance can be used representation of biomass concentration during the cell growth in exponential phase. The scattering in data points could be due to the specific media compositions as shown in

Appendix-C, Table C1-C6 or flocculation which resulted in 6% error in the calculated slope. This is generally a fairly small error and so the graph can be used to conveniently and accurately convert measured absorbance to biomass concentration. This would be very useful considering that measuring absorbance at 600 nm is much easier and faster and requires smaller sample volumes than direct measurement of biomass concentration.

4.4 Effect of Nitrate Concentration on Lipid Kinetics

4.4.1 Batch culture

S. dimorphus was grown in 3N-BB media (no vitamin) in 2 L bottles with working volume of 1 liter (section 3.2). The samples were obtained daily to measure A_{600}. Volumes of 200 mL of samples were taken for lipid and nitrate measurements, on days 13, 18, 20, 22 and 24. This experiment was performed to investigate the effect of sodium nitrate concentration on lipid content, mainly during the stationary phase. During the 0-1 days a normal lag phase was observed due to adjustment of algae to the new environment (Figure 4.5). This was followed by the exponential growth phase, which continued to day 10 while culture had adequate nutrient supply and was illuminated. The linear phase was observed from day 11-15 in this experiment, and the actual stationary phase from day 13-24.

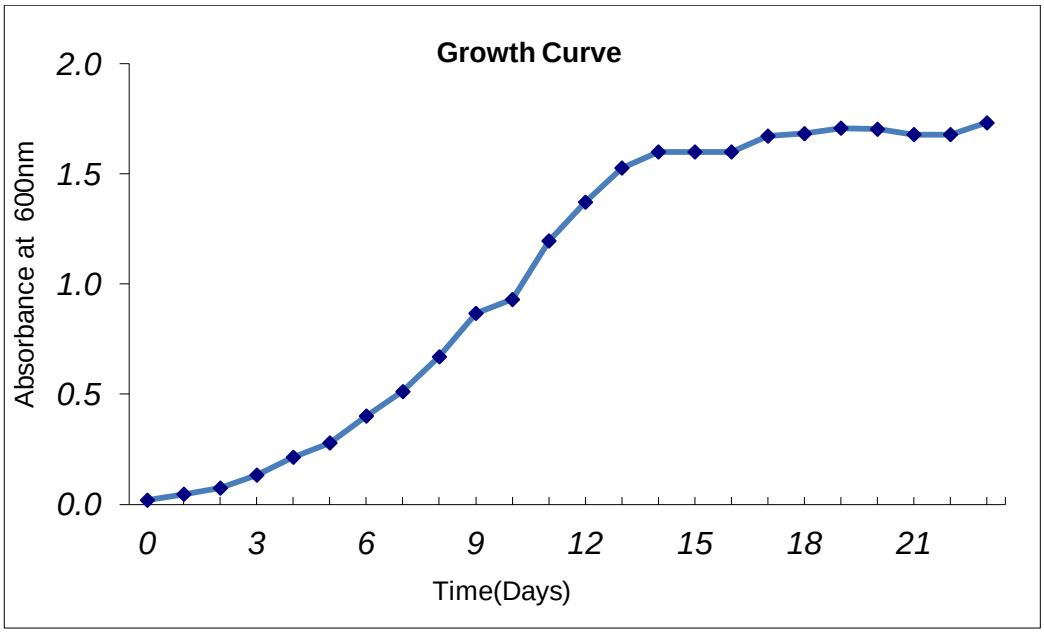


Figure 4.5. Growth data for *S. dimorphus* in 3N-BBM in 2L bottle.

Figure 4.6a shows the lipid content during the stationary phase. From day 13 to 20, during 5 days, the lipid content increased from 6 to 22% and after day 20 dropped to 16% which means that the culture was most likely dead and metabolic functions decreased. The measurement on day 20 could be an outlier, since there was only one measurement per time point. Figure 4.6b shows that sodium nitrate concentration decreased to essentially zero by the completion of the exponential phase on day 13. The results indicate that the maximum lipid content could be achieved during 8 days (days 13 – 20), upon depletion of nitrate by day 13.

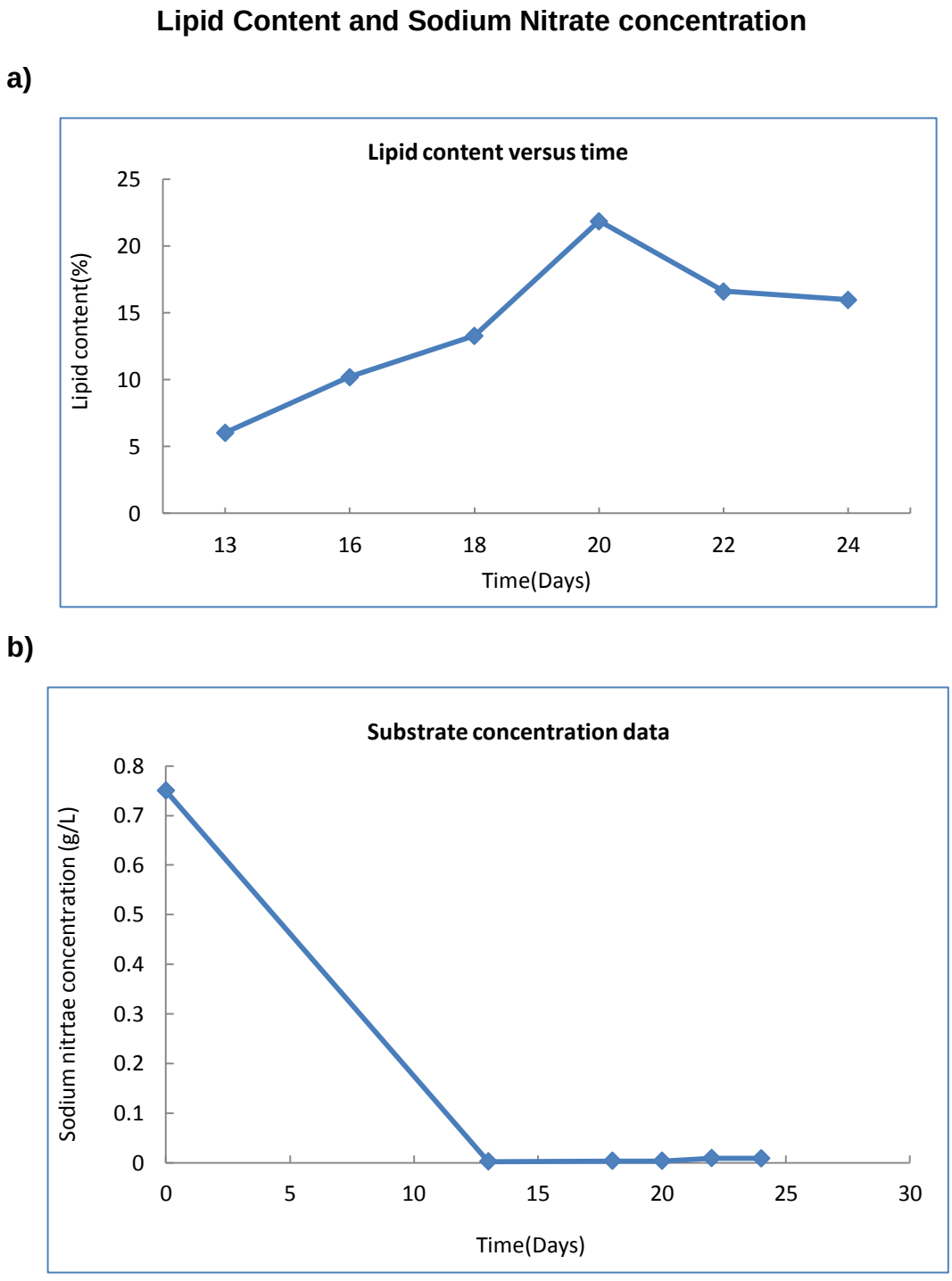


Figure 4.6. Lipid content and concentration of sodium nitrate against time of *S. d*imorphus in 3N-BBM media in batch culture. This experiment is in exponential and stationary phases. It also shows the consumption of nitrate during the experiment.

The growth rate was determined for the exponential and linear period of growth. The growth rate of cells during 15 days was determined to be 0.3± 0.02 day^{-1} for this experiment as shown in figure 4.7. It appears that there is no lag and the data from day 0 to 4 are in exponential phase and growth rate decreases after this point. It is therefore obvious that the maximum exponential growth rate should be evaluated from days 0 to 4 (see Figure 4.8).

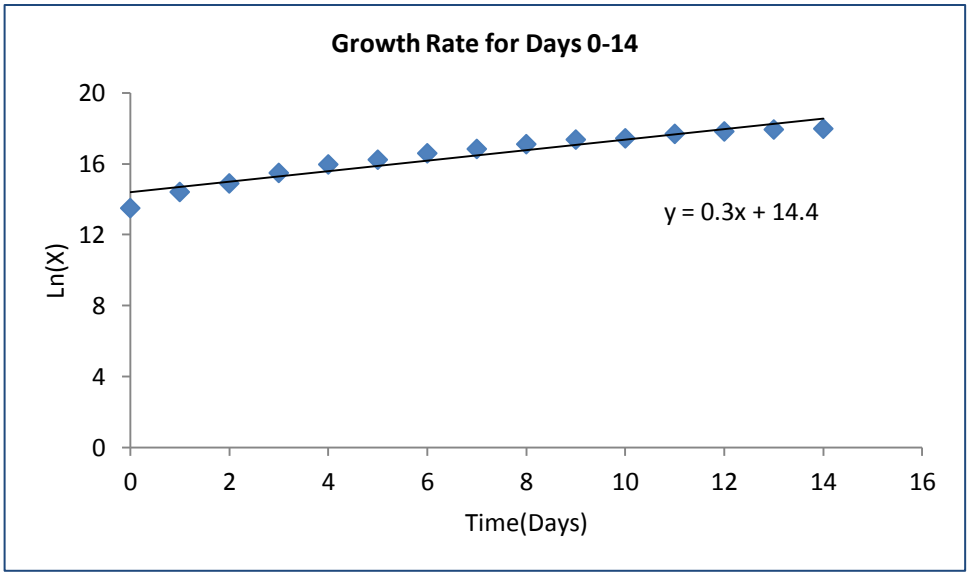


Figure 4.7.The growth rate in *S.dimorphus* in 3N-BBM from day 1 to day 15.

The growth rate was found to be 0.6±0.04 day^{-1} for 5 days. The yield coefficients for this experiment are shown at the end of this chapter.

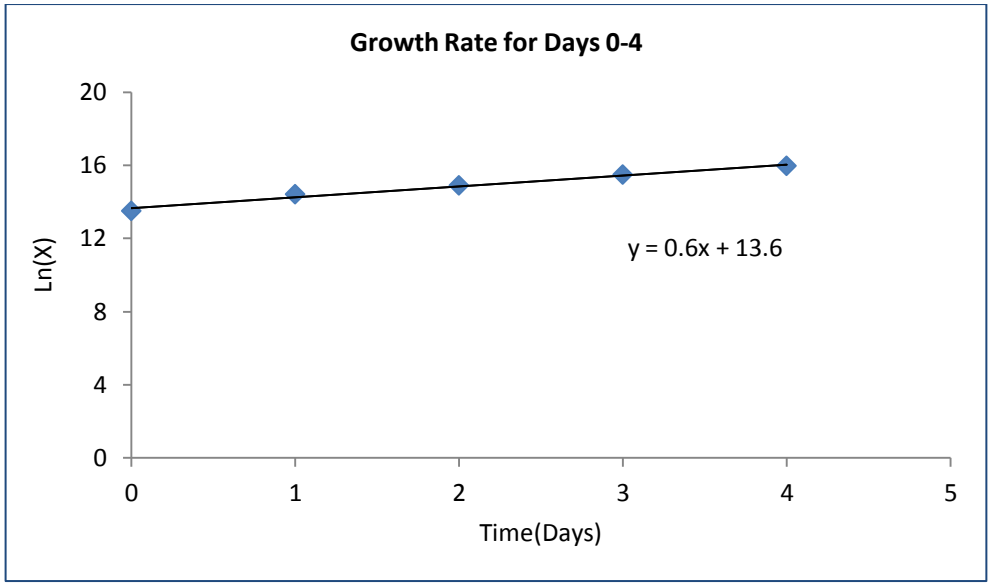


Figure 4.8.The growth rate in *S.dimorphus* in 3N-BBM from day 1 to day 5.

