Cultivation of *Tetraselmis suecica* Under Various Nutrient Concentrations and the Characterization of the Algal Biomass

by

Zoë Leigh Grabowski

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To Grandpa Joe and Auntie Kathy

Thank you for always believing in me

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ABSTRACT

Tetraselmis suecica is a candidate for producing 3-deoxy-D-*manno*-2-octulosonic acid (KDO), a high-value sugar. In this work, a cultivation system was implemented to maintain cultivation conditions and to investigate the effect of media composition on biomass growth and composition. Nitrogen-starved ($1.62x10^{-4}$ M nitrate), phosphorous-starved ($1.11x10^{-5}$ M phosphate) and nutrient-replete ($5.6x10^{-4}$ M nitrate and $3.24x10^{-5}$ M phosphate) cultivation conditions were investigated to probe these effects. Total starch, KDO and other sugars in the biomass were determined using enzymatic hydrolysis and GC/MS respectively. Biomass growth was found to be consistent over replicate cultures. For nitrogen-starved, phosphorous-starved and nutrient replete cultures, starch values by percent of total microalgal mass were 42±8%, 39±5% and 43±6% in the stationary phase and maximum cell counts were $4x10^5$, $8x10^5$ and $1.4x10^5$ cells/mL respectively. KDO per gram of lyophilized microalgae found were 20±2, 22±1 and 24±2 mg/g under nitrogen-starved, phosphorous-starved and nutrient-replete conditions respectively at the stationary phase.

LIST OF ABBREVIATIONS AND SYMBOLS USED

DI	Deionized
DHA	Docosahexaenoic acid
DIC	Dissolved inorganic carbon
DNS	Dinitrosalycylic
EH	Enzymatic hydrolysis
GC/MS	Gas Chromatography/Mass Spectrometry
KDO	3-deoxy-D-manno-2-octulosonic acid
LPS	Lipopolysaccharide
N-starved	Nitrogen-starved
P-starved	Phosphorous-starved
PEP	Phosphoenolpyruvate
Pi	Inorganic phosphate
PUFA	Poly unsaturated fatty acid
RT	Retention time
SS_b	Sum of squares between treatments
SS_w	Sum of squares within treatments
TMS	Trimethylsilyl
UTEX	University of Texas in Austin

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Chapter 1 : Introduction

1.1 Background

Microalgae are the source of many molecules with unexplored potential in many areas of research. Not only do they act as support for the entire aquatic ecosystem, but they are already used in a variety of industries. One large issue in implementation of useful products of microalgae is that, though these molecules can be very valuable, the process of culture growth and extraction can be costly.

Algae is responsible for a massive amount of biomass on the global scale and both seaweeds and microalgae together act as the support for the aquatic ecosystem. They are important for the fixation of carbon and production of 40 to 50 % of all atmospheric oxygen [1]. Microalgae can be used as livestock feed, cosmetics, nutrient supplements and the basis for new medicines. They are a valuable resource, and research into these molecules is useful for both fundamental research and for industrial applications.

Microalgae are notably used for aquaculture, particularly species from the genera *Tetraselmis*, *Pyramimonas*, *Micromonas*, *Olisthodiscus and Nannochloropsis* [2]. *Dunaliella salina* is produced to use its β-carotene as a food additive. *Haematococcus pluvialis* produces astaxanthin, which is used both for its anti-oxidant properties and as a food colorant [3].

Microalgae can be used for their polyunsaturated fats (PUFAs). For example, *Gymnodinium* is used to produce docosahexaenoic acid (DHA) which is used in baby formula [2].

Much of the research on microalgae focuses on their potential as a source of biomass for biofuel production. The US Department of Energy actively funded research in the field in the 1980s and, as such, significant research has gone into understanding the relationship of lipid cellular content and microalgal cultivation conditions [4] [3].

They were an attractive prospect because, unlike higher plants which require fertile soil, microalgae have no soil requirements and can be grown on otherwise uncultivatable land. Another advantage of microalgae-based technologies is the use of sunlight as a source of energy for the growth of these microorganisms. Different light exposures can affect growth, and light from the sun can be used in order to avoid electricity consumption [5] [6]. Nutritional and environmental conditions influence microalgal biomass concentration and composition, allowing for optimization of a chemical of interest through bioprocess engineering approaches. Other research has used these studies to enhance microalgae for various industrial applications such as biofuel, fertilizer, animal feed and pharmaceuticals [7].

Biochemical engineering approaches also provide opportunities to optimize the concentration of the desired product. For example, microalgal metabolites like carbohydrates and lipids tend to be overexpressed under adverse environmental conditions, while proteins are overexpressed under favourable growth conditions [8]. Nutrient stress can therefore be another way to force the microalgal cells into different metabolic pathways. This method allows for overexpression of different cellular components, such as lipids and carbohydrates.

Nitrogen-stress has been associated primarily with lipid overexpression, but can instead be responsible for additional carbohydrate accumulation. Dragone *et al.* 2011

studied the effect of different nitrogen concentrations on *Chlorella vulgaris.* They varied the range from nitrogen-rich media with 2.2 g/L urea to a nitrogen-deplete media with no urea in a batch column reactor in 40 mL of medium. *C. vulgaris* experienced an 8 fold increase in starch content in the nitrogen-deplete culture when compared with the control culture. The nitrogen-deplete culture reached 41.0% starch by dry weight [9].

Phosphorus-based stress leads to accumulation of both lipids and carbohydrates. Lynn *et al.* 2000 observed a 1.7 fold increase in lipids and 1.8 fold increase in carbohydrates when studying *Stephanodiscus minutulus* in phosphorous-deplete conditions. They performed this study by comparing 600 mL semicontinuous cultures of 10 μM P versus cultures of 50 μM P where the phosphorous source was dipotassium phosphate [10].

Under both these nutrient conditions, protein production is decreased. These effects vary from species to species, but by limiting access to nutrients like nitrate and phosphate, the cells will down regulate some processes and up regulate others. In order to implement nutrient stress on a commercial scale, it is important to first isolate which effect is most desirable at a smaller scale [8] [11].

Applications of microalgae for production of large volume commodity products is limited due to the low biomass concentration produced in algal cultivation systems. Biomass recovery through common harvesting technologies such as centrifugation, filtering, flocculation or forward osmosis are energy intensive and subsequently costly due to the low biomass concentration produced in the cultivation system [12].

Tetraselmis suecica is a green marine microalgae (Chlorophyceae) of industrial interest. It can grow under autotrophic, heterotrophic and mixotrophic conditions. It is grown at an industrial scale as aquaculture feed for brine shrimp [13]. As it is produced at an industrial scale, it is an attractive candidate for the exploration of secondary high-value products [14]. In addition to being a source of high protein content for aquaculture applications [15] [13], *T. suecica* contains a sugar of pharmaceutical interest, namely 3-deoxy-D-*manno*-2-octulosonic acid (KDO) [16]. Therefore, *T. suecica* is a potential feedstock for the simultaneous production of aquaculture feed and a high-value product (i.e. KDO).

The concept of derivation of multiple chemicals from microalgae in a biorefinery allows for maximizing the use of biomass and is particularly appealing in the area of microalgal production. This is because microalgal-product technology is challenged by low biomass concentration obtained in photobioreactors and subsequent energy intensive harvesting downstream processes. Therefore, either multiple products can be extracted, as with *T. suecica*, or the waste from one process can be used as a material for another. For example, a lot of farm waste is rich in nutrients and can be used for land plants and microalgae alike [17].

KDO has potential applications in antibiotic research as it is a necessary component in some pathogenic gram-negative bacteria [18]. These bacteria have negative health effects in humans, they can lead to ulcers, cancer metastasis and infections [19] [20]. KDO has been identified as a potential lead in the development of enzyme inhibitors [21] and could further be used as a starting material for complex oligosaccharide synthesis [22]. The concept of integrated biorefineries also plays an

important role in this research, as the cost is an important factor to consider in this venture. The ultimate goal of this research is to not only integrate both the aquaculture feed and KDO production, but also to develop technology that enables simultaneous recovery of both products [3].

The main objectives of this work were to produce consistent *T. suecica* biomass under controlled environmental conditions that can be further used to study the effect of cultivation conditions on the accumulation of cellular KDO. In this work, *T. suecica* was grown in a 1L photobioreactor under nutrient replete (5.6×10^{-4} M nitrate and 3.24×10^{-5} M phosphate), P-starved (1.11×10^{-5} M phosphate) and N-starved (1.62×10^{-4} M nitrate) conditions. Cultivation experiments in each nutrient condition were performed in duplicate and the biomass concentration was monitored throughout the culture. *T. suecica* biomass at the stationary phase was characterized with respect to both the cellular starch and sugar content. The results of biomass monitoring and characterization were used to demonstrate that replicates would be reproducible and consistent.

1.2. Research Objectives

The aim of this study is to investigate the marine microalgae *T. suecica* as a potential source of KDO by introducing cultures to nutrient stress. The specific goals of this study are:

1. Building a cultivation system in which production of consistent biomass can be achieved

2. To grow cultures using three different cultural conditions:
    - Nutrient Replete
    - Nitrogen-Starved (N-starved)
    - Phosphorous-Starved (P-starved)
3. To quantify carbohydrate content, KDO and other sugars, in *T. suecica* biomass using gas chromatography-mass spectrometry (GC/MS)

Chapter 2 : Literature Review

2.1 Microalgae:

Microalgae are single-celled organisms that can be found either individually or in colonies. They are able to photosynthesize, and some are able to consume organic carbon sources. They can inhabit a wide variety of environments, including fresh water (e.g. *Chlorella vulgaris*) [6], seawater (e.g. *Pavlova lutheri*) [6], and hypersaline water (e.g. *D. salina*) [23].

Since there are a variety of species and many have useful cellular components, there has been interest in industrial scale growth of microalgae. Currently a handful of species are grown for their desirable products. *D. salina* is grown for its β-carotene, used as a food additive; *H. pluvialis* for its astaxanthin, which acts as a food colorant and an anti-oxidant; and *Cypthecodinium cohnii* is grown for its PUFAs, DHA in particular, to be used for nutritional supplements and baby formula [23]. Several more species are used as aquaculture feed, an application that requires less processing as the whole biomass is typically used [2].

Although, microalgae are known as photosynthetic microorganisms and capable of using inorganic carbon as source of carbon and light as a source of energy, some microalgae species are also capable of growth under heterotrophic conditions in which they use organic carbon (e.g. glucose) as a source of carbon and energy. Microalgae capable of both heterotrophic and autotrophic growth, can be grown under mixotrophic conditions in which they use both organic and inorganic carbon and light as a source of energy [24].

Many microalgae are restricted to autotrophic growth, but some species are able to exploit different carbon sources depending on the cultivation conditions [24]. A notable species is *Galdieria sulphuraria*, which is able to grow heterotrophically on a large variety of different carbon sources [2]. *Chlorella protothecoides* [3] and *Porphyridium cruentum* [25] are among some of the other species that can experience heterotrophic growth. Some species experience less pigment production under heterotrophic growth [25].

Mixotrophic species, which are able to use both inorganic carbon with photosynthesis and organic carbon sources, tend to perform very well in overall biomass production [25] [2]. Species that can grow mixotrophically include *Platymonas subcordiformis* [26], *C. vulgaris* [27] and *T. suecica* [28].

Microalgae have several advantages over higher plants in both the production of biofuels and of other chemicals. They do not require arable land, and in the case of marine species, less fresh water is required in production [3].

2.2 *Tetralsemis suecica*:

The *Tetraselmis* genus is a group of green cordiform or "heart-shaped" microalgae which exist in fresh or marine water. This genus, as well as most chlorophytes, have subcellular compartments known as pyrenoids, which, compared to similar genera, are embedded in the chloroplast and surrounded by starch grains [29].

T. suecica is green microalga with a high content of nutritionally rich protein, comparable to eggs and soy [15], and relatively high specific growth rate [6] making

them appealing as aquaculture feed. *T. suecica* is capable of heterotrophic and mixotrophic growth, leading to higher biomass concentration compared to autotrophic growth alone, reaching growth rates of 0.90 per day (mixotrophic) [28] as opposed to 0.32 per day (autotrophic) [30].

When comparing its growth rate in a group of thirty strains growth autotrophically, *T. suecica* had a maximum biomass productivity of 0.32 g/L/day, with the best performing algae growing at a rate of 0.37 g/L/day (*P. cruentum*) and the worst performing algae growing at 0.04 g/L/day (*Chaetoceros calcitrans*). Notably, *T.suecica* also outperformed all strains of *Nannochloropsis* sp. and *C. vulgaris* [6], two other industrially relevant algae, in terms of biomass growth.

Go *et al.* 2012 studied 15 L batch cultures of *T. suecica* to optimize growth by varying both light intensity and nitrogen availability. Cultures reached maximum growth rates of 0.67 $days^{-1}$ at a light intensity of 108.9 μmol photon m^{-2} s^{-1} for a 24:0 hour light/dark cycle. Varying the nitrogen concentration was not shown to affect the growth rate [31]. Carballo-Cardenas *et al.* 2003 also subjected batch *T. suecica* cultures to 24 hour light, but in a 1.5 L reactor. The cultures were able to reach a growth rate of 1.2 $days^{-1}$ at a light intensity of 230 μmol photon m^{-2} s^{-1} [14]. Each studied used the modified f/2 medium as described by Guillard *et al.* 1962 [32]. These studies were able to achieve different maximum growth rates, but the growth rate was shown to have a relationship with the light available and not nutrient availability.

T. suecica is used as an aquaculture feed for organisms known as brine shrimp [33], which go on to be feed for larger aquatic species. *T. suecica* has many traits that are not only desirable as a feed, but make it an exemplary microalgal species in terms of

potential for secondary products. Like many microalgae, it is a source of PUFAs [34]. It is also a promising source of vitamin E [34]. Microalgae based nutrient supplements frequently cite their vegan nature and advertise themselves as an alternative to depleting the ocean's fish stocks [35], making microalgae such as *T. suecica* potential feedstocks for the nutraceutical industry.

Much of the research done on *T. suecica* focuses on finding potential feedstocks for biofuel production [31]. As such, there has been an emphasis on overexpression of lipids, particularly triglycerides, for biodiesel production. *T. suecica* has a variable lipid content based on the cultivation conditions. Nitrate-deplete conditions have increased its oil productivity while decreasing cell concentration and total biomass [31] [9]. As with many other species, to optimize for both lipids and sufficient biomass, a two-stage culture where biomass is first grown and then nutrient stress is introduced has been proposed [8]. This idea is explored further on the section on the effect of cultivation conditions.

Since *T. suecica* has industrial applications and its cultivation is an already established technology, there exists the opportunity to utilize the biomass produced for the derivation of novel value-added chemicals alongside the existing commercial product. The incorporation of a method which enables the derivation of a secondary, high-value product would add value to these already existing cultures. This makes *T. suecica* an appealing candidate for structuring a biorefinery around, which will be discussed further in the Process Integration portion of this review.

2.3 KDO:

KDO is an 8-carbon sugar and is of pharmaceutical interest because it is present in the cell wall of many gram-negative bacteria (Figure 2-1) [36] [37]. Included among these are *Escherichia coli, Neisseria meningitidis, Yersinia pestis, Helicobacter pylori* and the entire Enterobacteriaceae family [18] [37] [19]. Gram-negative bacteria can cause a whole array of problems in humans such as ulcers, cancer metastasis and bacterial infections [19] [20].

KDO and its associated enzymes are not present in human biology, allowing for selective drug development and as such chemical synthesis of KDO has raised significant interest [18] [38] [39]. As some gram-negative bacteria are pathogenic to humans, having a range from mild to severe negative health effects, and humans have no KDO or KDO enzymes in their cells, KDO has been suggested as a promising potential target for antibiotic development in medicinal chemistry [18].

KDO is relevant to this study as a secondary high-value product in *T. suecica* because, in *T. suecica,* KDO makes up 54% of the cell wall by mass, or 5% of the dry weight over all [16]. It is also present in other green algae such as *Tetraselmis tetrathele* and *Tetraselmis striata* [40] [41]. It is present in the majority of higher plants as part of the rhamnogalacturonan II pectin; however, its function remains unknown [42].

Furthermore, it is a critical component of the lipopolysaccarchide (LPS) of these bacteria, having been found in the innermost core of the LPS where it is anchored to lipid A (Figure 2-2). Mutants that have defective KDO biosynthesis show a cessation of LPS synthesis and are overall inviable [19].

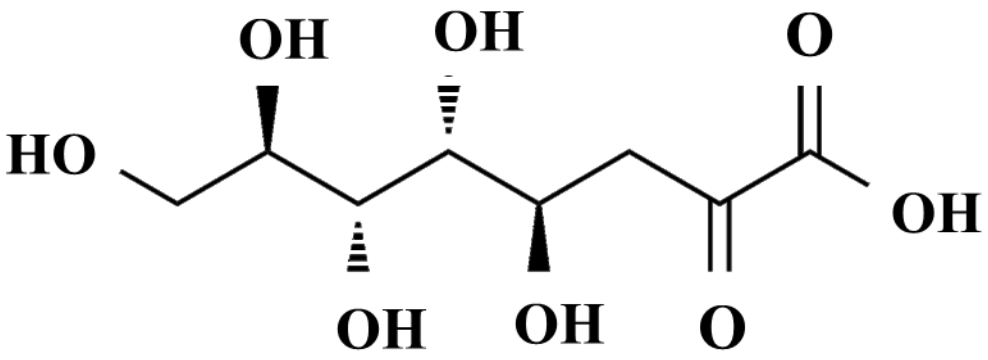


Figure 2-1: Linear 3-deoxy-D-*manno*-2-octulosonic acid

Some work on the potential medical utility of KDO has already been done. A KDO hydrolase that works to remove KDO units from the LPS has been investigated in *H. pylori* [19]. This process has the potential to reduce harmful activities of both *H. pylori* and other gram-negative bacteria especially with further research in the role of KDO and the importance of its position in the bacterial cell wall (Figure 2-2). It could be used as a lead in the design of enzyme inhibitors for KDO-containing bacteria [21].

