NUTRIENT RECYCLING OF SPENT BIOMASS AND LIPID-EXTRACTION WASTEWATER IN THE PRODUCTION OF THRAUSTOCHYTRIDS

by

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ABSTRACT

One of the most important challenges for the emerging sector of microalgae biotechnology is reducing the production costs by improving the efficacy of resource use, while minimizing environmental repercussions of the process. An avenue for accomplishing this goal is implementing a novel strategy of nutrient recycling, whereby the effluent wastewater produced is recycled internally for subsequent cultures. This concept is the foundation of this thesis where the prospect of nutrient recycling, and all of the corollaries associated with it, are explored in the context of the production process at Mara Renewables Corporation. Due to the vast majority of valuable nutrients residing in the lipid-extracted hydrolysate, that liquid waste material was the primary focus of the secondary fermentations conducted. Initial flask-scale secondary fermentations revealed that *Thraustochytrium* sp. T18 could achieve improved levels of biomass and lipid production in recycled hydrolysate with full media supplements (20.48 g cells L^{-1} and 40.9% (w/w) lipid) compared to the control (13.63 g cells L^{-1} and 56.8% (w/w) lipid). Furthermore, results indicated that the proteinaceous content of the recycled hydrolysate can offset the need to supply fresh nitrogen in a secondary culture, without any detrimental impact upon the produced biomass. In fact, the experiments employing the recycled hydrolysate with no nitrogen addition accumulated 14.86 g L^{-1} of biomass in 141 hours with 43.3% (w/w) lipid content in comparison to the control which had 9.26 g L^{-1} and 46.9% (w/w), respectively. Using the protocols developed in the flask experiments the nutrient recycling strategy was then applied to larger, laboratory bench top fermentors. In recycled hydrolysate, T18 amassed 63.68 ± 1.46 g L^{-1} of biomass with 50.8 ± 1.20% (w/w) of lipids compared to 58.59 g L^{-1} of biomass and 69.4% (w/w) in fresh media. When recycling the same hydrolysate for a tertiary fermentation, biomass reached 65.27 ± 1.15 g L^{-1} in 90 hours and 62.2 ± 0.3% (w/w) of lipids. The positive findings for nutrient recycling are an important opportunity that can aid in mitigating waste production and reducing costs as heterotrophic microalgae production progresses toward commercialization.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ATP	Adenosine triphosphate
BOD	Biochemical oxygen demand
CO_2	Carbon dioxide
COD	Chemical oxygen demand
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FAA	Free amino acids
FAME	Fatty acid methyl ester
FID	Flame ionization detector
FMOC	9-fluorenylmethyl chloroformate
$g\ L^{-1}$	Grams per liter
GC	Gas chromatography
GCR	Glucose consumption rate
GHG	Greenhouse gas
HCl	Hydrochloric acid
HMF	5-hydroxymethylfurfural
HPLC	High performance liquid chromatography
HTL	Hydrothermal liquefaction
LCA	Life cycle analysis
NaOH	Sodium hydroxide
NIR	Near infrared
ºC	Degrees Celsius
OPA	o-phthalaldehyde
PUFA	Polyunsaturated fatty acids
RID	Refractive index detector
SEM	Scanning electron microscopy

SPE	Solid phase extraction
T18	*Thraustochytrium sp.* T18
TAG	Triacylglycerol
TFA	Total fatty acids
TKN	Total Kjeldahl nitrogen
TOC	Total organic carbon
TS	Total solids
TSS	Total suspended solids
USD	United States dollars
VS	Volatile solids
$Y_{X/S}$	Cell yield
β-carotene	Beta carotene

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CHAPTER 1: INTRODUCTION

Current research areas pertaining to microalgae production are deeply varied in focus, but generally emphasize reducing production costs or improving product value in an effort to reach profitability. The preeminent interest in microalgae biotechnology remains harvesting the cellular lipids for biofuels and high-value oils (Chang et al., 2014; Lee Chang et al., 2013; Liang, 2013; Singh et al., 2011). It has been repeatedly demonstrated that an attractive avenue for maximizing the yield of microalgae oil, as a function of cell productivity and oil content, is by employing one subcategory of microalgae that undergo non-photosynthetic metabolism. These heterotrophic species are characteristically hyper-productive and can be produced using well-established industrial fermentation technology (Bumbak et al., 2011; Chang et al., 2014; Perez-Garcia et al., 2011). One specific heterotrophic microalgae, and the focus of this project, is the novel *Thraustochytrium sp.* (T18) that was isolated from the coastal waters of Nova Scotia, Canada (Burja et al., 2012, 2006).

Thraustochytrids are a group of heterotrophic microalgae that are commonly found in coastal marine environments that are characterized by high concentrations of decomposing organic material (Bongiorni, 2012). They are specifically attractive due to their capacity to produce tremendous amounts of biomass and cellular lipids, namely the high-value polyunsaturated fatty acids (PUFA). Owing their desirable attributes to their ecological role as primary producers and decomposers of organic matter, these versatile microorganisms can thrive in diverse conditions (Lee Chang et al., 2014; Liu et al., 2013; Raghukumar, 2008). A few research groups have spent considerable time and effort researching select thraustochytrids for the production of omega-3 fatty acids. This investment has yielded some of the most productive microalgae reported to date (Lewis et al., 1999; Perveen et al., 2006; Raghukumar, 2008; Yaguchi et al., 1997). The propensity for synthesizing large quantities of valuable fatty acids only further illustrates the commercial potential of these microalgae.

All microalgae production projects have struggled with the high costs and enormous resource demands of producing the oil-rich biomass (Chisti, 2013; Sun et al., 2011). This is particularly pronounced in heterotrophic conditions where instead of using

free sunlight and carbon dioxide, the cells require a significant input of organic carbon (i.e. sugar). Additionally, the rapid growth rates correspond to a voracious demand for nutrient inputs. Collectively, those media costs constitute the largest estimated expense in heterotrophic microalgae production (Liang, 2013; Yan et al., 2011). Furthermore, even in high density cultures, up to 85% of the final culture is water. After product extraction and recovery, this tremendous volume of residual water, now laden with unused nutrients and cell debris, represents a potential hazard to receiving water bodies or treatment facilities.

The overall **goal of this project** is to explore nutrient recycling as an emerging technique to improve the resource usage efficiency of microalgae production at Mara Renewables Corporation (Mara), where waste materials leaving the microalgae production system are captured and recirculated for use as a growth substrate for secondary culture. In doing so, water and residual nutrients are conserved, thereby aiding in reducing the raw material inputs and costs of microalgae production. Each microalgae production system employs a specific arrangement of species, production system, production conditions and extraction method – all of which have an influence of the viability and logistics of nutrient recycling. This project focuses on Mara's work with T18 cell biomass produced in glucose fed-batch fermentations with an enzymatic cell lysis and lipid extraction. The **specific objectives** for investigating this research goal are to:

i. Characterize the physicochemical composition of the waste products, both solid and liquid, remaining after enzymatic lipid-extraction processing of primary thraustochytrid fermentations;
ii. Determine suitability of waste products from lipid-extraction processes as a potential nutrient source for secondary applications;
iii. Develop a protocol for reusing spent media and hydrolysate from the lipid-extracted waste material in secondary fermentations and determine if supplementation with additional media elements (specifically carbon or nitrogen sources) is necessary;
iv. Develop a scaled-up protocol for use with commercially representative production vessels using recycled waste materials in secondary fermentations;
v. Evaluate the effect of multiple sequential recycles of the waste material in secondary fermentations using automated laboratory bench top fermentors.