In another experiment *S. dimorphus* was grown in 3N-BBM in two identical 2 L bottles with working volume of 1.5 L. This experiment was identical to the previous experiment; it was done to confirm previous results. The growth data is shown in Figure 4.9. To determine specific growth data, the log of biomass concentration versus time was used. The data of interest, which is the exponential growth phase, starts on day 1 and ends on day 7 for bottle 1 and starts at day 1 and continues to day 5 for bottle 2, as depicted in Figure 4.10.

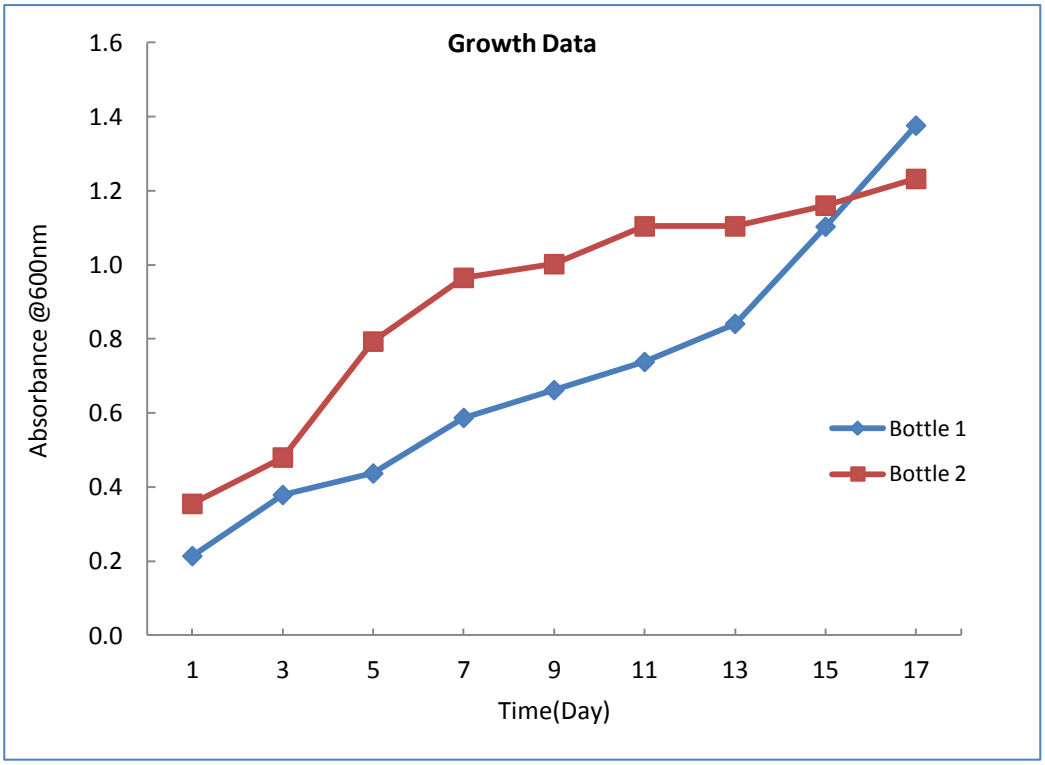


Figure 4.9. Measurement of absorbance in 2 identical bottles of *S.dimorphus* in 3N-BBM in different days.

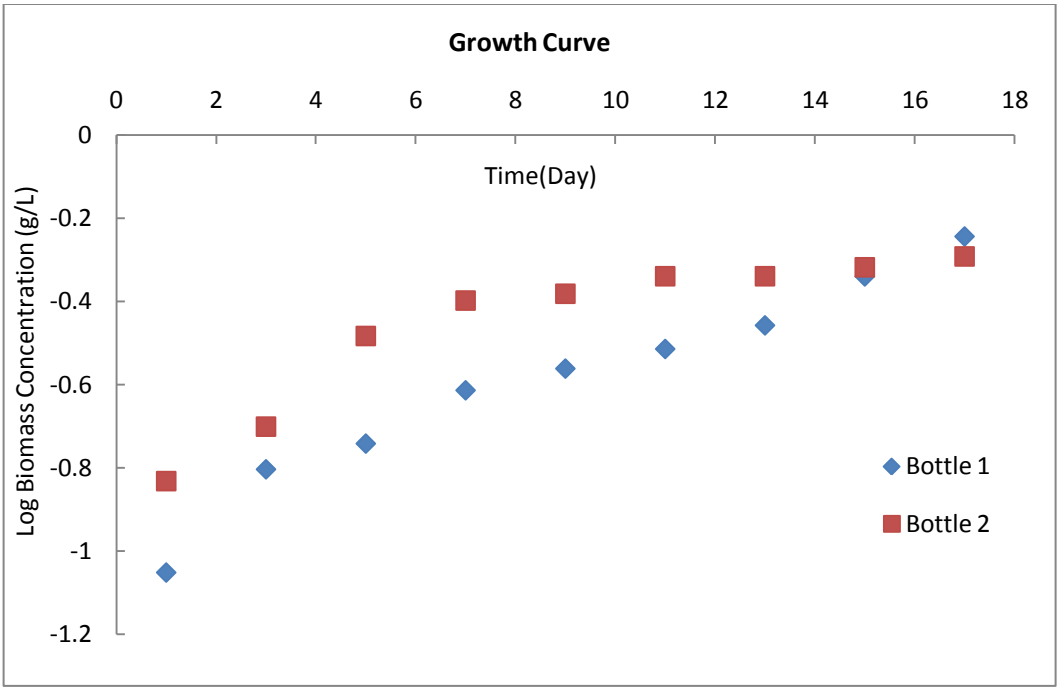


Figure 4.10. Log biomass concentration versus time data of *Scenedesmus dimorphus*.

The growth rates were calculated for the exponential phase for each bottle. The growth rate for bottle 1and 2 are 0.11±0.02 $day^{-1}$and 0.20±0.01 day^{-1}, respectively (Figure 4.11). The low values in specific growth rates may be due to flocculating of algae cells in two culture bottles or agitation or CO_2 flow rate. The yield coefficient for bottles 1 and 2 are shown at the end of this chapter.

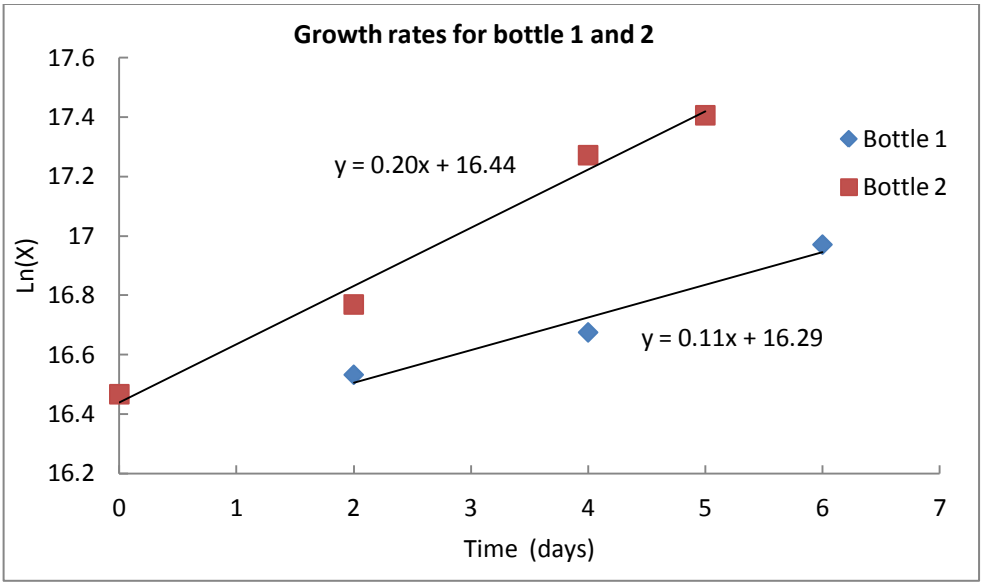


Figure 4.11. Growth rates in two identical bottles of *S dimorphus* in 3N-BBM.

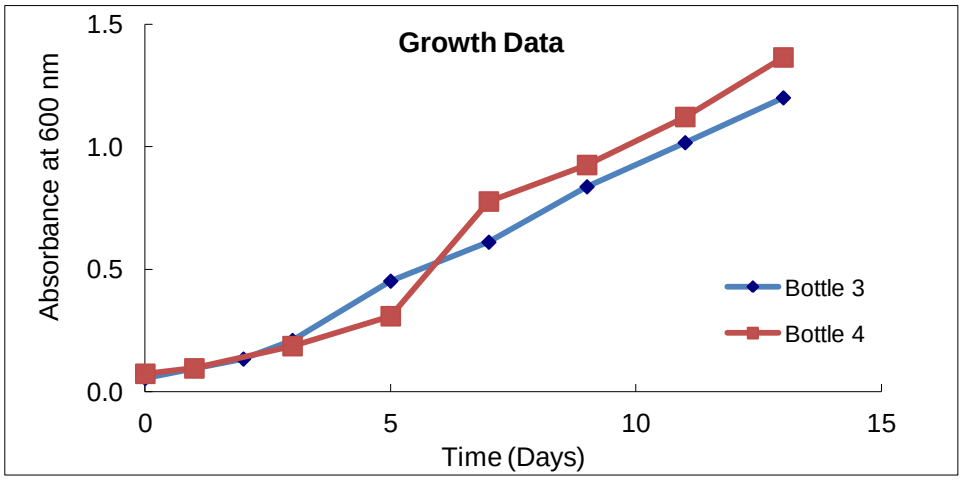
The next experiment was performed on *S. dimorphus* grown in 3N-BBM in two identical 2 L bottles with working volume of 1.5 L. This experiment has the same growth condition as previous two experiments. The purpose of this experiment was to get additional accuracy in relationship between growth and sodium nitrate concentration and yield coefficient. The growth data and sodium nitrate concentration for these bottles are shown in Figure 4.12. The exponential growth data starts on day 0 and ends on day 7 for bottle 4 and day 5 for bottle 3. Both bottles showed similar growth trends as shown by Figure 4.12a.

Sodium nitrate concentration shows a constant decrease throughout the experiments except for one measurement in each bottle, day 13 for bottle 3 and day 3 for bottle 4. This can be attributable to measurement errors or poor mixing at the sampling

port (Figure 4.12b). The sodium nitrate concentration in this experiment does not approach zero. Comparing growth data and sodium nitrate concentration, bottles 3 and 4 show growth up to day 13.