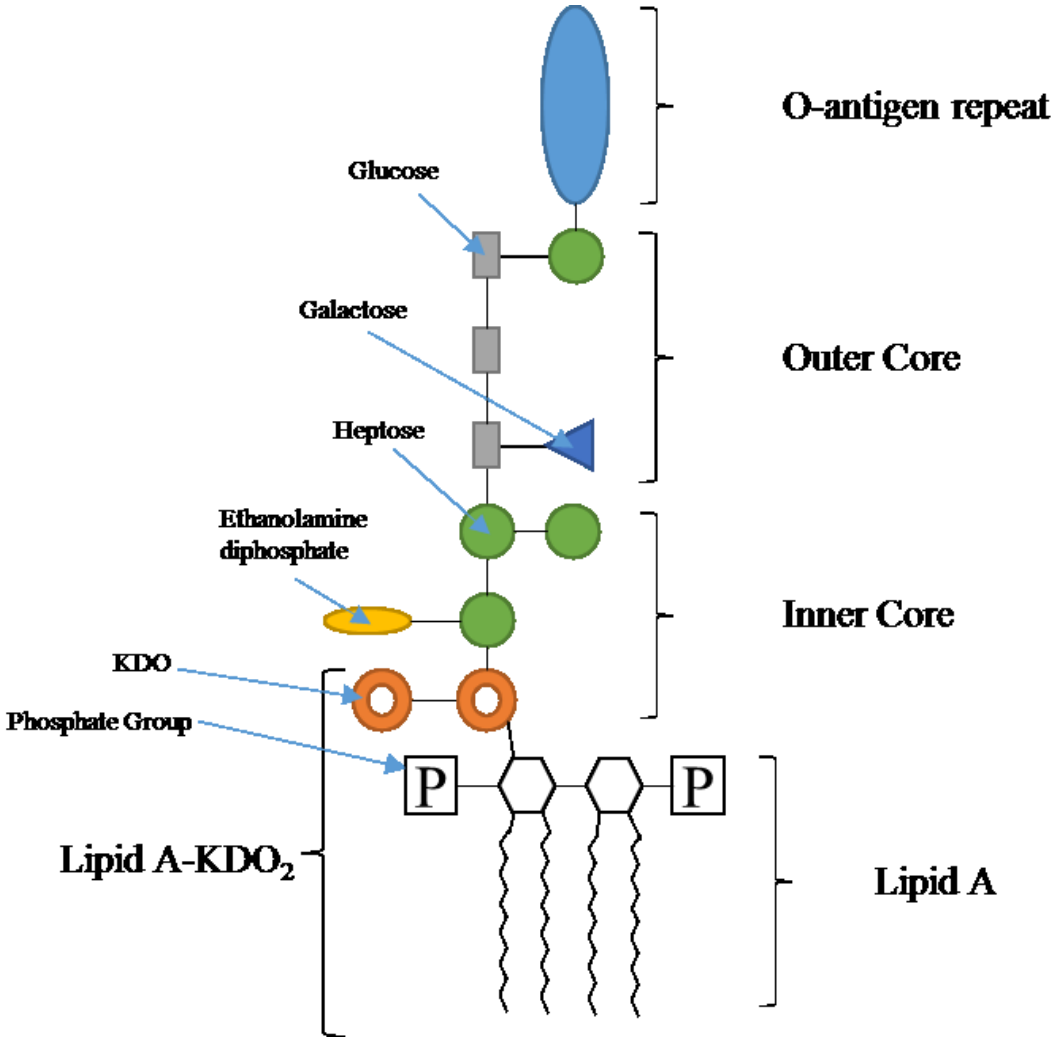


Figure 2-2: Inclusion of KDO in lipopolysaccharide as portion of the Lipid A-KDO_2 component

There have been a handful of chemical syntheses for KDO, typically either starting with either D-arabinose or D-mannose, the former resulting in a 66% yield (Figure 2-3) [43], the latter having several steps, one of which with only a 29% yield [38] [39]. The low to moderate yields obtained, coupled with expensive reagents, D-arabinose and oxaloacetic acid, and multi-step syntheses make synthetic production of KDO a very costly process. In some studies, KDO has been synthesized as a part of a KDO glycal

donor, but these are produced specifically as a precursor for the synthesis of β-KDO-containing oligosaccharides [22], while others make molecules with higher yields with similar characteristics to KDO [21] [44].

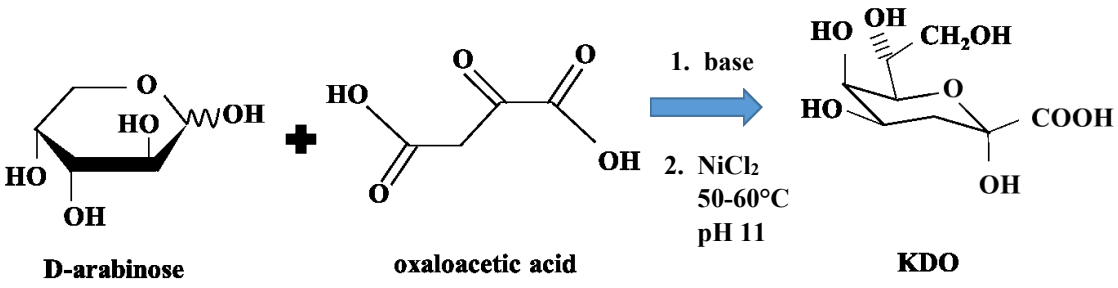


Figure 2-3: Synthesis of KDO from D-arabinose

The difficult chemical synthesis leads to a high cost of the final product (approximately 20 000 CAD per gram [45]) and stifles research that would otherwise make use of this sugar. This has largely been the source of interest for seeking out sources that naturally produced KDO as an alternative; one source that has already been investigated is *E. coli* [18].

The biosynthetic pathway for the production of KDO has yet to be explored in *T. suecica*, but has been identified in *E. coli* (Figure 2-4), and since the pathways in other KDO producing organisms, such as tomatoes [46], contain analogous enzymes, a similar KDO biosynthesis pathway is expected in *T. suecica*.

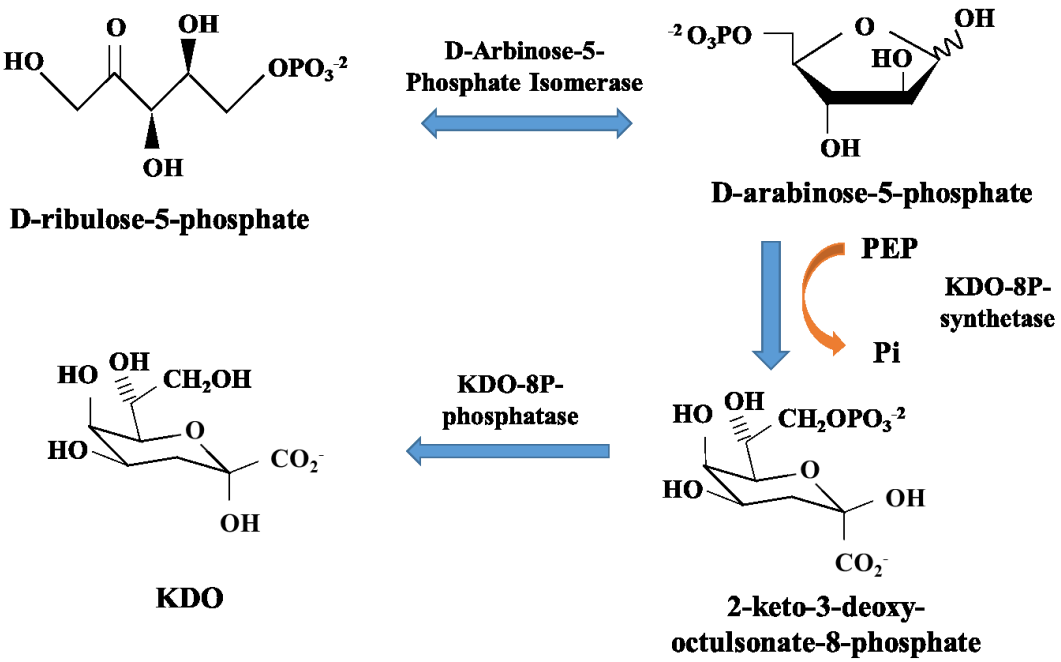


Figure 2-4: Biosynthesis of KDO in *E. coli*

There are three enzymes primarily involved in the biosynthesis of KDO: D-arabinose 5-phosphate isomerase; Kdo-8P synthetase; Kdo-8P phosphatase to KDO and two further enzymes to incorporate the KDO into the cell wall: CMP-Kdo synthetase and Kdo transferase. All of these enzymes are essential for the viability of *E. coli*, and, by extension, other bacteria requiring KDO [37]. Another area of investigation is into the genes responsible for the enzymes in order to better understand how to inhibit KDO development [47].

The formation of KDO_2-lipid A is also of interest because this is an essential step for KDO's inclusion of the LPS, providing another point in the process that may be considered for disruption [48].

2.4 Effect of Cultivation Conditions

The microalgal biomass composition, biomass concentration and biomass productivity are affected by cultivation conditions. Nutrient stress in particular, where a nutrient is in such short supply it is characterized as either being nutrient limitation or nutrient starvation, has shown effects where desirable products are overexpressed [11] [49]. Light, nonessential inorganic nutrients, and various organic carbon sources are other factors that affect biomass and cellular composition [50] [51] [30].

2.4.1 Nitrogen and Phosphorous Stress:

As nitrogen is a necessary element in many cellular components such as DNA and proteins, different nitrogen availability affects how microalgae are able to grow [8]. An overexpression of lipids is most commonly observed when a culture is under nitrogen-starvation, though some species instead accumulate carbohydrates. Photosynthetic pigments and biomass concentration decrease when nitrogen is not readily available [8]. Nitrate depletion leads to increases in oil content; Go *et al*. 2012 observed that oil production increased in *T. suecica* when growing nitrogen-free cultures as opposed to cultures containing 18.6 mg/L nitrogen sourced from sodium nitrate in 20L tubular reactors. The increase in lipids seen in the nitrogen-deplete culture was from 12.2 to 18.7 mg $g^{-1}day^{-1}$ [31]. Benvenuti *et al.* 2015 explored nitrogen-deplete conditions and its effect on lipid productivity in various microalgae species in 150 mL batch cultures. Sodium nitrate was used as the nitrogen source where the nitrogen-replete medium contained 70 mM $NaNO_3$ and the nitrogen-deplete medium contained did not contain $NaNO_3$. Lipid

productivity increased in *T. suecica* in nitrogen-deplete culture, though lipid content did not increase as much as it did in other microalgae, including *Nannochloropsis* sp. and *Chlorococcum littorale* [52].

Phosphorous is also critical in many roles in a cell. Carbohydrates and lipids are both affected under phosphorous starvation, accumulating more in microalgae than under nutrient-replete conditions. Protein content is reduced in response to phosphorous starvation [8]. Both *Anabaena flos-aquae* [53] and *Chlamydomonas reinhardtii* [54] in batch 200 mL cultures were subjected to a change in phosphorous concentration from 500 µg/L by dry weight to approximately 50 µg/L with phosphorous sourced from dipostassium phosphate. Both species saw increases in lipid and carbohydrates under phosphorous-stressed conditions when measured using Fourier transform infrared analysis.

In order to subject microalgae to nutrient stress, particularly when focusing on nitrogen and phosphorus, it is critical to not only look at the total concentration of either nutrient, but their relationship as the ratio of carbon, nitrogen and phosphorous (C:N:P). The ideal C:N:P ratio varies from species to species, but the popular f/2 medium has a ratio of 55:24:1 which ensures that carbon is exhausted before either nitrogen or phosphorous [49]. The metabolism will change to use minimal amounts of the limiting nutrient while taking advantage of the others which are present in relatively larger quantities. The exact ratios vary based on species and growth conditions, and CO_2 is typically bubbled through cultures to focus on nitrogen and phosphorous stress [49].

Both nitrogen and phosphorous starvation have the potential to affect carbohydrates, both in terms of total percent carbohydrate and the carbohydrate profile.

Nutrient stress has the potential in microalgae production to affect value-added products. The increased presence of value-added products is an important step towards optimizing cultures for industry. While nutrient stress does have the drawback of lower biomass accumulation, it has been suggested that a two stage cultures can make up for this and lead to appreciable amounts of desired products [8] [11] [55].

The first stage in a two-stage culture is where the microalgae is grown under optimal conditions for biomass production. The conditions of the culture are altered for the second stage, subjecting the microalgae to the nutrient stress that optimizes the desired product. This strategy is used to take advantage of nutrient stress, while producing a higher biomass concentration at the first stage [11]. When incorporating nutrient stress into microalgal production, it becomes important to not only maximize the cellular content of the high-value product, but to also optimize the biomass concentration and growth rate.

Aflalo *et al.* 2007 investigated a two stage system versus a one stage system by growing *H. pluvialis* and determining which worked better for producing astaxanthin. The one stage culture achieved the biomass growth and nutrient stress by using a continuous culture with a limiting nutrient stress condition. The two stage culture had a period of optimal growth and then a period of environmental stress. The two stage system saw an increase of astaxanthin, the desired product, by 4% of the dry weight [56].

2.4.2 Effect of Cultivation Condition on *T. suecica*

The different cell components in *T. suecica* are variable with the environment. Protein accumulation can be affected by the inorganic nutrients in media as well as organic carbon sources [30] [50]. The production of pigments predictably changes with the amount of light available [51]. Table 2-1 compares different autotrophic cultures and the targets products under investigation with respect to *T. suecica*. The carbohydrates, pigments, protein and lipids in *T. suecica* can all be varied by altering the cultivation conditions.

Carbohydrates in microalgae function as energy storage, and in *T. suecica* this has been enhanced by varying the organic carbon sources used under mixotrophic conditions. The carbohydrate content increases from 6.5 pg/cell in an autotrophic control culture to 25.38 pg/cell using a combination of peptone, glucose and yeast. Energy storage in terms of carbohydrates present in the cells responds dramatically when *T. suecica* can take advantage of organic carbon sources in addition to inorganic sources [50]. The effect of energy storage based on organic carbon sources is different for lipids; heterotrophically grown cultures have lower lipid levels than autotrophic cultures [57].

Pigments in *T. suecica* include carotenoids, chlorophyll and phaeopigments. Pigments, either photosynthetic or photoprotective, are expressed to facilitate light absorption and function to pass energy on to the plant or dissipate the absorbed energy. Autotrophic and mixotrophic cultures are green in colour, whereas heterotrophic cultures, which are completely deprived of light, end up being bright yellow in colour. As heterotrophic cultures have been shown to only express 1% of the chlorophyll level in comparison to autotrophic cultures, and 50% of the carotenoid content [25], the

heterotrophic cultures being visually distinct is not surprising. As heterotrophic cultures have acclimatised to darkness, these cultures use less energy to maintain light harnessing pigments [25].

T. suecica's protein content was what originally made it an appealing microalga for aquaculture feed. In aquaculture feed experiments, *T. suecica*'s protein could be linked linearly to the growth and survival rates of *Artemia* when semi-continuous cultures are maintained with a renewal rate of up to 40% [33]. The accumulation of protein can be affected when different inorganic nutrients, such as silicon and strontium, are added to the media, as well as organic carbon sources [30] [50]. Protein remains *T. suecica*'s most critical cell component due to its use in the aquaculture industry, and it is desirable that any secondary products do not impede the protein accumulation and its main use as feed.

Culturing conditions play a major role in the composition of any microalgae; *T. suecica* can vary in carbohydrate composition anywhere from 15 to 64% by dry weight [28] [16] [30]. Considering the variability of several cellular components of interest in the past, it is possible that KDO in *T. suecica* is affected as well and can be expressed in varying amounts.

Table 2-1: Autotrophic *T. suecica* experiments investigating product accumulation and biomass

Culture Volume	Target Product	Cellular Content of Product (% dry weight)	Biomass Productivity (g/L/hr)	Cell Density (cells /mL)	Ref.
100 mL	Lipids	8.5 to 12.9	0.07	-	[6]
30 mL	Pigments	2.03 chlorophyll 0.85 carotenoids (pg/cell)	-	$0.4x10^6$	[28]
-	Biomass	-	-	$2.3x10^6$	[58]
-	Biomass	-	-	$8.7x10^6$	[30]
500 mL	Biomass	-	-	$1.6x10^6$	[59]
200 mL	Carbohydrates	24 lipids 43 carbohydrates 17proteins 16 (β-glucans)	-	-	[60]
150 mL	Lipids	11 (Nutrient Replete)/ 17 (N-deplete)	0.12 (Nutrient Replete)/ 0.10 (N-deplete)	-	[52]
500 mL	Protein, Lipids, Pigments	94/25/4.6 pg/cell	0.008	$2.5x10^6$	[61]
500 mL	Lipids (PUFA)	31 (exponential growth phase)/ 23 (stationary growth phase)	-	-	[62]
5 L (glass reactor); 120 L (bag reactor)	Biomass	-	0.01	-	[63]
1.5 L	Pigment, Vitamins	2.8 chlorophyll a 0.06Vitamin E	-	-	[14]
590 L (Semi-continuous; panel reactor)	Lipids	22(N-starved), 27 (N&P-starved), 29 (Nutrient replete)	0.005	-	[64]

2.5 Process Integration:

The integration of multiple processes can be used to make the production of certain chemicals and biomass sources more feasible. The two principle ways this can be accomplished is to extract multiple products at once to extract more value from the feedstock or the incorporation of a waste product from one process as initial material into another process.

A microalgae biorefinery is one such approach, which relies on extraction of multiple chemicals from microalgae to enhance the process sustainability and economics. Given that the yield of biomass production in autotrophic microalgae cultivation is low, leading to energy intensive harvesting processes, optimizing the use of biomass through extraction of multiple chemicals can positively impact the economic feasibility of the products [3]. If the extraction of the high-value product can occur and the remaining biomass is in a condition to be of further use after this treatment, the use of microalgae on an industrial scale becomes a lot more attractive for production [65].

Another approach proposed to improve the economics of microalgal biofuel production is integration of wastewater treatment with microalgal biomass production. Microalgae can grow at the expense of nutrients present in wastewater. While the nutrients are being consumed, the water is being purified. By incorporating these processes, a costly purification process can be avoided and a product is obtained by taking advantage of a previously untapped nutrient source [17].

T. suecica has been shown to make efficient use of the waste from a fish farm, having removal efficiencies of 49.4% for nitrogen and 99.0% for phosphorous with an

original composition of 41.3 mg/L dissolved inorganic nitrogen and 4.96 mg/L dissolved inorganic phosphorous. When extra phosphorous is added, removal efficiency of nitrogen was increased to 95.7% [17]. This shows that microalgae a can use water that would otherwise need to be treated by other means. Incorporating microalgal growth with water treatment has the potential to reduce costs in both of these processes.