To sufficiently explore the aforementioned project goal, this thesis will cover the following series of topics with disclosure of the experimental work and the resultant outcomes. In **Chapter 2**, there is an introduction to the topic and an extensive evaluation of the current literature related to this research area. This literature review contains detailed information on microalgae production and challenges associated with cost, which transitions into discussing highly productive species and the potential for nutrient recycling to reduce costs.

Chapter 3 covers the first experimental stage centered on the production of the waste material used throughout the subsequent recycling experiments, as well as characterization of the important physicochemical parameters of those materials. This section provides pertinent information about the waste materials and analytical methods that will be used throughout the following chapters.

In **Chapter 4,** initial experimentation involving secondary fermentations of the recycled materials was conducted using small-scale growth studies in flasks. These studies establish feasibility and suitable protocols for nutrient recycling in this system, followed by some exploratory optimization of nitrogen provisions supplied to the secondary fermentations.

In **Chapter 5**, the techniques developed in Chapter 4 were further developed for increasing applicability to larger-scale, in laboratory bench top fermentors, which are similar to large scale commercial vessels. The experiments in this chapter provide sufficient basis for implementation of a nutrient recycling program in the production schematic currently used at Mara. In addition, subsequent recycling was explored to investigate the potential for repeated reuse of the waste materials.

Chapter 6 provides a summary of the work in this thesis with conclusions and recommendations for further research.

CHAPTER 2 : LITERATURE REVIEW

Sections of this chapter are derived from the published manuscript:
Lowrey, J., Armenta, R.E. and Brooks, M.S. (2015) 'Nutrient and Media Recycling in Heterotrophic Microalgae Cultures', Applied Microbiology and Biotechnology 100, 1061-1075. doi:10.1007/s00253-015-7138-4

2.1 INTRODUCTION

The purpose of this literature review is to provide the reader with a foundation of information relevant to the topics covered in the thesis. This section begins with some basic context to microalgae production and the significance of researching renewable energy sources. Some potentially attractive commercial applications for microalgae biomass that may contribute to improving production economics are then highlighted. A discussion of the challenges to microalgae production follows, and some attractive options for reducing costs are explored, which directs the discussion toward employing highly-productive heterotrophic microalgae (thraustochytrids). This chapter concludes by introducing the concept of nutrient recycling in heterotrophic cultures which is further explored in the subsequent experimental chapters throughout the remainder of this thesis.

2.2 MICROALGAE PRODUCTION

2.2.1 MICROALGAE AND THEIR ENVIRONMENT

Microalgae, in this case broadly defined to include over 35,000 species of phytoplankton and cyanobacteria, are a core foundational component to aquatic ecosystems (Borowitzka and Moheimani, 2012; Graham and Wilcox, 2000). These organisms have an essential role in nutrient cycling and energy conversion to sustain the complex network of organisms in their environment and beyond. Specifically, microalgae have a meaningful influence on global cycling of nitrogen and carbon (Graham and Wilcox, 2000). Accordingly, the impact of resource consumption and pollution from human society has enhanced the prevalence of microalgae in adjacent water bodies (Burkholder et al., 2008). This concept is evidenced by the occurrence of harmful algal blooms in waters receiving large concentrations of runoff pollutants. These blooms are

massive growths of algal biomass in response to an abundance of nutrients in the water, and effectively serve to sequester the nutrients into plant biomass. While harmful in these massive concentrations, the microalgae will normally provide a food source to higher order organisms, thereby passing the sequestered nutrients along the food chain (Graham and Wilcox, 2000).

Microalgae typically exhibit exceptional reproduction rates and possess an extremely dexterous metabolism due to species diversity and the evolutionary pressure of their natural environments and available substrates. Although most microalgae are photosynthetic, meaning they derive energy from solar radiation and carbon from CO_2, some other species derive carbon and energy from organic material present in water, without the requirement for light. This adaptation is particularly useful to bottom dwelling, detritivorous microalgae, such as thraustochytrids (Bongiorni, 2012). The innate capability of microalgae to use various carbon forms and nutritional substrates, as well as tolerating extreme environmental conditions, positions them in a pivotal role of energy conversion and nutrient cycling (Barsanti and Gualtieri, 2006). This ecological position encourages microalgae to accumulate large concentrations of stored energy, in the form of intracellular lipids, as well as valuable pigments and organic compounds. These compounds, concentrated within the algal cells, then serve as essential nutrients for higher order organisms. For example, the commercially desirable pink color of salmon is largely due to the accrual of astaxanthin from crustaceans, sourced originally from photosynthetic microalgae (Del Campo et al., 2007; Olaizola, 2003).

The propensity of microalgae to concentrate large amounts of valuable intracellular products is, as already stated, a product of their ecological role, but is also what makes these organisms appealing for research and development. Exploiting the natural ability of microalgae to produce useful materials for human consumption, and eliminating the intermediary network of ecosystem dynamics, presents a great opportunity for commercial applications. Successfully doing this requires careful investigation into optimizing growth rates and product yields, as well as maximizing efficiency of resource use and mitigating any adverse environmental repercussions of this technology.

2.2.2 A Brief History of Microalgae Production

The current prospect of widespread commercialization of microalgae-based bioproducts is indicative of the robust interest in research and development of the technology which owes its roots to decades of scientific inquiry. Some fundamental research on harvesting oils from mass-cultures of microalgae dates back to the 1930s and 1940s, which was a direct result of the realization of the strategic importance of alternatives to petroleum after WWII (Borowitzka and Moheimani, 2012). In the years following, focus shifted to alternative bioproducts derived from microalgae biomass, as well as the application of microalgae cultures for wastewater treatment (Borowitzka and Moheimani, 2012; Oswald and Gotass, 1957). However, in the 1970s when petroleum supply constraints reignited interest in alternative energy sources, substantial research focus returned to harvesting biofuels from mass-microalgae cultures. This trend was epitomized by the creation of the United States Department of Energy's Aquatic Species Program in 1978, operating under the directive to investigate the potential of using aquatic plants to provide renewable fuels to replace petroleum (Sheehan et al., 1998). In addition, significant research was executed in Australia, Europe, Japan, and Israel on various subjects including high-density cultures, extreme environments, bioreactor design, and nutritional bioproducts (Borowitzka et al., 1977; Borowitzka, 1980; Boussiba et al., 1987; Soeder, 1980). Collectively, this foundation of research enabled a surge in investigative interest in microalgae production technology in the last couple of decades.

2.2.3 Current Trends

The wide array of subjects within the algal biotechnology research sector is difficult to illustrate, much less quantify. Research topics include (but are not limited to) fundamental science in strain identification and isolation, gene profiling, algal physiology and metabolic pathway modelling (Burja et al., 2006; Wang and Chen, 2008; Yang et al., 2000). More applied research focuses on growth performance, alternative substrate usage, waste integration, large-scale studies and various economic analyses (Fu et al., 2012; Gallagher, 2011; Tabernero et al., 2012; Wu et al., 2013). With the diversity of applications for microalgae production technology there are many researchers contributing valuable information across many sectors. The focus of this work narrows the relevant research

trends to those pertaining to commercialization of microalgae production technology for biofuels.