Growth data and sodium nitrate concentration changes

a)



b)

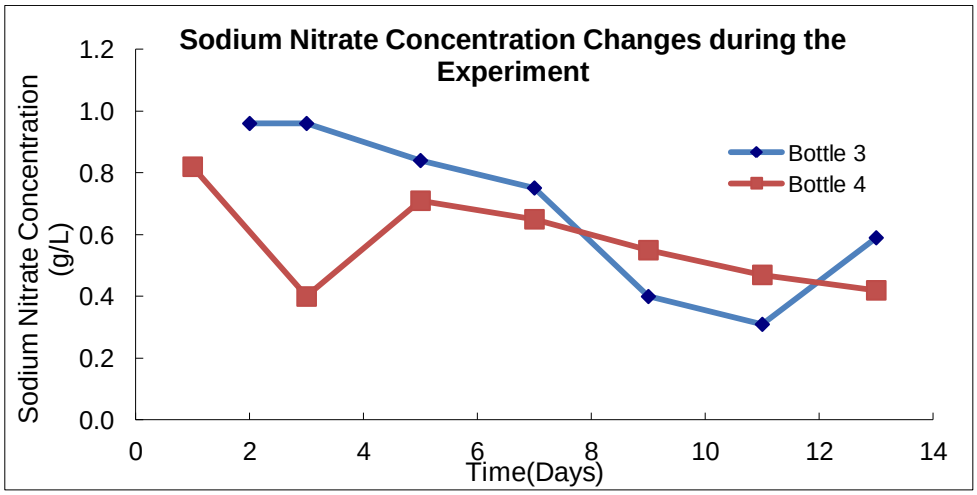


Figure 4.12. Measurement of absorbance and sodium nitrate concentration in 2 bottles of *S. dimorphus* in 3N-BBM with day ;(a) Cell growth data ;(b) Change in sodium nitrate concentration of two bottles with time.

The growth rate for bottle 3 during day 0 to day 5 was determined to be 0.43± 0.01 day^{-1} and for bottle 4 during day 0 to day 7 was determined to be 0.33±0.02 day^{-1} (Figure 4.13). The yield coefficients for these bottles are shown at the end of this section.

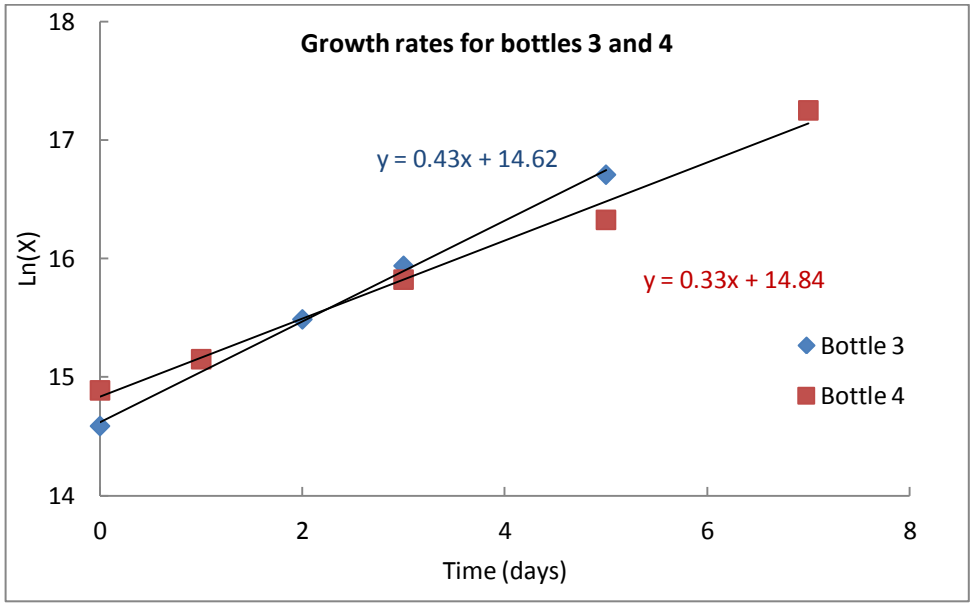


Figure 4.13. Growth rates of 2 bottles of *S.dimorphus* in 3N-BBM.

4.4.2 Fed-batch cultures

The fed-batch experiment was performed on *S. dimorphus* grown in 3N-BBM in four 2 L flasks with 1.5 L working volumes with different initial sodium nitrate concentrations. In order to keep the concentration at the target value for each flask, the sodium nitrate concentration was adjusted during the culture growth. Flask 1 has 0.75 g $NaNO_3$/L; flask 2 has 0.14 g $NaNO_3$/L, flask 3 has 0.07 g$NaNO_3$/L and 0.0 g $NaNO_3$/L

for flask 4 as described in section 3.5.2. The objective of these experiments was to identify the effect of sodium nitrate concentrations on lipid content of the green algae. Six samples of 175 mL volume where taken from each flask during this experiment to measure the sodium nitrate concentration and the lipid content. Lipid content was measured using the grinding method as described in section 3.4. The growth curves (Figure 4.14) indicate that the algae cells did not reach stationary phase during the 11-day experiment except for flask 4 (0.0 g $NaNO_3$/L). Higher sodium nitrate concentration results in higher cell densities at the final day, except for flask 1 with nitrate concentration of 0.75 g $NaNO_3$/L. The growth curve for flask 1(0.75 g $NaNO_3$/L) should be at the highest level but it is below that of the flask 3(0.07 g $NaNO_3$/L). This unexpected result could be a result of experimental or measurement errors or may be because of some unintended variation in the flask between day 7 to day 9 like the absence of carbon dioxide flow or change in air flow rate or clog of the vent tube or also can be due to volume change since we had to add more nitrate to this flask to keep the sodium nitrate concentration higher and inadvertently dilute the biomass.

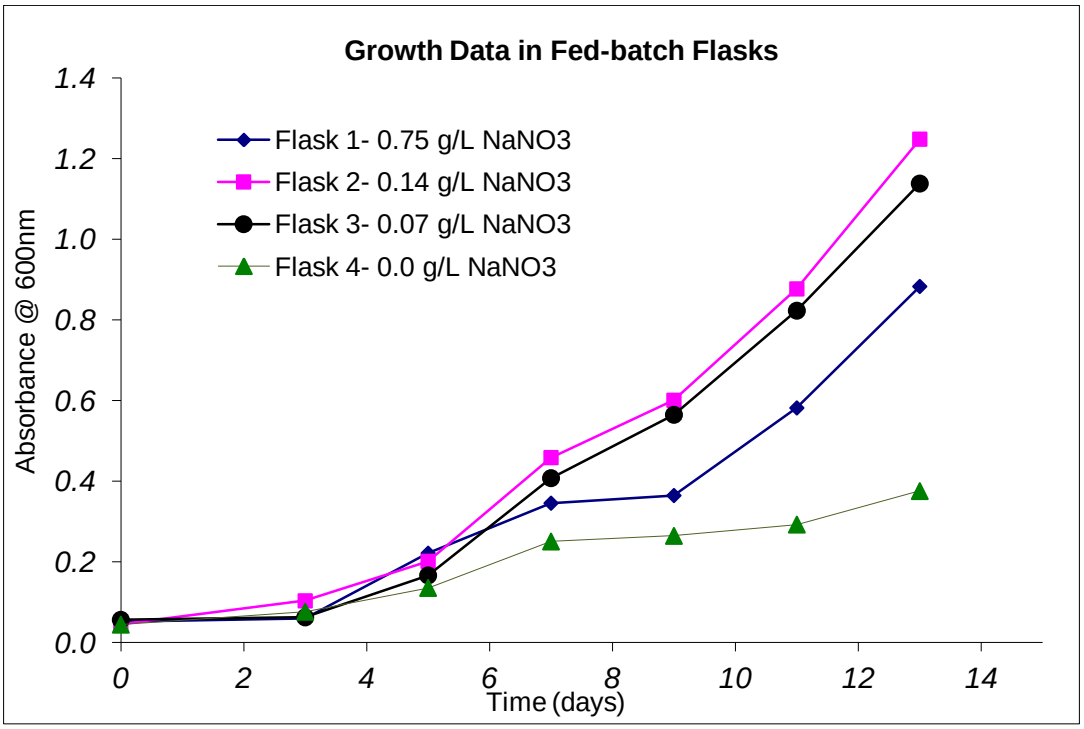


Figure 4.14. Growth data of *S.dimorphus* in 3N-BBM in four flasks, with different concentration of sodium nitrate.

Figure 4.15 shows the natural log of biomass concentration during days 0 to 13 for four flasks during growth phase. Figure 4.16 shows the average growth of exponential phase for each flask was found to be 0.27±0.02 day^{-1} for flask 1 at 0.75 g $NaNO_3$/L, 0.27±0.03 day^{-1} for flask 2 at 0.14 g $NaNO_3$/L, 0.47±0.01 day^{-1} for flask 3 at 0.07 g $NaNO_3$/L and 0.30±0.01 day^{-1} for flask 4 at 0.0 g $NaNO_3$/L. The lag phase can be seen clearly from days 0 to 3 for flasks number 1 and 3 with nitrate concentration of 0.75 and 0.07 g $NaNO_3$/L, respectively.

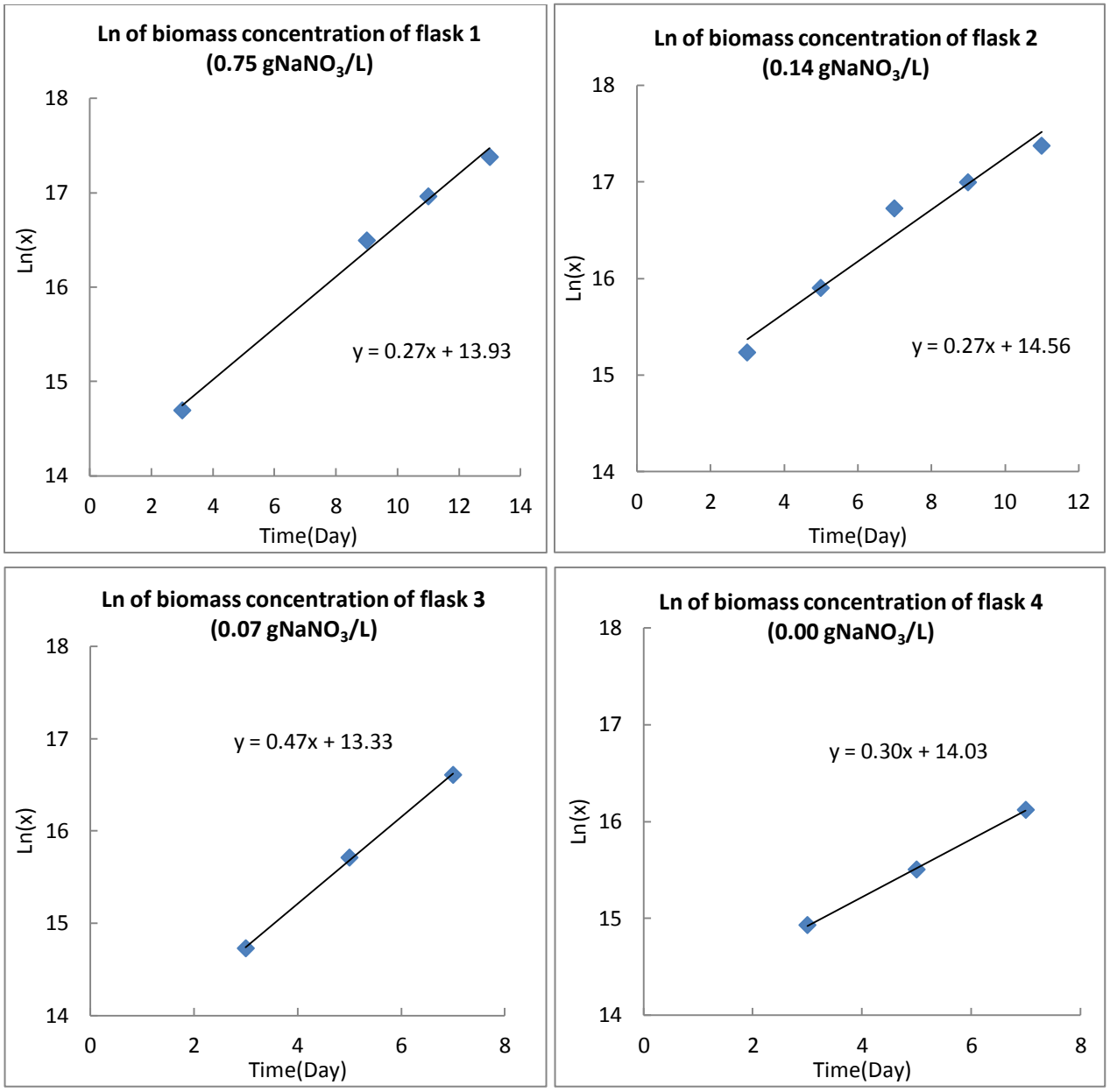


Figure 4.15. Ln of biomass concentration versus time for four flasks in *S. dimorphus* in N-BBM. Growth rates for flask 1 at 0.75 g $NaNO_3$/L for 11 days, flask 2 at 0.14 g $NaNO_3$/L for 9 days, flask 3 at 0.07 g $NaNO_3$/L for 5 days and flask 4 at 0.0 g $NaNO_3$/L for 4 days.