The strength of integrated biorefineries, regardless of what form they take, is that one overall process stream is being used for multiple objectives. This is both useful in cutting costs and in cutting infrastructure needed to take care of both processes separately. Though ideally additional products could be separated out, if wastewater can be cleaned with the controlled growth of microalgae, there is the benefit of preventing uncontrolled algal blooms. The integrated biorefinery is an intelligent strategy for most efficiently dealing with multiple problems in industries like microalgal culturing.

Chapter 3 : Materials and Methods

3.1 Cultivation

The algal strain used in this experiment was from the University of Texas at Austin's Culture Collection of Algae, UTEX LB 2286 *Tetraselmis suecica*. Seawater was obtained from the Northwest Arm, Halifax, NS, Canada, then filtered and autoclaved to grow *T. suecica.* Stock cultures in Enriched Seawater Medium prepared from 1 micron filtered and autoclaved seawater cultures were maintained in 250 mL closed vessels with a 12/12 hour light/dark period [66].

A photobioreactor was constructed according to the scheme in Figure 3-1. Three 1L bottles had their caps modified to add a gas inlet, gas outlet and sampling tubes. Glass tubing sheathed with silicone tubing was used for these three applications such that the silicone tubing prevented gas leakage. CO_2 and air lines were connected to a filter, and from this filter the gas was introduced to the three 1L bottles through their gas inlet tube [49]. Each bottle was placed on a stir plate and had a 1.5 inch stir bar through all cultivation to improve mixing. The stirring speed was varied to maintain homogeneity as necessary. Lights were set up on the back wall to give a light intensity of 250 μmol photons m^{-2} s^{-1} in the middle of the 1L bottles as measured when they contained 1L DI water.

The cultivation experiment consisted of two stages, the growth of the inoculum culture and the growth of the experimental culture. This experiment was modelled on an experiment performed by Kermanshahi-pour *et al.* 2014 [16]. For all media conditions, the inoculum culture remained the same and all culture manipulation was performed

under aseptic conditions. Seawater (970mL) filtered using 1 micron filter was autoclaved; 20 mL of enriched stock media (Table 3-1) and 10 mL stock culture were added to the autoclaved seawater [66]. The culture was grown under a 14/10 hour light/dark cycle, 250 μmol photons m^{-2} s^{-1} light, and 0.015 mL $culture^{-1}$ min^{-1} CO_2 with air flow to maintain proper aeration and mixing. The growth was monitored by measuring the optical density every second day at 750 nm in a Genesys 10 UV-Vis spectrophotometer with 10 mm optical glass cuvette and DI water blanks. It was observed that at day 14 the culture reached stationary phase, therefore at day 14 the culture was centrifuged down at 2000 rpm and the media was discarded, leaving primarily an algal pellet. The algal pellet was then used to inoculate an experimental culture.

All culturing took place in the set-up in Figure 3-1. A photograph of the operational set-up can be found in Figure E-1 in Appendix E. Each culture was performed in triplicate, performed simultaneously in 1L of media per bottle. Air flow and carbon dioxide fed into the system was passed through a filter containing activated carbon and was filtered further by 0.45 and a 0.2 micron syringe filters before being introduced to the culture. All cultures were subjected to continuous agitation via air flow in addition to magnetic stirring.

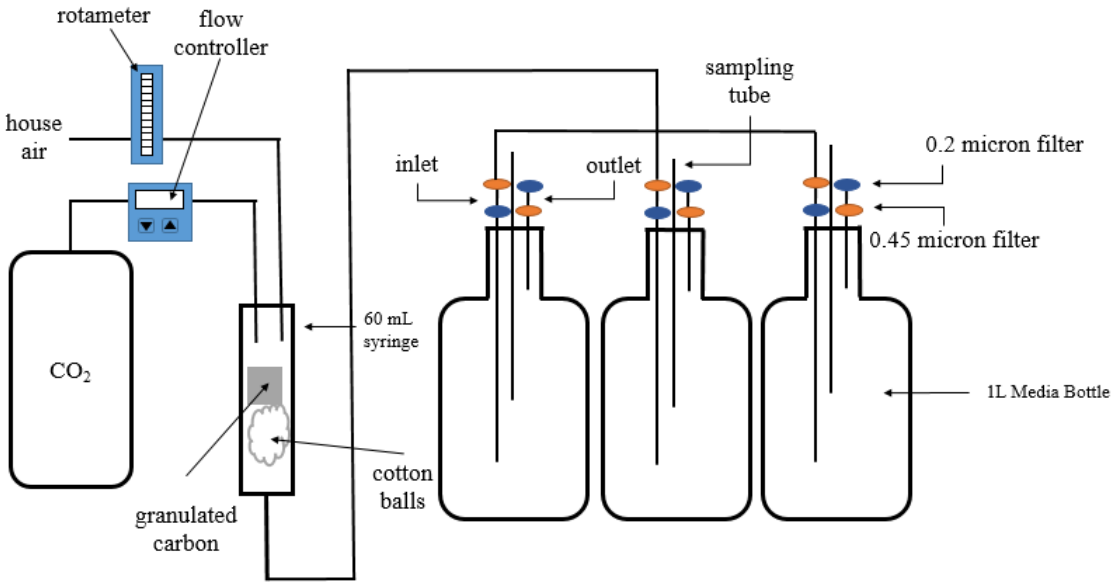


Figure 3-1: Experimental set-up for autotrophic batch cultures

The experimental culture consisted of one of three different cultivation conditions to investigate the effect of nutrient stress on *T. suecica*: nutrient-replete, N-starved, and P-starved. The nutrient-replete culture consisted of the same conditions as the inoculum culture. The N-starved and P-starved had less $NaNO_3$ or glycerophosphate present respectively as shown in Table 3-2. For more details on culturing refer to Appendix B.

Table 3-1: Basic UTEX enriched seawater stock solution, and N- and P-limited stock solution concentrations. The dilution of these stock solution into the media is 20 mL/L *[66]*

Components	**UTEX Enrichment Stock Solution (g/L)/(M)**	**N-limited UTEX Enrichment Stock Solution (g/L)/(M)**	**P-limited UTEX Enrichment Stock Solution (g/L)/(M)**
$NaNO_3$ (Sigma Aldrich, S5506)	2.35E+00 /2.77E-02	6.87E-01 /8.08E-03	2.35E+00 /2.77E-02
Na_2glycerophosphate·$5H_2O$ (Sigma Aldrich, G6501)	3.50E-01 /1.62E-03	3.50E-01 /1.62E-03	1.2E-01 /5.55E-04
$Fe(NH_4)_2(SO_4)_2·6H_2O$ (Sigma Aldrich, 215406)	1.14E-01 /2.90E-04	1.14E-01 /2.90E-04	1.14E-01 /2.90E-04
$Na_2EDTA·2H_2O$ (EMD, EX0539-1)	2.60E-01 /6.98E-04	2.60E-01 /6.98E-04	2.60E-01 /6.98E-04
H_3BO_3 (Fisher Scientific, BP168)	1.85E-01 /3.00E-03	1.85E-01 /3.00E-03	1.85E-01 /3.00E-03
$FeCl_3·6H_2O$ (Sigma Aldrich, 236489)	7.96E-03 /2.95E-05	7.96E-03 /2.95E-05	7.96E-03 /2.95E-05
$MnSO_4·H_2O$ (Sigma Aldrich, M8179)	2.67E-02 /1.58E-04	2.67E-02 /1.58E-04	2.67E-02 /1.58E-04
$ZnSO_4·7H_2O$ (Sigma Aldrich, Z0251)	3.58E-03 /1.24E-05	3.58E-03 /1.24E-05	3.58E-03 /1.24E-05
$CoCl_2·6H_2O$ (Sigma Aldrich, C8661)	7.80E-04 /3.28E-06	7.80E-04 /3.28E-06	7.80E-04 /3.28E-06
HEPES (Sigma Aldrich, H4034)	3.26E+00 /1.37E-02	3.26E+00 /1.37E-02	3.26E+00 /1.37E-02
Vitamin B12 (Sigma Aldrich, V6629)	2.03E-04 /1.49E-07	2.03E-04 /1.49E-07	2.03E-04 /1.49E-07
Thiamine (Sigma Aldrich, T1270)	5.03E-04 /1.67E-06	5.03E-04 /1.67E-06	5.03E-04 /1.67E-06
Biotin (Sigma Aldrich, B4639)	3.75E-05 /1.53E-07	3.75E-05 /1.53E-07	3.75E-05 /1.53E-07

Table 3-2: Final concentrations of sodium nitrate and glycerophosphate in 1L cultures

Components	**Basic UTEX Enriched Seawater media (g/L)/(M)**	**N-limited Enriched Seawater Media (g/L)/(M)**	**P-limited Enriched Seawater Media (g/L)/(M)**
$NaNO_3$	4.7E-02 /5.6E-04	1.37E-02 /1.62E-04	4.7E-02 /5.6E-04
Na_2glycerophosphate·$5H_2O$	7.00E-03 /3.24E-05	7.00E-03 /3.24E-05	2.40E-03 /1.11E-05

These cultures were maintained at the same light intensity and gas flow conditions as the inoculum cultures and were monitored until they reached stationary phase. They were sampled throughout growth for further analysis. Samples were collected for optical density measurements, cell counts, and freeze drying.

After sampling, where the volume removed ranged from 3 mL to 250 mL, the volume sampled was replaced with media. The nutrient-replete culture was replenished with more nutrient-replete media, the N-starved culture was replenished with N-free media and the P-starved culture was replenished with P-free media. N-free and P-free media differ from nutrient-replete media because $NaNO_3$ or glycerophosphate were not added, respectively.

A significant decrease in the volume of the reactor would affect the amount of volume available for investigation therefore the volume of samples was decreased as the culture became increasingly dense.

3.1 Culture Growth

All samples were affected by the concentration of the culture due to evaporation, which occurs through the gas outlet, and the dilution of the culture from the addition of new media. To adjust our measurements to take these factors into account, an evaporation correction and dilution correction were applied to the culture monitoring calculations: optical density, cell count and freeze dried mass.

To measure the evaporation correction, the culture level was measured before every sampling day. To measure the dilution correction, the volume before and after replenishment was recorded every sampling day.

$$M_1 * V_1 = M_2 * V_2 \quad (1)$$

$$M_2 = \frac{V_1}{V_2} * M_1 \quad (2)$$

Where M_1 is the value measured, V_1 is the volume at the time of measurement, V_2 is the volume before evaporation and dilution from the previous sampling day and M_2 is the measurement determined for V_2.

3.1.1 Optical Density

A Genesys 10 UV-Vis spectrophotometer was set at 750 nm wavelength and DI water was used to blank the instrument. Samples of 3mL were taken and dispensed into a 10 mm optical glass cuvette. This was place in a spectrophotometer and read at 750 nm.

3.1.2 Freeze Dried Mass

Dry weight was measure through lyophilisation rather than oven drying to avoid destroying biomass and to be able to use the biomass for further analysis. A known volume was taken from a culture and dispensed in a pre-weighed centrifuge tube. The sample was centrifuged down and rinsed with DI water three times. It was resuspended in DI water and frozen for freeze drying to take place. After freeze drying, the sample was weighed again and the value of the empty tube was taken from that of the tube with the freeze dried algae.

3.1.3 Cell Count

Samples of 1 mL were preserved with 10 µL of 25% glutaraldehyde (Sigma Aldrich, G5882), incubated for 15 minutes and refrigerated until analysis could take place [67]. Samples were analysed on a BD Accuri C6 flow cytometer in volumes of 200uL, in triplicate. DI water was used between samples to avoid carryover. The region of the plot associated with side scatter was omitted from the count.

3.2 Analysis of Cellular Content

3.2.1 Enzymatic Hydrolysis for total starch content

Freeze dried *T. suecica* was hydrolysed in 6 mL sodium acetate buffer at pH of 4.5 containing 15 µL amyloglucosidase (Sigma Aldrich A7095). The buffer itself was made

by adding 1.0254 g sodium acetate (Sigma Aldrich, S10) in 250 mL of DI water and adding acetic acid until the target pH of 4.5 was reached.

Pure starch from corn (Sigma Aldrich, S9679) was analysed along with microalgae samples to ensure all starch was being hydrolysed. The samples of approximately 3 mg were weighed out and dispersed in 6 mL sodium acetate buffer of pH 4.5 at 55°C with 15 µL amyloglucosidase.

Reducing sugar was analysed using dinitrosalicyclic (DNS) colorimetry and a Genesys 10 UV-Vis spectrophotometer at a wavelength of 540 nm [68]. More details can be found in Appendix C.

3.2.2 Derivatization of Sugars

It was important to derivatize the biomass as the sugars were initially integrated into the structure of the cell and had to be liberated. Additionally, sugars are not generally able to transition into the gas phase, therefore it was necessary to add trimethylsilyl (TMS) groups to the sugars to allow them to vaporize. The processes used for this are known as methanolysis and silylation. Methanolysis was used cleave sugars by their glycosidic linkages (Figure 3-2). Silylation added TMS groups to the monosaccharides, allowing them to vaporize (Figure 3-3).

Samples of approximately 5 mg freeze dried microalgae were weighed out into glass vials with Teflon lined caps. Approximately 3 mL anhydrous methanol (Sigma Aldrich, 322415) with 3 mmol acetyl chloride (VWR CAAA43262-AD) and a stir bar

were added to the samples. These samples were kept at 85°C in a reaction block for 24 hours being continuously stirred.

After 24 hours, to neutralize the reaction 0.812 g silver carbonate (Sigma Aldrich, 179647) was added to each sample and the samples were left to incubate in 50 µL acetic anhydride (VWR) for 18 hours at room temperature.

All samples were centrifuged and the supernatant was harvested. The remaining pellets were then rinsed with 1 mL anhydrous methanol and centrifuged again; this supernatant was added to the previous supernatant of the same sample. All samples were rinsed a total of 3 times. They were dried under nitrogen and then put in P_2O_5 (Alfa Aesar, A13348) desiccator overnight.

The next day, 200 µL pyridine (Sigma Aldrich, 270970) was added as a solvent and then 100 µL N,O-bis-(trimethylsilyl)trifluoro-acetimide with 1% trimethylchlorosilane (Sigma Aldrich, T6381) was added to begin the silylation step. The samples were then kept at 70°C in a reaction block for 4 hours and then prepared for GC/MS analysis [69].

Prior to GC/MS analysis, samples were dried under nitrogen, and 1 mL hexanes (Sigma Aldrich, 34859) was added.

An Agilent 7890B GC with Agilent 5977B MS detector was used for analysis. The column was a DB-5ms with an inner diameter of 0.320 mm and a length of 20 m. The column started at 80°C and had a hold time of 4 minutes. It was then ramped up at a rate of 20°C/min until it reached 280°C then it was heated at a rate of 10°C/min until it reached the final temperature of 300°C. Different split ratios were tested for this analysis;

splitless was used for KDO determination and a split ratio of 5:1 was used for the determination of the other sugars. For more details refer to Appendix D.

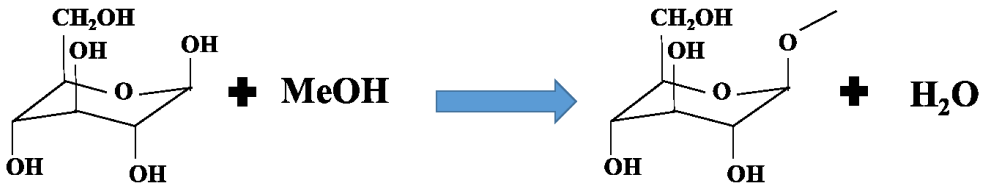


Figure 3-2: Methanolysis of glucose

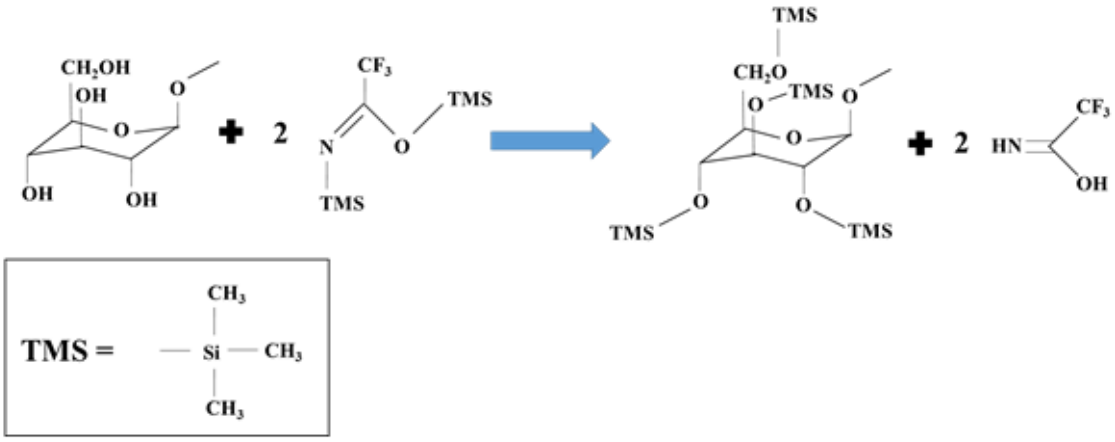


Figure 3-3: Silylation of methylated glucose

Chapter 4 : Results and Discussion

The purpose of this project was to establish a system for measuring the effect of nutrient conditions on growth and biomass composition of *T. suecica*. This was done to specifically test for the presence of KDO and to analyze its accumulation based on nutrient stress. Biomass growth was monitored during the cultivation and biomass was characterized with respect to carbohydrate content. All stages of the system were analyzed for their consistency including the inoculum cultures, the experimental cultures with varying nutrient conditions, and the methods of analysis.

4.1 Inoculum

The inoculum cultures grown for each experiment showed comparable growth as seen in Figure 4-1. Each replicate consisted of three reactors, which were each measured to give an average optical density for each time point. All cultures were grown for 14 days under 14/10 hour light/dark cycle, 250 μmol photons m^{-2} s^{-1}, 0.015 mL/min CO_2 per reactor with air flow to maintain proper aeration and mixing. The relationship between optical density and time was consistent for all inoculum cultures, with a lag phase lasting 4 days where optical density increased but not as rapidly as seen in the next stage, exponential growth for days 4 through 10, and all cultures harvested at the beginning of stationary phase at day 14. The comparable growth of the inoculum culture replicates demonstrates the robustness of the photobioreactor in maintaining consistent cultivation conditions as their standard deviations overlap, particularly from days 10 to 14.

