QUANTIFICATION OF *SCENEDESMUS DIMORPHUS* GROWTH AND
SUBSTRATE KINETICS FOR CONTINUOUS PHOTOBIOREACTOR DESIGN

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This thesis is dedicated to my husband, Abdulrhman Alboraihi, as a token of appreciation for all his encouragement and support throughout my academic pursuit.
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Increasing gas prices, limited fossil fuel resources and U.S. dependence on foreign oil make research in alternative fuels a priority. The feasibility of producing fuels from microalgae is economically dependent on improvements in lipid productivity by the algae. The research presented here focuses on the development of a mathematical model to describe the biomass and lipid productivity in a continuously-operated photobioreactor (PBR) system.

Five different cell lysing methods were evaluated for the purpose of improving the methods of analysis of lipid synthesis. The two most promising methods were found to be mortar and pestle and organic solvent cell fractionation methods.

Four types of batch experiments were performed with Scenedesmus dimorphus to determine key reactor model parameters: maximum cell growth rate ($\mu_{\text{max}}$), yield ($Y_{xs}$), and Monod constant ($K_s$). Two of the experiments were performed with varying initial sodium nitrate concentrations, for the purpose of more accurately obtaining the Monod parameters. The data was analyzed using three methods: differential/linear least-square, initial substrate and nonlinear. Nearly all of the results had non-reliable error results because most data yielded an ill-conditioned Covariance Matrix. Based on the results obtained by initial substrate method, the $K_s$ was determined to be about 0.005 +/- 0.01
g/L, the maximum growth rate to be $0.7 \pm 0.1$ day$^{-1}$ and the yield to range between 1.2 and 2.7 $g_{\text{cell}}/g_{\text{substrate}}$.

The values found in this research, although preliminary, were used to formulate an approximate steady-state model of a two- PBR system, with first reactor used for maximizing biomass and utilizing substrate, and the second reactor for accumulating lipids. The fed substrate concentration and the dilution rate of the first reactor were estimated to be ($S_0$ and $D$ here) 1g/L and 0.65 day$^{-1}$ for optimal biomass productivity. The dilution rate obtained for the second reactor suggests that the volumes ratio of the 2nd to the 1st reactor is about 3.25. A large second reactor would have a negative economic impact. Based on the results obtained here it is suggested that a concentration step be implemented between the first algae growth reactor and the second lipid accumulation reactor.

The growth and substrate kinetics along with experimental measurements of growth, nitrogen yield, and extraction methods are anticipated to help further microalgae based fuel R&D and thus become the foundation for scale-up of algae-based systems.
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NOMENCLATURE

General

PBR = photobioreactor
CSTR = continuously stirred tank reactor
OD = optical density

Reactor design

$A =$ Constant in lipid kinetic model ($g_{lipid}/g_{cell}/day$)
$D_1 =$ Reactor 1 dilution rate ($day^{-1}$)
$D_2 =$ Reactor 2 dilution rate ($day^{-1}$)
$F =$ Volumetric flow rate ($L/day$)
$K_S =$ Monod coefficient ($g/L$)
$K_D =$ cell death rate ($g/L/day$)
$L_0 =$ Feed lipid concentration ($g/L$)
$L_1 =$ Reactor 1 lipid concentration ($g/L$)
$L_2 =$ Reactor 2 lipid concentration ($g/L$)
$r_o =$ Rate of lipid increase with cell mass increase ($g_{lipid}/g_{cell}$)
$r_L =$ Lipid formation rate ($g/L/day$)
$r_{S1} =$ Substrate uptake rate in the first reactor ($g/L/day$)
$r_{S2} =$ Substrate uptake rate in the first reactor ($g/L/day$)
$r_X =$ Cell growth rate ($g/L/day$)
$S_0 =$ Feed $NaNO_3$ concentration ($g/L$)
$S_1 =$ Reactor 1 $NaNO_3$ concentration ($g/L$)
$S_2 =$ Reactor 2 $NaNO_3$ concentration ($g/L$)
$V_1 =$ Volume of 1st reactor ($L$)
$V_2 =$ Volume of 2nd reactor ($L$)
$X_0 =$ Feed cell concentration ($g/L$)
$X_1 =$ Reactor 1 cell concentration ($g/L$)
$X_2 =$ Reactor 2 cell concentration ($g/L$)
$\tau_D =$ Cell doubling time ($day^{-1}$)
$Y_{XS} =$ Yield of substrate uptake by the cells
$B =$ Constant used in lipid kinetic model ($L/g_{substrate}$)
$\beta =$ Constant in yield formula adjustment ($g_{substrate}/g_{cell}/day$)
$\mu =$ Growth rate in the 1st reactor ($day^{-1}$)
$\mu_2 =$ Growth rate in the 2nd reactor ($day^{-1}$)
CHAPTER I
INTRODUCTION

There are three major reasons that make research in an alternative fuel source a necessity in U.S. First, fossil fuel is a finite resource. Considering even the most optimistic estimate of oil peak production, somewhere near 2040 (Kerr, 2007) research in alternative fuels is needed now. Second, the U.S. imports most of its oil which poses a security problem. In 2009, the United States consumed a total of 6.9 billion barrels of oil (refined petroleum products and biofuels), from which 51% was imported from foreign counties, including 17% of that from unstable areas of Middle East (Figure 1) (EIA, 2010). Third, burning of fossil fuels forms carbon dioxide, a greenhouse gas, from carbon that has been outside of the carbon cycle for millions of years, which creates a net-positive increase in greenhouse gas in the Earth’s atmosphere. Thus, a fuel source with a net-zero greenhouse gas emission is required to stop mankind’s effect on the changing temperature of the Earth (Karl, 2003).

Explored sources for alternative fuels include plants, bacteria and algae. Microalgae have been regarded as the most promising renewable source as it can provide several different types of renewable biofuels: methane, oil, biohydrogen (Chisti, 2007).
Figure 1 - 2009 U.S. Net Petroleum Imports (EIA, 2010). (http://www.eia.doe.gov/energy_in_brief/foreign_oil_dependence.cfm).

Additional reasons that make algae an attractive source of biomass feedstock to produce energy, include: algae does not compete with food crops since algae can be grown on non-arable land in open ponds or photobioreactors (PBR), and are not limited by seasonal growth; algae, have higher energy yields per land area than terrestrial crops; have water advantages, because species grow in fresh, marine, or brackish water and algae consumes CO$_2$ and CO$_2$ from industrial sources can be recycled through algae growth (Ferrell et al., 2010) (Sheehan et al., 1998).

In the 1950s, the idea of producing methane from microalgae was first explored at the UC at Berkeley. Large scale cultivation of microalgae, mostly for food production, was first explored during WWII in Germany (Wolfgang, 1994). The oil situation in the 1970s, including the oil embargo and Iranian Revolution, brought the need for energy security to the forefront and led to renewed study of microalgae biofuels, hydrogen, and methane. This oil crisis shock resulted in the development of the Aquatic Species Program (ASP)
led by U.S. Department of Energy (DOE) from 1978 to 1996. In more recent years, with the major increase of oil prices (Figure 2), Research and Development (R&D) for fuels from microalgae has received renewed attention.

Figure 2 - Nominal crude oil prices between 1970 and 2010. (http://www.stlouisfed.org/).

One conclusion of the ASP program was that the feasibility of producing fuels from microalgae is economically dependent on overall productivity rates, which could be improved by increasing algae species’ ability to produce lipids. Studies on various algae species suggest that stressing the algae, by limiting a key nutrient, can increase lipid accumulation (Ferrell et al., 2010). The variability in lipid accumulation may depend on species and on other growth factors such as light or nutrients. A downfall of inducing lipid accumulation through nutrient stresses is that the synthesis rate of all other cell components is lowered (Sheehan et al., 1998). Finding a species that grows well and
stores large amounts of lipid at early stationary phase under nutrient starvation is therefore key.

For large scale production a continuous system is ideal. The purpose of this research is to determine the optimum operating conditions of a two photobioreactor (PBRs) continuous system. A PBR is a closed system that supports a biologically active environment by supplying light, nutrients, agitation, and heat to the culture (North American Algae Corporation, 2008). Continuous PBRs require a steady flow rate to allow for uninterrupted feed and harvest. The PBRs in this research are modeled as well-mixed continuous stirred tank reactors (CSTRs) in series. The design of the two-PBR system presented here involves: continuous media inflow into the first reactor to maintain the algae culture in growth phase; non-stop harvest of culture, containing low concentration of limiting nutrient, from the first reactor into the second reactor; and continuous harvest from the second reactor to a holding tank or a dewatering step. The design considers lipid synthesis stimulation in the second reactor by nutrient limitation. For designing purposes, a series of batch experiments were run to determine the key parameters such as: maximum cell growth rate ($\mu_{\text{max}}$), yield ($Y_{X/s}$), and Monod constant ($K_s$). In doing so, the continuous system dilution rate and the specific concentration of limiting nutrient in the feed could be determined.

To obtain a kinetic expression for the PBR model, the batch experiments were performed using nitrogen, as the limiting nutrient, and Scenedesmus dimorphus, as the algae species. Scenedesmus sp. was selected during the ASP research as one of the top 15 candidates from the 3000 species collection and was reported to have 44.7% lipid content after nitrogen limitation (Sheehan et al., 1998). Literature reports that S.
*dimorphus* can achieve lipid contents between 16% - 40% (Wolfgang, 1994). Although, microalgae can be grown in open pond (usually raceway), closed PBR, or a hybrid of the two, the *S. dimorphus* was cultured in closed PBRs at Cleveland State University (CSU) because of benefits such as high productivity rates and minimal contamination. A laboratory-scale lipid extraction method development, with a focus on cell fractionation, was carried-out in parallel to assist in the formulation of a kinetic expression for lipid production.

The continuous bioreactor model, along with experimental measurements of growth, nitrogen yield, and extraction methods, are the basis to assist in future microalgae based fuel research and development, and can also be considered the foundation for scale-up production. There are no large-scale plants that produce algae oil for the purpose of making alternative fuels in the world today, due to preliminary studies that forecast the process to be nonviable economically. Increasing the lipid content in algae will help nudge the economics towards an optimistic outcome, increasing the chances of successful pilot and industrial scale plants.
2.1 Lipids

Microalage stores lipids in order to conserve energy. Lipids are organic compounds, generally categorized into: simple, compound, and derived lipids (Figure 3).

Figure 3 – Lipid general classification. (http://redzuannorazlan.blogspot.com/2010/08/bbc1-k22-lipid.html).
Algae lipids vary with the species. Fatty acids, a fuel precursor in microalgae, are similar to those in terrestrial plant leaves. The portion of these acids that is different is that microalgae contain mostly C16 fatty acids and less C8 components than those reported for plant leaves (Gunstone, 2007). *Scenedesmus dimorphus* has mostly C18 components (Renaud, 1994).

The biosynthesis of saturated and monounsaturated fatty acid in plants occurs in plastids, also called chloroplasts in green algae (Houtz, 2007). Chloroplasts are the organelles in plants and algae in which photosynthesis occurs. They also are critical in starch and other product storage. The fatty acid synthesis involves two major enzyme reactions, as shown in Figure 4.

![Diagram of fatty acid biosynthesis](image)

*Figure 4 - Fatty acid biosynthesis.*


An Acetyl-CoA carboxylase catalysis is the first committed reaction in fatty acid synthesis: acetyl-CoA + CO₂ → malonyl-CoA. The second major reaction is performed
by fatty acid synthase (FAS) of type II which contains a multiprotein complex. There are several enzymes involved in FAS, depicted by the reoccurring reactions, in the cycle shown, in Figure 4. The reoccurring reactions continue six more times for C16 fatty acids and seven more times for C18 fatty acids (Hartzler, 2009).

Microalgae lipid extraction methodologies may depend on the selected species. The research discussed in this thesis is focused on finding a suitable lab scale cell lysing method. General lipid extraction methodologies from tissues and cells have been developed since the early 1800s for the purpose of understanding lipids and making soap, by famous chemists including: Tachenius, Braconnot, and Chevreul. One of the best described and commonly used methods to extract lipids used today was developed by Folch in 1957. Folch method extracts lipids from water-free tissue using chloroform/methanol (2:1 v/v). Other extraction methods were proposed by Bligh and Dyer (1959) and Sheppard (1963), which also used solvent mixtures made of chloroform/methanol and ethanol/diethyl ether, respectively (Leray, 2011).

When extracting lipids from plant tissue for the purpose of making fuel, lipase inhibition is required. Lipase is an enzyme that hydrolyzes phospholipids and glycolipids and increases the amount of free fatty acids (FFAs) in the extract. In biodiesel synthesis, the FFAs are not recovered when using the commonly used alkaline catalysts. An alternative acid catalysis can be used to recover the FFA and convert them into biodiesel, however this method has a water bi-product which inhibits esterification, the reaction that converts lipids into fuel (Canakci & Van Gerpen, 2001). To inhibit lipases the extraction methods at CSU were performed using isopropanol (Leray, 2011) in an isopropanol/hexane (2:3 v/v) solvent mixture.
Before extraction can be performed, the algae cells must be lysed or broken, to expose the lipids for removal. There is no standard protocol dedicated specifically to microalgae cell lysing. However, there are general fractionation methodologies for extracting various cell constituents. There are two ways the cells can be broken: physical and chemical. The physical methods of cell disruption are further broken down into solid and liquid shear methods. The solid shear method involves crushing the plant tissue between a mortar and pestle. The liquid shear methods of cell disruption rely on the shearing forces generated between the tissue and the liquid medium, which can be performed by various methods, including: cavitation, done using ultrasound; consecutive freeze/thaw sessions, in which the ice crystals formed during freezing disrupt the cells during thawing; and freeze-dry method. Chemical cell lysing is achieved through use of organic solvents such as chloroform/methanol, mineral acids, or detergents, which interact with the phospholipids bilayer of the cell wall.

Once the cells are lysed, the lipids are extracted using a solvent as previously discussed. After extraction is complete, a homogeneous top layer is formed that contains the solvent and lipids. The bottom layer has the residual biomass. Most methods result in a bi-layer liquid suspension of solvent/extract and residual biomass. To separate the biomass from the extract, filtration and centrifugation can be used (Dey et al., 1997).

In this work, the cell lysing methods experimented with were: freeze/thaw, sonication, mortar and pestle, freeze-dry and organic solvent.
2.2 Nitrogen Limitation in Algae

*Scenedesmus dimorphus* (Figure 5) is a unicellular algae in the class Chlorophyceae. This is one of the favored species for oil yield to produce biodiesel (Wolfgang, 1994).