An important area to observe in current research relates to the improvements being made to microalgae productivities in laboratory and large-scale studies. Here, it is important to distinguish between the two major metabolic states for microalgae cultivation: photoautotrophy and heterotrophy. Heterotrophic microalgae cultivation, as opposed to the traditional photoautotrophic (photosynthetic) metabolism, employs species that are capable of utilizing organic carbon for energy and cell synthesis, rather than carbon dioxide and light (Fig. 2.1-2.2).

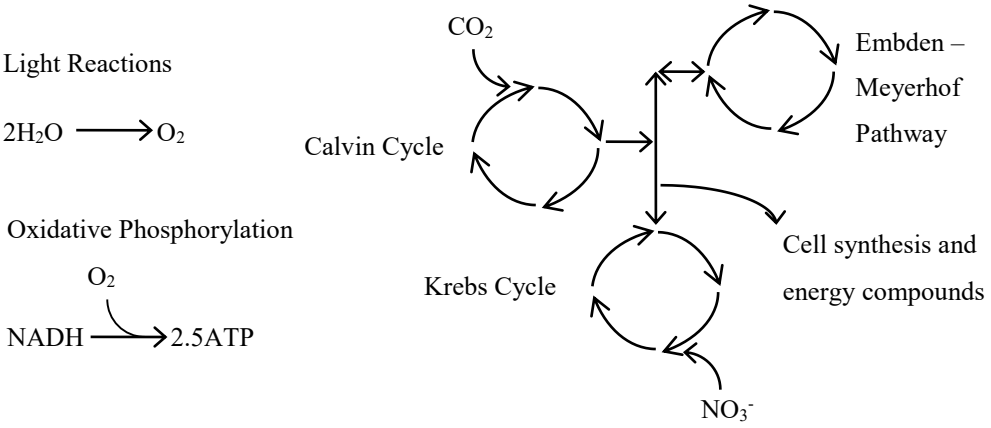

Figure 2.1 Generalized metabolic pathways for autotrophic microalgae growth. Source: Yang et al., 2000; Perez-Garcia et al., 2011.

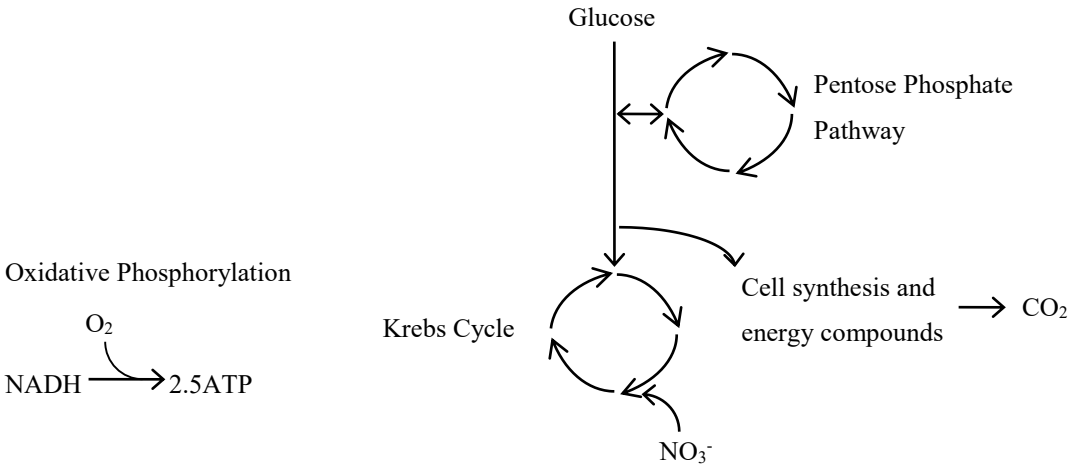

Figure 2.2 Generalized metabolic pathways for heterotrophic microalgae growth. Source: Yang et al., 2000; Perez-Garcia et al., 2011.

While numerous studies over the last 20 years have observed modest culture densities (0.5 g L^{-1} to 2 g L^{-1}), novel applications of heterotrophic strains and fed-batch growth systems have substantially improved the concentrations of biomass (>100 g L^{-1}) (Bohutskyi et al., 2014a; Lewis et al., 1999). These achievements, if verified at large-scale, may significantly increase the economic viability of the industry. In parallel to enhanced biomass productivity, the topic of preserving or increasing bioproduct concentrations is imperative. Contrary to the conclusions of the U.S. Aquatic Species Program, which suggested the conditions to promote rapid growth and lipid accumulation are "mutually exclusive", some research has observed attractive productivities of biomass and bioproducts (Bohutskyi et al., 2014a; Borowitzka and Moheimani, 2012; Doucha and Lívanský, 2012; Graverholt and Eriksen, 2007; Sheehan et al., 1998). This desirable combination of high productivity biomass and bioproducts is extremely favorable and will require further research and validation. Further, if the performance attained in these exemplary studies is due to unique strain characteristics or genetic engineered traits, a consolidation of viable research groups or companies with patent rights around such organisms may be observed in the coming years.

2.3 TARGET INDUSTRIES

Numerous potential industries have been proposed for commercial application of mass-produced microalgae biomass; each requiring different levels of processing to obtain the target product. Raw biomass can be used in many feed-based applications where the natural accumulation of protein, pigments, or oils is essential to the consumer market. For more specialized applications, extraction and concentration of the desired product is required, which adds to the operational costs. Consideration of the target product and industry is a vital element to logistical and feedstock designs, and in some cases, these feedstock concerns dictate the potential product options. This is well exemplified by integration of wastewater into production systems as a source of nutrition. Doing so gives rise to many regulatory and health concerns and effectively eliminates feed-based applications of the biomass and products.

Product flexibility (i.e. the ability to switch primary product for market dexterity) and co-product retrieval can also be important improvements to production schematics. In the lipid-focused industries, the target product can vary between targeting specific high-value polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid (DHA) or triacylglycerol (TAG) for transesterification to biodiesel (Armenta and Valentine, 2013; Demirbas and Demirbas, 2011). Using this advantageous flexibility can enable a commercial algae producer to coordinate feedstock logistics and production conditions with product demand and price changes. Furthermore, co-products such as proteins and pigments can present a valuable addition to the overall biomass utility for lipid-based applications.

2.3.1 BIOFUELS

Biofuels are a renewable energy resource derived from organic matter known as biomass, which can originate from many sources (Chisti, 2007). When looking specifically at fuels for the transportation sector, the term biofuels is generally restricted to bioethanol and biodiesel, although many other fuel types are available. Bioethanol (CH_3CH_2OH) is a renewable substitute for gasoline and is primarily derived from yeast fermentation of sugary biomass, while biodiesel replaces petroleum-based diesel and is sourced from bio-oils and fats (Hinrichs and Kleinbach, 2006). These alternative fuels markets have

increased steadily for decades, largely due to increases in petroleum prices (Ragauskas et al., 2006; USEIA, 2012). This demand is particularly pronounced for biodiesel, which has exhibited strong growth in consumption over the last decade worldwide (Fig. 2.3). In fact, year over year, global consumption has increased by an average of 37.2% between 2000 and 2011 (USEIA, 2014). In the United States, the primary biodiesel feedstock is soybean oil, which is complicated by requiring large areas of agricultural land and competing with human food resources (Chisti, 2007; USEIA, 2012). Even more, as demand continues to increase, diversifying feedstocks will be essential to maintaining price stability and fulfilling demand. Alternative biofuels feedstocks are abundant but many possible resources are plagued by high costs, food shortage concerns or technological deficiencies.