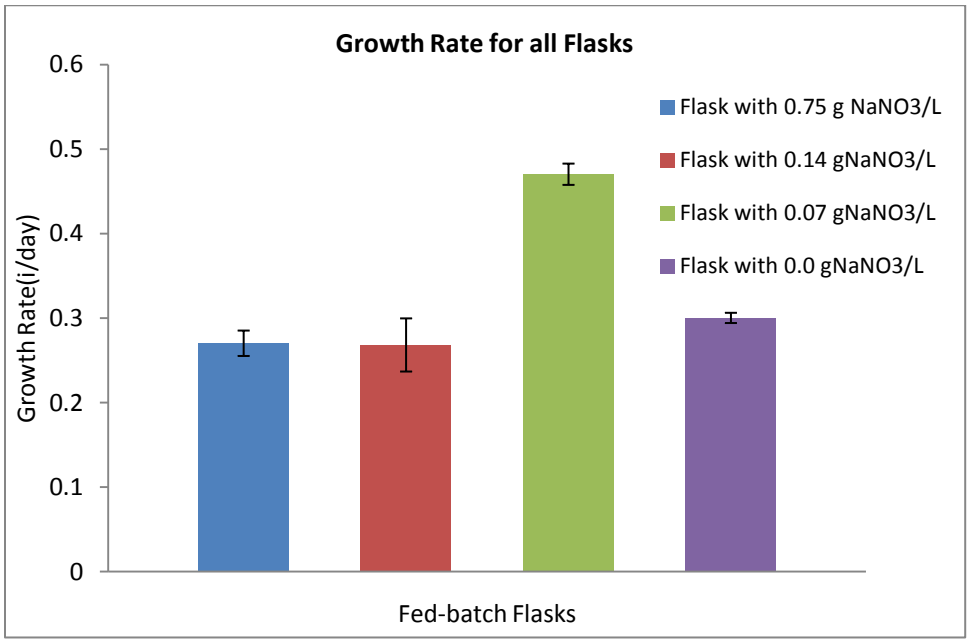


Figure 4.16. Growth rates for four flasks in *S. dimorphus* in N-BBM. Growth rates for flask 1 at 0.75 g $NaNO_3$/L is 0.27 ±0.02,flask 2 at 0.14 g $NaNO_3$/L is 0.27±0.03, flask 3 at 0.07 g $NaNO_3$/L is 0.47±0.01 and flask 4 at 0.0 g $NaNO_3$/L is 0.3±0.01.

A correlation curve between the growth rates and sodium nitrate concentration during the exponential phase is shown in Figure 4.17. Data shows that flask 2(0.140 g $NaNO_3$/L) and flask 1(0.75 g $NaNO_3$/L) have the same growth rates (μ=0.27 day $^{-1}$) but with different errors and flask 3(0.07 g $NaNO_3$/L) has the highest growth rate among the flasks (μ=0.47 day $^{-1}$). It shows that the flasks 2 and 3 with sodium nitrate concentration of 0.14 and 0.07 g $NaNO_3$/L, respectively, have higher growth than the rest.

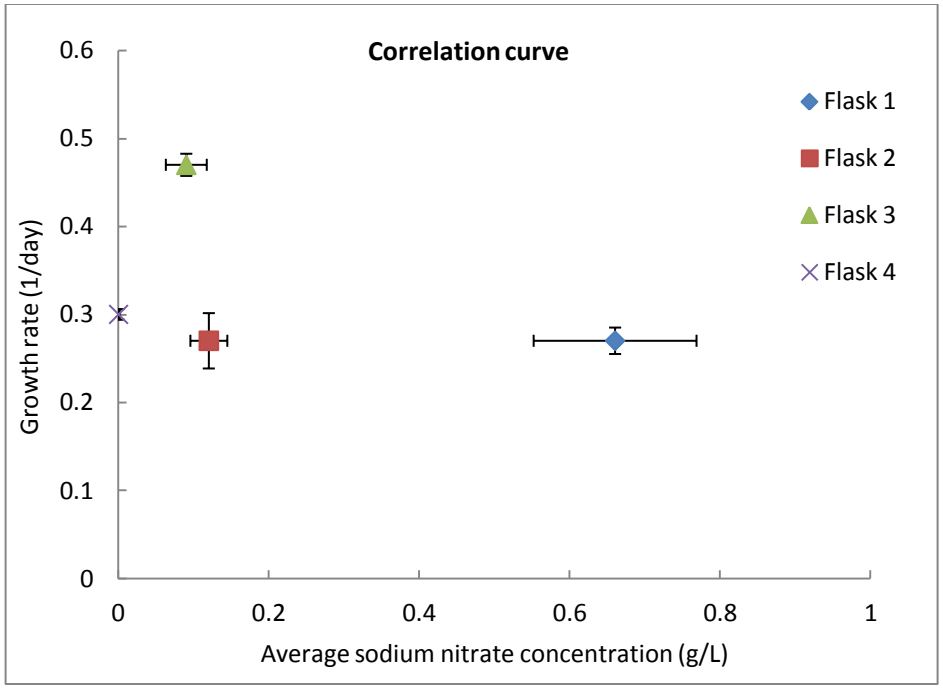


Figure 4.17. Graph of growth rate verses average sodium nitrate concentration for four flasks.

Figure 4.18 shows the concentration of sodium nitrate in each flask during the experiment. The concentration of sodium nitrate was measured with a nitrate probe every 2nd day and the volume of nitrate stock solution calculated and added to each flask as necessary to maintain the target concentration.

The concentration in flask 1 was observed to be constant up to day 7. At day 7, 0.54 mL sodium nitrate was added to this flask. At day 9 additional-sodium nitrate was calculated and added to the flask 1 to reach to the target concentration of 0.75 g $NaNO_3$/L. Despite the addition of 16.4 mL of stock solution to flask 1, the sodium nitrate concentration did not reach the target concentration; it increased from 0.48 to 0.54 g $NaNO_3$/L. This can be due to the nitrate probe reading, or lack of good mixing after addition of fresh solution. For flask 3 with 0.07 g $NaNO_3$/L, decrease in sodium nitrate

was observed in day 5 and 7 but adding 0.45 mL and 1.62 mL of sodium nitrate helped the media to go back to the target concentration. Table 4.2 shows the volumes of stock solution added to each flask during the experiment, at different specific days.

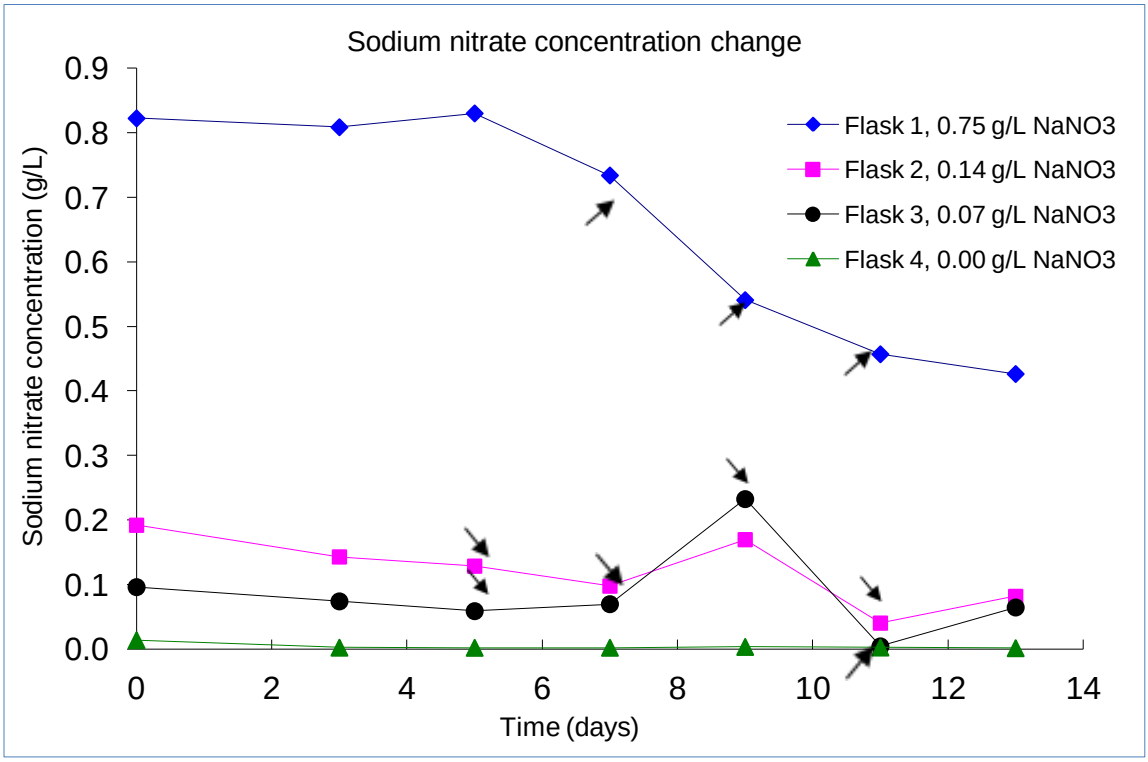


Figure 4.18. Newly measured values of sodium nitrate concentration in each flask after sodium nitrate addition in *S. dimorphus in 3N-BBM*. Arrows (↗) for flasks 1, 2 and 3, where the calculated volumes added to the related flask to keep the sodium nitrate concentration constant during the experiment.

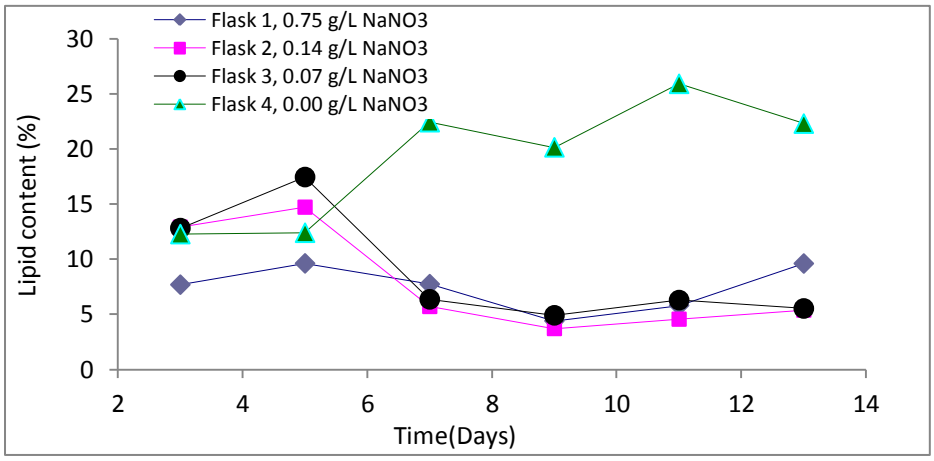
	Added volumes of stock solution (mL)			
Flask Number	**Day 5**	**Day 7**	**Day 9**	**Day 11**
Flask 1	**-**	**0.54**	**16.4**	**11.6**
Flask 2	**0.4**	**-**	**-**	**4**
Flask 3	**0.45**	**1.62**	**2.04**	**1.63**
Flask 4	**-**	**-**	**-**	**-**

Table 4.2. Added volumes of stock solution to each flask at each specific day in 3N-BBM.