![Figure 5 - Scenedesmus Dimorphus.](http://commons.wikimedia.org/wiki/File:Scenedesmus_dimorphus.jpg)

Each algae species prefers a specific nitrogen source. The few documented research papers to date using *S. dimorphus*, including: algae growth and lipid production (Ying et al., 2009) and waste water nitrogen removal (Gonzalez et al., 1997), discuss four different nitrogen sources. Ying and co-workers, (2009) research showed that nitrogen is a limiting nutrient in algae growth and that lipid accumulation is directly related to algae growth. Ying’s results for *S. dimorphus* showed that a preferential nitrogen source is urea, then nitrate, and a least favored source is glycine. Gonzalez and co-workers showed *S. dimorphus* to be an appropriate species to remove ammonia from wastewater. The nitrogen source used in the research presented here is sodium nitrate, one of the constituents of standard lab media.
No available sources to date report specific data on *S. dimorphus* lipid accumulation induced by nitrogen limitation. The ASP reports that *Scenedesmus TR-84* had a lipid content of 44.7 \% after 14 days of assumed nitrogen-limitation. Studies from 2008 by collaborating universities in Russia and Israel, of the species *Parietochloris incise*, showed that nitrogen deficiency promotes lipid biosynthesis. The strain was starved of nitrogen in the early stationary phase. The nitrogen limited *P. incise* produced almost double the lipids over a total growth of 14 days; 17.2 \% lipid content when grown under sufficient nitrogen conditions and 33.5 \% lipid content when grown under nitrogen deficient conditions (Solovechenko, 2008). Another group at Osaka University in Japan reported similar results for a *Nannochloris sp.*, where nitrogen-limited algae produced 20 \% more lipids than the algae that were fed adequate levels of nitrogen (Takagi, 2000). Additional sources present more moderate lipid accumulation under nitrogen-starvation, such as 15 \% higher, in *Neochloris oleoabundans* (Li, 2008).

### 2.3 Photobioreactor Design For Substrate Limited Cell Growth

The concept of a two-PBR continuous system for the purpose of growing algae and increasing lipid production is explored in this thesis. The model of a two-photobioreactor system is established by combining an exponential growth model with Monod kinetics in two CSTRs in series. The design consideres a continuous volumetric flow rate of media through a two-reactor system. The optimal ratio of the flow rate to reactor volume, called the dilution rate, is to be determined by the following design goals: maintaining the algae culture in growth phase in the first reactor, and inducing additional lipid synthesis in the second reactor by limiting nitrogen. The media flow from the first reactor into the second reactor should have virtually zero nitrogen concentration, so the
design needs to consider nitrogen yield and uptake in order to determine the appropriate limiting nutrient concentration in the media fed into the first reactor.

The simple exponential growth model was originally introduced by Thomas Robert Malthus in 1798. Microalgae follow the exponential growth model under batch conditions. The differential equation for a cell in terms of specific growth rate is:

\[ \mu_{net} = \frac{1}{\bar{X}} \frac{dX}{dt} \]

Where \( \mu_{net} \) is the specific growth rate, \( X \) is the cell concentration and \( t \) is time (Shuler, 2002). A typical batch culture of an organism has four phases as shown in Figure 6.

The lag phase occurs right after inoculation as the organism adjusts itself to the new environment. The growth phase occurs when the culture has plenty of nutrients, and thus the growth rate becomes independent of the nutrient concentration. Eventually, the
cells stop cell division, and enter the stationary phase. Cells begin transition to stationary phase when either an essential nutrient is depleted or toxic growth by-products have accumulated. During the stationary phase, metabolic functions, such as lipid production, are still active even though the specific growth rate is zero. The death phase occurs when all metabolic functions have stopped (Shuler, 2002).

The nitrate-limited growth can be modeled by the Monod equation:

\[ \mu = \frac{\mu_{\text{max}} S}{K_s + S} \]

where \( S \) is substrate, \( K_s \) is Monod’s constant, and \( \mu_{\text{max}} \) is the maximum growth rate. Monod describes the enzymatic Michaelis-Menten kinetics in cellular systems assuming one limiting nutrient as the substrate. As mentioned in Section 2.1, nitrate has been shown in past studies to be a limiting nutrient for algae. Previous studies on algae show that Monod kinetics to apply limited nutrient growth. Evidence of a Monod fit of growth data to nitrogen concentration have been presented in some algae species such as *Monochrysis lutheri* (Caperon, 1976) and *Isochysk galbanu* (Dugdale), but no studies on *S. dimorphus* have been found to date.

The net substrate rate of consumption can be related to the rate of cell growth as follows:

\[ -r_S = \frac{Y_X}{S} r_X \]

where \( Y_{X/S} \), known as the yield, is the ratio of consumed substrate mass over the mass of new cells formed. \( r_X \) is the rate of cells forming with time, or \( \frac{dx}{dt} \). Equation 3 also assumes
that all the substrate goes into cell formation. Knowing that \( \frac{Y_{S/X}}{Y_{X/S}} = \frac{1}{Y_{X/S}} \) \text{ Equation 3 } combined with Equation 1 gives the rate of substrate uptake as a function of cell growth:

\[
\text{Equation 4} \quad r_s = -\frac{Xu}{Y_{X/S}}
\]

The yield is species specific and it is affected by growth conditions, such as light. Information on the nitrogen limited growth and nitrogen uptake kinetics of *Scenedsoms dimorphus* are not available. As a result, the research herein is essential for future studies involving *S. dimorphus*. 
CHAPTER III
MATERIALS AND METHODS

3.1 Lipid Extraction and Cell Lysing Methods Development

The Scenedesmus dimorphus algae cells were lysed by two general types of disruption methods: physical (freeze/thaw, sonication, mortar and pestle, freeze-dry) and chemical (solvent fractionation). Prior to all extractions, preparatory centrifugation was done to concentrate the diluted culture samples to have more biomass available per extraction. Several 50 mL samples of culture were centrifuged for 30 minutes at 2000 RPM. The supernatant was discarded, and the remaining pellets were combined into a new centrifuge tube. DI water was used to ensure majority of biomass was transferred during this combination step. The centrifuge tube containing the combined biomass was centrifuged again for 30 minutes at 2000 RPM and the pellet was used for various cell lysing and lipid extraction experiments.

All extractions were performed in glass tubes using 5 mL of isopropanol/hexane (2:3 v/v) solvent mixture by shaking. After all extractions the lipid plus solvent layer was separated from the biomass layer by using a glass pipette and transferred into a new preweighed glass tube. The glass tube was placed in the fume hood because the solvent is
All extractions were performed in glass tubes using 5 mL of isopropanol/hexane (2:3 v/v) solvent mixture by shaking. After all extractions the lipid plus solvent layer was separated from the biomass layer by using a glass pipette and transferred into a new preweighed glass tube. The glass tube was placed in the fume hood because the solvent is a carcinogenic. A water aspirator was also experimented with, for two purposes: to aid in faster removal of solvent and to recover solvent. The aspirator system and vacuum manifold is shown in Figure 7.

![Figure 7 – a) Solvent recovery aspirator. b) Aspirator manifold.](image)

*Freeze/thaw cell lysing.* After the preparatory centrifugation the pellet was resuspended in DI water and placed in -20 °C freezer overnight. The next day, the samples were left at room temperature for 4 hours, then placed back in the freezer overnight. Following second freezing, the samples were thawed and then centrifuged. After removal of the supernatant, 5 mL of isopropanol/hexane (2:3 v/v) was added to the
wet pellet. Then the samples were placed on shaker at 170 RPM for 3 hours for the purpose of lipid extraction. After extraction, the samples were centrifuged again for 30 minutes at 2000 RPM to separate the solvent/lipid layer from the biomass/water layer. The top layer containing solvent and lipids was pipetted and transferred to new glass tubes, for solvent removal by aspirator.

*Sonication cell lysing.* Following the preparatory centrifuge step, the majority of supernatant was discarded and the pellet was resuspended in left over supernatant. A quarter of supernatant was left in the glass tube, to allow for sufficient total volume for the sonicator tip to reach into the liquid. After adding the extracting solvent the glass tubes were sonicated on ice for 1 minute at 40% amplitude. This was followed by 3 hours on the shaker at room temperature at 170 RPM. During shaking emulsion formed. The samples were centrifuged for 30 minutes at 2000 RPM and then the layers were allowed to settle. Four layers formed including a micelles layer. Top layer containing solvent and lipids was transferred to a new tube and placed in the hood for solvent removal. A second extraction for 3 hours at 170 RPM with an additional 5 mL of solvent was performed on the remaining layers. After the second extraction two layers were formed with the bottom containing biomass and the top containing lipid plus solvent. The top layer was transferred to the glass tube containing the first extract, then placed back in the hood for solvent removal.

*Mortar and pestle cell lysing.* The biomass pellets were rinsed with DI water after the preparatory centrifuge step and left to dry in an oven at 50 °C. Once dried the pellets were crushed into a fine powder using a mortar and pestle. The powder was then moved to a glass tube and the solvent added. Then the glass tube was placed on the shaker for 3
hour extraction. The top layer containing solvent and lipids was transferred to a new tube and placed in hood for solvent removal. A second 3 hour extraction using 5 mL of solvent was performed on the residual biomass, and the new extract added to the first extract for solvent evaporation.

*Freeze-dry cell lysing.* After pellets were combined during the centrifuge preparatory step, they were resuspended in original supernatant and placed in -80 °C freezer overnight. The next day, the frozen samples were placed in the freeze-dryer. The samples took about 20 hours to dry in the freeze-dryer. Extraction on the shaker proved troublesome as the samples were sticking to the side of the glass preventing the solvent from removing the lipids. Biomass stuck to the sides was scraped down into the solvent, but some biomass could not be removed from glass surface. Next the top layer containing solvent and lipids was transferred into a new tube and placed in the fume hood for solvent evaporation. A second 3 hour extraction with 5 mL solvent was performed on the remaining biomass, and the new extract was added to the first extract for solvent evaporation.

*Organic solvent cell lysing.* Following the centrifuge preparatory step, the wet pellet was vortexed for 1 minute, then the isopropanol/hexane (2:3 v/v) extracting solvent was added. Tubes were placed on shaker for 20 hours at 300 RPM. After shaking the solvent plus lipid layer was transferred to new glass tubes and placed in hood for solvent evaporation by vacuum. This extraction method was repeated with 6 hour extraction time instead of 20 hours. In the 6 hour extraction, a 3 hour extraction was performed followed by a second 3 hour extraction on the left over biomass.

A sample protocol for solvent cell lysing is available in Appendix A.
3.2 Batch Experiments

The *Scenedesmus dimorphus* algal cells were obtained from The Culture Collection of Algae from University of Texas. Cells were maintained in their growth phase by cultivation in a 1-L sterile fed-batch reactor. The fed-batch culture, also called the seed-jar, was used as inoculum for experiments. Periodically the seed-jar culture was restarted with fresh cells from cryostorage.

3.2.1 Growth medium

**Name:** Bold-Basal Medium with 3-fold Nitrogen and Vitamins, Modified (3N-BBM+V)

**Description:** Freshwater Algae

**Solutions, amounts per 1 liter H₂O**

1. NaNO₃ - 25.0 g
2. MgSO₄.7H₂O - 7.5 g
3. NaCl - 0.5 g
4. K₂HPO₄.3H₂O - 7.5 g
5. K₂PO₄ - 17.5 g
6. CaCl₂.2H₂O - 2.5 g
7. **Trace elements solution**
   Add to 1 litre of distilled water 0.75 g Na₂EDTA and the minerals below in exactly the following sequence:
   FeCl₃.6H₂O - 97.0 mg
   MnCl₂.4H₂O - 41.0 mg
   ZnCl₂.6H₂O - 5.0 mg
   CoCl₂.6H₂O - 2.0 mg
   Na₂Mo₄.2H₂O - 4.0 mg

8. **Vitamin B₁**
   0.12 g Thiaminhydrochloride in 100 ml distilled water. Filter sterile.

9. **Vitamin B₁₂**
   0.1 g Cyanocobalamin in 100 mg distilled water, take 1 ml of this solution and add 99 ml distilled water. Filter sterile.

Figure 8 – 3N-BBM+V media recipe.
(Source: [http://www.ccap.ac.uk/media/recipes/3N_BBM_V.htm](http://www.ccap.ac.uk/media/recipes/3N_BBM_V.htm)).
The growth medium used for all experiments is modified Bold-Basal Medium with 3-fold Nitrogen and Vitamins (3N-BBM+V). This growth medium contains all the necessary nutrients for algae cells to survive and grow. The 3N-BBM+V media recipe for one liter stock solutions is shown in Figure 8. The stock solutions were used as follows to make 1 liter of growth medium: 30 mL of NaNO₃; 10 mL of MgSO₄, NaCl, K₂HPO₄, KH₂PO₄ and CaCl₂; 6 mL of trace metal solution and 1 mL of each vitamin solution. The growth medium was prepared with DI water directly in the glass bottles or the Erlenmeyer flasks. The glass bottles and Erlenmeyer flasks containing media minus vitamins were sealed with foam stoppers, then covered loosely with foil, and autoclaved for 30 minutes at 15 psi. The foil was tightened around the neck of glass container post autoclave, during transfer from autoclave room to the laminar flow hood to prevent bacterial contamination from air. The media was left to cool in the laminar flow hood, and then the vitamins were added.

3.2.2 Growth conditions

Scenedesmus dimorphus is dense and forms thick sediments if not kept in constant agitation. The culture was kept in constant agitation using a water bath shaker. The cells were agitated at approximately 132 RPM. The bath water was kept at 30 °C using an automatic control temperature probe build into the shaker. Light was provided on a 12 hour dark/light schedule above the culture containers using seven fluorescent bulbs with a measured intensity of 515-550 ft-candles at the liquid surface. The type of fluorescent bulbs used were: 4X Coralite Aquapro T-5/10,000K/24"/14 watt bulbs, 3X Accupro AFL/F14TS/14W/830 bulbs and the most recent Coralife T-5/24"/24 watt bulbs. The
bulbs height above shaker bath was adjusted to obtain the desired intensity. See Figure 9 for shaker bath and lights set-up.

![Shaker water bath/lights set-up](image)

**Figure 9 – Shaker water bath/lights set-up**

### 3.2.3 CO₂ Delivery and Bubbling/Sampling System

A carbon dioxide – air mixture containing 5% industrial grade CO₂ was delivered to the cell culture via a bubbling/sampling system. The bubbling system was constructed in a rubber stopper, as show in Figure 10.

Three holes were made into the rubber stopper: one for delivery of CO₂, one for sampling and one for gas venting. The materials used in the bubbling/sampling apparatus were: 3/16 inch ID Tygon silicon tubing, 3/16 inch ID stainless steel tubing, two 0.2 µm syringe filter, one pipette tip and one T-connector. The vent was constructed of the
silicon tubing connected to a steel tube that penetrates all the way through the stopper. In the sampling system, a T-connector was used to attach two pieces of silicon tube. One side was used for culture to be pushed out of the container after pressuring the growth chamber. The other piece of tubing was used to push air through the tubing forcing the culture to go back into the growth container, using a syringe filled with air. The air was pushed through a syringe filter to prevent bacterial contamination. The sampling system was clamped with hemostats when not in use.