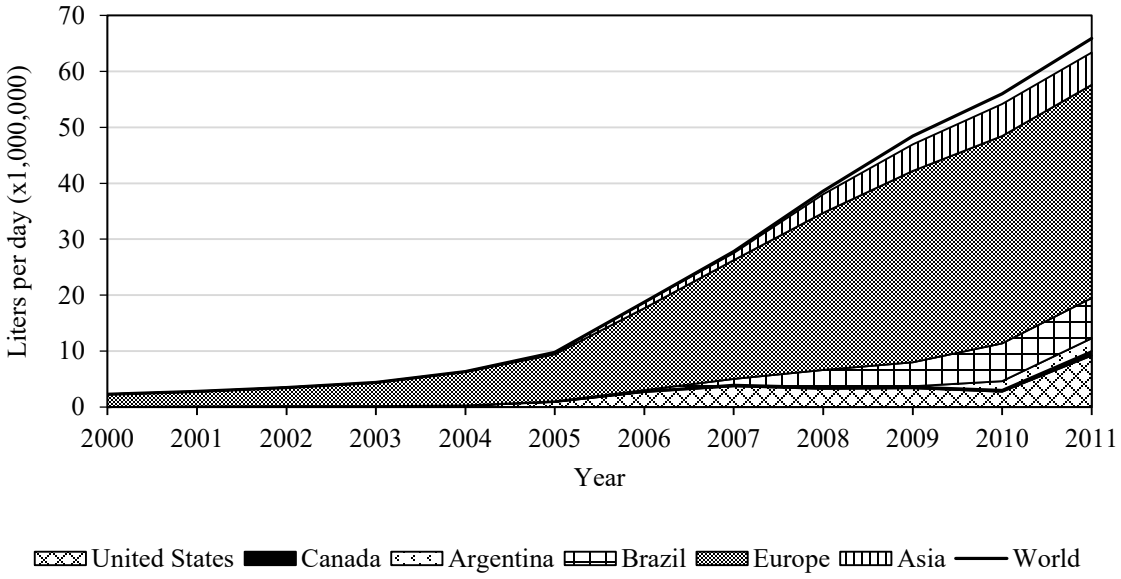

Figure 2.3 Biodiesel consumption from 2000-2011 in major markets worldwide (USEIA, 2014).

Microalgae have long been a promising potential source of renewable liquid fuels. This potential is attributable to many desirable characteristics including rapid growth rates and a predisposition for accumulating large quantities of intracellular oil. Furthermore, microalgae are not a major human food resource, therefore allocating microalgae resources toward biofuel production would not threaten existing food sources as is the case with soybean and sugarcane. The primary market of interest for the microalgae biomass is

production of biodiesel, derived from a relatively simple chemical conversion of naturally occurring oil products within the cells (Pienkos and Darzins, 2009). Once harvested, the microalgal oil can be extracted from the cells and converted to biodiesel. As shown in Fig. 2.4, this conversion process, known as transesterification, takes the triglycerides generated in the cells and cleaves the glycerol in the presence of methanol and a catalyst, resulting in methyl esters and a glycerol byproduct (Demirbas and Demirbas, 2011; Mata et al., 2010).

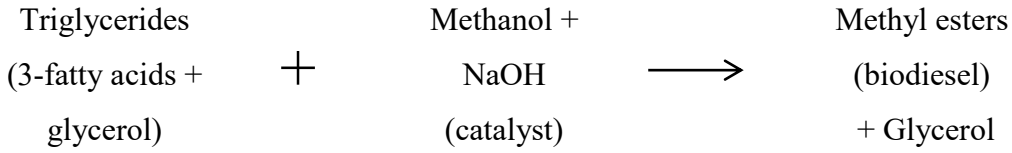

Figure 2.4 Methanolic transesterification of algal triglycerides to produce biodiesel.

The focus on biodiesel production from microalgae biomass generated a strong interest in identifying and enhancing the species with the greatest tendency to accumulate lipids. This trait varies tremendously between species and cultivation conditions, but tends to range between 15-40% for photoautotrophic microalgae and up to 70-80% for heterotrophic microalgae (Chisti, 2007; Demirbas and Demirbas, 2011; Liang, 2013). Further, advances in techniques for strain selection and highly controlled culture conditions have enabled researchers to reliably produce highly concentrated biomass. Not surprisingly, research on commercialization for biofuel tends to focus on the high-productivity oleaginous strains (see Table 2.1).

Table 2.1 Select microalgae species and their corresponding intracellular lipid contents (Demirbas and Demirbas, 2011).

Microalgae	Oil content (wt% of dry basis)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

2.3.2 NUTRACEUTICAL PRODUCTS

Additional high value bioproducts that can be derived from microalgae biomass are an array of pigments, nutritional oils, and proteins – collectively referred to here as nutraceutical products. To date, microalgae production at commercial scale has mostly been limited to production of high value nutraceutical products such as β-carotene and astaxanthin (Brennan and Owende, 2010; Del Campo et al., 2007). These specific bioproducts require production of a select microalgae biomass and extraction of the desired product. The resultant product, however, is a high purity, natural product that can be sold for significantly greater prices than would be obtained for biofuels (Brennan and Owende, 2010). Alternatively, whole algal biomass, typically in the form of *Chlorella* and *Spirulina,* has also become more prevalent in health food products such as fruit smoothies and supplements. In comparison to biofuel-targeted production, this whole-cell product stream allows for minimizing post-production processing requirements, thereby minimizing costs.

Commercial production of pigments from microalgae biomass has been successful in multiple locations in Australia, China, Israel, Japan and the United States (Del Campo

et al., 2007). These products are particularly attractive because they can be produced in outdoor systems using abundant sunlight with minimal operational expenses. β-carotene and astaxanthin are common pigments produced due to their known benefits to human health including decreasing risk of cancers and age-related disorders (Armenta et al., 2006; Olaizola, 2003). These beneficial characteristics also contribute to the very attractive prices (USD 2,500 kg^{-1} astaxanthin in 2008) attained for naturally sourced, pure products. Furthermore, the global market for astaxanthin alone was projected to reach USD 257 million in 2009 (Cysewski and Lorenz, 2008; Del Campo et al., 2007). This combination of market opportunity and high prices encourages the exploration of additional sources of nutritional pigments such as microalgae.

Multiple microalgae species are known to accumulate large concentrations of β-carotene or astaxanthin. *Dunaliella salina* has been produced for decades in highly saline outdoor ponds in Australia for β-carotene. The combination of high temperature, high solar input and saline ponds enables the microalgae to thrive and accumulate pigments without significant contamination concerns (Borowitzka, 1980). *Chlorella* has been produced both as whole-cell biomass for nutrition and as a targeted producer of astaxanthin in advanced heterotrophic cultures (Borowitzka, 1999; Iwamoto, 2008; Wang and Chen, 2008). To date, *Chlorella* remains the most extensively researched microalgae species due to its robust growth and high productivity. *Haematococcus pluvialis* production originally centered on aquaculture feed due to the contribution of the ubiquitous pink color of salmon from astaxanthin-rich diets (Cysewski and Lorenz, 2008; Del Campo et al., 2007). Naturally, the astaxanthin accumulation in *Haematococcus* made it an attractive species for the production of nutraceuticals for direct human consumption (Brennan and Owende, 2010).