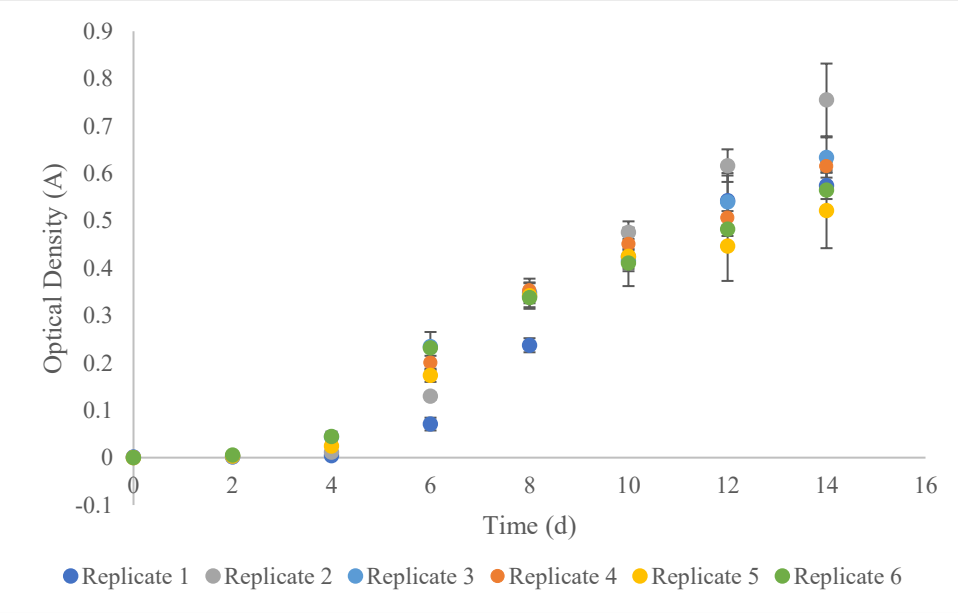


Figure 4-1: Comparison of the inoculum cultures grown over the course of the experiment, with standard deviation calculated from simultaneously grown cultures within each replicate

4.2 Experimental Cultures

T. suecica was grown under three cultivation conditions: nutrient-replete, P-starved and N-starved (Table 3-2). The photobioreactor set-up in Figure 3-1 was used to grow *T. suecica* under each of the above conditions to obtain triplicate samples in order to determine reproducibility with respect to microalgal growth and biomass composition. After the initial round of experiments was complete, a second replicate of each cultivation condition was grown. This resulted in a total two experimental cultures grown per condition, each done in triplicate. The first cultivation done in triplicate for each condition is referred to "Culture 1" and the second cultivation is referred to "Culture 2".

Each triplicate experimental culture was inoculated with the same algal inoculum with volumes from 450 to 600 µL to target an optical density range of 0.007 to 0.025 A.

The experimental cultures were monitored throughout growth with respect to their optical density measurements, cell concentration, and freeze dried mass per volume (biomass).

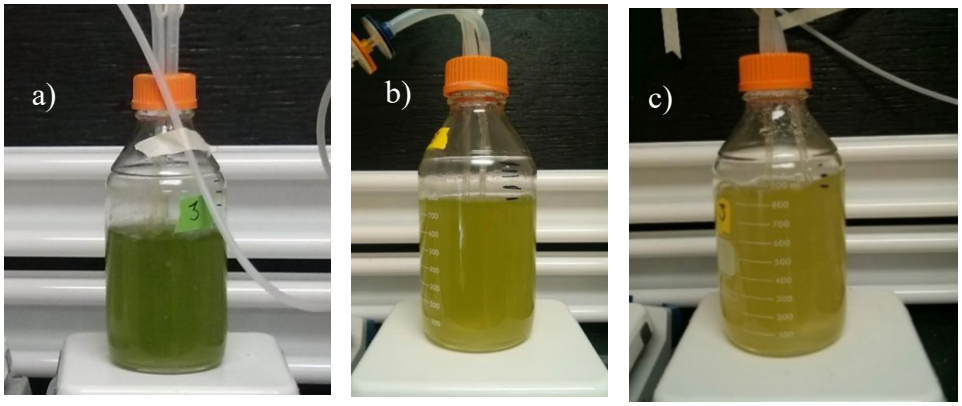


Figure 4-2: *T. suecica* biomass grown under a) Nutrient Replete, b) P-starved and c) N-starved cultures at stationary phase

The first most obvious difference between the three cultivation conditions was the difference in pigmentation as of the stationary phase, as can be seen in Figure 4-2. The Nutrient Replete culture was a green consistent with the inoculum cultures, whereas the P-starved and N-starved cultures were more yellow by comparison.

4.2.1 Monitoring Biomass Growth by Optical Density Measurements:

The biomass density depended heavily on the nutrient condition. The nutrient-replete cultures (Figure 4-3) showed a much larger increase in optical density than either P-starved (Figure 4-4) or N-starved (Figure 4-5), and maintained exponential growth for a longer period of time.

The nutrient-replete optical density in Figure 4-3 shows that stationary phase of growth was reached around day 8, followed by another slight increase in optical density before leveling off a second time at day 18.

The P-starved cultures (Figure 4-4) showed exponential growth over days 2 to 4 before leveling off. The N-starved cultures (Figure 4-5) showed the same trend, but did not reach as high an optical density as the P-starved cultures.

The trends between Cultures 1 and 2 stayed consistent between cultivation conditions. The nutrient-replete and P-starved conditions were often within standard deviation between Cultures 1 and 2. The N-starved cultures had a larger disparity. Temperature of the photobioreactor was not maintained and could have led to variation between Cultures 1 and 2, which were performed at different times.

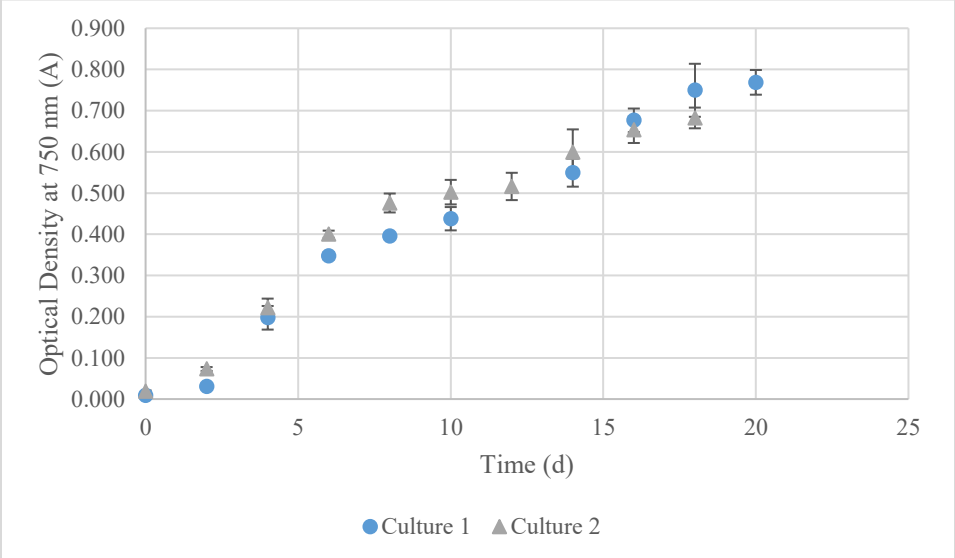


Figure 4-3: Optical density at 750 nm versus time under nutrient-replete cultivation condition

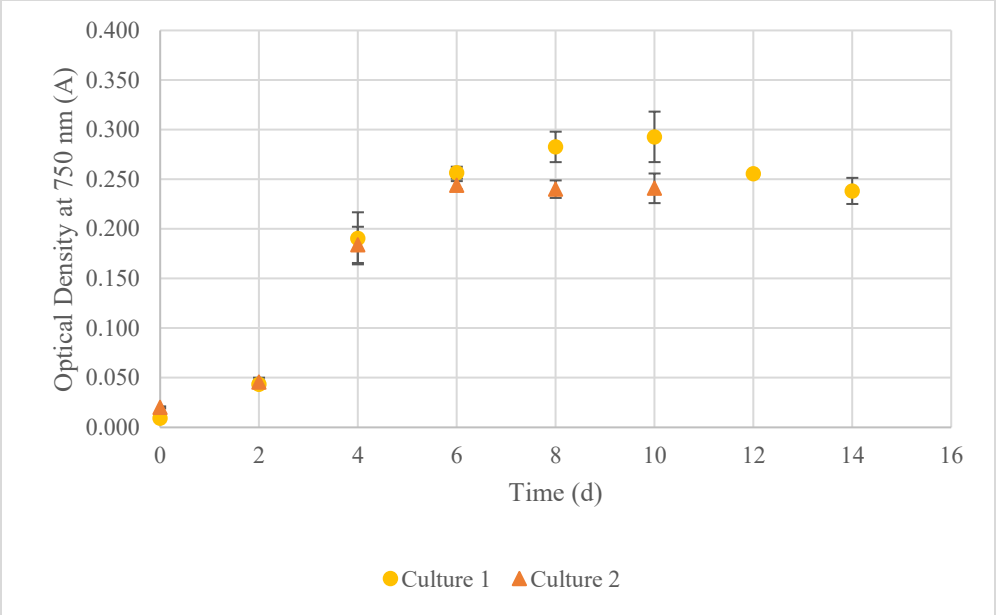


Figure 4-4: Optical density at 750 nm versus time under P-starved cultivation condition

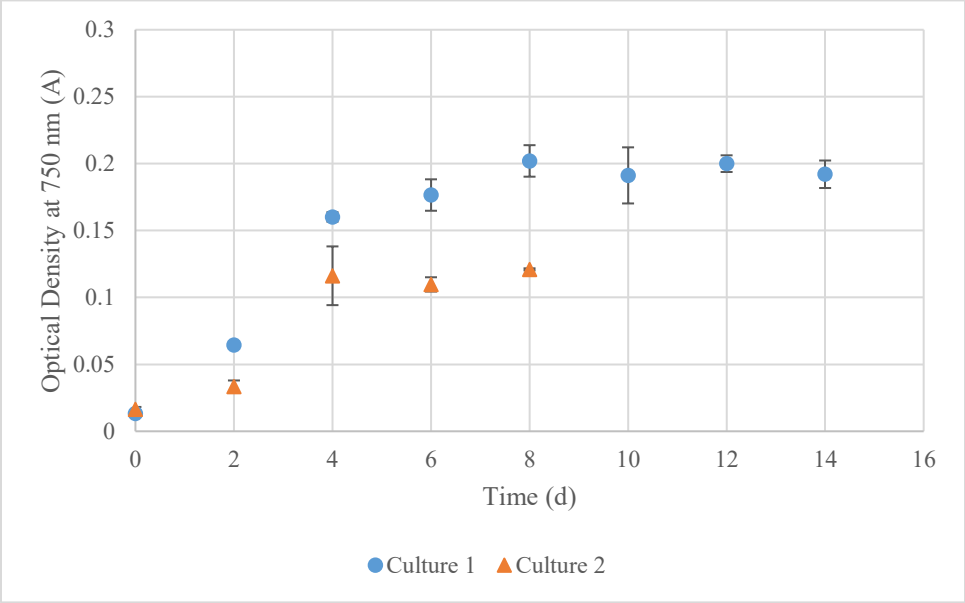


Figure 4-5: Optical density at 750 nm versus time under N-starved cultivation condition

4.2.2 Cell Count:

The cell concentration for the nutrient-replete cultures (Figure 4-6) were about three times higher than those of the N-starved cultures (Figure 4-8). The cell count relationship for the nutrient-replete cultures leveled off at day 8 and stayed at this level, unlike the trend seen in the optical density measurements. Here it was likely that the optical properties of the cells were changing, as this would not be reflected in cell count.

Cell concentration dropped off most dramatically in the P-starved cultures (Figure 4-7). Around days 4 or 6 the counts reached their peak and, unlike the other culture conditions, there was a steady decrease in cell count until harvest. This could have potentially been a result of cells dying off or the cells could have been adhering to the reactor walls or other cells and not being homogenously dispersed in the media.

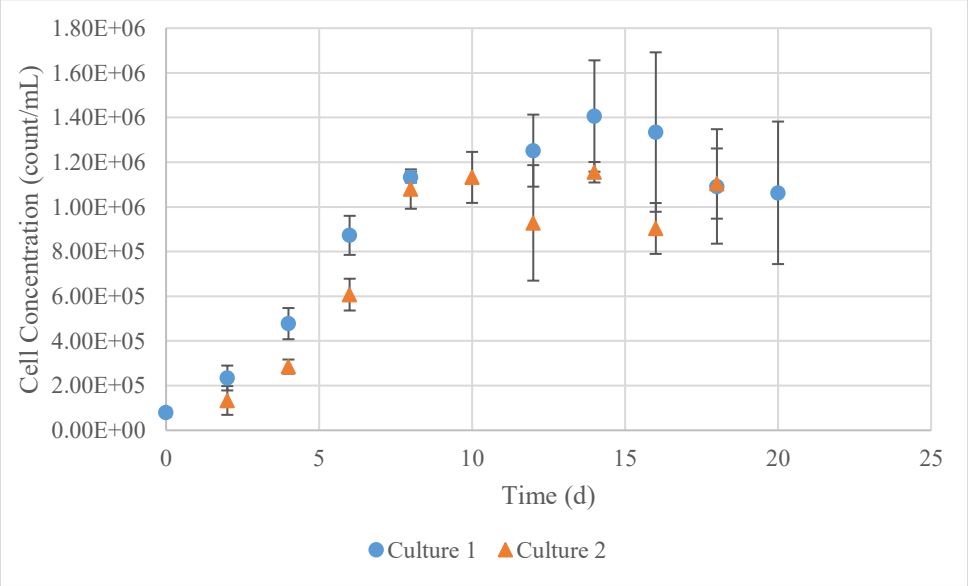


Figure 4-6: Cell count per mL as over time under nutrient-replete condition

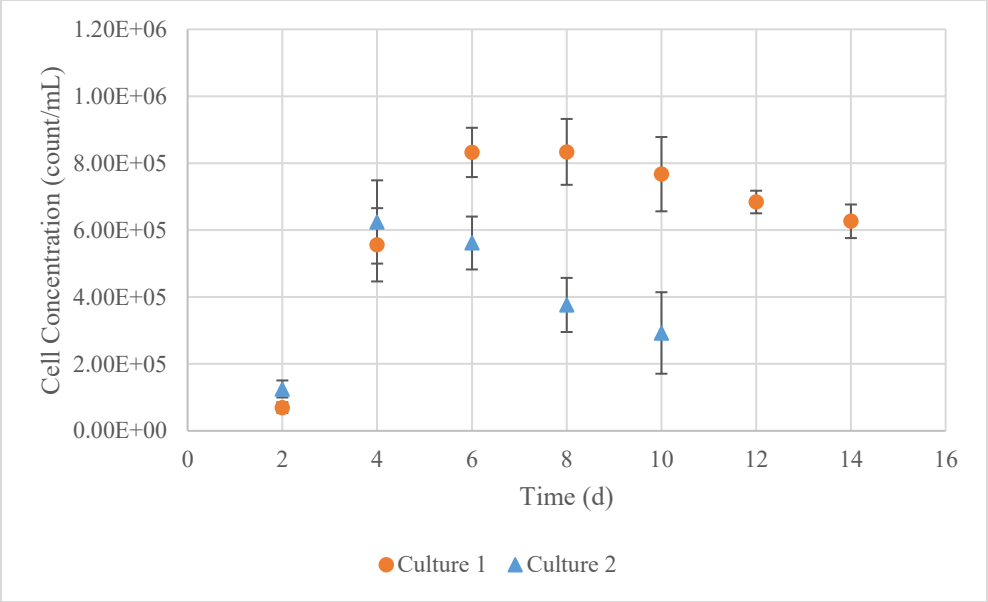


Figure 4-7: Cell count per mL as over time under P-starved condition

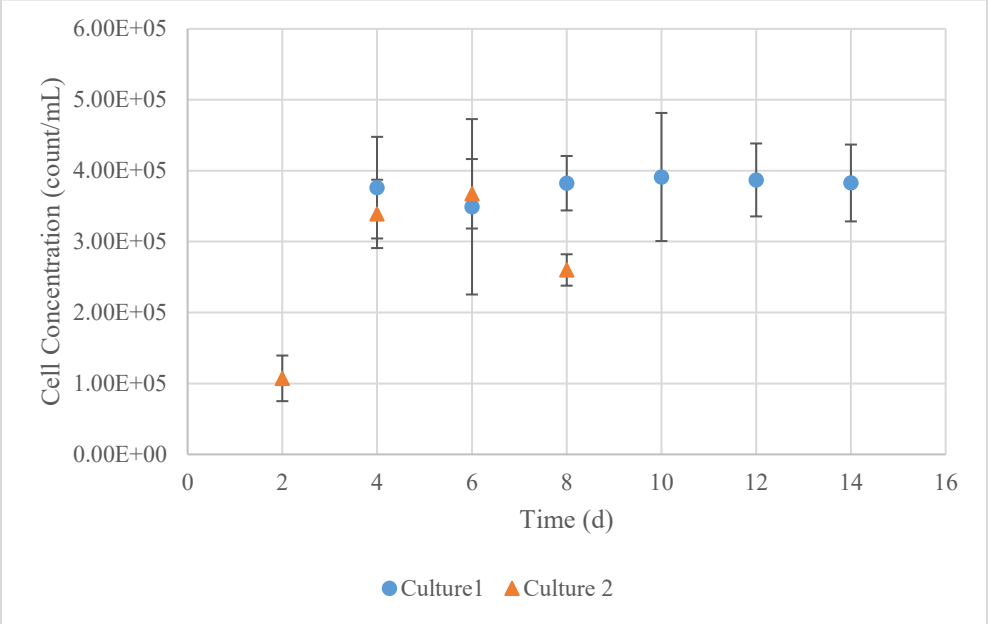


Figure 4-8: Cell count per mL as over time under N-starved condition

Together the optical density and cell count results confirmed that the nutrient-replete culture performed best from pure biomass production. It sustained the exponential

phase for a longer period of time, resulting in a denser culture. This was consistent with predictions as nutrient-deplete cultures result in lower biomass concentration as the limiting factor dictated growth, as stated in Leibig's Law of the Minimum [70]. The P-starved culture had the second highest cell concentration, double the highest cell concentration of the N-starved culture.

Nitrogen is necessary in cellular components like DNA and protein, and phosphorous is involved in components such as RNA, DNA, ATP and phospholipids; even with the changes in metabolism associated with nutrient stress, a limited amount of either nutrient results in a lower biomass [8]. For large systems the Redfield ratio, a C:N:P ratio of 106:16:1, is recommended for nutrient-replete microalgae and can be used as a guide when adjusting media compositions for nutrient limitation [49]. Therefore adjusting the C:N:P ratio such that nitrogen or phosphorus are limited negatively impacts the amount of biomass that can be produced.