Figure 10 – Bubbling/Sampling apparatus.

A long stainless steel tube going through the stopper connects to the gas source using the silicon tube and one syringe filter. A pipette tip was attached at the end of the
steel tube inside the growth container and angled at the bottom of the container to allow for smaller gas bubbles and better mass transfer into the liquid. The air and CO2 were mixed and then delivered to each growth container through a manifold (Figure 11) containing flow meters to adjust for even gas flow velocity to all culture samples.

The bubbling systems were autoclaved separately at 15 psi for 20 minutes. Each individual apparatus was wrapped in either foil or autoclave paper loosely. To prevent bacterial contamination post autoclave during transition from autoclave to laminar flow hood, the wrapping was tightened.

**3.2.4 Batch experiments overview**

Table 1 shows four types of batch experiments that were performed to determine Monod kinetics and nitrogen yield. Three columns of data were obtained at specific points: time, and cell and substrate concentration changes with time. All batch experiments were performed in either 250 mL glass Erlenmeyer flasks or 2 L glass bottles (Figure 10). The algae species, *S. dimorphus* was grown under the same
conditions for all experiments: 30˚C water shaker bath temperature, 5% CO₂ in air-CO₂ mixture, and 12 hour light/12 hour dark (unless specified in the experimental descriptions following). Light intensity of approximately 550 ft-candle at the surface of 250 Erlenmeyer flasks and 950 foot-candle at the liquid surface for 2 L bottles. Experiments were started at an inoculation concentration of approximately 0.01 OD.

<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Set-up</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2) 1500 mL working volumes – same initial NaNO₃ concentration</td>
<td>Nitrate yield, Monod parameters, lipid analysis</td>
</tr>
<tr>
<td>2</td>
<td>(7) 150 mL working volumes – varying initial NaNO₃ concentration</td>
<td>Monod parameters, nitrate yield</td>
</tr>
<tr>
<td>3</td>
<td>(4) 1600 mL working volumes – varying initial NaNO₃ concentration</td>
<td>Monod parameters, nitrate yield</td>
</tr>
<tr>
<td>4</td>
<td>(3) 1000 mL working volumes – same initial NaNO₃ concentration</td>
<td>Lipid free biomass, nitrate yield</td>
</tr>
</tbody>
</table>

The first type of experiment was run one time with the purpose of determining nitrate yield, growth rate and lipid accumulation during growth and early stationary phases. Two 2 L bottles were inoculated with S. dimorphus in 3N-BBM+V, at a working volume of 1500 mL. Daily OD samples were taken to monitor growth. Both bottles were inoculated at the same time and kept in the same water bath shaker. The culture was grown on a 12 hour light/12 hour dark cycle for the first 10 days, followed by 24 hour light for an additional 12 days. Twice a day 10 mL samples were taken from bottle 1. The samples were centrifuged for 30 minutes and supernatant cryostored in -80 ºC freezer for later nitrate testing. 300 mL samples were taken for lipid analyses from bottle 1, the last three days of experiment, during stationary phase. 200 mL samples were obtained mid-growth, early stationary and stationary phases (days 13, 18, 20, 22 and 24) for lipid
extraction from bottle 2. Every time samples were taken for lipid analysis, the supernatant was stored at -80 °C and later tested for nitrate content. The mortar and pestle method was used for extractions. The extractions were used for further method development and to obtain a preliminary *S. dimorphus* lipid percentage.

The second type of experiment was run with the purpose of determining maximum growth rate ($\mu_{\text{max}}$), Monod constant ($K_s$), and sodium nitrate yield ($Y_{X/S}$), under nitrogen limitation. The algae were grown in 3N-BBM+V media in seven 250 mL Erlenmeyer flasks at varying initial nitrate concentrations. The initial sodium nitrate concentrations were as follows: 0.3 g/L, 0.2 g/L, 0.13 g/L, 0.1 g/L, 0.06 g/L, 0.04 g/L, and 0.03 g/L. The working volume of each flask was 150 mL and all flasks were kept under the same growth conditions. Daily nitrate readings using NO$_3^-$ detection probe and daily OD readings were taken until the culture ran out of nitrogen from all seven flasks, which was typically about 5 days. Sampling was done about the time the lights were turned off every day.

In the third type of experiment, four bottles with working volume of 1600 mL were inoculated with nitrate-free algae culture. To obtain nitrate free algae culture, 600 mL of cell suspension from the seed jar was centrifuged and resuspended in 3N-BBM+V nitrate-free media. Fresh media was prepared with desired nitrate concentration prior to inoculation. The starting nitrate concentrations were as follows: 0.2 g/L, 0.1 g/L, 0.05 g/L and 0.01 g/L of sodium nitrate. The experiment was run for 15 days to allow for all the nitrogen to be used up in all four bottles.
The protocol for this experiment is shown in Appendix A. The sampling methods and the preparatory steps taken to start the experiment, such as autoclaving procedures, media, shaker bath and CO\textsubscript{2} preparations were similar for the other experiment types.

In the fourth type of experiment, three 2 L jars were inoculated at the same initial sodium nitrate concentration of 0.45 g/L in a working volume of 1 L from the same seed jar. The main purpose of this experiment was to monitor cell growth via OD and cell counting by hemocytometer, to differentiate between biomass accumulated through cell division and biomass accumulated through lipid storage due to stressed growth conditions.

### 3.2.5 Data Acquisition

Absorbance is a quick and simple way to determine cell growth, especially for smaller volume cultures where there is not enough volume to do dry biomass (Shuler, 2002). The algae growth was monitored by optical density (OD) measurement at 600 nm wavelength on the spectrophotometer. Using the spectrophotometer at CSU lab the biomass/OD correlation is approximately \( X \left[ \frac{g}{L} \right] = 0.62 \ OD \). Each measurement was read against a medium blank. For reading accuracy, the samples were diluted with DI water above an OD reading of 1.0. Measurements were taken twice a day, once shortly after lights were turned on and then twelve hours later after the lights were turned off.

The OD measurements became less accurate as lipid accumulation continued and cells stopped division in early stationary phase. Lipid accumulation increased the cell size not the number of cells, and OD readings do not account for this change. In some experiments, cell count using a hemocytometer was performed to determine the number of cells more accurately.
The nitrate concentration in the media was monitored daily using a Vernier Nitrate Ion-Selective Electrode Probe (Figure 12). The probe was calibrated using two point calibration from 1 ppm to 100 ppm nitrate. Go! Link software provided with the probe was used to read and record the nitrate concentration. The Ion-Selective Electrode only measures nitrate ion as nitrogen, so NaNO₃ concentration in g/L is determined as shown in Figure 13.

\[
\begin{align*}
\text{Example:} \\
\text{Nitrate probe Go! Link reading: } & 75 \text{ mg/L} \\
\frac{75 \text{ mg } N}{1 \text{ L}} & \times \frac{1 \text{ g } N}{1000 \text{ mg } N} \times \frac{85 \text{ g NaNO}_3}{14 \text{ g } N} = 0.455 \text{ g NaNO}_3/L \text{ of solution}
\end{align*}
\]

Figure 13 – NaNO₃ calculation sample from Vernier nitrate probe reading.

**Dry Mass and Percent Lipid Calculations**

All the glass tubes used for extraction and solvent evaporation were pre-weighed. After the extraction the left over biomass was placed in the oven to be dried at 50 °C. The glass tube containing the completely dried biomass was weighed. The glass tube
containing lipids was also weighed after all the solvent had evaporated. After the weight of glass tubes was subtracted, the following formula was used to determine percent lipid:

\[
\text{% Lipid} = \frac{\text{Lipid weight [g]}}{\text{Lipid weight [g]} + \text{Biomass weight [g]}}
\]
CHAPTER IV
THEORY

4.1 Continuous Photobioreactor Design

The photobioreactor system was modeled as two continuously stirred tank reactos in series, as shown in Figure 14. The variables used throughout this thesis are defined in the nomenclature section.

\[ \frac{F}{V_1} = D_1 \]

\[ \frac{F}{V_2} = D_2 \]

Figure 14 – PBR system.
The two CSTRs in series were modeled with the assumption of a constant volumetric flow rate, $F$, and constant volumes $V_1$ and $V_2$ in the two reactors, respectively. In reactor design the ratio of volume over the volumetric flow rate is known as the average residence time, $\tau$. For biological systems, the inverse of average residence time is known as the dilution rate, $D$, and this is the terminology used throughout the model presented here. Note that knowing the dilution rate and the volume of the reactor, the flow rate of the system can be determined. The reactor system is designed in two steps. The growth reactor is designed first to determine the inlet and outlet biomass and substrate concentrations and volumetric flow rate of the system for given $V_1$. Then the volume of the lipid synthesis reactor is designed by using the values obtained from modeling the growth reactor. Throughout the document the growth reactor is also referred to as the first reactor and the lipid synthesis reactor as the second reactor.

The mass balance on biomass in reactor 1 is given by:

\begin{equation}
FX_0 - FX_1 + V_1 r_X - V_1 k_p X_1 = V_1 \frac{dX_1}{dt}
\end{equation}

The cell growth rate, $r_X$, is defined in Equation 1. The feed cell concentration into the reactor, $X_0$, is equal to zero, because only fresh media free of algae cells is fed to the first reactor, when the system is operated in continuous mode. Assuming a steady state volumetric flow rate in and out of the reactor, the derivative term equals zero. Additionally, after assuming that there is no cell death the mass balance for the 1st reactor reduces to the specific growth rate being equal to the dilution rate:

\begin{equation}
\mu = \frac{F}{V_1} = D_1
\end{equation}
The specific growth rate is determined directly from experimental data as explained in the next section of this thesis. Because the cell growth is also assumed to be limited by substrate availability, the Monod equation, given by Equation 2, in the background chapter of this document, applies:

Equation 8 \[ \mu = \frac{\mu_{\text{max}} S_1}{K_s + S_1} = D_1 \]

Rearranging Equation 8, in terms of substrate, yields:

Equation 9 \[ S_1 = \frac{k_s D_1}{\mu_{\text{max}} - D_1} \]

According to Equation 9, to calculate the substrate concentration leaving the first reactor the following three parameter values must be determined: \( D_1 \) and Monod parameters, \( K_s \) and \( \mu_{\text{max}} \).

The denominator in Equation 9 should not be zero, meaning that the dilution rate in the first reactor should be smaller than the maximum growth rate of the algae cells. If \( D_1 \) is higher than \( \mu_{\text{max}} \) the culture will fail to sustain itself in the bioreactor. This situation of \( D_1 > \mu_{\text{max}} \) is known as “wash-out”.

The substrate mass balance in the first reactor is given by:

Equation 10 \[ F S_0 - F S_1 + V_1 r_S S_1 = V_1 \frac{d S_1}{dt} \]

Assuming steady state and substituting \( D_1 = \frac{F}{V_1} \) into Equation 10 yields:

Equation 11 \[ r_S S_1 = D_1 (S_0 - S_1) \]

The definition for \( r_S \) is given by Equation 4. Equation 11 is rewritten in terms of \( X_1 \):

Equation 12 \[ X_1 = Y_{X/S} (S_0 - S_1) \]

Substitution of Equation 9 into Equation 12, yields:
Equation 13 \[ X_1 = Y_{X/S} \left[ S_0 - \frac{D_1 K_s}{\mu_{\text{MAX}} - D_1} \right] \]

Using experimental data for cell growth and substrate uptake as functions of time, the yield and Monod parameters can be calculated. The equation to calculate the yield coefficient is obtained by combining Equation 1 and Equation 4, to get:

Equation 14 \[ -Y_{X/S} = \frac{\Delta X}{\Delta S} \]

Details on how the Monod parameters are obtained are given in the Kinetic Data section 4.2. Once the Monod parameters and yield are known, Equations 13 and 9 can be used to determine the biomass and substrate concentrations, \( X_1 \) and \( S_1 \), for a range of \( S_0 \) and \( D_1 \), using a computer program such as Excel or MatLab. To prevent washout, \( D_1 \) should be smaller than \( \mu_{\text{max}} \), so \( 0 < D_1 < \mu_{\text{max}} \). The feed substrate concentration can be between zero and the concentration given by the media recipe.

A coupled set of values for \( S_0 \) and \( D_1 \), should be obtained such that \( S_0 \) is low enough to allow for \( S_1 \) to be close to zero and yet desired cell productivity in the reactor is achieved. Cell productivity is defined as:

Equation 15 \[ \text{Cell productivity} = X_1 D_1 \]

The lipid mass balance in reactor 1 is given by:

Equation 16 \[ F L_0 - F L_1 + V_1 r_L = \frac{dL_1}{dt} \]

The mathematical model of lipid rate formation inside the algae cells used was:

Equation 17 \[ r_L = \left( \frac{A}{1 + \beta S} \right) X + r_o \mu X \]

where \( A = 0.04 \text{ g lipid/g cell/day}, \beta = 495 \text{ L/g substrate}, \) and \( r_o = 0.05 \text{ g lipid/g cell} \) \((\text{Kanani, 2011})\)

Assuming steady state the derivative term cancels. The feed lipid concentration into the reactor is equal to zero, because only fresh media free of algae cells is fed to the
first reactor, when the system is operated in continuous mode. Based on these assumptions, the lipid concentration in reactor 1 is represented by Equation 18:

\[
L_1 = D_1 \left( \frac{A}{1 + \beta S_1} \right) X_1 + r_0 D_1 X_1
\]

The mathematical model for the second reactor is derived in a similar fashion as reactor 1. In reactor 2 the cell mass balance is given by the equation:

\[
FX_1 - FX_2 + V_2 \mu_2 X_2 - V_2 k_P X_2 = V_2 \frac{dX_2}{dt}
\]

Assuming a steady state flow and no cell death, the derivative and \( V_2 k_P X_2 \) terms equal zero. Knowing that \( \frac{F}{V_2} = D_2 \), Equation 19 reduces to:

\[
\mu_2 = \frac{D_2 (X_2 - X_1)}{X_2}
\]

The cell growth in the second reactor is limited by substrate availability, so the Monod equation, given by Equation 2, in the background chapter of this document, applies as follows:

\[
\mu_2 = \frac{\mu_{max} S_2}{K_s + S_2}
\]

Combination of Equation 20 and Equation 21 gives the following equation in terms of the cell concentration in the effluent stream:

\[
X_2 = \frac{D_2 X_1}{D_2 - \mu_{max} \left( \frac{S_2}{K_s + S_2} \right)}
\]

The substrate mass balance in the second reactor is given by:

\[
FS_1 - FS_2 + r_2 V_2 = V_2 \frac{dS_2}{dt}
\]

Once again since steady state is assumed, the derivative term cancels out. The rate of nitrogen uptake is assumed to follow Equation 4. Knowing that \( \frac{F}{V_2} = D_2 \), after
applying the assumptions and substituting in Monod relationship, Equation 23 can be written in terms of $S_2$ implicitly as follows:

\[
0 = \frac{x_2 \mu_{\text{max}} S_2}{Y_{X/S}(K_S + S_2)} - D_2(S_1 - S_2)
\]

Equation 24

The lipid mass balance in reactor 2 is given by:

\[
FL_1 - FL_2 + V_2 r_L = \frac{dL_2}{dt}
\]

Equation 25

Dividing by $V_2$ and assuming the derivative term cancels out due to steady state, the following is obtained:

\[
r_L = D_2(L_2 - L_1)
\]

Equation 26

where the lipid rate of formation is as previously described by Equation 17.