Another very important and emerging nutraceutical product derived from microalgae biomass are nutritional oils, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These two nutritional oils are classified within the popular omega-3 fatty acids, which are increasingly demanded in consumer market. The health benefits of nutritional oils include reduced risk of macular degeneration, improved blood vessel flexibility, improved cold resistance in pregnant mothers and treatment of various diseases like schizophrenia and multiple sclerosis (Armenta and Valentine, 2013). Traditionally, omega-3 fatty acids are derived from fish oil, which is subject to supply

fluctuations and corresponding price spikes. To meet increasing market demand and achieve supply stabilization, alternative sources for such fatty acids are desirable. Single-celled microorganisms commonly accumulate very large quantities of intracellular oils, including the omega-3 fatty acids. Further, the fatty acid profiles tend to be simpler than those derived from fish oil, resulting in fewer impurities associated with unpleasant odors and flavors (Fan and Chen, 2011).

One commercially attractive group of microorganisms particularly adept at accumulating DHA and EPA are thraustochytrids. These single-celled marine protists commonly accumulate large portions of these specific fatty acids, sometimes in excess of 50% of the cell weight (Burja et al., 2006; Yang, 2011). Some of the most commercially attractive thraustochytrids include *Schizochytrium* sp. and genetically modified variants of *Thraustochytrium* sp. (Armenta and Valentine, 2013; Chang et al., 2012). In both species, values have been reported with total oil contents of 70-77% of cell weight and around 37% of that comprised of DHA (Burja et al., 2006; Lewis et al., 1999; Scott et al., 2011). The great potential of harnessing the ability of select microalgae to accumulate these nutritional oils has been recognized by multiple large companies trying to optimize the technology (Scaife et al., 2015).

2.3.3 PROTEIN FEED

Typical microalgae biomass has a significant protein composition (up to 71%) that can have valuable applications as animal and human feed supplements (Becker, 2007). These applications of microalgae biomass are among the oldest and most commercially lucrative despite the stringent regulatory considerations for human nutrition (Brennan and Owende, 2010; Mata et al., 2010). However, the success of microalgae protein additives for human consumption is mostly limited to health food markets and has certain limitations due to concerns regarding color, taste, and smell that are caused by non-protein constituents of the biomass (Becker, 2007). When whole biomass is employed rather than protein extract, there are many beneficial nutritional compounds beyond the protein (e.g., carotenoids, nutritional oils). This whole biomass presents a challenge due to the protective cell wall, which can inhibit bioavailability of the protein due to poor digestibility compared to traditional protein sources (Becker, 2007). To mitigate the challenges with undesirable

qualities many methods have been developed to extract and isolate the protein from the microalgae biomass, thereby making it a more viable protein source for human nutrition (Schwenzfeier et al., 2011).

Estimates suggest that 30% of microalgae production is dedicated to animal feed supplements (Becker, 2007). Specifically, microalgae biomass has long been a primary source of nutrition in aquaculture industries. This arrangement simulates the natural ecosystem dynamics that would place microalgae as primary producers and a food source for lower order marine organisms. Using microalgae biomass as a feed source in aquaculture also allows customization of specific nutritional properties of the feed by species selection (Borowitzka, 1997). Additionally, specific compounds like astaxanthin can be produced in high concentrations within the cells resulting in a high-value aquaculture product (Brennan and Owende, 2010). It is important to note that aquaculture operations typically use the whole cell biomass rather than extracts, which may decrease the attractiveness for co-production applications.

Experts suggest that recovering microalgal protein after lipid extraction for biofuels could serve as a valuable co-product to assist in production profitability (Brennan and Owende, 2010; Wijffels and Barbosa, 2010). The co-production model maximizes the utility of the microalgae biomass and enhances the revenue generation via multiple marketable bioproducts. However, successfully extracting and isolating multiple product streams from a single microalgae biomass requires careful cell disruption and use of specific chemicals that are compatible with nutritional applications, without destroying the target proteins (Wijffels and Barbosa, 2010). It is important to note that the production of lipids or proteins, and the enhancement of one of those corresponding cellular fractions, typically results in the reduction of the other. This dichotomy would likely lead to co-production being a secondary consideration for commercial producers of microalgae biomass.

2.4 COMMERCIALIZATION CONSTRAINTS

Despite the numerous potential applications for microalgae biomass and the significant progress that has been made improving productivity and yields, there still exist major impediments to commercialization of the technology when intended for use as a

renewable biofuel feedstock. Multiple economic analyses have concluded that in order for prices to be competitive with petroleum fuels, further research and development in the areas of productivity improvement, waste stream integration, waste carbon dioxide usage and inexpensive oil extraction are required (Chisti, 2013; Norsker et al., 2011; Singh et al., 2011; Sun et al., 2011). Alternative applications of microalgae biomass, particularly in the nutritional markets, also struggle with high production cost due to the purity requirements of process feedstocks and aseptic culture conditions. As previously mentioned, these industries are currently the only successful applications of microalgae biomass at commercial scale (Del Campo et al., 2007). However, as increasing supply enters the nutritional markets, it is imperative for research to improve production economics by reducing the demand for expensive material inputs.

2.4.1 Feedstock Requirements

Feedstocks are defined as the raw material inputs that are required for an industrial process. In microalgae biotechnology, there are a few key feedstocks required for production of useful and abundant biomass. In photoautotrophic (photosynthetic) microalgae cultures, the major required inputs are water, light, nutrients, and carbon dioxide (Lee, 2008). Nutrients and carbon dioxide (CO_2) need to be imported from external sources, many times coming from pure sources which correspond to higher costs. Alternative heterotrophic (fermentation) microalgae cultures do not require light or carbon dioxide, but demand organic carbon which is typically provided in the form of pure glucose. In either metabolic arrangement, the demand for raw material inputs represents an important cost to commercial producers.

Pate et al. (2011) conducted a comprehensive assessment of resource requirements for commercial scale, autotrophic microalgae production for biofuels in the United States and concluded that carbon dioxide and nutrients, in the form of nitrogen and phosphorus, were the most limiting factors to financial viability. Carbon dioxide can contribute up to 50% of the cost of producing microalgae biomass, therefore making it prohibitively expensive unless obtained at minimal cost (Chisti, 2013). Although carbon dioxide is increasingly a greenhouse gas of concern, due to excessive atmospheric concentrations, single-point sources of sufficient purity are not available for microalgae co-production in

quantities sufficient to substantially replace fossil fuels (Chisti, 2013; Pate et al., 2011). Single-point sources, such as coal-fired power plants, could theoretically provide convenient sources of abundant carbon dioxide, however these are still insufficient for generating meaningful quantities of microalgae (Chisti, 2013). Of those single-point sources that are available, there are also concerns with additional compounds present in the flue gases as they can have potentially toxic effects upon the microalgae culture (McGinn et al., 2011). It is also worth considering that for effective uptake of waste carbon dioxide resources, the microalgae production facility would also have to be located adjacent to the emission source, thereby potentially diminishing any advantages of strategic facility placement.

Nutrient requirements for microalgae culture can be met from many substrates, however they must contain sufficient quantities of nitrogen, phosphorus, and a suite of secondary nutrients (Lee, 2008). In laboratory studies and aseptic nutritional applications, it may be necessary that these nutrients are provided by raw chemical sources. In one economic assessment, the cost of the culture media that contains the nutrients was estimated to account for approximately 35% of the biomass production costs (Molina Grima et al., 2003). When considered at commercial scales capable of supplanting a large percentage of fossil fuels, this demand of nutrients could perilously compete with agriculture for fertilizers (Chisti, 2013; Pate et al., 2011). However, these nutrients can also be obtained from various agricultural and industrial waste streams which can contain significant concentrations of nutrients at little or no cost (Chisti, 2013). Employing wastewaters for microalgae culture does, however, introduce regulatory and toxicity concerns. Furthermore, the supply of wastewater may not be reliable or consistent in composition, possibly resulting in varied productivities.