4.2.3 Freeze Dried Mass:

Samples, ranging in volume from 50 to 250 mL, were taken from the photobioreactors and lyophilized. The profile of biomass concentration, measured as lyophilized biomass versus time (Figures 4-9, 4-10, 4-11), show a trend similar to the trends seen in the profile of cell concentration versus time (Figures 4-6, 4-7, 4-8).

For the nutrient-replete condition, biomass (Figure 9) and cell concentration (Figure 4-6) did not show the trend seen in optical density (Figure 4-3), suggesting that

the stationary phase was reached around day 10 and that the secondary increase in optical density was not associated with an increase in biomass.

The P-starved culture, shown in Figure 4-10, shares the same trend as seen in Figure 4-4. The decrease in cell count around day 6, seen in Figure 4-7, was not observed in the profile of lyophilized biomass concentration and was consistent with how the cells would experience agglutination. This would result in a seemingly lower cell concentration as cells may have adhered to one another.

The N-starved culture had the lowest biomass. The trend seen in Figure 4-11 agreed with the optical density results seen in Figure 4-5, which had a lower maximum optical density when compared with P-starved and nutrient-replete cultures.

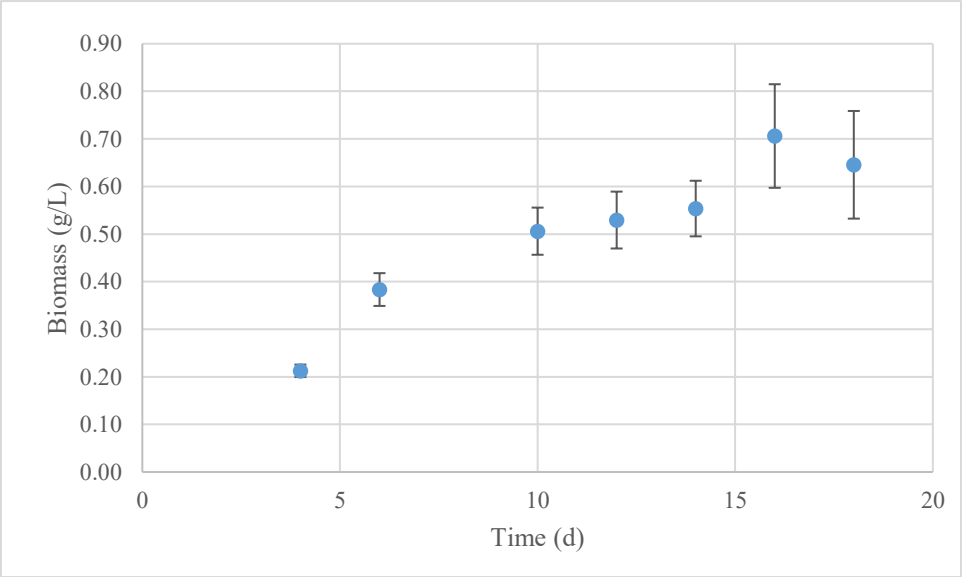


Figure 4-9: Concentration of lyophilized algal biomass of Culture 2 versus time under nutrient-replete condition

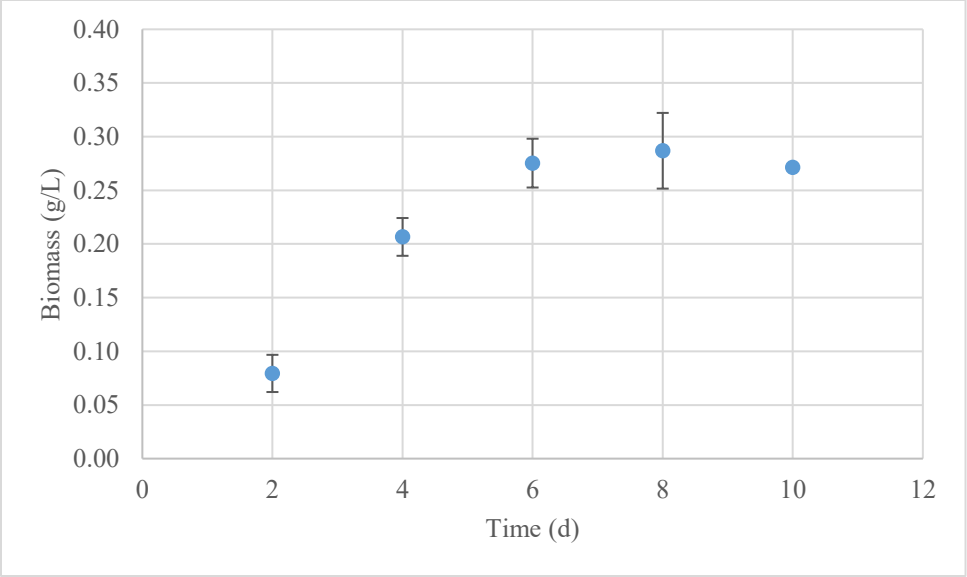


Figure 4-10: Concentration of lyophilized algal biomass of Culture 2 versus time under P-starved condition

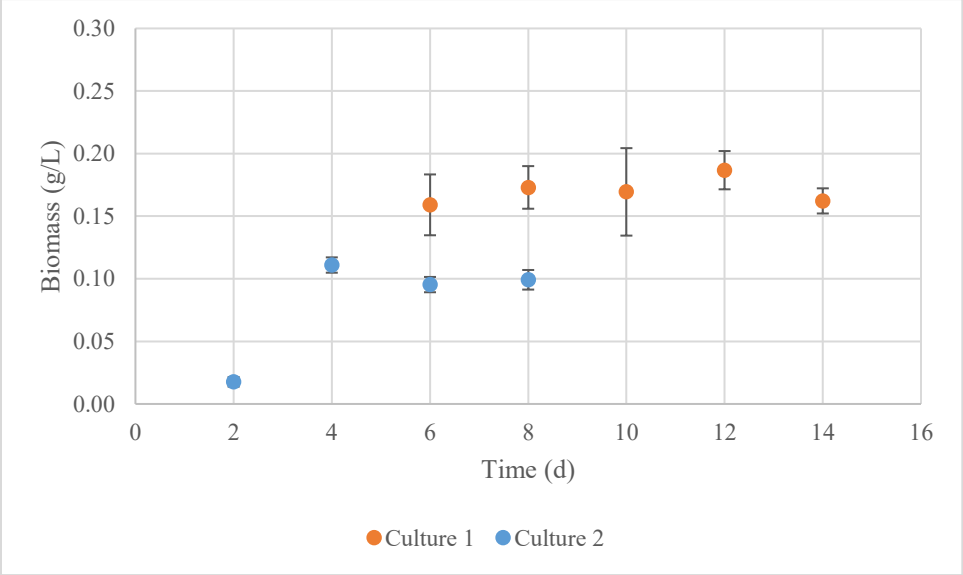


Figure 4-11: Concentration of lyophilized algal biomass of Cultures 1 and 2 versus time under N-starved condition

Similar trends in the optical density, cell concentration and biomass were found, especially during the exponential growth phase. However the profile of the optical density did not consistently follow the trend yielded by the cell concentration. In all

nutrient-replete cultures, after an initial plateau associated with the stationary phase, optical density values began to increase. This did not correspond to an increase in cell count or in an increase in biomass. These results suggest that an optical property aside from biomass density was affecting these results, possibly from a change in cell shape or size [49].

Go *et al.* 2012 was able to achieve a maximum biomass of 0.89 g L^{-1} by the stationary phase [31]. Comparatively the nutrient-replete culture in this experiment achieved a maximum biomass of 0.71 g L^{-1}. The P-starved and the N-starved reached 0.29 and 0.19 g L^{-1} respectively. There was a clear drawback in biomass production when implementing nutrient stress, as the nutrient-replete cultures were able to approach an optimized biomass value whereas the nutrient-stressed conditions were not.

In Figures 4-12 and 4-13 the relationships between the various monitoring methods are shown. In all conditions, the exponential phase, characterized by increasing optical density, shows a linear relationship with biomass and cell concentration. Despite the notable secondary increase in optical density seen in the nutrient-replete culture, the relationship between optical density and biomass remains linear at higher optical densities. For all conditions, these comparisons indicate that the optical density and freeze dried biomass are in agreement throughout growth, but cell concentration is less consistent as the culture becomes denser due to agglutination, particularly in the P-starved condition.

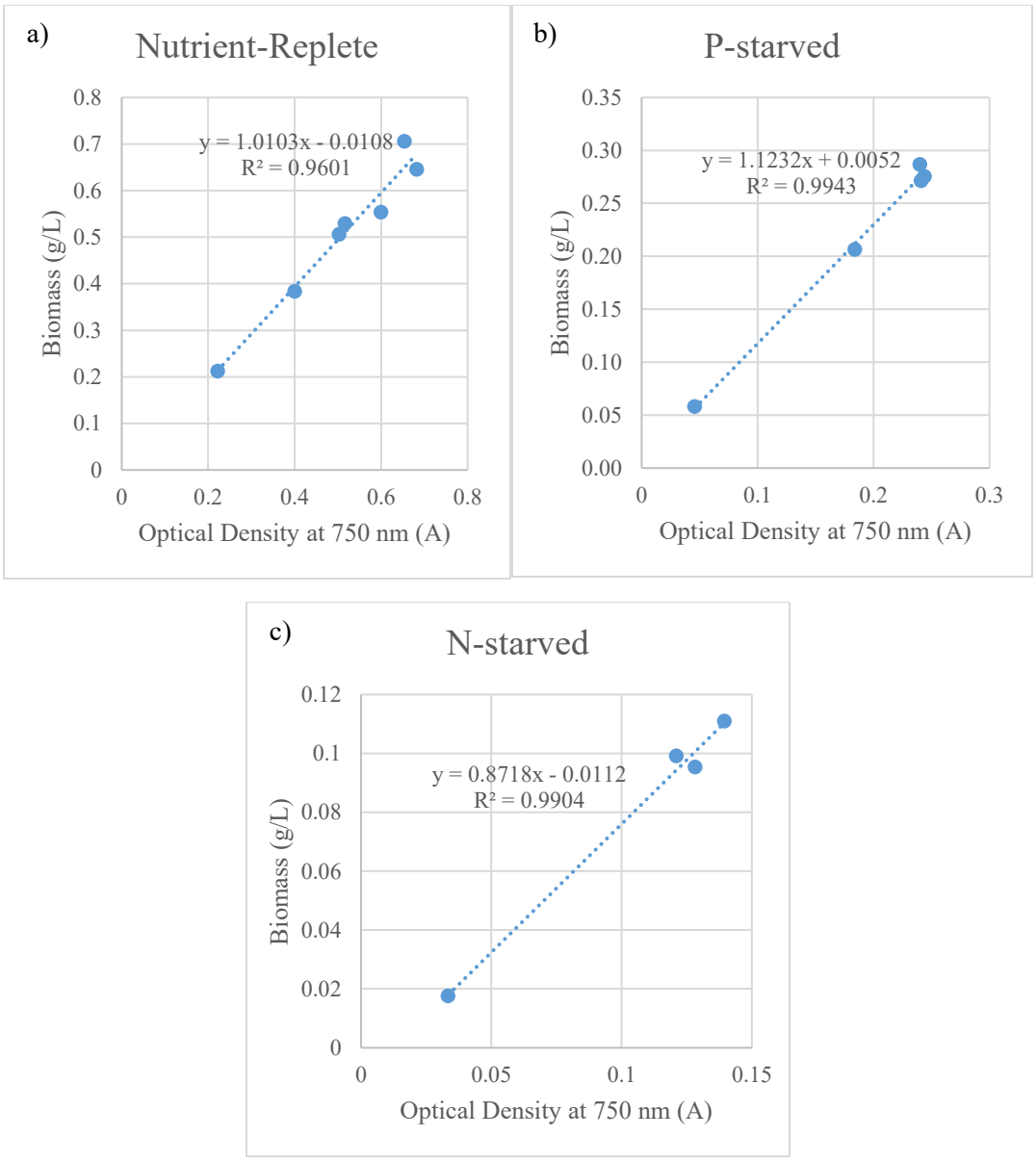


Figure 4-12: Optical density versus biomass for a) nutrient-replete, b) P-starved and c) N-starved cultivation conditions

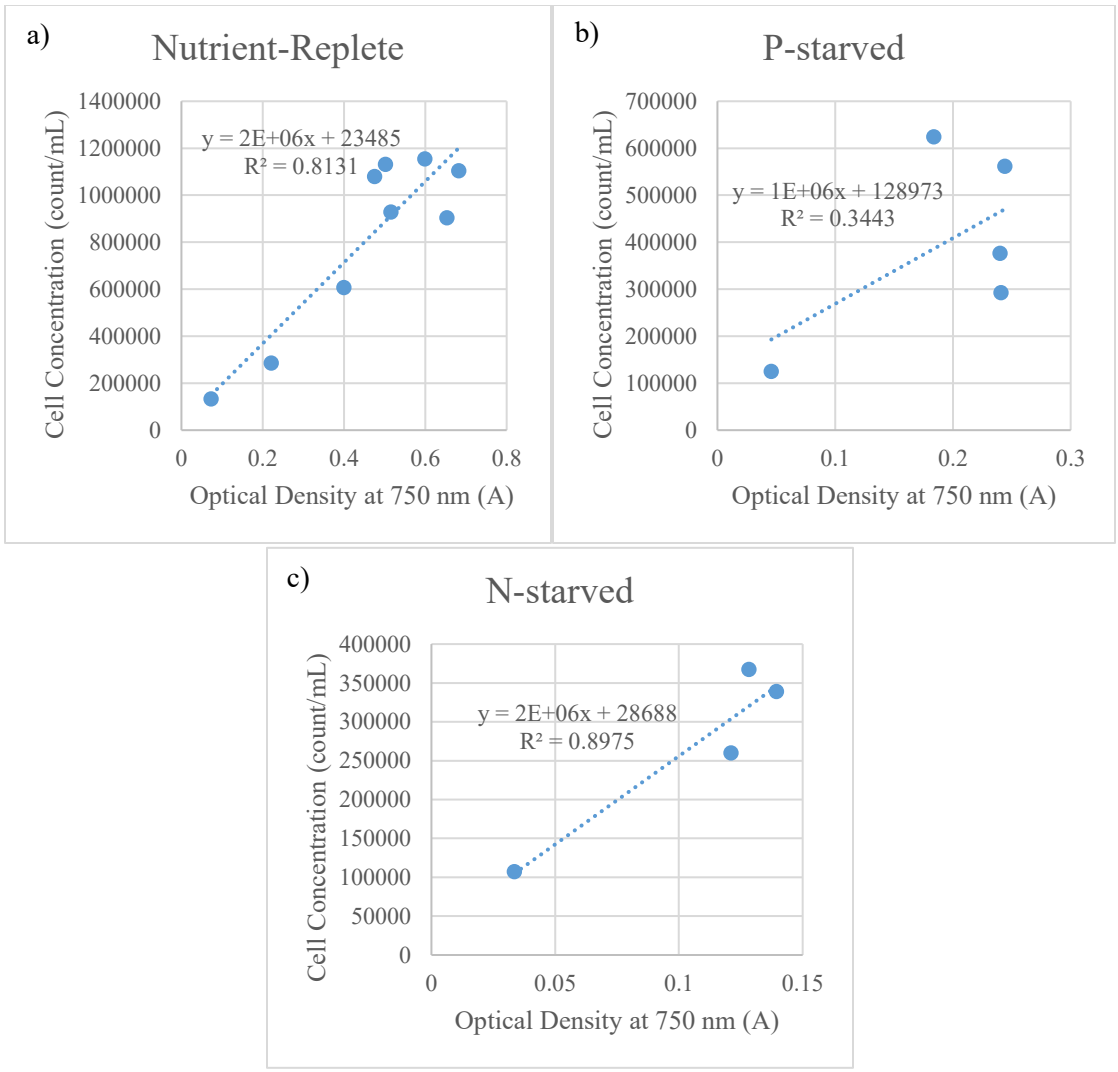


Figure 4-13: Optical density versus cell concentration for a) nutrient-replete, b) P-starved and c) N-starved cultivation conditions

4.3 Carbohydrate Analysis

4.3.1 Enzymatic Hydrolysis:

Enzymatic hydrolysis of corn starch was carried out to ensure the complete hydrolysis of starch. A calibration curve constructed from glucose standard solutions of concentrations ranging from 0.1 to 3.5 g L^{-1} was used for the calibration of DNS analysis

and can be found in Appendix F (Figure F-1). The glucose levels as determined by DNS analysis in the starch samples levelled off around day 4 and the average of the last three values was 98.5% (Figure 4-14), which was within the standard deviation of the expected value of 100%. The results showed that the enzymatic hydrolysis condition (pH 4.5, 55°C and 15µL amyloglucosidase) was suitable for measuring starch content in microalgae.

Starch content analysis of the algal samples from each of the nutrient conditions at their stationary phases was performed. It took just over 24 hours for the starch content of the algae samples to hydrolyze completely and level off (Figure 4-15). The total starch content determined for the nutrient-replete, P-starved and N-starved conditions were 43 ± 6%, 39 ± 5%, and 42 ± 8% starch of the total mass of the lyophilized microalgae (Figure 4-16).