The lipid content and lipid concentration as a function of time are shown for each flask in Figure 4.19. The graph shows that during the exponential growth phase, all flasks showed increases in lipid content and concentration. During day 7 to day 13 periods, based on our theory, the flask with high amount of sodium nitrate concentration (flask 1, 0.75 g $NaNO_3$/L), should have the higher lipid content. In these two graphs, the maximum lipid content and concentration was achieved in flask 4 (0.0 g $NaNO_3$/L). This shows that the zero concentration of sodium nitrate has highest lipid content.

Lipid Values of *S. dimorphus* in 3N-BBM

a)



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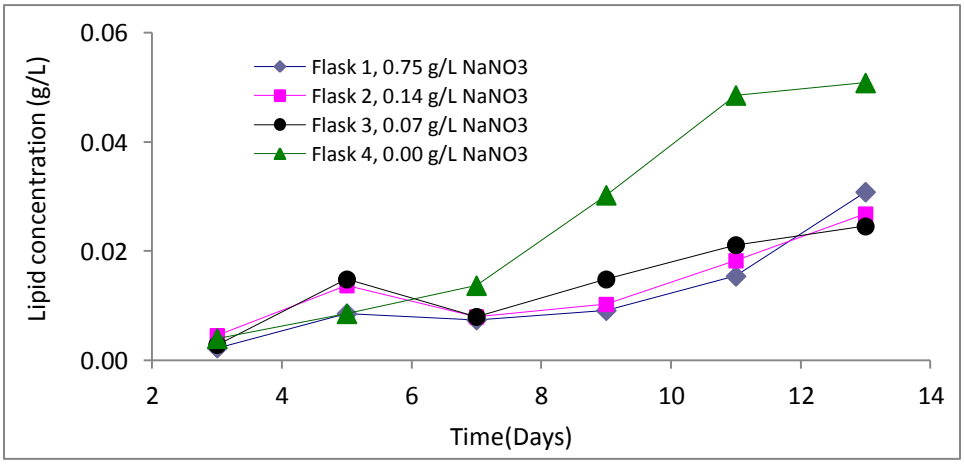


Figure 4.19. Experiment with fed-batch flasks, 4 flasks with different concentration of $NaNO_3$; (a) lipid content in each flask against days; (b) Amount of lipid concentration with day for 4 flasks in *S.dimorphus* in 3N-BBM.

The combination of all the lipid content data versus actual sodium nitrate concentration from the 4 fed-batch flasks is shown in Figure 4.20. It indicates that there is an inverse relationship between lipid content and sodium nitrate concentration. The algae cells which did reach stationary phase have high lipid content. It also shows that flask 4 (0.00 $gNaNO_3/L$) has the highest lipid content among the flasks. The best trend line fitted this graph is logarithmic one with R^2=0.42.

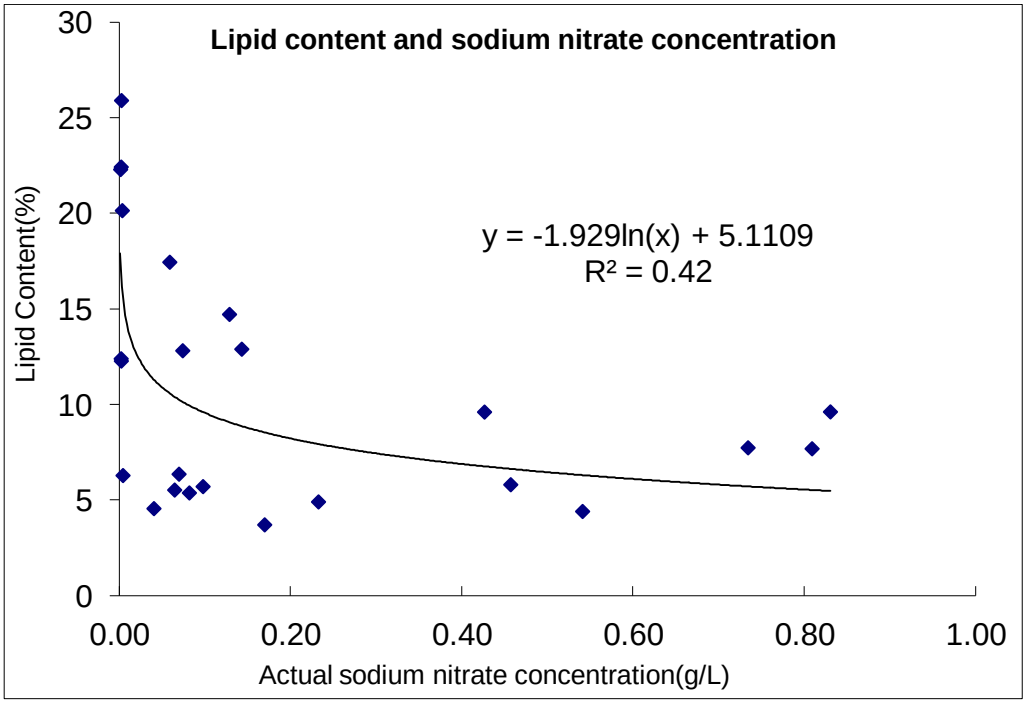


Figure 4.20. Effect of actual measurement of sodium nitrate concentration on lipid contents of *S.dimorphus* in 3N-BBM, combined from 4 fed-batch flasks data from Figures 4.18 and 4.19.

In comparing sodium nitrate concentration of the flasks to the expected target concentration as measured throughout the experiment, flask 1 (0.75 g $NaNO_3$/L) exhibits a scattered distribution (Figure 4.21). The error could be due to inaccurate amount of added stock solution.

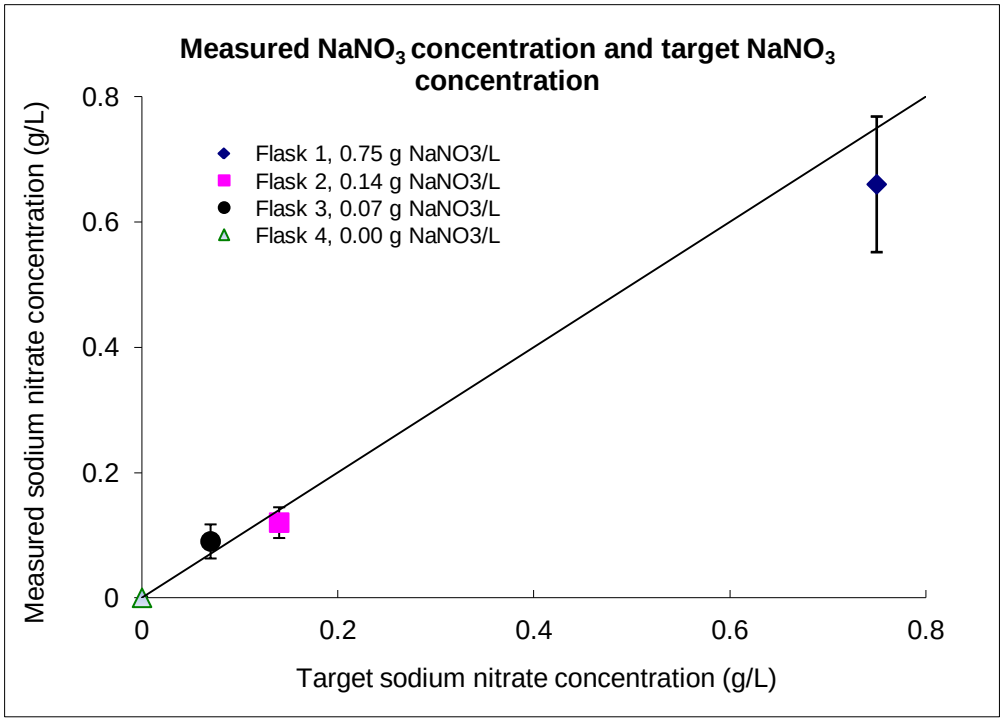


Figure 4.21. Measured and target concentration of sodium nitrate in four flasks in 3N-BBM. The measured values are the averages.

4.4.3 Yield coefficients

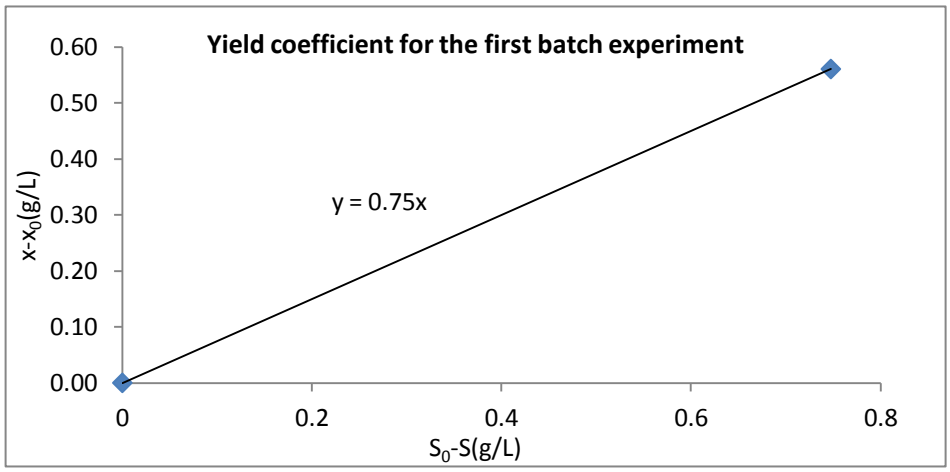
The data obtained in this research was also used to determine sodium nitrate yield. Yield coefficient was determined by using equations 7 and 8 in section 3.5.2. A graph for first and third experiments (bottle 3 and 4), in batch mode, is shown in Figure 4.22. The yield coefficient which is the slope of the linear curve, calculated to be 0.75, 0.58±0.12, 1.23±0.10 g cell/ g substrate for experiment one, experiment three (bottle 3) and experiment three (bottle 4), respectively. The results are shown in Table 4.3, which indicates a range of values for yield coefficients from 0.58 g cell/ g substrate to 1.23 g cell/ g substrate for the three experiments in section 4.3. The initial concentrations of sodium nitrate for the experiments were approximated and not measured with the probe, due to problems with sample storage conditions. Sodium nitrate was assumed to be the sole nitrogen source in all experiments.

Table 4.3. Yield coefficient values from batch cultures in $g_{dry\ Wt\ cells}/\text{-}g_{NaNO3}$ with standard errors (except for first experiment with two points).