To calculate $X_2$, $S_2$ and $L_2$, Equations 22, 24 and 26 must be solved simultaneously using the aid of a computer, because $S_2$ cannot be expressed explicitly.

The overall lipid productivity can be calculated with respect to the second reactor, using the following formula:

\[
\text{Overall productivity} = \frac{L_2 D_2}{1 + \frac{L_1}{D_2}}
\]

Equation 27

Based on the equations derived in this section, the design of the two PBRs system can only be done, if the following parameters are known: $Y_{X/S}$, $K_S$, and $\mu_{\text{max}}$. The yield coefficient and Monod constants can be determined by performing batch experiments, as explained in section 3.2.4. The methods used to obtain these parameters from the batch experimental data is explained in the next section. Once these parameters were approximately determined, the reactor system equations were solved in Excel.
4.2 Kinetic Data

The three tabulated sets of data from batch experiments were analyzed to determine growth rates, cell growth and substrate uptake trends, as well as the substrate yield coefficient, and Monod constants. The first step in analyzing the data was to look at the growth curves and determine the lag, exponential and stationary phases of cell growth (Figure 6). Only the exponential phase data should be included. One of Monod equation’s assumptions is that there is no significant lag phase, so any lag phase data must be excluded from the analysis. To determine when the stationary phase starts, two graphs were compared against each other: cell growth curve (Figure 6) and substrate concentration versus time. Knowing that the cells do not proliferate when media is free of substrate, any cell growth data obtained from samples taken when the media was free of substrate, was not used in determining Monod parameters and yield coefficient. The OD method, used in the experiments associated with this thesis, does not provide an accurate measurement of cell growth when the nitrogen is depleted from the media as this method gives a false cell growth since the cells increase in size due to lipid accumulation not cell division. In comparing the cell growth curve and substrate uptake with time, the point where substrate is depleted is considered as beginning of stationary phase, unless the growth curve indicates a stationary phase before the substrate is exhausted. Once the exponential phase data is selected, the specific growth rates, Monod constants and yield coefficient can be determined.

To determine the specific growth rate, $\mu$, two methods were used: differential and integral.
1) **Differential**

The differential of Equation 1 was taken to yield:

\[
\text{Equation 28} \quad \frac{\Delta x}{\Delta t} = \mu X
\]

Then, using Equation 28 and backwards difference method, \( \mu \) was calculated at each time point.

2) **Integral**

In the second method, Equation 1 was rearranged and the integral was taken with respect to cell concentration and time, between feed cell concentration, \( X_0 \), and final cell concentration, \( X_1 \), and \( t=0 \) days and \( t=t_1 \) days:

\[
\text{Equation 29} \quad \ln \frac{X_1}{X_0} = \mu (t_1 - t_0)
\]

Equation 29 is linear in the parameters, where the growth rate is the slope. A graph of natural log of cell concentration versus the change in time was created in Excel, to determine the exponential phase of growth, which is the linear portion of the graph. Then \( \mu \) is calculated for the exponential phase using a linear least square fit to Equation 29. In this method, \( \mu \) is assumed to be constant over the interval \( t_0 \leq t \leq t_1 \).

The yield coefficient was determined by a plot of the change in biomass concentration (\( X-X_0 \)) versus the change in substrate concentration (\( S-S_0 \)), with respect to initial biomass and substrate concentrations. The negative of the intercept value is the yield, as shown by Equation 14. The slope was calculated by linear least-square method.
To determine Monod’s parameters, maximum cell growth rate, $\mu_{\text{max}}$, and Monod constant, $K_s$, three methods were used:

1) **Differential/linear LSQ method**

This method was applied to all four experiments (Table I). In this method, the slope and intercept of the Lineweaver-Burk plot is calculated using linear least square method (LSQ), using $\mu$ calculated by backwards differential method. The Lineweaver-Burk plot is obtained by inverting Monod’s equation (Equation 2) to give:

\[
\frac{1}{\mu} = \frac{1}{\mu_{\text{max}}} + \frac{K_s}{\mu_{\text{max}}S}
\]

Equation 30 is in a linear format, and a plot of the reciprocal growth rate versus the reciprocal of the substrate concentration gives a straight line with an intercept $\frac{1}{\mu_{\text{max}}}$ and slope $\frac{K_s}{\mu_{\text{max}}}$, as shown in Figure 15:

---

Figure 15 – Lineweaver-Burk plot sample.
(Web source: http://themedicalbiochemistrypage.org/enzyme-kinetics.html).
2) **Initial substrate method**

This method was applied to experiments 1 and 2, in which the initial substrate concentration was varied. In this method, the $\mu$ calculated using integral method was plotted versus the initial substrate concentration using the Lineweaver-Burk, Equation 30. The slope and intercept were obtained by linear least square method.

3) **Nonlinear least-square method**

This method was applied to all four experiment types (Table I). This method was performed by fitting the dynamic model to the original biomass time-dependent data. The combination of batch and Monod equation yields the following mathematical model:

$$\frac{dX}{dt} = \frac{\mu_{\text{max}} + S}{K_s + S} X$$

Equation 31

The substrate time dependent data was interpolated using piecewise cubic interpolation to create a continuous fit. These data along with numerical integration was used to approximate $\mu_{\text{max}}$ and $K_s$ through nonlinear regression estimates. Initial values close to the actual values must be entered into the program. The approximated initial values of the two parameters were those obtained by differential/linear least-square and initial substrate methods, performed in Excel.

The linear least-square analysis of Lineweaver-Burk, which helps determine Monod parameters, is flawed because the best fit is generated between observed and calculated values of $\frac{1}{\mu}$, rather than $\mu$. Taking the double reciprocal causes an increase in small errors, and thus a greater uncertainty in the results. Other similar graphs, such as
the Hanes-Woolf plot, could be used but the same error issue exists with these alternatives. Lineweaver-Burk is one of the more popular approaches and this why it was used here. A more reliable solution could be nonlinear regression because it gives equal weight to all the data in any range of growth rate or substrate concentration.

The specific growth rate, yield, and Lineweaver-Burk intercept and slope, were calculated in Excel by the linear least-square method with a Student t-test error analysis using a 95% confidence level. In the linear least-square method, the parameter, p, was determined using the following mathematical expression:

\[
\text{Equation 32} \quad p = [A^T A]^{-1} X [A^T B]
\]

Where A is the matrix of x values, B is the matrix of y values, p is the matrix of the parameters m and b, in the expression of \( y = mx + b \). \([A^T A]^{-1}\) is known as the covariance matrix.

The following statistics were used to find the error:

<table>
<thead>
<tr>
<th>M</th>
<th>Number of data points</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>Number of parameters</td>
</tr>
<tr>
<td>df</td>
<td>M - N (degrees of freedom)</td>
</tr>
<tr>
<td>Risk</td>
<td>5%</td>
</tr>
<tr>
<td>( t )</td>
<td>( \text{tinv}(df, \text{risk}); (\text{tinv} = \text{Inverse of Student T-test distribution}) )</td>
</tr>
<tr>
<td>( s^2 )</td>
<td>sum square of the difference between experimental and calculated values</td>
</tr>
</tbody>
</table>

The mathematical expression to find the error is as follows:

\[
\text{Equation 33} \quad \varepsilon = \sqrt{\frac{[A^T A]^{-1} s^2}{df} \times t}
\]
CHAPTER V
RESULTS AND DISCUSSION

5.1 Lipid Extraction Methods

Lipid extraction experiments were performed on three separate batches of *Scenedesmus dimorphus* culture. All culture samples reached stationary phase, but the amount of nutrient available or the exact amount of time the cells have been in stationary phase was not known. Samples from the same batch were grown in separate growth containers, meaning that variations in nutrients and lights could have affected the lipid accumulation in the cell. According to the results shown in Table III, the solvent extraction of the wet pellet gave the highest lipid return, however the mortar and pestle method of cell lysing followed by dry pellet extraction gave the most consistent lipid return.

Table III – Lipid Biomass Fractionation Methods. Values shown are lipid content as % of total dry biomass

<table>
<thead>
<tr>
<th>Batch</th>
<th>Phase of growth</th>
<th>Solvent</th>
<th>Freeze/Thaw</th>
<th>Sonication</th>
<th>Freeze-Dry</th>
<th>Mortar and Pestle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stationary phase</td>
<td>11.5% - 33%</td>
<td>15.8%</td>
<td>5.4%</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>2</td>
<td>Stationary phase</td>
<td>3.1% - 5.1%</td>
<td>N/A</td>
<td>N/A</td>
<td>3.1% - 6.4%</td>
<td>4.1%</td>
</tr>
<tr>
<td>3</td>
<td>Early stationary phase</td>
<td>9.70%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>3.2% - 3.5%</td>
</tr>
</tbody>
</table>
The mortar and pestle and freeze-dry method were performed on dry biomass, while the remaining exactions were performed on wet biomass. The freeze/thaw, sonication and freeze-dry methods were not pursued further due to emulsion and stickiness issues.

The most consistent technique was the mortar and pestle cell lysing method. This method was also the least complicated approach among the physical type cell disruption techniques, due to the simplicity of lipid removal. Because the sample was completely dried before extraction, complications such as emulsion did not occur. One issue with this method is the amount of time it takes to complete the extraction process, attributed mostly to the drying before and after the extraction is performed, which takes a couple days. Another concern is that biomass is easily lost once in powder form. The amount of biomass lost was determined to be $23.51\% \pm 0.14\%$, by obtaining a dry biomass weight prior to and after extraction. Compared to the other physical types of cell lysing methods, the mortar and pestle approach seems to be most accurate, because in the absence of micelle formation, less amount of lipid was lost.

Both sonication and freeze/thaw methods resulted in multiple liquid layers sample post extraction, including an emulsion layer. Because, the residual biomass and the lipids were not fully recovered, the lipid percentage was difficult to quantify, in both of these methods.

A promising method seems to be the non-physical lysing method using only organic solvent. In this method, most of the extracellular water was removed prior to extraction, with the goal of eliminating or minimizing any possible micelles formation. Perhaps due to the gentle way of breaking open the cells, no emulsion formed, and the
solvent/lipid layer was easy to separate from the biomass layer. This method was performed on all three batches of cultures. The results varied greatly from 3 % to 33 % lipid. The inconsistency in results could be attributed to any of the following: amount of lipid present in the algae cell due to the growth stage or growth conditions, or some overlooked inconsistency in the extraction method.

The freeze-dry method proved troublesome because the algae stuck to the upper sides of the glass tube while extracting during shaking, so the solvent did not have continuous contact with the samples, making the extraction inefficient. Further, the samples warmed up to room temperature during the long freeze-drying process encouraging cells to undergo metabolic changes, which could include lipid alterations, and perhaps even causing the cells to break open similarly to the freeze/thaw procedure. Ice was packed around the container holding the samples in attempt to maintain the cells frozen, but that was inefficient especially since the samples took about 20 hours to dry.

When cells undergo stress they release polysaccharides, causing the cells to stick to each other or other surfaces (Guerrini et al., 1998). Perhaps this was the reason the freeze-drying method was inefficient. Stickiness was also observed in the freeze/thaw cell lysing method, which may indicate that polysaccharides were released due to the freezing of cells. Further, the sonicated algae also stuck to the sides of the extracting glass tube, which can be an indication that the sonication may be too harsh on the cells and overfractionate them causing them to released polysaccharides.

Based on the results of the experiments run here, it is preliminarily suggested that either organic solvent or mortar and pestle methods could provide satisfactory and consistent results for quantifying lipid content in future experiments. Other methods may
still prove to be useful if procedures to prevent or deal with emulsions are implemented and if operating parameters are optimized such as: duration and power of sonication, number of time the samples should undergo freezing/thawing, and a better way to keep samples chilled during the freeze-drying, to name a few.

It has been suggested that unicellular algae must be extracted rapidly with a minimum of preparative mechanical treatments such as centrifugation and filtration. The centrifuge used in the preparatory steps was old, so a centrifuge that can go to higher speeds should be used to minimize centrifugation time in the future.

The results in Table III are based on the assumption that all the extract is lipid. To better quantify the results, in the future, the extract could be analyzed through analytical methods such as chromatography or other oil analysis instrumentations or techniques. Analytical methods would help quantify only lipids relevant for fuel synthesis. Further, to ensure the reproducibility and accuracy of the method, a dried biomass with known lipid content should be used as a control. Further, as suggested by Nichols in (Leray, 2011) in methods for plant tissue, the algae should be fractionated with isopropanol only at first to inhibit lipases. One experimental option is to perform the mortar and pestle grinding in isopropanol after the pellet is dried; similarly isopropanol can be used with the other fractionation methods. Further, to increase the recovery of lipids, the separation of biomass from the lipid/solvent layers should be filtered, instead of pipetted.

5.2 Kinetic data

5.2.1. Experimental data and growth rate calculations

The three tabulated columns of experimental data (time, cell and substrate concentrations as functions of time), were used to graphically compare and contrast the
growth curves and substrate uptake trends of *Scenedesmus dimorphus* for the four types of experiments performed (Table I), for the purpose of determining the exponential phase. The exponential growth phase data was used to calculate yields and Monod constants.

Experiment 1 was performed in two 1.5 L working volume bottles with *S. dimorphus* in 3N-BBM+V media and under similar growth conditions. The biomass obtained from bottle 2, was used to further develop the lipid extraction methods and obtain preliminary results on *S. dimorphus* lipid content. The samples obtained from bottle 1, were used to determine sodium nitrate yield and Monod kinetics. Due to limitations of culture volume availability, bottle 1 was mainly used for nitrate sampling and the other bottle mainly for lipid sampling. The growth curves for both bottles are shown in Figure 16.

![Experiment 1 - Growth Data](image)

Figure 16 – *Scenedesmus dimorphus* cell growth data in 3N-BBM+V media (Experiment 1). Bottle 2 data obtained from: Kanani, 2011.
Figure 17 – Log cell concentration and substrate cell concentration versus time data of *Scenedesmus dimorphus* (Experiment 1-Bottle 1).

To determine specific growth rate, yield and Monod parameters the log cell concentration and substrate cell concentration versus time curves were compared. The data of interest, exponential growth phase during substrate availability, starts with day 0 and ends with day 9, as depicted by the two curve comparison in Figure 17. The specific growth rate of cells during this time period was determined to be $0.60 \pm 0.05 \text{ day}^{-1}$ for bottle 1 and $0.51 \pm 0.05 \text{ day}^{-1}$ for bottle 2, by using Equations 28 and 30. The error was determined by Equation 33. Both bottles have a similar biomass growth trend as shown by Figure 18.
Figure 18 – Experiment 1 growth rates.