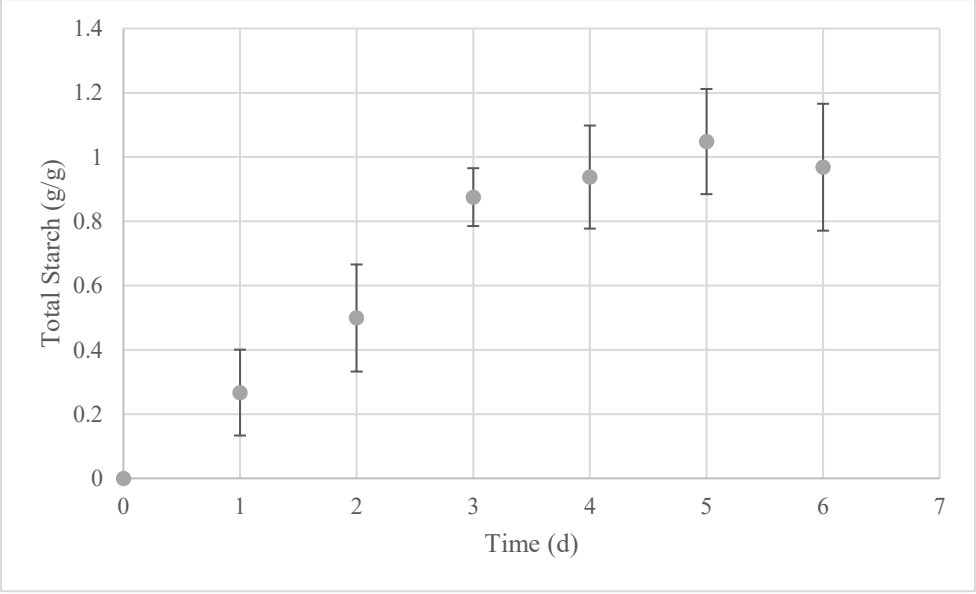


Figure 4-14: Enzymatic hydrolysis of corn starch shown as starch hydrolyzed to the total starch over time

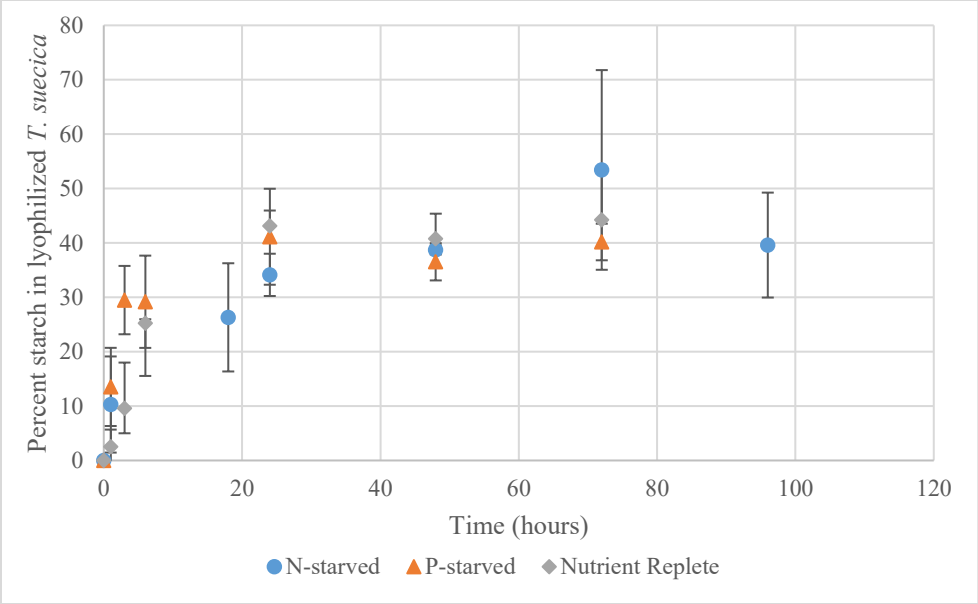


Figure 4-15: Enzymatic hydrolysis of *T. suecica,* at stationary phase grown under nutrient-replete, P-starved and N-starved conditions

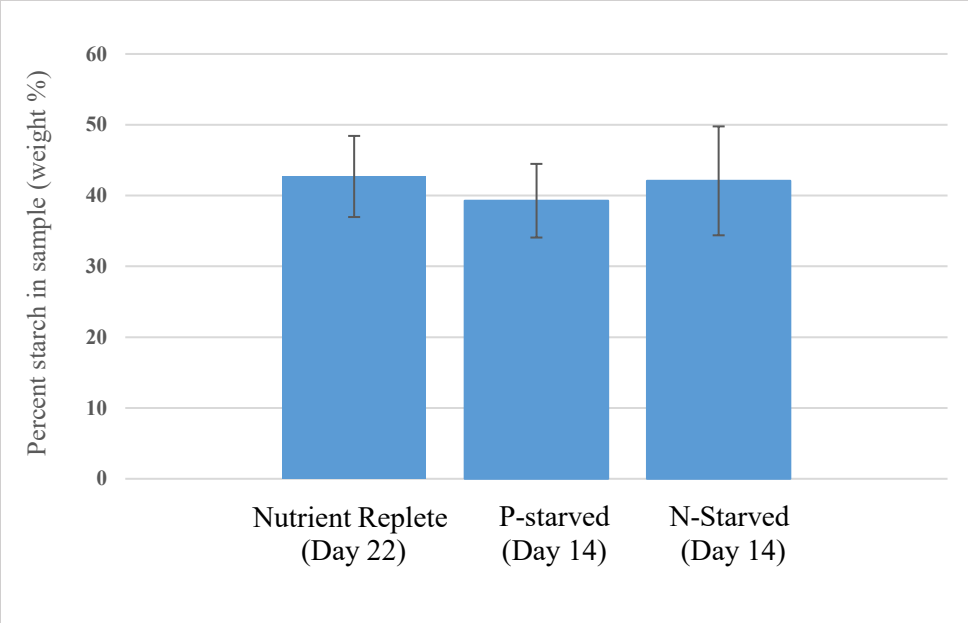


Figure 4-16: Amount of starch in lyophilized *T. suecica* shown as a percent of starch in lyophilized biomass at stationary phase of growth (harvest day is shown in parentheses).

Figure 4-15 shows some differences in initial amounts of starch hydrolyzed over time, which is likely due to slight variations in the amounts of amyloglucosidase in each sample.

Starch content of *T. suecica* at stationary phase at the three cultivation conditions studied was similar. When the analysis of variance is considered, there was no detectable difference in starch hydrolyzed due to the cultural conditions. In other studies of *T. suecica* cultures there is a range of total starch from 15-61% [28] [16] [30]. In Kermanshahi-pour *et al.* 2014 we see similar values to what was produced in this study after reaching the stationary phase.

To assess the significance of the different results for enzymatic hydrolysis, a one-way analysis of variance was performed (Table 4-1). The treatment was the three cultivation conditions, and the replicates were the times 24, 48 and 72 hours where starch had leveled off. The F-value determined for these results was smaller than the critical value, therefore there was no detectable difference at the 95% confidence level for the percent starch under different cultivation conditions.

Table 4-1: Analysis of variance for enzymatic hydrolysis measurements. SS_w is the sum of squares within treatments, SS_b is the sum of squares between treatments, F is the value used for the F-test and $F_{0.05,\,2,\,6}$ is the critical value for a one tailed F-distribution.

	Replicate				
Treatment	**1**	**2**	**3**	**Mean**	**SS_w**
Nutrient-replete	0.449	0.382	0.450	0.427	0.00152
P-starved	0.339	0.403	0.436	0.393	0.00243
N-starved	0.509	0.37	0.383	0.421	0.00589
SS_b	0.001001				
F	0.305143			**Total Mean**	0.413444
$F_{0.05,2,6}$ [71]	5.173			**Pooled SS_w**	0.003282

4.3.2 KDO and Other Sugars:

In Appendix F, the standard curves of carbohydrates investigated using GC/MS are shown in concentrations ranging from 0.1 to 1.5 mg/mL. Figure F-2 shows the calibration curves of glucose, galactose and galacturonic acid at a split ratio of 5:1 and Figure F-3 shows KDO at split ratios of 10:1, 5:1 and splitless. The splitless ratio was used for the quantitation of KDO, and the 5:1 split ratio was used for the other sugars. All relationships were linear between these concentrations, therefore samples that fall in this range were determined reliably.

The peaks observed in the chromatogram of *T. suecica* (Figure 4-17) were accounted for by the monosaccharides: glucose, galactose, galacturonic acid and KDO. This suggests that the sugars used for standards accounted for most of the sugars derivatized from *T. suecica*.

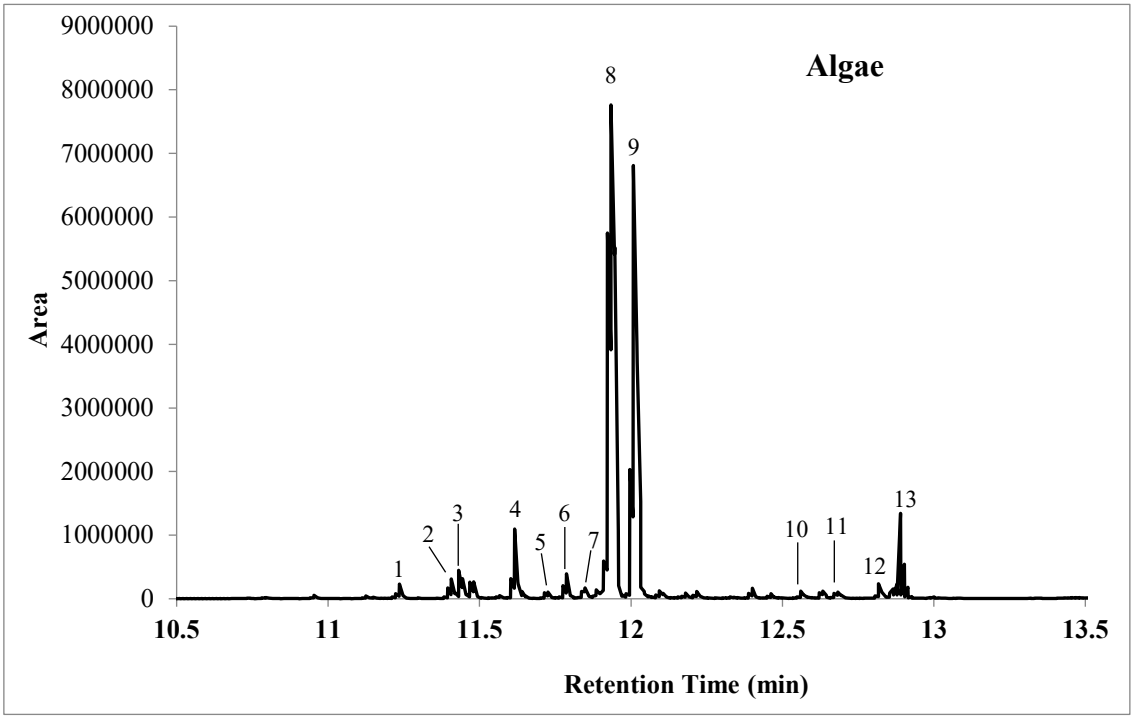


Figure 4-17: Chromatogram for methanolyzed *T. suecica* sample. Monosaccharides are labelled as follows: galacturonic acid at 1, 3, 6, 7; galactose at 2, 4, 5; glucose at 8, 9; KDO at 10, 11, 12, 13 (see Table 4-3 for retention times)

Sugars present in *T. suecica* were identified by comparing the MS fragmentation pattern of the TMS-derivatives of methanolyzed sugars derived from *T. suecica* to the corresponding TMS-derivatives of methanolyzed standard sugars. In the case of KDO, four main peaks were identified that each have the fragmentation pattern consistent with the m/z from the algae, which can be seen in Table 4-4 along with rest of the monosaccharides.

The m/z fragments of 73 and 133 were characteristic of TMS derivatives are both rearrangements of the trimethylsilyl groups [72]. The 147, 204 and 217 fragments were consistent with TMS ether derivatives of carbohydrates [72]. As these fragments were

seen in the peaks associated with KDO, glucose, galactose and galacturonic acid. This acted as confirmation that the TMS derivatization step was successful.

Figure 4-18 shows the sugar content in *T. suecica*. Glucose values differed by a range of 50 mg, with 396, 383 and 355 mg per gram of microalgae for nutrient-replete, N-starved and P-starved conditions respectively. Galactose, galacturonic acid and KDO content were all within standard deviation across the different cultivation conditions. The nutrient-replete culture has the highest overall content, with the four sugars investigated accounting for 450 mg per gram of microalgae.

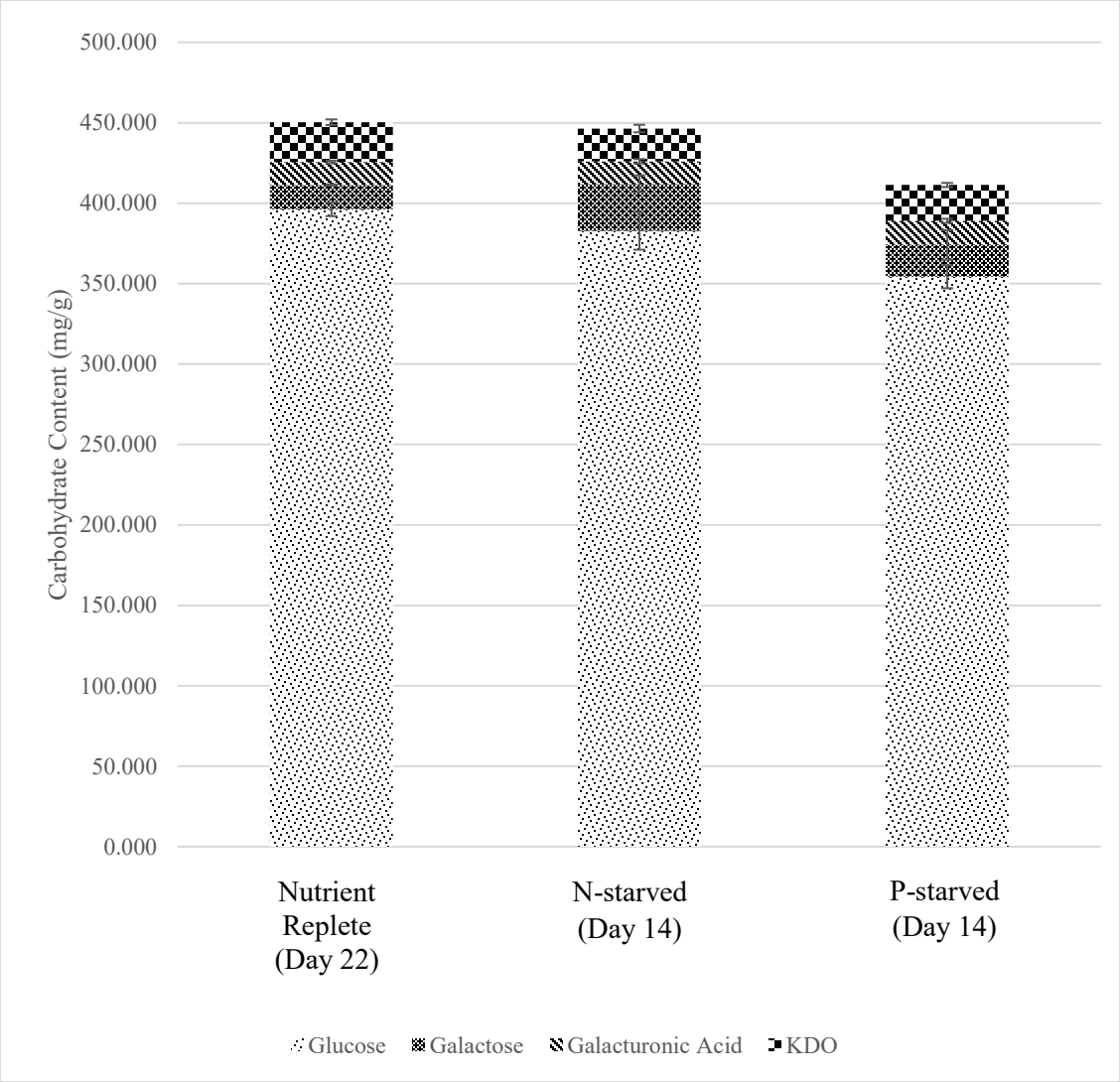


Figure 4-18: Total carbohydrate content determined as a function of mg per g of dry mass by GC/MS

To assess the significance of the different results of KDO content, a one-way analysis of variance was performed (Table 4-2). The treatment was the three cultivation conditions, and the replicates were the KDO content determined for each reactor from Culture 2. It was determined that the F-value calculated for these results was smaller than the critical value, therefore the effect of different cultivation conditions was not significant at the 95% confidence level. The increase in carbohydrates that has been seen

in different studies was not seen when samples from the stationary phase were measured [30].

Table 4-2: Analysis of variance for the mg KDO per g dried mass as determined GC/MS results based on nutrient condition. SS_w is the sum of squares within treatments, SS_b is the sum of squares between treatments, F is the value used for the F-test and $F_{0.05, 2, 6}$ is the critical value for a one tailed F-distribution.

	Replicate				
Treatment	**1**	**2**	**3**	**Mean**	**SSw**
Nutrient-replete	25.39	25.95	22.65	24.66	3.110
P-starved	18.18	22.82	19.98	20.33	5.484
N-starved	22.24	21.03	23.61	22.29	1.671
SS_b	14.16				
F	4.138			**Total Mean**	22.43
$F_{0.05, 2, 6}$ [71]	5.173			**Pooled SS_w**	3.422

In Figure 4-19 below, the total monosaccharides measured both with enzymatic hydrolysis and by GC/MS are presented. As in Kermanshahi-pour *et al.* 2014, the values from both methods estimate similar amounts of carbohydrates per gram of dry mass [16]. Enzymatic hydrolysis was therefore suitable for an estimation of carbohydrate content, whereas GC/MS was suitable for use in identification and quantitative analysis of carbohydrates.

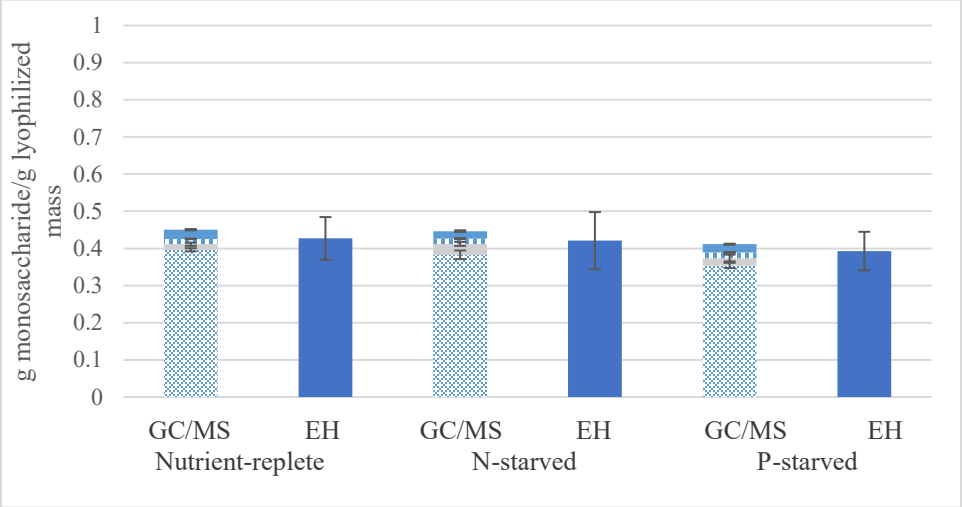


Figure 4-19: Comparison of total monosaccharides per freeze dried algae sample in both GC/MS and enzymatic hydrolysis (EH) for nutrient-replete, N-starved and P-starved cultivation conditions

At the stationary phase, all three cultures had similar carbohydrate compositions. As nutrients such as phosphate and nitrate would be depleted at the stationary phase, the cells at this point were subjected to approximately the same nutrient conditions.