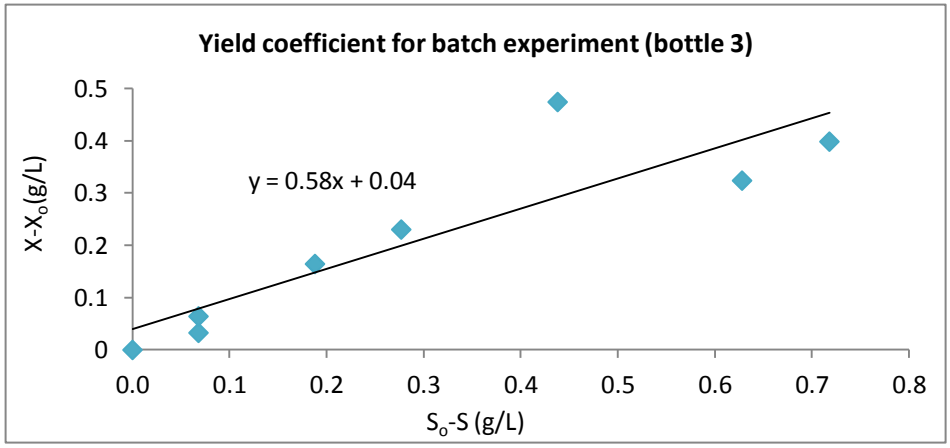
Experiments in section 4.3	**Figure numbers**	**Sample**	**$Y_{X/S}$ (g/g)**
First experiment	4.6	Bottle 1	0.75
Third experiment	4.12	Bottle 3	0.58±0.12
Third experiment	4.12	Bottle 4	1.23±0.10

Larger yield coefficients show that less mass of sodium nitrate was necessary to produce an equivalent mass of biomass. Yield coefficients for the third experiment (bottle 4) in section 4.3 were higher than those of other experiments (Y=1.23± 0.10 g/g). Different growth factors such as temperature, light, agitation, size of container and contamination have influenced nitrogen consumption as well as on the yield coefficients.

a)



b)



c)

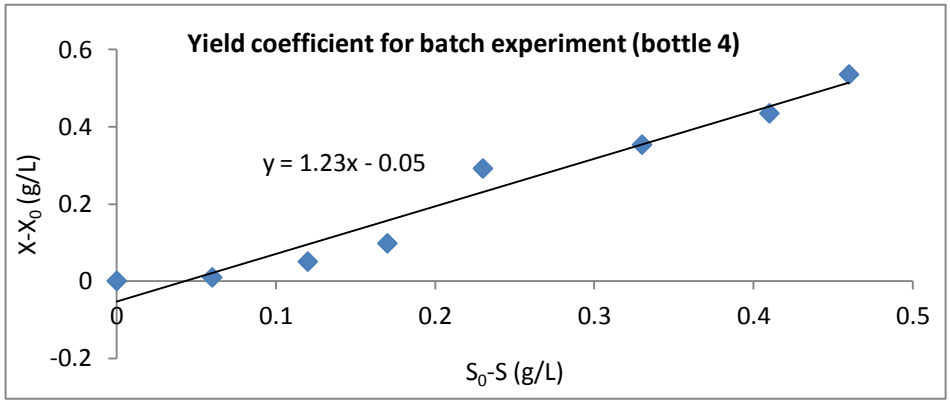


Figure 4.22. Sodium nitrate yield coefficient by *Scenedesmus dimorphus* applied on experiments one and three.

4.5 Discussion

4.5.1 Permeabilization and lipid extraction

On vegetable powder, effect of various permeabilization methods on lipid recovery was investigated. Four different permeabilization methods was applied on vegetable powder including wet pellet, freeze-dry, grinding and wet pellet+ paper filter. Comparing student T-test results show that wet pellet, grinding and freeze-dry methods can be applied on *S. dimorphus* and because of some difficulties with other methods freeze-dry and freeze-thaw methods were no longer used in further investigations. The lipid content measured from the lipid extraction of vegetable powder was above 20% which can be a result of fine vegetable powder which ended up in lipid tube and resulted in higher percentage of lipids with solvent solution. Performing permeabilization on *S. dimorphus* resulted in choosing grinding method as the best method to determine lipid content among all five permeabilization methods. This method gave the most consistent lipid recovery. This was also an easy method among all permeabilization methods. But there are some limitations with this method; one issue is that it takes long time to complete the extraction process; another issue is loss of biomass in the powder form.

Since the choice of solvent solution is a key factor in lipid extraction from algae, permeabilization methods were limited to use with hexane/isopropanol (3:2, v/v) as solvent solution. Hexane is the most popular solvent and is safe, relatively cheap, largely un-reactive, volatile and non-polar. It is an organic solvent and is shown to be a good candidate for lipid extraction (Gouveia 2009). Alcohols such as isopropanol are very

good solvent for most lipids. There are of course other solvent extractions methods on algae like Bligh and Dyer (Bligh 1959), which were not ever used in this research which can be considered in future work.

4.5.2 Batch growth

The results from the first and second experiments of the biomass concentration and adjusted absorbance shows that there is a linear relationship between these two parameters and higher absorbance means higher biomass concentration and indicates that absorbance is an adequate representation of biomass concentration during the growth phase. There is 6% error in the slope of this graph but the results still prove to be useful in predicting the biomass concentration. Since absorbance at 600 nm is so much easier and quicker and uses smaller sample sizes than biomass concentration. The scattering in data could be due to agitation, CO_2 flow rate, evaporation and the sample taking process. It should be noted that this correlation holds for data in exponential growth phase.

The growth rates in 2L bottle in batch experiment was calculated to be 0.3 and 0.6 for days 0-14 and 0-4, respectively. From Figure 4.7, it is evident that, during the stationary phase, when the sodium nitrate concentration is close to zero, the lipid content increases and it decreases in during decline phase. Next experiments (in bottles 1, 2, 3 and 4) were done to confirm previous results. The growth rates are shown in Figure 4.11 and 4.13. The growth rate varies from 0.11 to 0.43 day^{-1}. Since the four bottles in the two experiments are identical, the significant difference in growth rate should be due to factors such as evaporation, CO_2/air flow rate, agitation or even small contamination in

the media. As it has shown in Figure 4.13, growth rates almost follow the same trend, whereas in Figure 4.11 that is not the case. In the first experiment in section 4.3, the specific growth rate is calculated to be 0.6±0.04 1/day which is unusually higher than the previous experiments. It could be due to the flocculation of cells in this bottle.

4.5.3 Effect of nitrate concentration on growth rate and lipids

In this work, we investigated the effect of reducing the sodium nitrate concentration in the media on lipid content and growth rate. Previous studies have shown the reduction of sodium nitrate concentration would increase the lipid content on *Nannochloropsis oculata* and *Chlorella vulgaris* (Converti 2009). In our research, the sodium nitrate concentration for *S. dimorphus* in fed-batch growth experiment were reduced and kept constant during the experiment. The result of reduction of sodium nitrate on *S. dimorphus* is shown in Figure 4.17. It shows that although specific growth rate was not significantly affected by reduction of sodium nitrate concentration, increase in lipid content took place in the lowest sodium nitrate concentration (Figure 4.18). Our research confirms the results from literature and our hypothesis.

It should be noted that in our experiments the algae still grew if sodium nitrate was zero. The possible reason may be because of the chemical composition used in the media ($NaNO_3$ in the media). For most microalgae such as *Dunaliella tertiolreta*, the lipid content increased under nitrogen deficiency (Fabregas 1989) and the lipid of *Phaeodactylum tricornutum* increased as nitrogen decreased (Thomas 1984). The lipid

content of *Chlorella sp*. reached 70% to 80% (dw) when nitrogen was insufficient and lipid content of *Isochrysis galbana* decreased from 22.0 to 16.9% dw when the nitrogen content was increased from 0.04 to 0.70 mmol/L (Utting 1985). Also Figure 4.8 in batch experiment showed the reduction of sodium nitrate concentration during the experiment which was almost zero at day 13 to day 24. But the lipid content increased even up to day 20 because there was sodium nitrate in inoculums source.

4.5.4 Yield coefficient

The yield coefficient values and standard errors are shown in Figure 4.22 and Table 4.3. The data in experiments 1 and 3 are different. Environmental growth factors such as light, small temperature variations, agitation or even small contamination can affect the nitrogen uptake. But since all the experiments might have changes of light intensity, small temperature variations or agitation or small contamination, it could have resulted in different yield coefficients in our system. Based on these results, it could be concluded that first and third experiment (bottle 3) have almost same yield coefficient values which means same mass of substrate was necessary to produce an equivalent mass of biomass. The constant value of yield coefficient cannot be determined from these data.

A theoretical sodium nitrate yield can be calculated from biomass composition. Base on an old study, the molecular ratio of carbon, nitrogen and phosphorus of marine organic is constant and equal to 106:16:1, respectively (Redfield 1934). Using this ratio for algae and assuming that all of the nitrogen comes from sodium nitrate, the sodium nitrate yield coefficient was calculated by another group to be $Y_{X/S}$ = 2.62 g mass/g substrate (Elbeling 2006). The experimental yield obtained from three different

experiments as reported in Table 4.3, are between 0.58±0.12 to 1.23±0.10 g cell/ g $NaNO_3$ which are below the theoretical value as expected.

In a study previously done by a colleague to obtain the yield coefficient values for *S. dimorphus*, the large errors in results practically prevented them from reliable calculation of the yield (Ribita 2011). The yield values from that study were between 0.57±1.71 g cell/g substrate to 2.67±4.41 g cell/g substrate which some of them falls outside the theoretical range. But as shown, the yield coefficient values of all three experiments in this present work agree with the theoretical values.

To improve the measurement of the yield coefficient even further, it would be recommended that future experiments are performed in a better controlled environment to avoid fluctuations and unintentional changes in the experimental conditions and also sodium nitrate concentration might be measured using other methods for higher precision.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Dry vegetable powder and *Scenedesmus dimorphus* were used in this research. This microalgae strain was selected because it has been shown to be a good candidate for lipid production. Microalgae have many potential applications in the realms of biofuels and biotechnology but commercial viability can only be achieved by optimizing process parameters with respect to time and cost. When considering the application of algal lipid as a biofuel source, the effect of sodium nitrate and lipid content are the most important aspects of the process. This work has succeeded in discovering an effective lipid extraction method and the effect of sodium nitrate on lipid content

Lipid extraction on dry vegetable powder was performed to analyze the effect of different permeabilization methods and compare the measured lipid content with what was reported by the manufacturer. In this research, the effect of five permeabilization

methods including wet- pellet, sonication, freeze-dry, freeze-thaw and grinding followed by hexane/isopropanol (3:2, v/v) solution as solvent system on lipid recovery of *Scenedesmus dimorphus* UTEX 746 was studied. Optimal permeabilization and lipid extraction technique for *S. dimorphus* was found to be grinding method. Our results indicate that permeabilization of the dry biomass with mortar and pestle, followed by the 2-stage solvent extraction, had the most consistent results.

The results also show that sodium nitrate concentration has an effect on growth rate and lipid content and is inversely proportional to the lipid content. Based on our hypothesis which was confirmed by our results, the lipid content was increased under sodium nitrate starvation. Higher lipid content might be achieved in future work by varying the nutrient starvation limits.

In all four batch experiments on *S. dimorphus*, growth rates were also determined and varied from 0.11 day^{-1} to 0.6 day^{-1}. In fed-batch experiment, the growth rates varied from 0.27 day^{-1} to 0.47 day^{-1}. Three of four bottles in this experiment had almost the same growth rate values and it seems that concentration of sodium nitrate did not have any effect on growth rate. In all experiments, after harvesting algae, it took 2-3 days to measure lipid content.

This work has shown the variation of $Y_{x/s}$ in all three batch experiments performed with *S. dimorphus*. Yield coefficient values of sodium nitrate concentration of experiments 1 and 3 (bottle 3) in section 4.3, are almost similar and experiment 3 (bottle 4) has the highest amount of yield coefficient of sodium nitrate among three experiments. The yield coefficient values were between 0.58±0.12 to 1.23±0.10 (g/g).

5.2 Recommendation

Further research is recommended, including:

- Continue to investigate the effect of higher and lower sodium nitrate concentration on growth rate and lipid content.
- Investigate alternative nitrogen source like ammonium or urea instead of sodium nitrate.