Experiment 2 was performed on *S. dimorphus* grown in 3N-BBM+V media in seven Erlenmeyer flasks, each having a working volume of 150 mL. The cell growth data for the seven cultures of varying initial substrate concentration are shown in Figure 19.

The nitrogen concentration changes in each flask are shown in Figure 20. For the first 3 days, when substrate was still present in the media, all cultures grew similarly as they all had sufficient nitrogen available. The lowest initial substrate concentration culture, 0.03 g NaNO₃/L, grew on day 4, after seemingly having entered stationary phase in day 3. Regardless of the growth spurt, the lack of nitrogen clearly had an effect on the cell concentration reached; higher initial substrate cultures reached cell concentrations as high as 0.26 g/L within a 5 day period, while the lowest initial substrate concentration only reached 0.15 g/L during the same sample time period.
Figure 19 – *Scenedesmus dimorphus* growth data in 3N-BBM+V media with varying initial sodium nitrate concentrations (Experiment 2).

Figure 20 – Change in sodium nitrate concentration with time (Experiment 2). The legend identifies each flask by initial NaNO₃ concentrations.
None of the flasks underwent a lag phase, as indicated by the linearity of the log plot shown in Figure 21. The beginning of the stationary phase was determined by the point where the substrate was used up in each flask, because the cells still show growth even when they are substrate limited when cell concentration is measured by OD. Growth during limited nitrate conditions is an indication that the cells are increasing their mass through lipid accumulation, not by division. The flasks that started with low sodium nitrate concentrations of 0.03 g/L NaNO₃ and 0.04 g/L NaNO₃, entered stationary phase by day 3 because this is the point when majority of the substrate was spent (Figure 20).

The three flasks that started with highest substrate concentrations were still in growth phase at the time the experiment was stopped on day 5. Looking at the trends of the other flasks, these three flasks would require from one to about three days, to reach stationary phase.

The increase and decrease in sodium nitrate concentration for the higher initial substrate concentration cultures, in Figure 20, could be due to an error in the nitrate probe readings or the range of values used during calibration. Or it could also be due to a phenomenon where the *S. dimorphus* expels nitrogen back into the media as part of adjusting to the new environment right after inoculation (Subramanian et al., 1986). The approximate nitrate probe readings for the initial substrate values were read between 4 ppm to 50 ppm nitrate, and the calibration was performed between 1 ppm to 100 ppm nitrate, so calibration threshold error could be ruled out. Further investigation is required for an accurate assessment of this phenomenon.
The growth rates, calculated using integral method are shown in Table IV. The time period used to calculate the growth rate excludes the data points obtained during nitrogen depravation.

![Experiment 2 - Growth Rates](image)

Figure 21 – *Scenedesmus dimorphus* growth rates (Experiment 2).

Although the cells started at different initial substrate concentration, the growth rate does not seem to be affected greatly by the amount of nitrogen available at the time of inoculation.

Table IV – Specific growth rate [day\(^{-1}\)] results for Experiment 2.

<table>
<thead>
<tr>
<th>Method (Equation)</th>
<th>0.3 g NaNO(_3)/L</th>
<th>0.2 g NaNO(_3)/L</th>
<th>0.15 g NaNO(_3)/L</th>
<th>0.1 g NaNO(_3)/L</th>
<th>0.05 g NaNO(_3)/L</th>
<th>0.04 g NaNO(_3)/L</th>
<th>0.03 g NaNO(_3)/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integral (Equation 29)</td>
<td>0.7 ± 0.05</td>
<td>0.7 ± 0.06</td>
<td>0.69 ± 0.045</td>
<td>0.7 ± 0.03</td>
<td>0.7 ± 0.05</td>
<td>1 ± 1</td>
<td>0.6 ± 0.2</td>
</tr>
</tbody>
</table>

Experiment 3 was performed on *S. dimorphus* grown in 3N-BBM+V media in four jars of 1.6 L working volume. The media recipe was adjusted such that each jar had
a different initial substrate concentration, varying from 0.01 g/L to 0.2 g/L NaNO₃. The purpose of this experiment was to gain additional accuracy in measurements of Monod kinetics and yield coefficient. The substrate concentration changes with time are shown in Figure 23.

Figure 22- *Scenedesmus dimorphus* growth data in 3N-BBM+V media with varying initial sodium nitrate concentrations (Experiment 3).

Figure 23 - Change in sodium nitrate concentration with time (Experiment 3).
Jar 4, which started with the smallest amount of sodium nitrate, 0.01 g NaNO₃/L ran out of substrate and reached stationary phase very quickly compared to the other three jar cultures. This jar grew for approximately three days before entering stationary phase. Although a log plot indicates a longer period of growth, about 5 days, the early stationary phase is specified by the point where the substrate was used up, which is close to day 3. Jar 3 ran out of substrate day 9, while jars 1 and 2 ran out of substrate day 15. In comparing the growth data graph and substrate graphs, jars 1, 2 and 3 show some growth after the sodium nitrate has run out, indicating that lipid accumulation may be taking place. None of the jars went through a lag phase as shown by the linearity in Figure 24. The data obtained during the first 5 days of experiment was used to determine specific growth rate, yield and Monod constants for jars 1, 2, and 3 because this time period represents the exponential growth phase. The yield and Monod parameters are discussed in sections 5.2.3 and 5.2.2.

The dips in substrate concentration observed during the first couple days of experiment may be due to either inaccurate readings by the probe or a phenomenon where the *S. dimorphus* expels nitrogen back into the media. The calibration threshold could be an issue as all the readings were done in the lower part of the calibration curve; the approximate nitrate probe readings for the initial substrate values here read between 1.5 ppm to 33 ppm nitrate, and the calibration was performed between 1 ppm to 100 ppm nitrate. Additionally the algae cells in all four culture jars clumped together, forming large aggregations of cells, which caused the cells to settle at the bottom regardless of the continuous shaking. This phenomenon could be another reason for nitrogen concentration variations.
Figure 24 – *Scenedesmus dimorphus* growth rates (Experiment 3).

The growth rates for experiment 3 were determined using linear least-square analysis of Equation 29. The data points are graphically shown in Figure 24 and the slope of the equation of the trendline through origin gives the specific growth rate. The errors associated with these values are shown in Table V.

Table V - Specific growth rate [day⁻¹] results for Experiment 3.

<table>
<thead>
<tr>
<th>Method (Equation)</th>
<th>Jar 1: 0.2g/L NaNO₃</th>
<th>Jar 2: 0.1g/L NaNO₃</th>
<th>Jar 3: 0.05g/L NaNO₃</th>
<th>Jar 4: 0.01g/L NaNO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integral (Equation 29)</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>0.9 ± 0.1</td>
<td>1 ± 0.2</td>
</tr>
</tbody>
</table>

One reason the specific growth rates and errors calculated for Experiment 3 are unusually higher than the previous experiments could be due to the algae cells flocculating in all four culture jars.

Experiment 4 was performed with the purpose of monitoring cell growth via OD and cell counting by hemocytometer, to differentiate between biomass accumulated through cell division and biomass accumulated through lipid storage due to stressed
growth conditions. All three jars were inoculated at the same time with an initial NaNO$_3$ concentration of 0.45 g/L.

Figure 25 - *Scenedesmus dimorphus* growth data in 3N-BBM+V media (Experiment 4).

Figure 26 - *Scenedesmus dimorphus* growth data in 3N-BBM+V media using cell count data from hemocytometer (Experiment 4).
The growth data for the three cultures obtained through OD is shown in Figure 25 and the data obtained through cell counting using a hemocytometer is shown in Figure 26. Both methods show similar growth trends. A correlation curve between the cell counts and OD measurements is shown in Figure 27.

The correlation between the cell count using the hemocytometer and those returned via OD measurements is close to linear by inspection. Since the culture did not run out of substrate (Figure 28), this observed correlation is an indication that OD measurements are a good representation of cell growth during cell proliferation. If the culture would have run out of substrate, the cells would have stopped reproducing but continued a lipid accumulation process. The data points obtained by hemocytometer would be expected to reach a plateau when the cells ceased division, and the relation between cell count and OD would become nonlinear.

![Correlation Curve](image)

Figure 27 – Cell count and OD correlation curve during days 2 to 10 (Experiment 4).
Figure 28 - Change in sodium nitrate concentration with time (Experiment 4).

The substrate concentration in experiment 4 (Figure 28) follows the same increasing/decreasing trend as seen in experiments 2 and 3, which again it could be due to either probe reading errors or a phenomena of algae species itself. The first data point was calculated, not measured, and is based on the expected media composition of 0.45 g NaNO₃/L.

Comparison of the growth curve and substrate concentration with time graphs indicates that the algae cells did not reach stationary phase during the 9-day experiment in any of the jars. The growth rate graphs in Figure 29 and 30 were constructed using all data points. Following the method used in the previous three types of experiments, the slope through origin is equal to the specific growth rate. The specific growth rate values
with errors were calculated by linear least-square method and Student t-test, and the results are shown in Table VII.

Table VI - Specific growth rate [day⁻¹] results for Experiment 4.

<table>
<thead>
<tr>
<th>Method (Equation)</th>
<th>Cell Concentration Method</th>
<th>Jar 1</th>
<th>Jar 2</th>
<th>Jar 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integral (Equation 29)</td>
<td>OD</td>
<td>0.5 ± 0.4</td>
<td>0.5 ± 0.4</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Cell count</td>
<td>0.31 ± 0.03</td>
<td>0.23 ± 0.03</td>
<td>0.29 ± 0.03</td>
</tr>
</tbody>
</table>

The optical density method consistently yields higher growth rates compared to the cell count method. Error in specific growth rate values may be due to individual cells being hard to identify and count since the cells were sticking together due to flocculation.

Figure 29 – Growth rates using OD cell concentration measurements (Experiment 4).
Figure 30 - Growth rates based on cell counts using hemocytometer (Experiment 4).

One of the things accomplished in this section was to measure specific growth rates to see how the cells grow in different size containers and different initial substrate concentrations. Table VII summarizes the specific growth rate values obtained by integral and differential methods for each sample.

Comparing the values from experiment 1 and 2, the size of the container does not seem to affect growth rate. These observations are based on the assumption that all other environmental effects such as light and temperature are exactly the same in both containers. Experiments 3 and 4 are not used in the comparison because the cells were flocculating.

The other thing accomplished in this section was the identification of the exponential growth phase data for each set of experimental data. The following, Table
VIII, is a summary table showing which experimental days were used to determine yield and Monod parameters based on the comparison of growth curves and substrate concentration with time. The approximate calculations to obtain yield and Monod parameters are discussed in the next two sections.

Table VII – Specific growth rate values summary table.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Growth Containers Working Volume</th>
<th>Sample</th>
<th>$\mu_{\text{specific}}$ [day$^{-1}$] Integral Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5 L</td>
<td>Bottle 1</td>
<td>0.6 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bottle 2</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>150 mL</td>
<td>0.3 g NaNO3/L</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 g NaNO3/L</td>
<td>0.7 ± 0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15 g NaNO3/L</td>
<td>0.69 ± 0.045</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1 g NaNO3/L</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05 g NaNO3/L</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.04 g NaNO3/L</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.03 g NaNO3/L</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>1.6 L</td>
<td>Jar 1 - 0.2 g/L NaNO3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jar 2 - 0.1 g/L NaNO3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jar 3 - 0.05 g/L NaNO3</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jar 4 - 0.01 g/L NaNO3</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>4*</td>
<td>1.0 L</td>
<td>Jar 1</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jar 2</td>
<td>0.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Jar 3</td>
<td>0.5 ± 0.4</td>
</tr>
</tbody>
</table>

*Specific growth rate values obtained using OD measurements
5.2.2. Monod parameters

Two approaches were employed to determine Monod’s parameters. In the first approach, $\mu_{max}$ and $K_s$ were determined by Lineweaver-Burk plot along with linear least-square analysis. In the second approach, nonlinear least-square method was used with the purpose of eliminating some of the uncertainty in the results obtained by the linear method.

The specific rate growth values obtained using Equation 28 (differential method), from Experiment 1 were used to construct a Lineweaver-Burk plot (Figure 31). The slope and intercept values along with errors are shown in Table IX. Using Equation 29, $\mu_{max}$
and $K_s$ were calculated to be approximately $0.9 \text{ day}^{-1}$ and $0.08 \text{ g/L}$ respectively, from the slope and intercept values.

![Experiment 1 - Lineweaver-Burk Plot](image)

Figure 31 – Lineweaver-Burk plot using data from algae grown in batch under full nutrient standard 3N-BBM+V media.

Table IX – Monod parameters calculated by linear least-squares of double reciprocal linearized Monod equation (Experiment 1).

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope ($k_s/\mu_{max}$)</td>
<td>$0.09 \pm 0.05$</td>
</tr>
<tr>
<td>Intercept ($1/\mu_{max}$)</td>
<td>$1 \pm 0.6$</td>
</tr>
<tr>
<td>$\mu_{max}$</td>
<td>$0.9 \pm 0.5$</td>
</tr>
<tr>
<td>$K_s$</td>
<td>$0.08 \pm 0.06$</td>
</tr>
</tbody>
</table>
The data from experiment 1 was also used to calculate $\mu_{\text{max}}$ and $K_s$ by nonlinear regression using MatLab programming, as described in section 4.2. The numerical results are shown in Table X. Using methods described in section 4.2, a Michaelis-Menten curve is obtained. The resulting curve is shown in Figure 32.

![Experiment 1 - Monod Kinetics](image)

Figure 32 – Graphical representation of Monod kinetics obtained through linear and nonlinear regression using Experiment 1 data.

The results from the two methods obtained similar maximum growth rate values: 0.9 day$^{-1}$ using the differential/linear LSQ method and 0.87 day$^{-1}$ using the nonlinear regression method, but different $K_s$ values of 0.07 g/L and 0.2 g/L respectively. According to Figure 32, the model generated by the Lineweaver-Burk was surprisingly much better in fitting the data than the nonlinear model.
Figure 33 – Lineweaver-Burk plot created using initial substrate method (Experiment 2).

The purpose of experiments 2 and 3 was to obtain better approximations of the Monod kinetics. For experiments 2 and 3, two sets of Lineweaver-Burk plots were created, one for each of the two methods used to find the specific growth rates, as explained in the Theory section of this thesis. For the initial substrate method one Lineweaver-Burk plot was created for the entire experiment, as shown in Figure 33. Linear least-square method was used to find the slope and intercept of the double reciprocal of Monod equation. For the differential/linear LSQ plot method individual plots were created for each flask in each experiment. An example graph is shown since all plots have similar trend: Figure 34 for Experiment 3. A summary table of results with errors is available in the appendix (Table B. II).
Figure 34 - Lineweaver-Burk plot created using differential/linear LSQ plot method (Experiment 3).