Lynn *et al.* 2000 maintained a semi-continuous culture, which resulted in the exponential phase of growth being prolonged. The use of a semi-continuous culture meant that the analysis took place at a point in growth where the phosphorous stress was observable. The effect of the phosphorous stress was a 1.7 and 1.8 fold increase in lipids and carbohydrates respectively in *S. minutulus* [10]. Dragone *et al.* 2011 implemented a batch culture, and varied the nitrogen concentration. The nitrogen-deplete culture was able to achieve the highest starch content at 41.0% by dry weight. Dragone *et al. 2011* observed an 8-fold increase in starch content in *C. vulgaris* when subjecting cultures to nitrogen stress [9].

Harvesting during exponential growth may make the difference in composition based on nutrient conditions more pronounced as both N-stress and P-stressed algae cultures have experienced increased carbohydrate content in studies such as those done by Dragone *et al.* 2011 and Lynn *et al.* 2000 [9] [10]. Several microalgal species experience carbohydrate accumulation in response to both nitrogen and phosphorous stress [8].

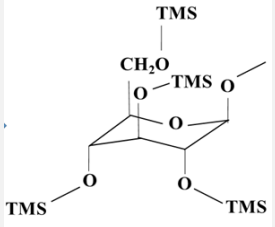
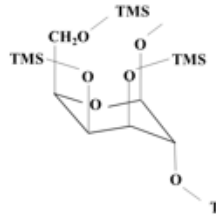
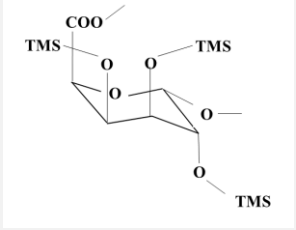
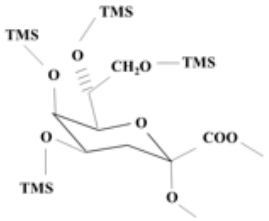
Due to KDO's high cost, the amount measured in this experiment could contribute to the value of *T.suecica* cultures. KDO is approximately 20 000 CAD per gram and this value was used to calculate the value of the various cultures [45]. Table 4-3 displays the value of each cultivation condition as a function of the amount of KDO present and the biomass production. The nutrient-replete condition was the highest in value at 350 CAD L^{-1}.

The nutrient-replete culture still has a much higher biomass yield than the nutrient-stressed cultures, so at the time of harvest it was the most productive culture. The exponential growth phase needs to be investigated to see if there are significant differences in cellular composition that justify nutrient-stressed conditions.

Table 4-3: Determination of value of *T. suecica* cultures based on mg of KDO per litre

	Nutrient-Replete	**P-starved**	**N-starved**
Biomass Production (g L^{-1})	0.71	0.29	0.19
mg KDO per g lyophilized mass	24.66	20.33	22.29
Value of *T. suecica* culture (CAD L^{-1})	350	118	85

Table 4-4: TMS derivatives of sugars found in *T. suecica* and their GC/MS characteristics (RT = Retention time)

Compounds	Molecular Formula	Molecular mass	Molecular Mass + TMS	Molecular Structure	m/z	RT in minutes (Peak number)
Glucose	$C_6H_{12}O_6$	180	482		73, 133, 147, 204, 217	11.94 (8), 12.02 (9)
Galactose	$C_6H_{12}O_6$	180	482		73, 133, 147, 204, 217	11.42 (2), 11.63 (4), 11.80 (5)
Galacturonic acid	$C_6H_{10}O_7$	194	438		73, 133, 147, 204	11.25 (1), 11.48 (3), 11.85 (6), 11.92 (7)
KDO	$C_8H_{14}O_8$	238	554		73, 133, 147, 204, 217	12.57 (10), 12.70 (11), 12.83 (12), 12.99 (13)

Chapter 5 : Conclusion

There were some key differences that emerged when studying *T. suecica* grown under different cultivation conditions. In terms of pure biomass, the nutrient-replete cultures far outperformed any of the nutrient-stressed cultures. This is unsurprising as it is expected that the limited nutrient will limit the total growth [70] and has been seen in studies on microalgae before this one [11] [56]. What has also been observed in other studies, however, is that microalgae under nutrient stress accumulate intracellular metabolites differently than microalgae that have been grown under growth favourable conditions [9] [10]. Favourable growth condition vary in their exact C:N:P ratio, but generally a C:N of 53:8 as found in the Redfield ratio is consistent with nutrient-replete conditions and N:P is more variable and can be either lower or higher than the 16:1 value in the Redfield ratio [73]. This experiment sought to produce consistent triplicate cultures for each cultivation condition and explore the differences in carbohydrate composition based on different cultivation conditions.

Each inoculum culture followed the same trend in optical density over time; the exponential growth phase lasted from day 4 to day 10 and growth leveled off around day 14. Culture 1 and Culture 2 of the nutrient-replete condition saw the same features emerge in their optical densities, even in the second spike in optical density at day 12, which was not represented in the biomass. This is indicative of both cultures undergoing the same changes, both in biomass and in other cellular features that can affect optical density measurements. The nutrient-replete and nutrient-stressed conditions had optical densities that shared a consistent trend with the results from the freeze dried mass, as

demonstrated in the linearity of the relationship when these strategies were compared. Cell count encountered issues where agglutination caused the count to drop dramatically.

A consistent amount of KDO across all cultivation conditions was found as of the stationary phase. It is worth further investigating the cellular accumulation of KDO at other points in the growth cycle. In addition to the stationary phase, it would be useful to investigate the KDO content in both the early and late points of the exponential phase where the difference in nutrient availability would be more pronounced.

An issue that exists with nutrient-stressed conditions is the significant decrease in biomass in comparison to nutrient-replete cultivation conditions. One strategy for increasing biomass could be to grow heterotrophic or mixotrophic cultures. These generally outperform autotrophic conditions in pure biomass production, though the effects of nutrient stress differ. In addition to exploration of nitrogen and phosphorous stress, the effect of different organic carbon sources on growth rate and cellular accumulation in nutrient-stressed conditions would also have to be considered.

Optimization of extraction strategies are also necessary. This set of experiments only sought to extract derivatized KDO along with the other carbohydrates, but it is critical to determine extraction efficiency if *T. suecica* is going to be a feedstock for KDO production at industrial scales.

Limitation, as opposed to starvation, could be another avenue to explore, so that a low amount of a nutrient is continually introduced and not simply exhausted. This has been shown in some cultures to be an effective means of optimizing desired metabolites [8].

The results from this study find that different cultivation conditions of *T. suecica* at their respective stationary phases do not differ significantly in their overall starch content or in their composition KDO. To fully explore the effects of cultivation conditions on KDO in *T. suecica*, it is necessary to analyze the composition of the biomass at several different points in the cultures, before all the relevant nutrients are depleted.

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Appendix A: Culture Maintenance for *Tetraselmis suecica*

Note: Culture maintenance of *Tetraselmis suecica* was performed according to the following procedure. The culture manipulation was always conducted in the laminar flow hood.

Initial Culture Maintenance:

1. Upon receiving the culture (day 0), it was spiked with 0.5 mL dissolved inorganic carbon (DIC) made from 0.02 M sodium bicarbonate, syringe filtered using a 0.2 µm pore size and stored in incubator at 18ºC. The light level in the incubator where the culture is then placed was set to 15 to 20 µmol/m^2/s photons of light. (the incubator shelf was at 40 µmol/m^2/s photons of light and was filtered using two layers of mesh screen. It is ideal to test the light intensity with a vial full of water with a light probe to determine how much screen achieves the desired intensity). The reason for addition of DIC solution is that algae has a molar C:N:P ratio in nutrient-replete growth of about 100:20:1. The ratio of bicarbonate:nitrate:phosphate in f/2 in seawater is about 50:22:1 so the culture's growth will be limited by carbon availability if there is no additional source.
    a. DIC is only needed for the first initial transfer, however, frequent transfers of stock culture is what replenishes the carbon in the culture
2. On day 1, the culture was divided into 3 portions of 5 mL each into 50 mL glass vials and 3 mL UTEX media was added to each vial aseptically.
3. On day 3, 3 mL UTEX media was added again to each culture vial.

4. On day 4, 29 mL UTEX media was added, to make a total of 40 mL and the light intensity was increased to 40 μmol/m^2/s photons of light (mesh screen was removed).
5. When the 40 mL of culture grew (visibly green), 5 mL of each vial was taken and put into to a new vial of 35 mL media for a total of 40 mL of culture again. Once these cultures begin to be visibly green (try to do the transfers before a scum appears on the water surface of the culture as this means the culture has begun to die) the last transfer before regular maintenance can ensue.
6. Two stock culture bottles (250 mL media bottles) were filled with 100 mL media, one culture vial and half of a second were put into the first bottle. The second half of the half emptied vial and the final culture vial were emptied into the second stock bottle. Each bottle should have roughly 160 mL in total volume.

Regular maintenance:

1. 12 hours light/dark cycle, with the lighting coming from the side
2. Cultures grown in 250 mL sterile glass Pyrex bottles
3. Bottles cleaned by a 24 hour bath in 4% micro solution followed by 24 hour bath in 10% HCl
4. Transfers done in sterile flow hood
5. 0.5 mL culture should be transferred into 150 mL fresh media every two to three weeks to maintain cultivation conditions and CO_2 availability
6. The light intensity should be 60 to 70 μmol/m^2/s photon of light.

Procedure for cleaning the glassware:

Bottles sterilized by a 24 hour bath in 4% micro solution followed by 24 hour bath in 10% HCl. Micro 90® (International Product Corporation) is a concentrated detergent that we use in a 2-step cleaning process. Glassware should be soaked in it, rinsed with DI water, soaked in 10% HCl, rinsed with DI water, and dried in an inverted position. It's important not put certain plastics (black rubber in particular) in the Micro 90 solution as they leach plasticizers that can kill the cultures. The following plastics are safe but do age and become brittle after repeated cycles of soaking and autoclaving: Teflon, polycarbonate, high-density polyethylene, clear polystyrene.

Appendix B: Cultivation and Culture Monitoring

Inoculant

1. Autoclave 970 mL seawater in 3 1L media bottles each containing a magnetic stirrer with caps on loosely
2. Temporarily tighten the caps, then move the bottles to a laminar-flow hood and loosen them again while the media cools. Then tighten the cap. If you tighten right away, the contraction of the fluid can cause enough of a pressure difference in the head space that it sometimes cracks a bottle.
3. The next day, wrap the modified caps with glass and silicone tubing in aluminum foil and autoclave
4. In flow hood, aseptically add 20 mL of the UTEX Enrichment Solution (Table 1).
5. Invert the stock solution 10 times to target homogenous sampling and add 10 mL stock UTEX LB 2286 *T. suecica* to each bottle. Try to do homogenous sampling
6. Close bottles with modified caps, ensuring that the outlet tubing sits above water level
7. Place pinch valves, that have been sterilized with 70% ethanol prior to entry in flow hood, on the sampling tubes and close them; place pinch valves on outlet tubes, leaving them open
8. Cover the tops of the tubing with the aluminum foil they were autoclaved in
9. Remove from flow hood and move to the rest of the set up (light, stir plates, gas lines, etc.) and set each bottle on a stir plate

10. Put the filters on the inlets and outlets in the proper orientations (female luer lock facing downward for inlet and upwards for outlet, such that the luer lock to tubing attachment fits in appropriately)
11. Set CO_2 flow rate to 0.045 mL/min (0.015 mL/min for each bottle) and set air flow rate such that the medium is disturbed by the bubbling.
12. The light intensity should be maintained at 250 µmol m^{-2} s^{-1} with a light/dark cycle of 14/10 hours
13. Optical density should be measured on daily basis at 750 nm by attaching a syringe to the sampling tubing, withdrawing approximately 3 mL per bottle and measure the samples in optical glass cuvettes in the spectrophotometer. The optical density should be monitored until the absorption levels of at approximately 10 days.
14. Prior to Harvesting, do first three steps of Cultivation procedure
15. Transfer the photobioreactor to the flow hood and transfer the content to several centrifuge tubes of 50 mL. Harvest algae using centrifugation, discard supernatant and transfer all pellets to one centrifuge tube. Sterile centrifuge tubes should be used. Proceed to fifth step of cultivation procedure on the same day, as soon as possible. **Note on centrifugation: Perform at 200 – 3000 rpm and at room temperature (<u>NOT</u> 4°C) to avoid killing cells**

Cultivation

1. Autoclave 980 mL seawater in 3 1L media bottles each containing a magnetic stirrer with caps on loosely.

2. Temporarily tighten the caps, then move the bottles to a laminar-flow hood and loosen them again while the media cools. Then tighten the cap. If you tighten right away, the contraction of the fluid can cause enough of a pressure difference in the head space that it sometimes cracks a bottle.
3. The next day, wrap the modified caps with glass and silicone tubing in aluminum foil and autoclave
4. In flow hood, aseptically add 20 mL of the Enrichment Solution that targets (Original UTEX formula/N-limited/P-limited) N concentration to each bottle (Either original UTEX concentrations, N- or P- limited based on which experiment is being performed, refer to table 1 for specifics)
5. Add the inoculum 100 µL at a time to get to 0.05-0.06 of the optical density at 750 nm (should be approximately 600 µL of the concentrated inoculum). Invert the bottle 10 times before taking samples to measure the optical density.
6. Close bottles with modified caps, ensuring that the outlet tubing sits above water level
7. Place pinch valves, that have been sterilized with 70% ethanol prior to entry in flow hood, on the sampling tubes and close them; place pinch valves on outlet tubes, leaving them open
8. Cover the tops of the tubing with the aluminum foil they were autoclaved in
9. Remove from flow hood and move to the rest of the set up (light, stir plates, gas lines, etc.) and set each bottle on a stir plate

10. Put the filters on the inlets and outlets in the proper orientations (female luer lock facing downward for inlet and upwards for outlet, such that the luer lock to tubing attachment fits in appropriately)
11. Set CO_2 flow rate to 0.045 mL/min (0.015 mL/min for each bottle) and set air flow rate such that the medium is disturbed by the bubbling.
12. The light intensity should be maintained at 250 μmol m^{-2} s^{-1} with a light/dark cycle of 14/10 hours.
13. Sample throughout culture growth through sampling tubes using sterile 50 mL syringes (day 0 (day of inoculation), 2, 4, 6, 8, 10, 12)
14. For first sampling, take 3 mL for optical density.
15. For every subsequent sampling measure the optical density then dispense the appropriate volume between the tube designated for dry weight and the tube designated for freeze drying. Centrifuge the tube, then pour off and save the supernatant. Using a syringe filter, filter the supernatant and keep in the freezer for nitrate analysis
16. Take 1 mL for cell count, 3mL for OD, 20 mL for dry weight and 30 mL for KDO, starch, lipid analysis. Decrease volume sampled as concentration of algae increases, noting these changes
17. Centrifuge the samples taken for KDO/starch/lipid, save the supernatant for nitrate analysis and rinse the pellet with DI water and repeat centrifugation. This should be done a total of 3 times, but the supernatant at this point should be discarded. Save the pellet for the designated biomass characterization.

18. Replace sample volume with fresh media (N-free for N-starvation experiment/P-free for P starvation experiment, refer to table 4 for specifics)
19. Sample volume may be decreased as concentration of algae increases, noting these changes, though the volume replaced also must then be adjusted
20. Take sample every 2 days. Sample volume should be decided
21. Harvest the whole culture bottles on day 14, taking samples for optical density and dry weight and centrifuging the rest to be freeze dried.

Sampling

22. For sampling, take sterile 60 mL syringe, open the package and quickly attach to sampling tube
23. Open the pinch valve for the sampling tube and pull up the necessary volume of sample into the syringe (1mL for cell count, 3mL for OD, initially 20mL for dry weight, initially 30 mL for freeze drying (dry weight and freeze drying volumes will decrease with increase in culture density)
24. Close pinch valve
25. Dispense into 50mL centrifugation tubes for dry weight and freeze drying
26. Dispense into 10mL centrifugation tube for cell count
27. Analyze OD immediately
28. Dispose of Syringe
29. Add the volume that was withdrawn back to the reactor, bring appropriate media
30. For media preparation, refer to UTEX website (http://utex.org/products/enrichment-solution-for-seawater-medium-recipe). The

stock culture preparation should be altered according to the composition shown in Table 3. Table 3 shows the composition of the enriched stock solution.