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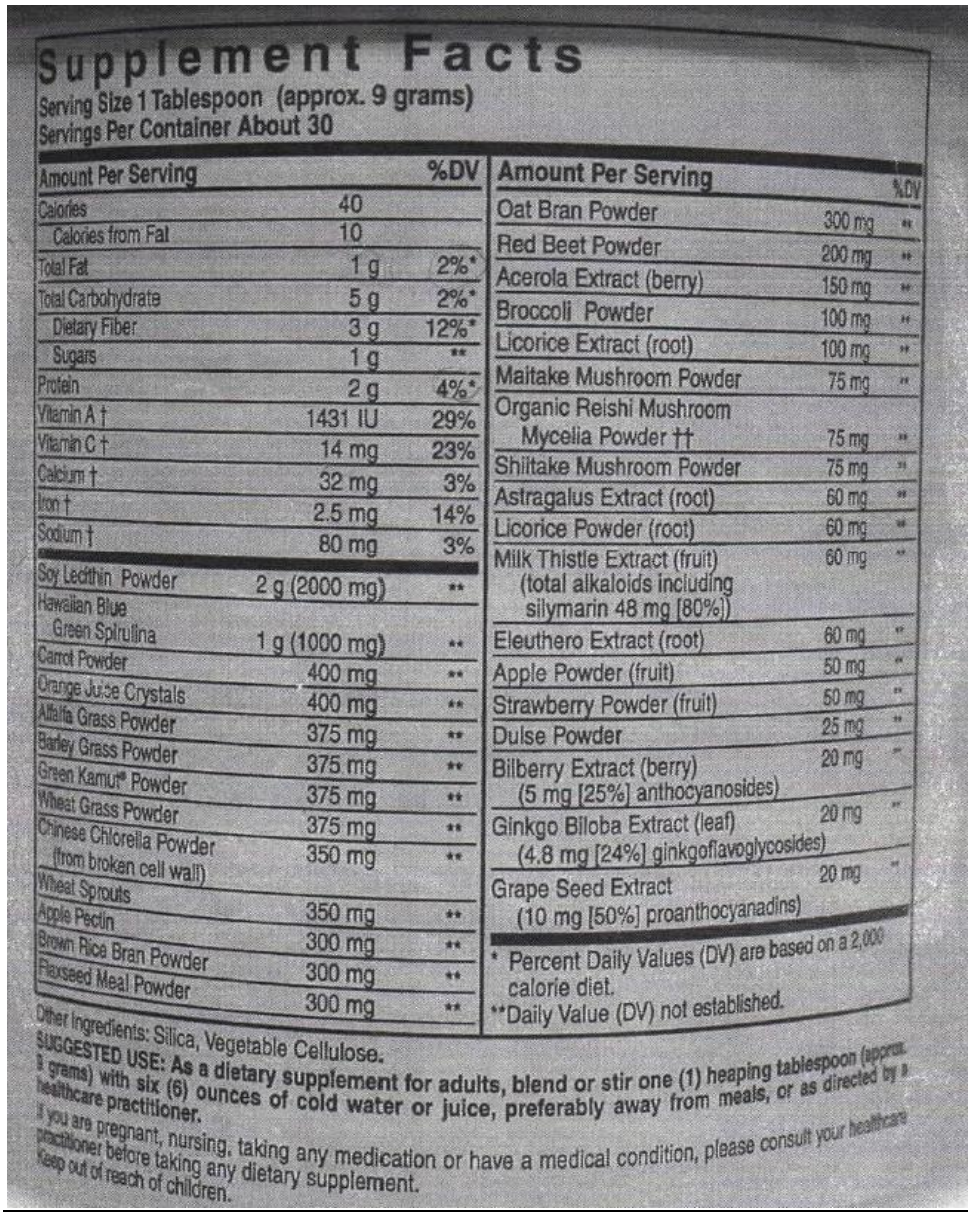
APPENDICES

APPENDIX-A – VEGETABLE POWDER COMPOSITION

The supplement facts for dry vegetable powder, EARTH SOURCE Greens and More from Solgar Company as shown below:



Supplement Facts

Serving Size 1 Tablespoon (approx. 9 grams)
Servings Per Container About 30

Amount Per Serving		%DV
Calories	40	
Calories from Fat	10	
Total Fat	1 g	2%*
Total Carbohydrate	5 g	2%*
Dietary Fiber	3 g	12%*
Sugars	1 g	**
Protein	2 g	4%*
Vitamin A †	1431 IU	29%
Vitamin C †	14 mg	23%
Calcium †	32 mg	3%
Iron †	2.5 mg	14%
Sodium †	80 mg	3%
Soy Lecithin Powder	2 g (2000 mg)	**
Hawaiian Blue Green Spirulina	1 g (1000 mg)	**
Carrot Powder	400 mg	**
Orange Juice Crystals	400 mg	**
Alfalfa Grass Powder	375 mg	**
Barley Grass Powder	375 mg	**
Green Kamut® Powder	375 mg	**
Wheat Grass Powder	375 mg	**
Chinese Chlorella Powder (from broken cell wall)	350 mg	**
Wheat Sprouts	350 mg	**
Apple Pectin	300 mg	**
Brown Rice Bran Powder	300 mg	**
Flaxseed Meal Powder	300 mg	**
Oat Bran Powder	300 mg	**
Red Beet Powder	200 mg	**
Acerola Extract (berry)	150 mg	**
Broccoli Powder	100 mg	**
Licorice Extract (root)	100 mg	**
Maitake Mushroom Powder	75 mg	**
Organic Reishi Mushroom Mycelia Powder ††	75 mg	**
Shiitake Mushroom Powder	75 mg	**
Astragalus Extract (root)	60 mg	**
Licorice Powder (root)	60 mg	**
Milk Thistle Extract (fruit) (total alkaloids including silymarin 48 mg [80%])	60 mg	**
Eleuthero Extract (root)	60 mg	**
Apple Powder (fruit)	50 mg	**
Strawberry Powder (fruit)	50 mg	**
Dulse Powder	25 mg	**
Bilberry Extract (berry) (5 mg [25%] anthocyanosides)	20 mg	**
Ginkgo Biloba Extract (leaf) (4.8 mg [24%] ginkgoflavoglycosides)	20 mg	**
Grape Seed Extract (10 mg [50%] proanthocyanadins)	20 mg	**

* Percent Daily Values (DV) are based on a 2,000 calorie diet.
**Daily Value (DV) not established.

Other Ingredients: Silica, Vegetable Cellulose.

SUGGESTED USE: As a dietary supplement for adults, blend or stir one (1) heaping tablespoon (approx. 9 grams) with six (6) ounces of cold water or juice, preferably away from meals, or as directed by a healthcare practitioner.

If you are pregnant, nursing, taking any medication or have a medical condition, please consult your healthcare practitioner before taking any dietary supplement.
Keep out of reach of children.

Table A.2. Comparison of different extraction procedures using dry vegetable powder.

Experiment 1	**solvent**		**freeze-dryer**	
Biomass (lipid free)(g)	**0.182**	**0.1314**	**0.1823**	**0.1737**
Lipid (g)	**0.0549**	**0.0357**	**0.0496**	**0.0399**
Total Biomass (after extraction) (g)	**0.2369**	**0.1671**	**0.2319**	**0.2136**
Lipid %	**23.17**	**21.36**	**21.39**	**18.68**
Experiment 2	**solvent+grinding**			
Biomass (lipid free)(g)	**0.47**	**0.4766**	**0.4831**	**0.4801**
Lipid (g)	**0.1407**	**0.1497**	**0.1482**	**0.1442**
Total Biomass (after extraction) (g)	**0.6107**	**0.6263**	**0.6313**	**0.6243**
Lipid %	**23.04**	**23.90**	**23.48**	**23.10**
Experiment 3	**solvent+filtering**			
Biomass (lipid free)(g)	**0.2442**	**0.25**		
Lipid (g)	**0.0531**	**0.0547**		
Total Biomass (after extraction) (g)	**0.2973**	**0.3047**		
Lipid %	**17.86**	**17.95**		

APPENDIX-B – CHECK LIST FOR EXPERIMENT

1) Preparation List Before Each Experiment
    a) Media
    b) Seed jar
    c) Autoclave
    d) CO_2 tank
    e) Lights
    f) Temperature probe
    g) DI water
    h) Prepare bubbler assemblies
    i) Prepare clean bottles/ jars/Erlenmeyer
2) Preparation Steps for Each Experiment
    a) Wrap all the bubbler equipments in autoclavable paper.
    b) Autoclave one DI water bottle for future need.
    c) Autoclave the media bottle using media recipe without vitamins shown in Table I.2.
    d) Prepare 1500 mL media in 2L bottles, some of them containing the various amount of sodium nitrate ($NaNO_3$) as it has shown in the following table.
    e) Cover all the bottles/jars loosely with a screw cap and Aluminum foil.
    f) Turn on the autoclave machine.
    g) Autoclave time is 30 minutes.

h) After finishing the autoclave time, let the glass containers be cool at enough to be touched and then unwrap the screw caps and foils.

i) Put them under hood to reach to room temperature before starting any experiment.

APPENDIX-C – MEDIA COMPOSITION

Table C.1. Experimental design; Factors A, B, and C are defined as follows: A: % Digestate, at levels of 1% (-1) and 2% (1) with a center point value of 1.5% (0). B: mL of KH2PO4/K2HPO4 solution, at levels of 0 mL added (-1), 3 mL added (1), with a center point value of 1.5 mL (0). C: mL of 0.0305 M MgSO4 solution, at levels of 0 mL added (-1), 3 mL added (1), with a center point value of 1.5 mL (0). High and low levels for magnesium and phosphorus are based upon prior experiments with 3N-BB. Actual concentrations of Mg2+ and PO43- in each flask derived from additions of MgSO4 and KH2PO4/K2HPO4 solutions. Note that digestate already contains magnesium and phosphate, but there is considerable uncertainty in the exact amount in this particular batch of digestate. The concentrations shown in the table below do not reflect basal amounts contributed by the digestate (from Jacob Shwank Master thesis, Cleveland State University).

			Factors			Actual Concentrations		
Flask #	CenterPt	Blocks	Factor A digestate	Factor B KH_2PO_4/K_2HPO_4	Factor C $MgSO_4$	Digestate%	PO_4^{3} (mM)	Mg^{2+} (mM)
1	1	2	1	1	1	2	3.44	0.61
2	0	2	0	0	0	1.5	1.72	0.305
3	1	2	-1	-1	1	1	0	0.61
4	1	2	1	-1	-1	2	0	0
5	1	2	-1	1	-1	1	3.44	0
6	0	2	0	0	0	1.5	1.72	0.305
7	0	2	0	0	0	1.5	1.72	0.305
8	0	2	0	0	0	1.5	1.72	0.305
9	1	1	-1	-1	-1	1	0	0
10	1	1	-1	1	1	1	3.44	0.61
11	0	1	0	0	0	1.5	1.72	0.305
12	1	1	1	-1	1	2	0	0.61
13	0	1	0	0	0	1.5	1.72	0.305
14	0	1	0	0	0	1.5	1.72	0.305
15	0	1	0	0	0	1.5	1.72	0.305
16	1	1	1	1	-1	2	3.44	0

Table C.2. Media composition on *S. dimorphus* grown in 3N-BB+V media of 100 mL working volume on set 1.

Flask#	**$NaNO_3$**	**$CaCl_2$**	**$MgSO_4$**	**K_2HPO_4 / KH_2PO_4**	**P-IV**	**Vitamins (B1/B12/H)**	**NaCl**
α	***0.75 ml***	***0.25 ml***	***0.25 ml***	1.5 ml / 1.5 ml	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
β	4.5 ml	***0.25 ml***	1.5 ml	***0.25 ml / 0.25 ml***	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
γ	***0.75 ml***	1.5 ml	1.5 ml	1.5 ml / 1.5 ml	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
δ	4.5 ml	1.5 ml	***0.25 ml***	***0.25 ml / 0.25 ml***	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
ε	4.5 ml	1.5 ml	***0.25 ml***	1.5 ml / 1.5 ml	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
θ	***0.75 ml***	1.5 ml	1.5 ml	***0.25 ml / 0.25 ml***	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
σ	4.5 ml	***0.25 ml***	1.5 ml	1.5 ml / 1.5 ml	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
φ	***0.75 ml***	***0.25 ml***	***0.25 ml***	***0.25 ml / 0.25 ml***	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml

Table C.3. Media composition on *S. dimorphus* grown in 3N-BB+V media of 100 mL working volume on set 2.