Heterotrophic microalgae, which are attractive because of their tendency toward higher productivity, have different feedstock requirements than photoautotrophic microalgae. In heterotrophic cultures of microalgae there is the added requirement of providing an organic carbon source to offset the need for light and carbon dioxide. This organic carbon source is commonly provided by the universal carbon source of many microorganisms, glucose (Perez-Garcia et al., 2011). Typically heterotrophic cultures exhibit higher growth rates and oil content, but the added glucose requirement introduces

a substantial cost to commercial-scale production. One estimate suggests that the cost of glucose in a medium for *C. protothecoides* represented up to 80% of the total media cost (Li et al., 2007). Any potential avenue for minimizing or eliminating this substantial feedstock cost would be paramount to the economic viability of commercial microalgae production. There are many possible alternative sources of organic carbon derived from waste material or lower cost sugars, however these must be explored in detail for each microorganism of interest (Lowrey et al., 2014).

2.4.2 Resource Demands

Expanding the production of microalgae systems to meet any significant portion of biofuel demand will require the appropriation of immense resources beyond the aforementioned feedstocks. These resources include water, land and energy and vary in intensity depending upon the production conditions. The principal natural resource of concern is the demand for water, especially in the case of operations using freshwater microalgae. Considering that reported biomass concentrations rarely exceed 150 g L^{-1} (85% water), and are much more likely in the range of 10 to 20 g L^{-1} (99% and 98% water, respectively), the water volume required is considerable (Brennan and Owende, 2010). Accordingly, one study determined that in order to produce 220×10^9 L yr^{-1} of microalgae oil it would require three times the amount of water used for irrigated agriculture in the U.S. (Wigmosta et al., 2011). These figures translate to roughly 1,400 liters of water required per liter of algae oil produced. Another study estimates the evaporative loss of water, to maintain production volumes, to account for 900 - 2,200 liters per liter of algae oil (Pate et al., 2011). These massive quantities of water required for production of a renewable fuel suggest that alternative strategies should be employed to use non-potable water resources.

Among the advantages of microalgae as a biofuel crop is the fact that fertile agricultural land is not required. Theoretically, production facilities could be placed in low-value, otherwise unusable land with high solar inputs. However, due to the sheer quantity of microalgae required to supply any appreciable amount of biofuels, the land requirements are still enormous, regardless of the type (Borowitzka and Moheimani, 2010). Although the availability of land is not a severely limiting factor for deployment of commercial

microalgae production facilities, a greater concern is identifying locations that combine desirable engineering characteristics and do not infringe upon social, economic or environmental interests in the area (Wigmosta et al., 2011).

A study conducted by Borowitzka and Moheimani (2010) illustrates the resource requirements of producing algae oil at various productivities (Table 2.2). These estimates, focusing on outdoor pond-produced photoautotrophic microalgae biomass, indicate water demands in accordance with previously cited volumes (Pate et al., 2011; Wigmosta et al., 2011). The theorized nutrient requirements are summarized by nitrogen (N as $NaNO_3$) and phosphorus (P as NaH_2PO_4). Raw material demands of this magnitude further emphasize the potential for competition with agricultural fertilizers if commercial microalgae production reaches significant levels.

Table 2.2 Estimated resource requirements for an algae plant producing 100,000 bbl per year algal lipids (15,898,700 L lipids) in open ponds at experimentally proven productivities and lipid contents. Source: Adapted from Borowitzka and Moheimani, 2010.

Production Parameter	**Estimated Value**					
Productivity (g m^{-2} d^{-1})	20	30	40	20	30	40
Lipid Content (% dry wt)	30	30	30	40	40	40
Biomass Production (t ha^{-1} $year^{-1}$)	73	109.5	146	73	109.5	146
Lipid Production (L ha^{-1} $year^{-1}$)	24,333	36,500	48,667	32,444	48,667	64,889
Total Pond Area (ha)	653	436	327	490	327	245
Total Pond Water - 30 cm deep (ML)	1,960	1,307	980	1,470	980	735
Evaporative Make-up (ML)	1,307	871	653	980	653	490
Harvest Loss Replacement (ML)	35,772	23,848	17,886	26,829	17,886	13,415
Total Water (GL)	3.9	2.6	2	2.9	2	1.5
Water/Lipids (L L^{-1})	2334	1553	1165	1749	1165	875
Total Biomass (t)	47,696	47,696	47,696	35,772	35,772	35,772
N (as $NaNO_3$) at 5% (t)	14,477	14,477	14,477	10,858	10,858	10,858
P (as NaH_2PO_4) at 0.1% (t)	219	219	219	164	164	164
CO_2 fixed (t)	85,853	85,853	85,853	64,390	64,390	64,390

Another important aspect to evaluating the feasibility of microalgae biofuel technology at commercial scale involves considering the energetic efficiency of the production process. Life cycle assessments (LCA) conducted on the subject incorporate such energy consumption/production figures (Khoo et al., 2011; Lardon et al., 2009; Passell et al., 2013). Considering that generating an energy product for the biofuels industry is a major ambition of microalgae production, it is essential that more energy is generated in the final product than that which is required to produce it. This energy ratio is commonly estimated as above parity given traditional microalgae production techniques, meaning more energy was required than produced (Chisti, 2013; Lardon et al., 2009). In fact, in a study comparing a conducted LCA against others existing in literature showed that the energy ratio (MJ required per MJ in produced biodiesel) estimates ranged from 0.9 to 6.3 MJ MJ^{-1} and averaged approximately 3.3 MJ MJ^{-1} when all processes were included. The results originating from that study even suggested that a likely estimate was 4.4 MJ MJ^{-1} and an optimistic case was only 1.9 MJ MJ^{-1} (Khoo et al., 2011). Within these estimates a sensitivity analysis indicated that the largest contributing factor to the overall energy ratio was the lipid extraction process, which, in this study was a conventional homogenization and solvent extraction technique. Alternative, low energy lipid extraction methods (i.e. enzymatic hydrolysis) may have the potential to reduce the energy requirements of the overall production process and possibly contribute to a favorable energy ratio.

2.4.3 Product Extraction and Recovery Technology

One of the most challenging areas for microalgae biotechnology as a renewable source for biofuels or nutritional products is extraction of the target compounds from within the cells. This process is considered a challenge not because of a lack of available techniques, but rather because of a difficult tradeoff between extraction efficiency, cost, and environmental impact (Mercer and Armenta, 2011). Many physical, chemical, and biological methods do exist for rupturing algal cells to release intracellular products, however, they vary tremendously in difficulty and effectiveness. Before proceeding to elaborate on existing product extraction methods, it is important to distinguish between two distinct and generalized phases of product recovery in microalgae biotechnology: i) biomass harvesting or solid-liquid separation and ii) product extraction (Pragya et al.,

2013). In many product recovery methods an initial separation step is required to dewater the microalgae biomass prior to further product extraction. These “dry route” techniques have the distinct disadvantage of additional processing required to remove the liquid, resulting in added energy demand and cost (Xu et al., 2011). While the separation step is important, this section focuses on “wet route” product extraction from the cells due to the fact that some extraction techniques do not require the solid-liquid separation step. Further, rather than providing detailed descriptions of these extraction methods, special attention will be paid to the drawbacks of the methods most relevant to this project, which will factor into future discussion on nutrient recycling.