The Monod parameters $\mu_{\text{max}}$ and $K_s$ obtained by both methods for Experiments 2 and 3 are shown in Table X.

Additionally, the parameters obtained by the initial substrate method, were used to visualize the results by constructing Monod model, and comparing the results to the experimental data (Figure 35).

The maximum growth rate for the two experiments using the initial substrate method are very similar: $0.7\pm0.1 \text{ day}^{-1}$ for experiment 2 and $0.7\pm0.05 \text{ day}^{-1}$ for experiment 3. The Monod constant is also about the same, $0.005\pm0.01 \text{ g/L}$ and $-0.005\pm0.002 \text{ g/L}$ respectively; the negative sign is probably due to noise in the data. The Monod constants obtained by the differential/linear LSQ method differ significantly among the samples within the same experiment (Table X) and no clear pattern can be
observed, suggesting that the initial substrate method for samples grown in low substrate concentration may be more appropriate.

![Monod Kinetics - Initial substrate method](image)

Figure 35 – Graphical representation of Monod kinetics obtained through initial substrate method (Experiments 2 and 3).

Because experiment four was not carried through the depletion of substrate, the Monod parameters cannot be determined accurately using the differential/linear LSQ method because the smallest substrate value measured on day 10 of the experiment of 0.26 g/L NaNO₃ is much larger than the expected Monod constant value of approximately 0.005 g/L. The method was attempted and the results were used as initial guesses into the MatLab algorithm.

Experiments 2, 3 and 4 were also analyzed by nonlinear regression in MatLab. The $\mu_{\text{max}}$ and $k_s$ values obtained from previous analyses (differential/linear LSQ and initial substrate methods), were used as initial guesses for the nonlinear algorithm. All of
the data sets were also run with one constant set of initial guesses: 0.7 day\(^{-1}\) for \(\mu_{\text{max}}\) and 0.009 g/L for \(k_s\), which were chosen based on the initial substrate method results from Experiments 2 and 3.

Table X – Summary of results for Monod parameters

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sample</th>
<th>Method to find (\mu_{\text{specific}})</th>
<th>Maximum growth rate (\mu_{\text{max}}) [day(^{-1})]</th>
<th>Monod constant (k_s) [g/L]</th>
<th>Maximum growth rate (\mu_{\text{max}}) [day(^{-1})]</th>
<th>Monod constant (k_s) [g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bottle 1</td>
<td>Differential</td>
<td>0.9 +/- 0.5</td>
<td>0.005 +/- 0.01</td>
<td>0.87</td>
<td>0.191</td>
</tr>
<tr>
<td>All</td>
<td>Integral</td>
<td>0.7 +/- 0.1</td>
<td>0.005 +/- 0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.3 g NaNO3/L</td>
<td>Differential</td>
<td>2 +/- 5</td>
<td>0.3 +/- 0.9</td>
<td>4.17</td>
<td>1.120</td>
</tr>
<tr>
<td>All</td>
<td>Differential</td>
<td>2 +/- 5</td>
<td>0.2 +/- 0.7</td>
<td>18.60</td>
<td>3.230</td>
<td></td>
</tr>
<tr>
<td>0.15 g NaNO3/L</td>
<td>Differential</td>
<td>0.9 +/- 0.4</td>
<td>0.026 +/- 0.021</td>
<td>1.25</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>0.1 g NaNO3/L</td>
<td>Differential</td>
<td>0.7 +/- 0.2</td>
<td>0.001 +/- 0.002</td>
<td>0.79</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>0.05 g NaNO3/L</td>
<td>Differential</td>
<td>0.7 +/- 0.6</td>
<td>-0.0002 +/- 0.005</td>
<td>0.74</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>0.04 g NaNO3/L</td>
<td>Differential</td>
<td>1 +/- 3</td>
<td>0.004 +/- 0.03</td>
<td>1.25</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>0.03 g NaNO3/L</td>
<td>Differential</td>
<td>0.4 +/- 3</td>
<td>-0.001 +/- 0.03</td>
<td>0.56</td>
<td>-0.001</td>
<td></td>
</tr>
</tbody>
</table>

| 3          | All        | Integral                        | 0.7 +/- 0.05                   | -0.005 +/- 0.002 | 14.30                       | 3.35            |
| Jar 1 - 0.2g/L NaNO3 | Differential | 1 +/- 0                     | 0.1 +/- 2                    | 4.00            | 0.34                         |
| Jar 2 - 0.1g/L NaNO3 | Differential | -0.2 +/- 0.2                | -0.09 +/- 0.1                | 2.70            | 0.07                         |
| Jar 3 - 0.05g/L NaNO3 | Differential | -0.3 +/- 0.2              | -0.04 +/- 0.03               | 2.93            | 0.006                        |
| Jar 4 - 0.05g/L NaNO3 | Differential | 2 +/- 2                    | 0.004 +/- 0.008              | 1.91            | 1.320                        |

| 4          | Jar 1 | Differential                        | Experimental data not suitable |                  |                               |                 |
| Jar 2 | Differential | 1.24                           |                               |                 |                               |                 |
| Jar 3 | Differential | 2.76                           |                               |                 |                               |                 |

*Errors shown in Appendix B. Errors not reported because error analysis unreliable, since most of the data yielded an ill-conditioned Covariance Matrix.

The nonlinear least-square method results shown in Table X are generally those obtained by using the differential/linear LSQ results as initial guesses. For the complete MatLab results see appendix Table B. I. The results were often a strong function of the initial guess. Most results suggest that there is a strong correlation between \(\mu_{\text{max}}\) and \(k_s\). Regardless of the initial guess, as long as the program converges, the interpolated substrate concentration values and the model predictions for the cell concentration were fairly close to the experimental data for all experiments. See Figure 36 and Figure 37 as examples, since most data fit similarly.
In comparing the results given in this section for Monod parameters, it can be noted that there are large differences among most results. The linear least square based methods gave $\mu_{\text{max}}$ results ranging from -0.2 day$^{-1}$ to 2 day$^{-1}$ and $K_s$ results ranging from -0.0002 g/L to 0.313 g/L. The nonlinear least-squares method gave $\mu_{\text{max}}$ results ranging from 0.74 day$^{-1}$ to 16.80 day$^{-1}$ and $K_s$ results ranging from -0.001 g/L to 3.35 g/L. The error percentages associated with these values had large variance (Table B.1). Most of
the data yielded an ill-conditioned Covariance Matrix, making the error analysis unreliable. Some $K_s$ and $\mu_{max}$ values are negative probably due to scatter in data. The statistical analysis needs to be refined in the future.

Although a pattern exists among some results, overall the results are not very reliable. To find the Monod parameters additional experiments should be designed and performed to ensure no additional factors such as light, temperature or contamination, other than substrate, are affecting the algae cell growth. Monod kinetics is based on the assumption that the growth is solely limited by substrate concentration. Future experiments should also ensure that the culture enters stationary phase before the experiment is stopped. It was difficult to find a pattern among the results obtained by nonlinear regression. Some literature sources claim that the use of least-square methods cannot retrieve the Monod parameters and yield coefficient, and that an alternative method called, optimal design theory, is much more efficient (Dette et al., 2003), (Dette et al., 2005), (Strigul et al., 2009). Thus, alternative data analysis methods such as the optimal design theory should be explored.

In the future, both substrate and biomass concentration as functions of time should be analyzed simultaneously by using Equation 31 along with the following equation:

Equation 34  \[ \frac{dS}{dt} = \frac{\mu_{max}}{K_s+S} \frac{1}{Y_{X/S}} \]

In doing so, the Monod parameters and yield are calculated at the same time. In this thesis the yield was calculated separately as discussed in the next section.
5.2.3. **Yield coefficient**

The data obtained from bottle 1 in Experiment 1, was used to determine sodium nitrate yield. All yield values in this thesis are expressed in units of g\textsubscript{cell}/g\textsubscript{substrate}. As expected the nitrate concentration decreased as the cell concentration increased (Figure 17). Data prior to day four was unreliable due to problems with the storage conditions of the samples and was excluded from data analysis. The yield was calculated to be 1.37 ± 2.07 grams biomass produced per grams substrate used, using linear least square and an approximated initial substrate concentration.

![Graph](figure38.png)

Figure 38 – Sodium nitrate uptake yield by *Scenedesmus dimorphus* over growth phase from 0.08 g/L to 0.27 g/L. Experiment 2, 0.3 g NaNO\textsubscript{3}\%/L sample.

The initial substrate concentration for the samples in experiment 4 were also approximated and not measured with the probe, due to problems with the storage conditions of the samples. The linear least square method was used to determine the yield coefficient for each sample in the four experiment types. A sample graph is shown in Figure 38. The results with errors are shown in Table XI. The results Table XI indicates a range of values for the yield from about 1 g\textsubscript{cell}/g\textsubscript{substrate} to about 3 g\textsubscript{cell}/g\textsubscript{substrate} for the four
experiments. There seems to be no changing trend in yield values with varying initial substrate concentrations.

A theoretical sodium nitrate yield can be calculated from biomass composition. Alfred Redfield, an oceanographer, found the molecular ratio of carbon, nitrogen and phosphorus of marine organic matter was constant: C:N:P = 106:16:1 (Redfield, 1934). Saltwater algae can be represented by a Redfield Ratio of C\(_{106}\)H\(_{263}\)O\(_{110}\)N\(_{16}\)P (Ebeling et al., 2006). Using this stoichiometry and assuming that all of the nitrogen in the cell comes from NaNO\(_3\) the yield coefficient is \(Y_{X/S} = 2.62\), where \(S\) is NaNO\(_3\) concentration and \(X\) is the biomass concentration. *Escherichia coli* elemental composition is as follows: 50 % carbon, 20 % oxygen, 14 % nitrogen and 3 % hydrogen (Duran, 1995). Assuming all of the nitrogen in the cell comes from NaNO\(_3\), the yield is calculated to be \(Y_{X/S} = 1.03\) g cell/g substrate. The experiments presented here were all performed with NaNO\(_3\) as the nitrogen source.

The yield values of all four experiment fall within the theoretical range just discussed for *E. coli* and saltwater algae, 1.03 and 2.62 respectively. However the large errors indicate that yield sensitivity limits fall partially between and partially out of this theoretical range.

The substrate rate equation assumes a constant fraction of the consumed substrate being converted into biomass. An accurate yield value for the species of interest, *Scenedesmus dimorphus*, cannot be determined from these data due to the large errors. Experiment 1 and 0.05 g NaNO3/L sample from Experiment 2, had a better fit of data. Based on the results of these two samples the yield in future experiments is expected to be between 1.2 and 2.7, which fits the theoretical range discussed above. In this design it
was assumed that all of the substrate consumed from the media goes into making biomass. However, the consumed nitrate could be used for other cellular functions such as maintenance. Growth environmental factors such as light, size of container, small temperature variations, agitation or even slight contamination of media could have had an effect on the nitrogen uptake. Additionally, all the samples in experiments 3 and 4 were flocculated and it could be that this has caused some of the variation in substrate uptake.

Table XI – Yield coefficient values in $g_{cell}/g_{substrate}$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sample</th>
<th>$Y_{X/S}$ (Linear LSQ*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bottle 1</td>
<td>1.4 +/- 0.2</td>
</tr>
<tr>
<td></td>
<td>0.3 g NaNO3/L</td>
<td>1 +/- 0.8</td>
</tr>
<tr>
<td></td>
<td>0.2 g NaNO3/L</td>
<td>2 +/- 0.9</td>
</tr>
<tr>
<td></td>
<td>0.15 g NaNO3/L</td>
<td>2 +/- 0.6</td>
</tr>
<tr>
<td></td>
<td>0.1 g NaNO3/L</td>
<td>2 +/- 0.7</td>
</tr>
<tr>
<td></td>
<td>0.05 g NaNO3/L</td>
<td>1.9 +/- 0.8</td>
</tr>
<tr>
<td></td>
<td>0.04 g NaNO3/L</td>
<td>2 +/- 0.8</td>
</tr>
<tr>
<td></td>
<td>0.03 g NaNO3/L</td>
<td>1 +/- 1</td>
</tr>
<tr>
<td>2</td>
<td>0.2 g/L NaNO3</td>
<td>1 +/- 1</td>
</tr>
<tr>
<td></td>
<td>0.1 g/L NaNO3</td>
<td>1 +/- 1</td>
</tr>
<tr>
<td></td>
<td>0.05 g/L NaNO3</td>
<td>1 +/- 1</td>
</tr>
<tr>
<td></td>
<td>0.01 g/L NaNO3</td>
<td>1 +/- 2</td>
</tr>
<tr>
<td>3</td>
<td>Jar 1</td>
<td>3 +/- 0.7</td>
</tr>
<tr>
<td>4</td>
<td>Jar 2</td>
<td>3 +/- 0.7</td>
</tr>
<tr>
<td></td>
<td>Jar 3</td>
<td>2 +/- 0.7</td>
</tr>
</tbody>
</table>

*Initial substrate concentration value for Experiments 1 and 4 were approximated, not measured with the probe.
Based on these results future research should consider experiments where the samples are grown in complete isolated environment and in 24 hour light, to minimize metabolic cell changes that could occur in the dark that can have an effect on the constant yield uptake value. Samples should also be taken several times per day and the nitrate concentration should be measured using a more reliable method, such as an assay.

The mathematical model for substrate uptake may need to be reconsidered as the data does not seem to support Equation 14. One alternative is to modify Equation 4, by introducing a constant $\beta$, as follows:

$$\text{Equation 35} \quad \frac{ds}{dt} = \left( \frac{\mu}{\nu_{x_s}} + \beta \right) X$$

Additionally the yield should be calculated at the same time as the Monod constants as previously suggested at the end of the Monod parameters section of this chapter, section 5.2.2.

### 5.3 Continuous Photobioreactor Design

The effect of dilution (D) on substrate, biomass concentration, cell and lipid productivities was determined. In doing so, an optimal dilution rate value could be identified, which in turn can help determine the flow rate of the system according to Equation 7.

The equations described in the Theory section were solved using Excel. Reactor 1 was modeled first. Throughout the reactor 1 calculations, the dilution rate was kept to a range between 0 and 0.75 day$^{-1}$. It’s necessary that D is smaller than $\mu_{\text{max}}$ to prevent washout. Monod parameters used in the design described here were: $K_s = 0.005$ g/L and $\mu_{\text{max}} = 0.8$ day$^{-1}$ and a yield of 2 g$_{\text{cell}}$/g$_{\text{substrate}}$. 

71
Figure 39 – Substrate concentration versus dilution rate (Reactor 1).

A graph of the substrate concentration versus the range of dilution rates was created, as shown in Figure 39. The substrate concentrations were determined using Monod’s equation rearranged for S, as shown by Equation 9.