31. Add 20 mL of the stock solution to 980 mL autoclaved seawater.
32. Table 4, shows the final concentration of components in seawater, which is the media that should be added to the reactor after samples were taken.
33. Bring sterile beaker, sterile 60mL syringe and syringe needle sterilized with 70% ethanol to flow hood.
34. For N-limited culture place N-free media and for P-limited place P-limited media as stated in table 4.
35. Dispense the appropriate media (as stated in #33) into beaker inside the flow hood
36. Draw up appropriate volume (volume removed that same day) into syringe with the needle on inside the flow hood.
37. Cover needle in aluminum foil
38. Bring to bioreactor
39. Open pinch valve to sampling tube and put the needle into sampling tube
40. Dispense media into culture
41. Close pinch valve

Optical Density Measurements

42. Turn on spectrophotometer by using the switch on the back of the instrument
43. Hit Esc twice and change programme to absorb at 750 nm
44. Use optical glass cuvettes

45. Fill cuvette three quarters full of DI water and wipe the outer faces with a kimwipe, holding the cuvette on the clouded sides
46. Place cuvette in spectrophotometer so a clear face faces the opening, and close the lid
47. Use the DI water blank to set 0 absorbance
48. Empty the cuvette and fill three quarters full with sample from Flask 1
49. Place in spectrophotometer and allow it to read a steady value
50. Record three readings
51. Empty sample into waste beaker
52. Rinse with DI water
53. Repeat Steps 41 to 45 for samples from Flask 2 and 3
54. Leave cuvette on portion of kimwipe to dry
55. Turn of spectrophotometer

Sample preparation for freeze drying

56. For sample preparation for freeze drying, centrifuge at 4°C for 10 minutes, ensuring that the centrifuge is balanced
57. Remove tubes from centrifuge, discard the supernatant, rinse the pellet with DI water and disperse the pellet with vortexing
58. Repeat centrifugation until samples have been rinsed 3 times
59. Transfer the centrifuged samples to a new, sterile centrifuge tube, and make sure the algae is dispersed in DI water for freezing at -80 C
60. Start pump for freeze dryer and wait an hour

61. Remove caps from centrifuge tubes with frozen algae, cover with aluminum foil and poke holes in the top
62. Store in freezer until freeze dryer is ready
63. After 1 hour, put samples in freeze dryer chamber, close and turn on vacuum
64. Wait one to two days for the samples to dry completely
65. Turn off freeze dryer, release vacuum by pinching one of the rubber seals and remove samples and replace their caps
66. Allow pump to run for an additional hour and then shut it off

Sample preparation for dry weight measurement

67. Mark aluminum weighing dishes, put them in oven at 70°C for 24 hours and weigh them
68. For sample preparation for dry weight measurement, centrifuge a known volume (to be determined) at 4°C three times. Between centrifugation, discard the supernatant, rinse with DI water and disperse the pellet with vortexing.
69. Put pellet in pre-weighed weighing dish
70. The dishes are then put in oven at 70°C for 48 hours, then weighed

Sample preparation for cell count

71. Add 2.5 µL glutaraldehyde to 1 mL sample that was taken for cell count
72. Allow to incubate for 15 minutes at room temperature
73. Freeze the sample until analysis can take place

Table B-1: Enrichment N-free and P-free stock solution composition. Twenty milliliters of this solution will be added to 980 mL of autoclaved seawater to be added to the photobioreactor after sampling.

Components	**N-free UTEX Enrichment Stock Solution (g/L)/(M)**	**P-free UTEX Enrichment Stock Solution (g/L)/(M)**
$NaNO_3$	0	2.35E+00 /2.77E-02
Na_2glycerophosphate·5 H_2O	3.50E-01 /1.62E-03	0
$Fe(NH_4)_2(SO_4)_2·6H_2O$	1.14E-01 /2.90E-04	1.14E-01 /2.90E-04
$Na_2EDTA·2H_2O$	2.60E-01 /6.98E-04	2.60E-01 /6.98E-04
H_3BO_3	1.85E-01 /3.00E-03	1.85E-01 /3.00E-03
$FeCl_3·6H_2O$	7.96E-03 /2.95E-05	7.96E-03 /2.95E-05
$MnSO_4·H_2O$	2.67E-02 /1.58E-04	2.67E-02 /1.58E-04
$ZnSO_4·7H_2O$	3.58E-03 /1.24E-05	3.58E-03 /1.24E-05
$CoCl_2·6H_2O$	7.80E-04 /3.28E-06	7.80E-04 /3.28E-06
HEPES	3.26E+00 /1.37E-02	3.26E+00 /1.37E-02
Vitamin B12	2.03E-04 /1.49E-07	2.03E-04 /1.49E-07
Thiamine	5.03E-04 /1.67E-06	5.03E-04 /1.67E-06
Biotin	3.75E-05 /1.53E-07	3.75E-05 /1.53E-07

Appendix C: Starch Determination

1. Add 1.025375 g CH_3COONa (sodium acetate) in 250 mL of DI water (50 mM) and add CH3COOH (acetic acid) to target pH of 4.5 (one droplet may be enough). This buffer solution can be stored at room temperature for subsequent uses
2. Weigh out 3 mg freeze dried *T. suecica* in a glass vial with lid
3. Add 6 mL buffer solution to *T. suecica* and a stir bar
4. Add 15 µL amyloglucosidase (A7420, Sigma Aldrich) to your sample
5. Set up reaction block on hot plate at a temperature of 55°C Put the sample, along with a vial with just the buffer solution and enzymes (this is the control), in the reaction block
6. Take samples every 24 hours and follow steps 8 through 14 for each time point with both the sample and the control until the absorbance values plateau
7. When taking sample from reaction vials, take it as they are still continuously stirring. Take 0.2 mL and put each into a tube for centrifugation, including for the control.
8. Once the samples have been centrifuged down, take 60 µL of the supernatant of each sample and control and dispense each into its own 1.5 mL micro- centrifuge tube. Discard the algal pellet.
9. Add 180 µL of DNS solution to each 1.5 mL micro- centrifuge tube
10. Boil water in a 100 mL beaker. Be careful when dealing with boiling water. Be present in the lab for the duration of the experiment and set up a sign to notify others in front of the water.

11. Place the micro-centrifuge tubes in a portion of aluminum foil with holes and place it so the tubes are being held in the boiling. It should be already boiling when the samples are placed. Let the samples boil for 10 minutes
12. Remove the samples from boiling water with long tweezers, taking caution not to get burnt.
13. After the samples were cooled to room temperature, 960 μL of DI water should be added to the samples
14. Use the control sample (hydrolytic solution which has gone through the method as the samples) described in step 6 to zero the absorbance of the spectrophotometer at 540 nm
15. Measure absorbance of sample in spectrometer at 540 nm

Procedure for Spectrophotometer usage

1. Switch on the power supply.
2. Press ‘Esc’ button twice after the main screen appears.
3. Change the wavelength to 540nm.
4. Clean the cuvette and fill with the control sample. Gently wipe off the walls of the cuvette with tissue.
5. Place this cuvette in inside the spectrophotometer and press blank. This will give a reading of 0.000A (zero absorbance).
6. Next, clean the cuvette and pour the above sample from sugar analysis into it and wipe off the walls of the cuvette with tissue paper.

7. Place the cuvette back into the chamber and note the reading of absorbance.

DNS reagent

1. Dissolve 2 g of 3,5-dinitrosalicylic acid (Sigma D-0550) in about 160mL of water
2. With continuous magnetic stirring, gradually add 3.2 g of NaOH and let it dissolve
3. Add 60 g of Rochelle Salt (K-Na-tartrate, Merck 8087) in small portions with continuous stirring. The solution may be cautiously warmed to a maximum temperature of 45ºC
4. Cool to room temperature and make up to 200 ml with water in a volumetric flask. If the solution is not clear, filter through Whatman 1 filter paper.
5. Store in a dark bottle at room temperature. The DNS solution should be stored in a glass bottle covered with an aluminum foil at room temperature.

Appendix D: Methanolysis Method

1. Measure out at record the amount of samples being investigated, for freeze dried algae this should be at least 5 mg
2. Put in glass vials with Teflon lined screw cap and add a small magnetic stir bar
3. Use syringe with needle to extract 3 mL anhydrous methanol through the bottle's membrane
4. Add 3 mmol (0.2133 mL) acetyl chloride slowly
5. Purge acetyl chloride bottle with nitrogen for storage
6. Purge glass vials with nitrogen
7. Put on reaction block at 85°C (by thermometer), set it to stir (at stirring speed 1), and leave for 24 hrs
8. After 24 hrs, neutralize the reaction mixture with 3 mmol (0.812 g) silver carbonate, and add 0.53 mmol (50 μL) acetic anhydride
9. Purge both silver carbonate and acetic anhydride with nitrogen for storage
10. Purge glass vials with nitrogen
11. Incubate the samples with the acetic anhydride added in step 12 at room temperature for 18 hrs, as methanolysis causes deacetylation therefore reacetylation must take place.
12. The mixture is centrifuged and the supernatant is collected using a Pasteur pipette.
13. The pellet is rinsed with 1 mL anhydrous methanol, then centrifuged, and the supernatant is collected and added to the supernatant from step 16. This rinsing step is repeated for a total of three rinsings.

14. The 6 mL of supernatant that has been collected is then dried under nitrogen in a hot water bath, being careful to ensure that the nitrogen flow is not to vigorous as to cause splashing. This process takes 90 minutes to 2 hours.
15. After evaporating the solvent, the sample is kept in a P_2O_5 desiccator overnight
16. Derivatization of the sugars generated in methanolysis is performed by first dissolving the residue in 100 to 200 µL pyridine, then by adding 100 µL *N,O*-bis-(Trimethylsilyl)Trifluoroacetamide with 1% TMCS
17. Purge both pyridine and *N,O*-bis-(Trimethylsilyl)Trifluoroacetamide with 1% TMCS with nitrogen for storage
18. Purge glass vials with nitrogen and keep them at 70°C for 3 hrs
19. At this point, remove the samples, evaporate them to dryness and add approximately 1 mL of hexane.
20. New filter and using new Pasteur pipettes each time to avoid contamination.
21. Prior to gas chromatography, the sample is evaporated to dryness under nitrogen to then be redissolved in 1 mL hexane for injection.
22. After the sample is run, the vial can be durasealed to be kept for later analysis, or if the sample needs to be run again after a couple days and the hexanes have evaporated, it can be dried under nitrogen, and another 1 mL hexane can be added, so that the volume (and the concentration) will be reliable.

Appendix E: Photobioreactor Set-up

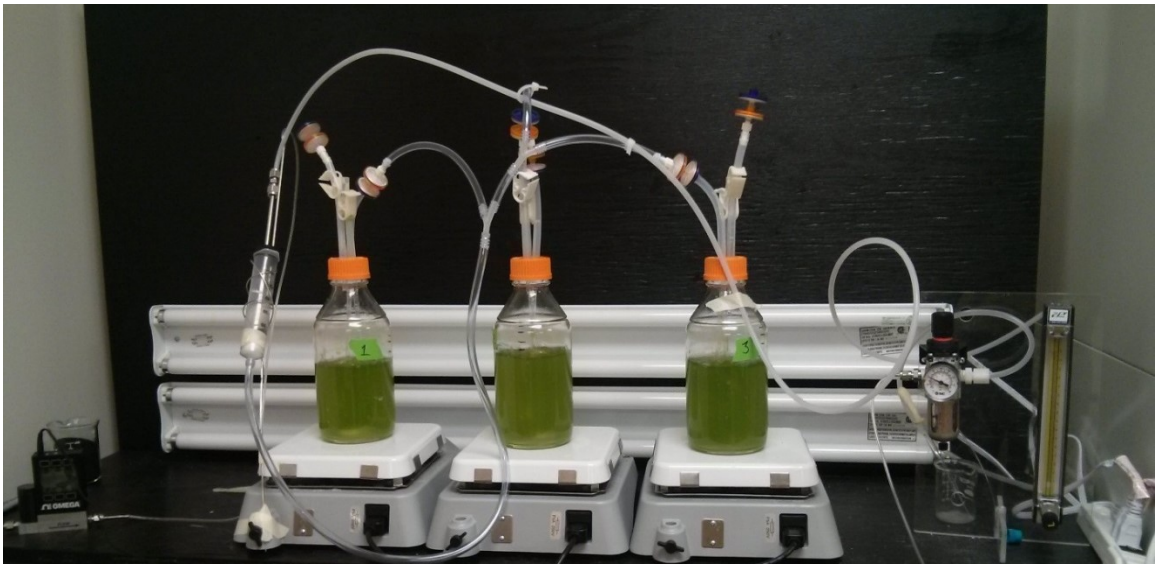

Figure E-1: Photobioreactor set-up for autotrophic cultivation condition experiments

Appendix F: Calibration Curves for Carbohydrate Analysis

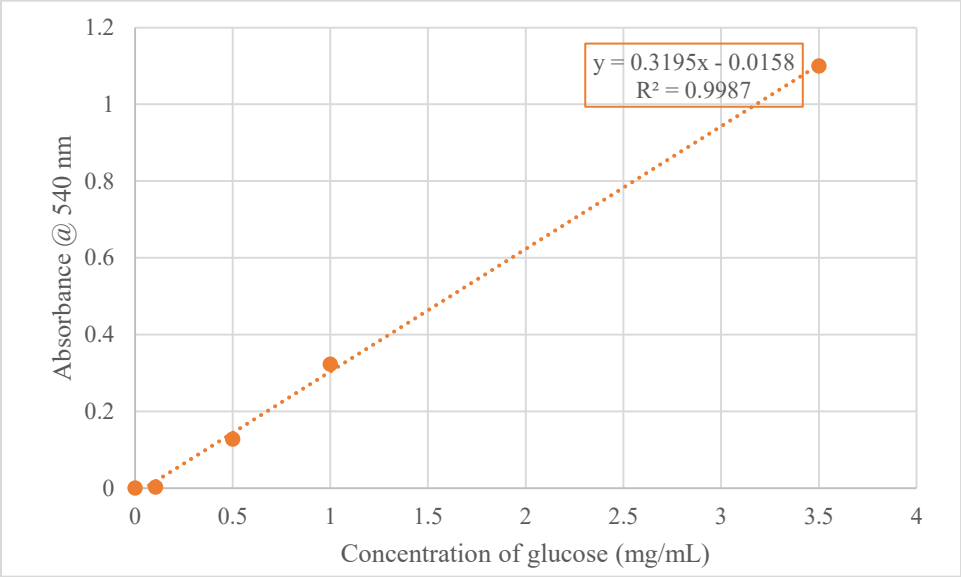

Figure F-1: Glucose calibration curve prepared using DNS colorimetric method at an absorbance of 540 nm

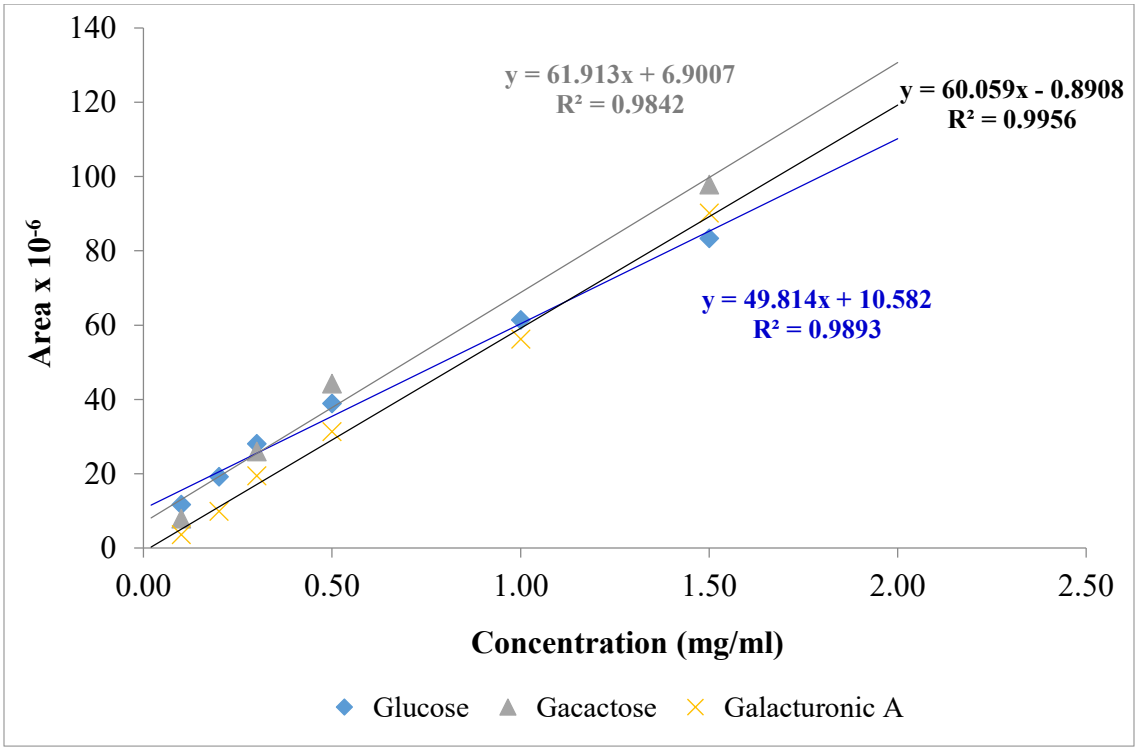

Figure F-2: Standard curves for sugars commonly found in *T. suecica* (glucose, galactose, galacturonic acid) under a 5:1 split ratio

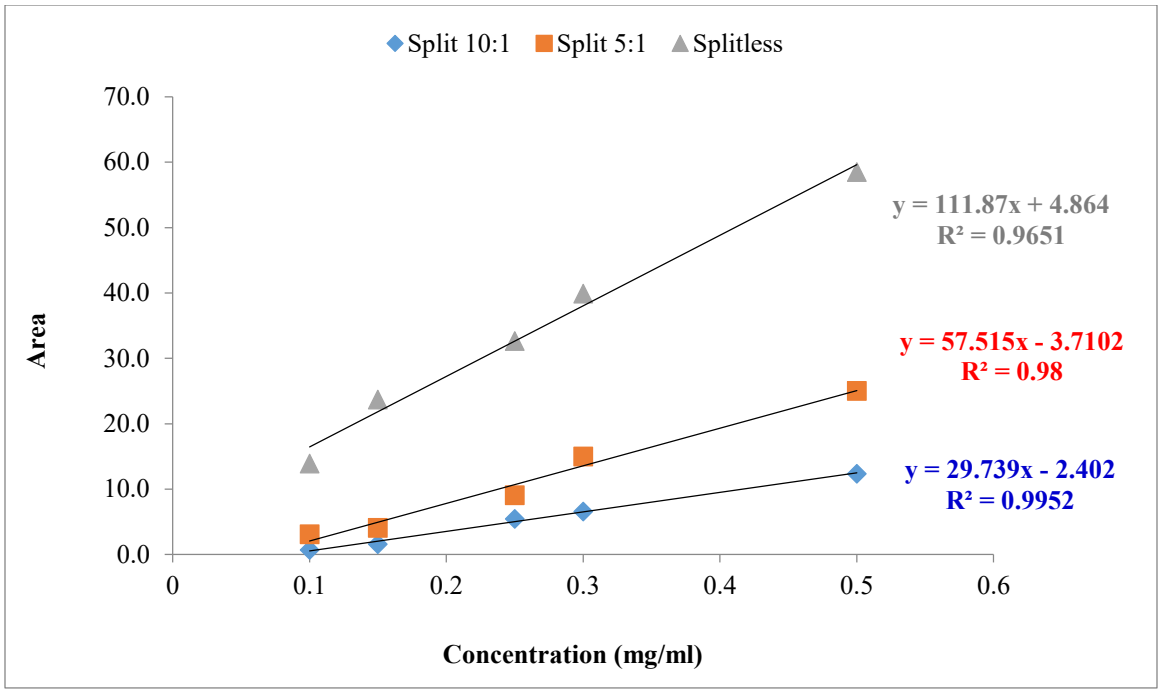

Figure F-3: Standard curves for KDO in T. suecica under different split ratios