Flask#	**$NaNO_3$**	**$CaCl_2$**	**$MgSO_4$**	**K_2HPO_4 / KH_2PO_4**	**P-IV**	**Vitamins (B1/B12/H)**	**NaCl**
α	***0.75 ml***	1.5 ml	1.5 ml	1.5 ml / 1.5 ml	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
β	4.5 ml	1.5 ml	***0.25 ml***	***0.25 ml / 0.25 ml***	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
γ	***0.75 ml***	***0.25 ml***	***0.25 ml***	1.5 ml / 1.5 ml	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
δ	4.5 ml	***0.25 ml***	1.5 ml	***0.25 ml / 0.25 ml***	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
ε	4.5 ml	***0.25 ml***	1.5 ml	1.5 ml / 1.5 ml	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
θ	***0.75 ml***	***0.25 ml***	***0.25 ml***	***0.25 ml / 0.25 ml***	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
σ	4.5 ml	1.5 ml	***0.25 ml***	1.5 ml / 1.5 ml	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
φ	***0.75 ml***	1.5 ml	1.5 ml	***0.25 ml / 0.25 ml***	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml

Table C.4. Media composition on *S. dimorphus* grown in 3N-BB+V media of 100 mL working volume on set 3.

Flask #	**$NaNO_3$**	**$CaCl_2$**	**$MgSO_4$**	**K_2HPO_4 / KH_2PO_4**	**P-IV**	**Vitamins (B1/B12/H)**	**NaCl**
α	4.5 ml	***0.25 ml***	1.5 ml	1.5 ml / 1.5 ml	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
β	***0.75 ml***	***0.25 ml***	***0.25 ml***	***0.25 ml / 0.25 ml***	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
γ	4.5 ml	1.5 ml	***0.25 ml***	1.5 ml / 1.5 ml	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
δ	***0.75 ml***	1.5 ml	1.5 ml	***0.25 ml / 0.25 ml***	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
ε	***0.75 ml***	1.5 ml	1.5 ml	1.5 ml / 1.5 ml	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
θ	4.5 ml	1.5 ml	***0.25 ml***	***0.25 ml / 0.25 ml***	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
σ	***0.75 ml***	***0.25 ml***	***0.25 ml***	1.5 ml / 1.5 ml	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
φ	4.5 ml	***1.5 ml***	1.5 ml	***0.25 ml / 0.25 ml***	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml

Table C.5. Media composition on *S. dimorphus* grown in 3N-BB+V media of 100 mL working volume on set 4.

Flask#	**$NaNO_3$**	**$CaCl_2$**	**$MgSO_4$**	**K_2HPO_4 / KH_2PO_4**	**P-IV**	**Vitamins (B1/B12/H)**	**NaCl**
α	4.5 ml	1.5 ml	1.5 ml	1.5 ml / 1.5 ml	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
β	***0.75 ml***	1.5 ml	***0.25 ml***	***0.25 ml / 0.25 ml***	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
γ	4.5 ml	***0.25 ml***	***0.25 ml***	1.5 ml / 1.5 ml	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
δ	***0.75 ml***	***0.25 ml***	1.5 ml	***0.25 ml / 0.25 ml***	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
ε	***0.75 ml***	***0.25 ml***	1.5 ml	1.5 ml / 1.5 ml	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
θ	4.5 ml	***0.25 ml***	***0.25 ml***	***0.25 ml / 0.25 ml***	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
σ	***0.75 ml***	1.5 ml	***0.25 ml***	1.5 ml / 1.5 ml	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
φ	4.5 ml	1.5 ml	1.5 ml	***0.25 ml / 0.25 ml***	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml

Table C.6. Media composition on *S. dimorphus* grown in 3N-BB+V media of 100 mL working volume on set 5.

Flask #	**$NaNO_3$**	**$CaCl_2$**	**$MgSO_4$**	**K_2HPO_4 / KH_2PO_4**	**P-IV**	**Vitamins (B1/B12/H)**	**NaCl**
α	4.5 ml	***0.25 ml***	***0.25 ml***	1.5 ml / 1.5 ml	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
β	***0.75 ml***	***0.25 ml***	1.5 ml	***0.25 ml / 0.25 ml***	0.9 ml	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
γ	4.5 ml	1.5 ml	1.5 ml	1.5 ml / 1.5 ml	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
δ	***0.75 ml***	1.5 ml	***0.25 ml***	***0.25 ml / 0.25 ml***	***0.15 ml***	0.15 ml / 0.15 ml / 0.15 ml	1.5 ml
ε	***0.75 ml***	1.5 ml	***0.25 ml***	1.5 ml / 1.5 ml	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
θ	4.5 ml	1.5 ml	1.5 ml	***0.25 ml / 0.25 ml***	0.9 ml	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
σ	***0.25 ml***	***0.25 ml***	1.5 ml	1.5 ml / 1.5 ml	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml
φ_5	4.5 ml	***0.25 ml***	***0.25 ml***	***0.25 ml / 0.25 ml***	***0.15 ml***	***0.025 ml / 0.025 ml / 0.025 ml***	1.5 ml

APPENDIX-D – SODIUM NITRATE CONCENTRATION EFFECT ON EXPERIMENTS

During this research equations about the effect of nitrate concentration on lipid, used were:

$$NaNO_3\left(\frac{g}{L}\right) = 25\left(\frac{g}{L}\right) NaNO_3\,(Stock) \times 30mL \times 99\% = 0.74$$

$$NO_3\left(\frac{g}{L}\right) = NaNO_3\ \left(\frac{g}{L}\right) \times 62g\ NO_3/85\ g\ NaNO_3 = 0.54$$

$$NO_3\left(\frac{g}{L}\right) = Reading\ N\left(\frac{mg}{L}\right) \times 62g\ NO_3/14\ g\ N \times 1g/1000mg$$

Where, 25 g/L $NaNO_3$, according to stock solution concentration (Figure 3.1):

W_{NO3}=62 g, W_{NaNO3}=85 g, W_{N}=14 g

Nitrate experiment sampling

This experiment was done in 3N-BB media. Samples were taken during 11 days from 3 different bottles. Each sample is used to be examined the concentration of nitrate with the nitrate probe. Figures of the "theoretical concentration of nitrate versus measured concentration of

nitrate" and "theoretical concentration of nitrate versus percentage of error" shown in the following Table and Figures. The error percentage is defined as below:

$$(Mean - Theoretical\ amount)/Theoretical\ amount \times 100 = Error\%$$

Table D.1. Measured amount of nitrate in *S.dimorphus* in 3N-BBM. To calculate the amount of error in the experiment.

Measured NO_3 in bottle 1	Measured NO_3 in bottle 2	Measured NO_3 in bottle 3	Mean	NO_3-theor.(g/l)	Error (%)
0.817	0.928	1.000	0.915	0.542	68.96
0.402	0.466	0.489	0.452	0.271	66.95
0.176	0.211	0.221	0.203	0.135	49.72
0.083	0.108	0.106	0.099	0.068	46.29
0.044	0.049	0.055	0.049	0.034	45.20
0.022	0.024	0.027	0.024	0.017	43.35
0.010	0.012	0.012	0.011	0.008	34.10
0.005	0.006	0.007	0.006	0.004	44.66
0.004	0.004	0.005	0.004	0.002	102.48
0.004	0.004	0.004	0.004	0.001	282.21
0.003	0.003	0.003	0.003	0.000	408.41

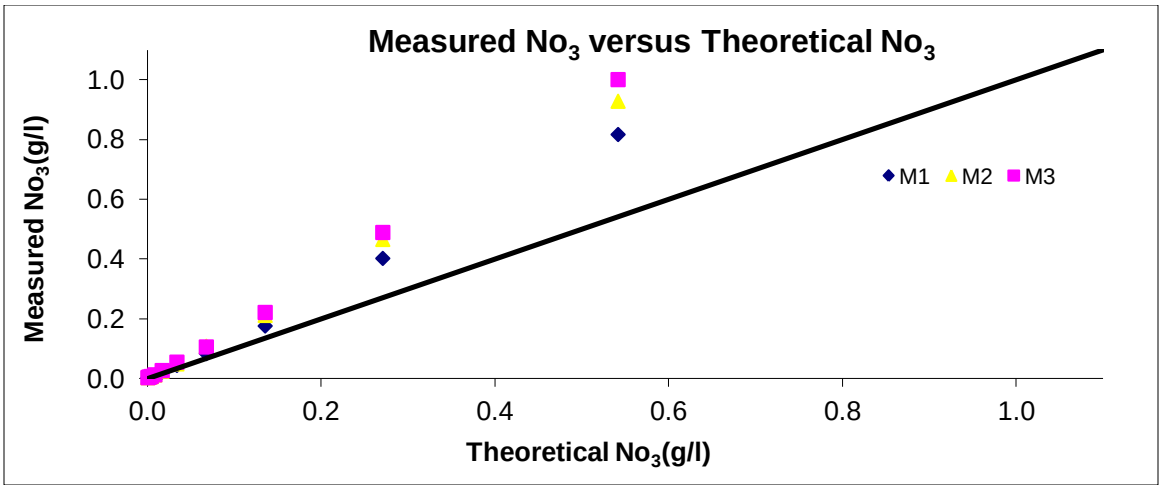


Figure D.1. Calibration of nitrate in 3N-BB media. The calibration of measured nitrate versus theoretical nitrate.

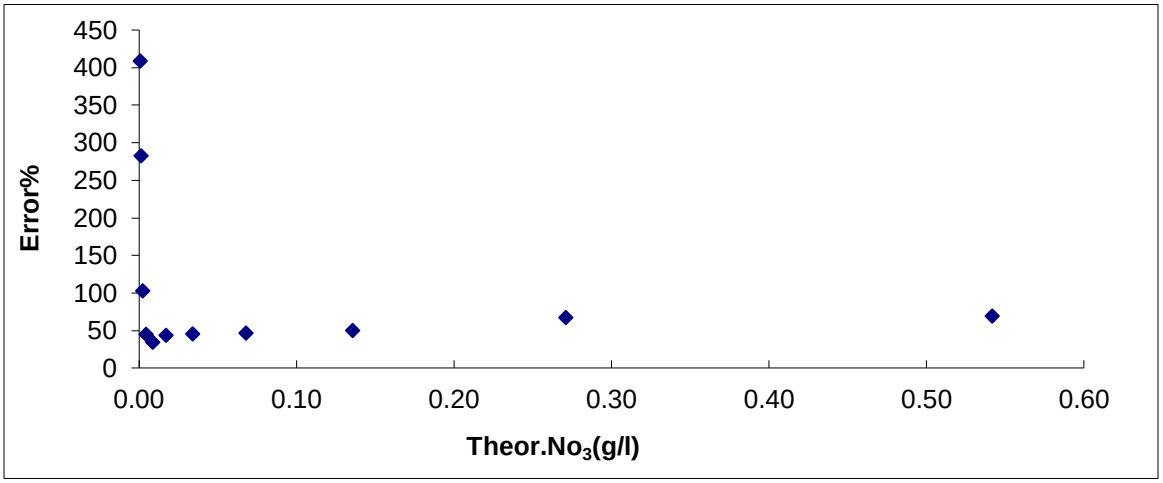


Figure D.2. Calibration of nitrate in 3N-BB media. Comparison of percantage of error with theoretical amount of nitrate.