Ideally the dilution rate should be as high as possible without potential of washout because at a steady state, that dilution rate will be the same as the specific growth rate of the cells. A dilution rate of approximately 0.65 day$^{-1}$ should be satisfactory, in preventing washout, maintaining a low enough substrate concentration in the feed to the second reactor, and still achieve a reasonable specific growth rate.

A second graph (Figure 40) of cell concentration versus dilution rate was generated. The cell concentration was calculated using Equation 13, which combines the Monod model and substrate mass balance equations. The same dilution rate range was used as mentioned above, and the range of feed substrate concentration was varied from 0.25 g/L to 1.75 g/L to calculate the biomass concentration, $X_1$. 
In the four types of batch experiments discussed here, the highest biomass concentration obtained was about 1 g/L. Some, in the literature, report cell concentration as high as 5 g/L cell concentration for algae grown in PBRs. Although increasing the feed NaNO₃ concentration appears to increase the cell concentration in Figure 40, based on experimental observations the culture would be light-limited above 2 g/L. For a 2 g/L cell concentration, a feed sodium nitrate concentration of approximately 1 g/L is required. Notice that as the dilution rate increases, the cell concentration starts to decrease, so the dilution rate of 0.65 day⁻¹ remains satisfactory.

The X values obtained above using Equation 13 were multiplied by the same range of dilution range, 0 and 0.79 day⁻¹, to obtain the cell productivity. The cell productivity (X₁D₁) versus D₁ was graphed (Figure 42) to demonstrate a close to maximum productivity at the chosen dilution rate.

Figure 40 – Cell concentration versus dilution (Reactor 1). All the NaNO₃ values shown in the graph are feed substrate concentration.
Figure 41- Cell productivity versus dilution rate (Reactor 1). All the NaNO₃ values shown in the graph are feed substrate concentration.

Using 1 g/L NaNO₃, for a dilution rate of 0.65 day⁻¹ a cell productivity of 1.27 g_cell/L/day was calculated. Knowing the dilution rate and the volume of first reactor, a flow rate for the two-PBR system can be determined using Equation 7.

The lipid formation in the algae cells was calculated using Equation 18. Using the feed substrate and cell concentrations determined above, the lipid productivity, defined as L₁D₁, is shown as a function of D₁ in Figure 42. As expected the lipid production increases with dilution rate or specific cell growth rate, as the lipid productivity graph indicates. Using 1 g/L NaNO₃, for a dilution rate of 0.65 day⁻¹ a cell productivity of 0.03 g_lipid/L/day was calculated.
Figure 42- Lipid productivity versus dilution rate in Reactor 1, using feed NaNO$_3$ concentration of 1 g/L.

Different Monod parameters could have a negative impact on the cell and lipid productivities. For example, keeping all parameters the same, but changing the Ks value to 0.05 g/L from 0.005 g/L, calculates a lower cell productivity of 1.02 g$_{cell}$/L/day, and a lower lipid productivity of 0.022 g$_{lipid}$/L/day. A smaller value of $\mu_{max}$ would cause the cell and lipid productivities to decrease. Thus, a large Ks combined with a small $\mu_{max}$ value could be disadvantageous to biomass production in the first reactor. A larger value of substrate yield would have a positive effect on cell productivity. If the yield value is halved to 1 g$_{cell}$/g$_{substrate}$, both the cell and lipid productivities values would be cut in half.

In the scenario where the initial substrate is assumed to be higher, say 1.5 g/L instead of 1 g/L, the cell productivity is expected to reach 1.92 g$_{cell}$/L/day and the lipid productivity is expected to increase to 0.09 g$_{lipid}$/L/day. Lowering the sodium nitrate
concentration, to 0.5 g/L, the productivity of cells would decrease to 0.6 g\text{cell}/L/day and the productivity of lipids would decrease to 0.15 g\text{lipid}/L/day. Having sufficient substrate is important because the purpose in the first reactor is to produce as much biomass as possible.

Going into the second reactor it is important that the substrate concentration in the media coming from the first reactor is low enough to induce additional lipid synthesis within the biomass produced in the first reactor. Fortunately the sodium nitrate flowing out the first reactor is dependent on the dilution rate, as indicated by Equation 9. For the assumed Monod constants of 0.8 µ\text{max} and 0.005 k\text{S}, and a 0.65 day\textsuperscript{-1} dilution rate, the expected sodium nitrate concentration leaving reactor 1 is 0.02 g/L. Lowering the dilution rate would lower S\textsubscript{1}, as illustrated in Figure 39. However, lowering the dilution rate would cause a decrease in biomass productivity. The inputs and outputs of the PBR 1 in the series are shown in Table XII.

The values for the streams leaving reactor 1 are used to determine the outflows of the second reactor. The Monod kinetics and yield used to model the second PBR are the same as those used for the first reactor: K\text{s} = 0.005 g/L, µ\text{max} = 0.8 day\textsuperscript{-1}, and Y\text{X/S} = 2. The purpose of the second reactor design is to determine a suitable residence time that would allow for utmost lipid synthesis. No additional biomass is expected in the second reactor as the substrate concentration is close to zero. The most important aspect of the second reactor is the additional lipid productivity.

To determine the lipid productivity and dilution rate in the second reactor, Equations 22, 24 and 26 must be solved simultaneously, as the Theory section indicates. Because the equation to solve for substrate is nonlinear, the Solver function in Excel was
used, by setting the target value of the leaving substrate concentration equal to zero and setting a maximum constrain value on the leaving concentration to be smaller or equal to the substrate concentration entering the second reactor. The dilution rate is determined by trial and error by first guessing a value and then looking at the lipid production versus dilution correlation. It is strongly suggested to perform these calculations with a programming program, such as MatLab.

<table>
<thead>
<tr>
<th>Table XII – Reactor 1 Mass Flows.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>S [g/L]</td>
</tr>
<tr>
<td>D [day⁻¹]</td>
</tr>
<tr>
<td>X [g/L]</td>
</tr>
<tr>
<td>L [g/L]</td>
</tr>
</tbody>
</table>

When deciding the dilution rate for the second reactor, two parameters need to be considered: volume of the second reactor and lipid production in the second reactor. The lipid productivity increases with dilution rate, as indicated by Figure 43. The dilution rate in the second reactor determines the volume of the second reactor; the smaller the dilution rate, the larger the volume and longer average residence time in the second reactor.
According to Figure 44, a dilution rate of about 0.2 day\(^{-1}\), or an average residence time of approximately 5 days, in the second reactor could be satisfactory to allow for additional lipid synthesis. Determining the dilution rate in the second reactor will aid in determining the volume ratio of the two reactors. For the case presented here where the dilution rate in the first reactor is 0.65 day\(^{-1}\) and the dilution rate of the second reactor is 0.2 day\(^{-1}\), the volume of the second reactor would need to be 3.25 larger than the first reactor. This surprising result suggests that the two-reactor system for the purpose of increasing lipid accumulation is not economically favorable because of the large reactor volumes requirements. Alternative options could include, partially dewatering the algae culture first and inducing lipid accumulation by means of other stresses such as light.
Figure 44 – Lipid concentration versus dilution rate in Reactor 2.

The overall lipid productivity as a function of dilution rate in the second reactor (Figure 45), indicates that the lipid productivity levels off above a certain dilution rate value. In the case presented here, above $D_2=0.7 \text{ day}^{-1}$ the lipid productivity is increasing slower than before this point. The overall lipid productivity is lower than the productivity achieved in the first reactor (0.03 g/L/day), because the overall productivity is divided by the sum of the two reactors volume, not just the volume in reactor one.

The two-PBR system has a lower productivity than one single reactor. Having a larger second reactor will have a negative impact on the overall economics of producing fuel from algae. Alternatives need to be explored, such as feeding algae sugar in the dark, or finding more profitable ways to use the lipids and other constituents from algae to make pharmaceutical or other product and co-products.
Figure 45 – Overall lipid productivity versus dilution rate in the second reactor.
CHAPTER VI
CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The lipid extraction method with the most consistent results used mortar and pestle for cell lysing. The other promising method is organic solvent cell lysing. All methods described here extracted chlorophyll and perhaps other fat-soluble cell components that could be a hindrance in the refining process of fuel.

From the four types of batch experiments performed with *Scenedesmus dimorphus* to determine the necessary parameters for the reactor model (maximum cell growth rate ($\mu_{\text{max}}$), yield ($Y_{x/s}$), and Monod constant ($K_s$)), two of the experiments were performed with varying initial substrate concentrations, here sodium nitrate. The data sets were analyzed by three methods: differential/linear least-square, initial substrate method, and non-linear regression. The results obtained by the three methods varied so much that it is difficult to identify accurate values for the three parameters.
The differential/linear least square and initial substrate methods gave $\mu_{\text{max}}$ results ranging from $-0.2$ day$^{-1}$ to $2$ day$^{-1}$. but a more common result was $0.7$ day$^{-1}$. Based on the results obtained by the initial substrate method, $K_s$ is expected to be $0.005 \pm 0.01$ g/L maximum growth rate to be $0.7 \pm 0.1$ day$^{-1}$. The data analyzed by non-linear square method yielded an ill-conditioned Covariance Matrix, which made the error analysis unreliable. Some $K_s$ and $\mu_{\text{max}}$ values obtained by both methods were negative; probably due to scatter in data.

The yield was determined using linear least-square data analysis. The results for the yield values of all four experiment fall between $1.2$ and $2.7 \text{ g}_{\text{cell/g substrate}}$, which is within the theoretical range discussed for E. coli and saltwater algae, $1.03$ and $2.62 \text{ g}_{\text{cell/g substrate}}$ respectively. However the large errors indicate that yield sensitivity limits fall partially between and partially out of this theoretical range.

The equations derived in the theory sections along with approximate values for $\mu_{\text{max}}$, $Y_{x/s}$, and $K_s$, were used to create a two-PBR system mathematical model. For the case presented here where the dilution rate in the first reactor is $0.65$ day$^{-1}$ and the dilution rate of the second reactor is $0.2$ day$^{-1}$, it was determined that the volume of the second reactor would need to be $3.25$ larger than the first reactor. This surprising result suggests that the two-reactor system for the purpose of increasing lipid accumulation is not economically favorable because of the large reactor volumes requirements.

### 6.2. Recommendations

1. Better lipid methodology development is suggested by analysis to extract lipids using a Gas Chromatograph or other oil analysis instrumentations or techniques.
2. To validate your predictions of the CSTR model using experimental CSTR system. Experimental results may indicate the need for refinement of the model.

3. To refine this model, continuous mode instead of batch mode experiments at laboratory scale should be tested.

4. Additional batch experiments with cell counts and lipid extraction could better determine lipid kinetics.

5. In this thesis, the yield was calculated independently of Monod parameters. In the future, both substrate and biomass concentration as functions of time should be analyzed simultaneously and all three parameters ($\mu_{\text{max}}, K_s, Y_{x/s}$) be determined together.

6. It is recommended that the mathematical model for substrate uptake should also be reconsidered. Future experiments should also use better methods, such as an assay instead of a probe, to measure sodium nitrate concentration more accurately.

7. Future experiments should include counting the cells to help differentiate between cell mass increase by cell proliferation and lipid accumulation, instead of only optical density measurements.

8. It is strongly recommended that the statistical analysis be refined in the future. It is also suggested here that alternative data analysis methods, such as optimal design theory, be researched and tried, based on literature suggestions because the least-square method does not seem to be an accurate method to calculate the Monod parameters and yield.
9. Alternatives need to be explored that can have a positive impact on the economics of growing algae, such as introducing a concentration step between the two reactors discussed here, stressing the algae by other means such as light, feeding algae sugar in the dark, or finding more profitable ways to use the lipids and other constituents from algae to make pharmaceutical or other product and co-products.
REFERENCES


APPENDICES
APPENDIX-A - PROTOCOLS

Experiment Protocol Sample

Light schedule for this experiment is 12-hour light/12-hour dark.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mL sterilized centrifuge tubes</td>
<td>4 (time zero sampling) – can reuse these for daily sampling.</td>
</tr>
<tr>
<td>Nitrate Probe</td>
<td>1</td>
</tr>
<tr>
<td>2 L bottles</td>
<td>4</td>
</tr>
<tr>
<td>Large stoppers-bubblers-sampler</td>
<td>4</td>
</tr>
<tr>
<td>Foil</td>
<td></td>
</tr>
<tr>
<td>Autoclave paper or bags</td>
<td>4</td>
</tr>
<tr>
<td>50 mL sterilized centrifuge tubes</td>
<td>2 (1 for balance)</td>
</tr>
<tr>
<td>Cuvettes</td>
<td>8</td>
</tr>
<tr>
<td>DI water</td>
<td>~6L</td>
</tr>
<tr>
<td>1000 uL automatic Pipette</td>
<td>1</td>
</tr>
<tr>
<td>10 mL Pipette</td>
<td>6 (one for each nutrient)</td>
</tr>
<tr>
<td>Graduated cylinder</td>
<td>1 (for DI water for making media)</td>
</tr>
<tr>
<td>250 mL Erlenmeyer Flask</td>
<td>1 (for extra nitrate free media)</td>
</tr>
<tr>
<td>Glass bottle</td>
<td>1 (for extra DI water)</td>
</tr>
<tr>
<td>Cuvettes</td>
<td>4 (reusable)</td>
</tr>
</tbody>
</table>

1. Checklist before experiment:
   a. CO2 tank
   b. Lights
   c. Temperature probe
   d. Timer
   e. Autoclave
   f. Seed vessel
   g. Media
   h. DI water
   i. Have all needed materials / clean glassware if necessary
   j. Lab manual
   k. Add tubing and filters to new bubbler assemblies
   l. Double check nutrient volumes for nitrate free and low nitrate media.

2. Experiment steps:
   a. Wrap in autoclavable paper 4 stopper-bubblers-sampling systems.
   b. Prepare one extra glass container of DI water to be used as make-up water if one not in hood.
   c. Make ~100 mL of media NaNO3 free – for inoculation (see step j) in a 250 mL Erlenmeyer flask using media recipe shown in Table 1 below. Depending
on starting cell concentration, the amount of media NaNO₃ free needed will vary (see step k and change accordingly). Note that extra media is needed for diluting samples for absorbance.