Traditionally the benchmark method for product recovery is mechanically-assisted solvent extraction, following some derivative of the Bligh and Dyer method (Bligh and Dyer, 1959; Mercer and Armenta, 2011). This technique is incredibly popular due to the relative ease of execution and very high efficiency of oil recovery. While some methods achieve 20 – 80% recovery of intracellular oils, the Bligh and Dyer derived methods can recover upwards of 95% (Mercer and Armenta, 2011; Pragya et al., 2013). However, concerns do exist surrounding the energy requirements of some mechanical cell lysing methods and, more importantly, the use of large volumes of hazardous solvents. The appropriate solvents include organic compounds like hexane, chloroform/methanol, ethanol and acetone that can effectively break lipid bonds and release the oil. These solvents may be recovered to some extent, but at a significant expense, and waste materials are still hazardous (Mercer and Armenta, 2011).

For effective product recovery, solvent extraction is commonly paired with a mechanical cell lysis technique to allow passage through the cell wall. Some of the available mechanical cell disruption methods available include bead milling, high-pressure homogenization, microwave digestion, ultrasound cavitation and autoclaving (Mercer and Armenta, 2011; Pragya et al., 2013). Advanced enzymatic methods are also becoming increasingly popular, with specifically catered enzymes efficiently lysing microalgae cells and thereby releasing valuable products (Mercer and Armenta, 2011; Zheng et al., 2011). For example, Zheng et al. (2011) employed a cellulase to degrade cellulose in *C. vulgaris* into simple sugars, and in the process releasing the intracellular oil. This technique has a dual purpose of accessing the cell oil and converting challenging cellulosic compounds

into fermentable simple sugars. Although more expensive, enzymatic product extraction has distinct advantages of being solvent-free and low in energy demand. The targeted nature of enzymatic cell lysis also minimizes the solubilization of other cell components (i.e. proteins) which may be of value for secondary applications.

Another very promising method of product recovery from microalgae biomass involves exposing the whole wet biomass to elevated pressures (5 to 15MPa) and temperatures (250 to 350 ºC) resulting in the conversion of the cellular components into a dark, viscous, energy-dense product called biocrude (Biller et al., 2012; López Barreiro et al., 2013). During this hydrothermal liquefaction (HTL), the entirety of the cell material is solubilized resulting in the target biocrude as well as gaseous, liquid and solid by-products. This relatively rapid process has potential for integration into the production schematic without excessively intensive processing. However, due to the non-specificity of HTL, low-lipid or lipid-extracted biomass is most appropriate to avoid loss of high-value fatty acids (Vardon et al., 2012). Further, the complexity of the thermal degradation process in HTL can yield a variety of potentially harmful products in the resultant waste streams (Biller et al., 2012).

Each cell lysis technique intends to rupture cells to access the internal products and in the process solubilizing much of the remaining cell biomass. The result is a liquid waste stream with potentially high concentrations of solubilized cell carbohydrates, proteins, and minerals. If solvents are used to aid in product extraction, there is an additional hazard of residual compounds in the effluent waste streams that must be treated or disposed of properly. These generated wastes are an added concern atop the considerable cost of product extraction, and if possible should be minimized. Altogether, the product extraction and recovery phase of microalgae production constitutes a significant energetic, cost, and environmental impact.

2.4.4 Environmental Implications

As with most industrial processes, commercial-scale microalgae production will have environmental repercussions. Due to the complexity of the full-circle production and consumption of microalgae-based biofuels or bioproducts, there are many layers of environmental benefits and consequences. As shown in Figure 2.5, biodiesel derived from

renewable feedstocks generates lower emissions profiles (except for nitrogen oxides) than petroleum fuel emissions (Janaun and Ellis, 2010). Greenhouse gas (GHG) emissions, particularly CO_2, are also similar between biodiesel and petrodiesel (USEPA, 2002). Taking into account the similarity of petroleum fuel CO_2 emissions to those of microalgae-based biofuel, the focus on assessing environmental impacts shifts to the biomass production aspects rather than the consumption side. As previously mentioned there are resource demands associated with microalgae production that will require large volumes of water, significant land development, various nutrient provisions, processing and purification chemicals, and considerable energy consumption. Beyond these resource demands the environmental concerns are primarily carbon dioxide (CO_2) uptake or emission and the quantity and severity of wastewater generated.

When assessing the carbon balance of microalgae production it is essential to revisit the distinction between the metabolic states of the organisms being produced. This refers to whether the microalgae being produced are metabolizing energy and carbon via photosynthesis (photoautotrophs) or fermentation (heterotrophs). This distinction is important because photoautotrophic microalgae consume CO_2 as captured carbon for cell synthesis whereas heterotrophic microalgae consume organic carbon (e.g., glucose) for cell synthesis, but emit carbon dioxide (Fig. 2.1-2.2) (Perez-Garcia et al., 2011). These complex metabolic pathways are highly variable depending upon the organism, culture conditions, substrate composition, and the growth stage. However, a study by Yang et al. (2000) traced the carbon through photoautotrophic and heterotrophic growth of *Chlorella pyrenoidosa* and developed some insightful observations about metabolic differences. Regarding carbon balance specifically, photoautotrophic *C. pyrenoidosa* consumed 1.75 g CO_2 per gram of cells produced whereas in a cyclic heterotrophic state, it produced 2.57 g CO_2 per gram of cells produced (Yang et al., 2000). This difference is indicative of the greenhouse gas consideration pertaining to commercial microalgae production in heterotrophic culture as opposed to photoautotrophic.

Various life cycle assessments (LCAs) conducted to evaluate microalgae-based biofuels have also suggested a lack of consensus regarding the carbon balance of commercial production (Chisti, 2013). This variability is expected due to the multitude of model-specific factors (e.g. species, metabolic state, growth vessel, culture media, growth

conditions, etc.) that must be included in the analysis. According to a compilation of LCA results by Chisti (2013) the reported values for carbon demand per unit of energy produced range from 78 to 351 g CO_2 per MJ. In comparison, the U.S. Energy Information Agency (EIA) reported greenhouse gas emissions of petroleum diesel is 75.15 g CO_2 MJ^{-1}, indicating some concern regarding elevated GHG from microalgae-based biofuels (USEIA, 2013). However, it is important to remember that petroleum diesel CO_2 emission estimates are unlikely to include extraction and refining factors, and certainly not the tremendous bio-geological investment of energy required to formulate those hydrocarbons; meanwhile every stage of production of microalgae-based biofuels is incorporated into many LCAs.

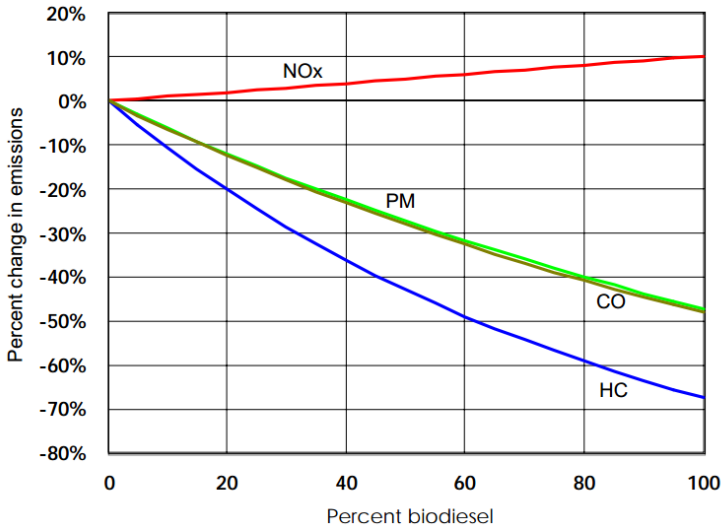

Figure 2.5 Percent change in major biodiesel emissions compared to petroleum-based diesel at increasing blends of biodiesel to petrodiesel. NO_x = nitrogen oxides, PM = particulate matter, CO = carbon monoxide, HC = hydrocarbons. Source: USEPA, 2002.