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1</td>
</tr>
<tr>
<td>NaCl</td>
<td>1</td>
</tr>
<tr>
<td>P-IV</td>
<td>0.6</td>
</tr>
<tr>
<td>DI H₂O (mL)</td>
<td>44.4</td>
</tr>
</tbody>
</table>

Table 1

d. Turn on shaker bath to warm up to 30°C.
e. Turn on autoclave machine to warm up.
f. Prepare four 1600mL media in 2 L jars, containing various amounts of diluted NaNO₃ solution as shown in the following table.

g. Cover all the glass jars (including extra DI and extra NaNO₃ free media) loosely with a screw cap and a loose piece of foil. Ensure the piece of foil is long enough to go down past the screw cap.
h. Mark the volume on all glass jars with a piece of tape to help determine make-up volume after autoclave due to evaporation.
i. Autoclave for 30 minutes
j. Once autoclaved glass containers are cool enough to touch, tighten foil around screw cap and move to hood to further cool.
k. Prepare seed
   - Take absorbance of seed jar and determine approximate volume needed for 0.01 absorbance inoculation.
   - Sample out 50 mL (amount changes with initial cell concentration in step k) of seed from seed stock under hood in a sterilized 50mL centrifuge tube (cap tube before removing from hood to maintain sterilization). Note that more amount of seed sample may be needed
depending on seed jar concentration and desired inoculation concentration.

- Centrifuge cells for 30 minutes or until all cells concentrated into a pellet.
- Spray the centrifuge tube with 70% isopropanol and work under hood for the remaining steps.
- Remove supernatant under laminar hood to maintain cell sterilization.
- If more than 1 centrifuge tube used, combine cells into one centrifuge tube!
- Resuspend cells in sterilized NaNO₃ free media.
- Measure absorbance of resuspended cells to determine volume needed for inoculation.

1. Inoculate at approximately 0.01 absorbance each 2L jar (1.6L working volume).

m. Upon inoculation take 7 mL of samples into 15mL sterilized centrifuge tubes and measure zero-point absorbance and nitrogen concentration of all 4 jars.

n. Transfer the four 2L jars to shaker bath. Connect CO₂/air to all samples at 5% CO₂. Ensure shaker bath water is 30°C.

o. Sampling for Monod parameters determination:
   - Take absorbance data every approximately 24 hours (6 pm) for ten days. Record exact time.
   - Daily sample size approximately 7 mL of sample into 15 mL centrifuge tube. Approximately 3 mL used to take sample absorbance at 600nm. Remaining volume will be used to measure nitrogen concentration using probe (see additional protocol on sampling). At least 3mL needed for nitrogen probe.
   - Data analysis for each 2L jar:
     - ln(X/X₀) vs t to obtain μ (do not force through 0 or graph lnX vs t.
     - Then plot μ vs C°ₙaNO₃ (average over days 0-3) to graphically determine the Monod equation constant ks and maximum growth rate (μ_max).
     - Plot C vs C°ₙaNO₃ to get yield coefficient. Then plot Yₓ/NaNO₃ vs C°ₙaNO₃

p. Sampling for lipid vs lipid and lipid free biomass data
   - Each sample is to be centrifuged and dried, then stored in -80°C until all samples are taken. All the lipid extractions will be done at the same time.
   - Every time a sample is taken, also measure the absorbance and nitrate concentration.
   - All jars
     - 1ˢᵗ sample out of seed jar (Day 1): 600 mL (split into 3 samples of 200mL)
     - Jar 1 - 0.2g/L NaNO₃
       - Expected ~7 days to reach close to zero N₂ concentration
- Sample days 2, 3, 4 and 6, 200mL samples
- The remaining volume take 200mL samples every other day (ex. Days 8, 10…)

- Jar 2 - 0.1g/L NaNO₃: Expected ~4 days to reach close to zero N₂ concentration
  - Sample days 1, 2, 3, 4 samples of 200 mL
  - The remaining volume take 200mL samples every other day (ex. Days 6, 8)

- Jar 3 - 0.05g/L NaNO₃: Expected ~3 days to reach close to zero N₂ concentration
  - Sample days 1, 2, 3, 4 samples of 200 mL
  - The remaining volume take 200mL samples every other day (ex. Days 6, 8)

- Jar 4 - .001g/L NaNO₃: Already close to zero Nitrogen!
  - Sample days 1, 2, 3, 4 samples of 200 mL
  - The remaining volume take 200mL samples every other day (ex. Days 6, 8)

- Data analysis for each 2L jar:
  - Extract lipids and measure mass of dry biomass and lipid for each sample

---

**Monod Experiment Sampling**

1. Leave nitrate probe in 100mg/L standard solution for approximately 30 minutes.
2. Have ready 4 15mL clean centrifuge tubes. Label them (1,2,3,4).
3. At about 30 minutes after nitrogen probe is been soaking in standard solution, sample out 7mL of cells into centrifuge tube (Jar 1 sample to centrifuge tube 1, etc)
4. Transfer a couple mL to a cuvette from each centrifuge tube, read absorbance and record in provided table.
5. Calibrate the nitrate probe.
6. Record nitrogen probe readings for each sample using software for about 30 seconds. Cap the centrifuge tube and mix well before reading.
7. Rinse well with DI water between readings and pat dry with paper towel.
8. Once all samples have been read, spray probe with 70% isopropanol and rinse well with DI water.
9. Store probe in provided storage moisture bottle.
Lipid Extraction Protocol – Centrifuge/Solvent

Materials

4 50mL centrifuge tubes
2 or 3 glass tubes
25 mL pipettes
5 mL pipettes
50 mL Isopropanol/Hexane (2:3 v/v) Solvent
Drying oven
Shaker

Procedure

1. Observe the cells under microscope and record growth stage.
2. Pipette approximately 37.5 mL of sample into 50 mL sterile centrifuge tubes from the 150mL sample bottle. Repeat until all sample in the bottle is exhausted. 4 50mL centrifuge tubes will be needed.
3. Centrifuge the 50 mL tubes for 25 minutes at 2000 rpm.
4. After centrifugation remove most of the supernatant. Leave a couple milliliters.
5. Weigh a glass tube and record the weight. (If more than one tube is needed number the tubes and record appropriate weights)
6. Combine the pellets from the 50 mL tubes into the glass tube. Rinse the 50 mL tube with few millimeters of water and add to the glass tube.
7. Spin the tube for 30 minutes at 2000 rpm.
8. Leave a couple milliliters of supernatant and discard the rest of the supernatant.
9. Add 5 mL of isopropanol/hexane (2:3 v/v) solvent to the glass tube.
10. Place the tube in a shaker and leave for 3 hours.
11. Leave to settle for a couple of hours.
12. Using a glass pipette remove the top layer containing solvent and lipids and put the contents in another preweighed glass tube. Place tube uncapped under hood or under vacuum until second extraction is completed.
13. Centrifuge the remaining contents in the original glass tube, resuspend and repeat extraction steps 9-11. When extraction is completed pipette the top layer containing solvent and lipids into the glass tube containing previous extract.
14. Using a water aspirator evaporate the solvent from both glass tubes (the one containing the biomass and the one with lipids).
15. After solvent is evaporated dry in oven at 50 °C until constant weight.
16. To determine the total dry weight, add the dry weight from both tubes.
### APPENDIX-B - ADDITIONAL DATA AND GRAPHS

Table B. I – Maximum growth rate and Monod constant results from MatLab using nonlinear regression method.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Trial</th>
<th>Results (\mu_{\text{max}} \text{ [day}^{-1}])</th>
<th>Initial guesses</th>
<th>Error (Significant Figures)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(K_s) [μL]</td>
<td>(\mu_{\text{max}}) [day}^{-1}]</td>
<td>(K_s) [μL]</td>
</tr>
<tr>
<td></td>
<td>day and night data</td>
<td>0.87</td>
<td>0.191</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>day data</td>
<td>0.71</td>
<td>0.156</td>
<td>0.90</td>
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<td>0.3 g NaNO3/L</td>
<td>4.17</td>
<td>1.120</td>
<td>0.74</td>
<td>0.004</td>
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<tr>
<td></td>
<td>20.10</td>
<td>6.320</td>
<td>1.87</td>
<td>0.315</td>
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<td>0.875</td>
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<td>0.009</td>
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<td>0.2 g NaNO3/L</td>
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<td>0.000</td>
<td>0.74</td>
<td>0.004</td>
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<td>6.28</td>
<td>1.120</td>
<td>1.75</td>
<td>0.203</td>
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<tr>
<td></td>
<td>-1.63</td>
<td>-0.466</td>
<td>0.70</td>
<td>0.009</td>
</tr>
<tr>
<td>0.15 g NaNO3/L</td>
<td>0.00</td>
<td>0.000</td>
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<td>0.004</td>
</tr>
<tr>
<td></td>
<td>1.26</td>
<td>0.007</td>
<td>0.91</td>
<td>0.026</td>
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<tr>
<td></td>
<td>1.95</td>
<td>0.170</td>
<td>0.70</td>
<td>0.009</td>
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<tr>
<td>0.1 g NaNO3/L</td>
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<td>0.000</td>
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<td>0.004</td>
</tr>
<tr>
<td></td>
<td>0.79</td>
<td>0.006</td>
<td>0.70</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>0.79</td>
<td>0.006</td>
<td>0.70</td>
<td>0.009</td>
</tr>
<tr>
<td>0.05 g NaNO3/L</td>
<td>0.74</td>
<td>0.001</td>
<td>0.74</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>No convergence</td>
<td>0.66</td>
<td>0.655</td>
<td>0.70</td>
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<tr>
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<td>No convergence</td>
<td>0.70</td>
<td>0.009</td>
<td>0.70</td>
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<tr>
<td>0.04 g NaNO3/L</td>
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<td>0.013</td>
<td>0.74</td>
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<tr>
<td></td>
<td>1.25</td>
<td>0.013</td>
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<td>No convergence</td>
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<td>0.009</td>
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<tr>
<td>0.03 g NaNO3/L</td>
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<td>0.007</td>
<td>0.74</td>
<td>0.004</td>
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<tr>
<td></td>
<td>0.56</td>
<td>-0.001</td>
<td>0.70</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>No convergence</td>
<td>0.70</td>
<td>0.009</td>
<td>0.70</td>
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<tr>
<td>0.2 g NaNO3/L</td>
<td>14.30</td>
<td>3.350</td>
<td>0.87</td>
<td>0.006</td>
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<td></td>
<td>35.30</td>
<td>3.830</td>
<td>0.79</td>
<td>0.149</td>
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<td>28.70</td>
<td>6.860</td>
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<td>0.1 g NaNO3/L</td>
<td>1.00</td>
<td>0.880</td>
<td>0.87</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>-0.17</td>
<td>-0.088</td>
<td>-0.17</td>
<td>-0.088</td>
</tr>
<tr>
<td></td>
<td>4.00</td>
<td>0.336</td>
<td>0.70</td>
<td>0.009</td>
</tr>
<tr>
<td>0.05 g NaNO3/L</td>
<td>0.56</td>
<td>0.007</td>
<td>0.87</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>2.70</td>
<td>0.067</td>
<td>0.70</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>No convergence</td>
<td>1.58</td>
<td>0.004</td>
<td>0.70</td>
</tr>
<tr>
<td>0.01 g NaNO3/L</td>
<td>2.93</td>
<td>0.006</td>
<td>0.87</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>No convergence</td>
<td>0.70</td>
<td>0.009</td>
<td>0.70</td>
</tr>
<tr>
<td>1 - 0.45 g NaNO3/L</td>
<td>4.45</td>
<td>3.630</td>
<td>0.18</td>
<td>0.657</td>
</tr>
<tr>
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<td>0.009</td>
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<tr>
<td>2 - 0.45 g NaNO3/L</td>
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<td>0.000</td>
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<td></td>
<td>5.03</td>
<td>4.200</td>
<td>0.70</td>
<td>0.009</td>
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<tr>
<td>3 - 0.45 g NaNO3/L</td>
<td>0.00</td>
<td>0.000</td>
<td>0.12</td>
<td>0.463</td>
</tr>
<tr>
<td></td>
<td>5.78</td>
<td>4.940</td>
<td>0.70</td>
<td>0.009</td>
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</table>
Table B. II – Summary of Linear Least-Squares Analysis of double reciprocal linearization of Monod.

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1</th>
<th>Experiment 2 -0.3 g/L NaNO3L</th>
<th>Experiment 2 -0.2 g/L NaNO3L</th>
<th>Experiment 2 -0.15 g/L NaNO3L</th>
<th>Experiment 2 -0.1 g/L NaNO3L</th>
<th>Experiment 2 -0.05 g/L NaNO3L</th>
<th>Experiment 2 -0.04 g/L NaNO3L</th>
<th>Experiment 2 -0.03 g/L NaNO3L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope ($k/k_m$)</td>
<td>0.21 ± 0.01</td>
<td>0.17 ± 0.24</td>
<td>0.12 ± 0.19</td>
<td>0.13 ± 0.62</td>
<td>0.01 ± 0.03</td>
<td>-0.0044 ± 0.01</td>
<td>0.004 ± 0.33</td>
<td>-0.002 ± 0.08</td>
</tr>
<tr>
<td>Intercept ($V_{max}$)</td>
<td>6.54 ± 0.28</td>
<td>5.87 ± 0.63</td>
<td>5.89 ± 0.62</td>
<td>1.42 ± 0.46</td>
<td>1.03 ± 1.29</td>
<td>1.04 ± 1.73</td>
<td>1.04 ± 3.44</td>
<td>2.37 ± 17.73</td>
</tr>
<tr>
<td>$K_s$</td>
<td>2.05 ± 0.34</td>
<td>1.87 ± 1.95</td>
<td>1.75 ± 0.91</td>
<td>0.70 ± 0.68</td>
<td>0.68 ± 1.04</td>
<td>0.42 ± 1.73</td>
<td>0.42 ± 3.04</td>
<td>0.20 ± 1.08</td>
</tr>
</tbody>
</table>

*Data for pret obtained by initial substrate method

<table>
<thead>
<tr>
<th></th>
<th>Experiment 3 - Jar 1 - 0.2 g/L NaNO3</th>
<th>Experiment 3 - Jar 2 - 0.15 g/L NaNO3</th>
<th>Experiment 3 - Jar 3 - 0.15 g/L NaNO3</th>
<th>Experiment 3 - Jar 4 - 0.15 g/L NaNO3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope ($k/k_m$)</td>
<td>-0.01 ± 0.002</td>
<td>1.49 ± 1.35</td>
<td>0.37 ± 0.2</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>Intercept ($V_{max}$)</td>
<td>-0.87 ± 0.16</td>
<td>-8.3 ± 0.64</td>
<td>-3.44 ± 3.64</td>
<td>1.73 ± 1.4</td>
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<tr>
<td>$K_s$</td>
<td>0.0086</td>
<td>-0.2172</td>
<td>-0.1071</td>
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<table>
<thead>
<tr>
<th></th>
<th>Experiment 4 - Jar 1</th>
<th>Experiment 4 - Jar 2</th>
<th>Experiment 4 - Jar 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slope ($k/k_m$)</td>
<td>3.84 ± 2.93</td>
<td>0.46 ± 7.04</td>
<td>1.37 ± 4.42</td>
</tr>
<tr>
<td>Intercept ($V_{max}$)</td>
<td>-6.53 ± 7.87</td>
<td>3.7 ± 18.8</td>
<td>-3.68 ± 12.06</td>
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<td>$K_s$</td>
<td>-0.16</td>
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<td>0.1236</td>
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