The other major environmental impact that should be considered for microalgae production is the enormous volumes of water that will likely be discharged, potentially laden with residual nutrients and chemicals required for product extraction and purification. Considering that even the upper end of reported biomass concentrations attained in culture (100 to 150 g L^{-1}) correspond to 85 to 90% water, water consumption at commercial-scale has tremendous implications for water consumption and discharge

(Bumbak et al., 2011). Consumption of these quantities of water is important, but this section will explore the effluent water and potential for significant pollutant concentrations. For simplicity, one major area for water contamination will be considered: spent production water. This aspect of microalgae production is currently largely unexplored therefore minimal literature examples exist.

Spent production water, or spent media, is the effluent liquid that remains after cells have been removed and/or extracted for the intracellular products. The significance of this waste stream is primarily due to the significantly large volumes in comparison to other wastes (e.g., extraction processing water). Depending on the specific production and extraction schematic, the composition may include cell debris and trace solvents (Fon Sing et al., 2014). In addition, this material will contain any residual nutrients that were not depleted during the growth phase (Rösch et al., 2012). These residual nutrients, however low in concentration, pose some threat to receiving waters if they trigger eutrophic conditions. Ideally a nutrient recycling regime would be employed to maximize the nutrient depletion in discharge water to the benefit of the receiving water ecology and process economics (Fon Sing et al., 2014).

It is difficult to approximate the exact concentration of nutrients in the spent media waste stream because it is largely system-specific and few studies explore this material in depth. Estimates of the probable composition of spent media after microalgae cultivation can be inferred by observing studies that used microalgae cultures to remediate various wastewaters. One study demonstrated an 87.6% reduction in chemical oxygen demand (COD), 89.2% reduction in total nitrogen, and 95.5% reduction in total phosphate after 10-day growth of *Chlorella zofingiensis* (Zhu et al., 2013). These results suggest a strong depletion of primary nutrients in an effluent solution due to microalgae growth. Another similar study showed reduction in secondary nutrients and minerals after cultivation of *Scenedesmus sp.* AMDD in secondary treated domestic wastewater (Table 2.3). These findings indicate the fractional percentage of initially available elements that were observed in the microalgae cells after cultivation. While iron (Fe), cadmium (Cd) and Zinc (Zn) were efficiently taken up by the cells, it is apparent that many other minerals remain in the effluent in significant concentrations (McGinn et al., 2012).

Table 2.3 Secondary nutrient and mineral uptake into cells by *Scenedesmus* sp. AMDD during the stationary phase of growth. Source: Adapted from McGinn et al., 2012.

Trace Element	Wastewater Concentration	Fractional Uptake/Adsorption by Microalgae			Remaining Concentration[a]
	($\mu g\ L^{-1}$)	(%)			($\mu g\ L^{-1}$)
		pH 6.2	pH 6.6	pH 7.0	
Fe	95.2 ± 2.1	91.7	99.1	88.1	6.7
Cd	0.026 ± 0.003	96.4	95.3	90.9	0.002
Zn	28.5 ± 3.1	89.2	92.9	91.6	2.5
Al	10.2 ± 0.55	69.5	83.2	97.1	1.7
V	0.467 ± 0.006	40.5	49.3	50.2	0.249
Pb	0.166 ± 0.006	40.9	30.9	45.5	0.101
Co	1.88 ± 0.08	14.4	14	24.6	1.55
Mn	195.6 ± 5.1	9.8	12.4	20.9	167.5
Mg	4679 ± 183	14.5	14.3	12	4043
Mo	1.38 ± 0.03	8.8	10.5	8.2	1.25
Cr	1.57 ± 0.05	5.7	9.1	9.1	1.45
Ba	13.88 ± 0.51	1.7	1.9	2.8	13.58

[a] Calculated from averaged available data

The findings from the aforementioned studies suggest that there is serious potential for remaining nutrient concentrations existing in effluent water from microalgae production. When production volumes are extrapolated to commercial-scale, a residual concentration of a few percent can represent a substantial concentration into receiving waters as well as a significant financial waste to the producer. However, there are some important caveats regarding this data; first, the data are provided from effluent concentrations of substrates that were originally wastewaters of complex matrices. When specialized culture media are used instead of wastewater, there will be no components added that are not vital to the metabolism of the microorganism, therefore there will be smaller concentrations in the effluent. The second consideration is that these studies were demonstrations of wastewater remediation and tolerance by microalgae, not results from optimized industrial processes which would surely have minimal inefficiencies regarding material inputs and losses.

2.5 HIGH-PRODUCTIVITY SOLUTIONS

Despite the challenges to commercialization of microalgae production, it is clear that cost reduction is paramount for successful adoption of the technology for production of biofuels or bioproducts (Chisti, 2013; Norsker et al., 2011; Singh et al., 2011; Sun et al., 2011). The most obvious and attainable pathway for improved economics is enhancement upon cultivation techniques and product recovery. This has been the subject of enduring research attention, with advanced cultivation strategies resulting in extremely high-density cultures and correspondingly improved production economics (Bohutskyi et al., 2014a; Doucha and Lívanský, 2012; Sansawa and Endo, 2004). Among those advanced techniques are specialized heterotrophic cultures, fed-batch growth regimes and effective strain selection resulting in improved biomass and bioproduct concentrations. This combination has demonstrated considerably improved yields (up to 150 g L^{-1} biomass and 80% lipid content) as compared to the conventional batch-cultivated photoautotrophic culture (up to 40 g L^{-1} biomass, 15 to 40% lipid content) (Bumbak et al., 2011; Raghukumar, 2008). However, these increasingly specialized conditions narrow the population of compatible microalgae that can thrive in these environments. Researchers have identified some highly valuable microalgae species that have exhibited desirable growth characteristics in heterotrophic conditions which are summarized in Table 2.4.

The premier attraction of heterotrophically cultured microalgae is the increased potential for high-density cultures that can simultaneously accrue large concentrations of intracellular lipids. As reported by Scaife et al. (2015), the highest reported photoautotrophic yields of *Chlorella* sp. are 40 g L^{-1} dry cell weight and productivity of 3.3 g L^{-1} d^{-1}, in comparison to a fed-batch heterotrophic culture of *Chlorella vulgaris* which produced 117.2 g L^{-1} dry cell weight and productivity of 87.9 g L^{-1} d^{-1} (Doucha and Lívanský, 2012, 2006). It is also commonly reported that heterotrophic cultures are characterized by an enhancement to the lipid content of the microalgae cells. For example, *Chlorella protothecoides* contained 14.6 % intracellular lipids in photoautotrophic culture as opposed to 55.2 % in heterotrophic culture (Miao and Wu, 2006). These cellular lipids produced include valuable omega-3 fatty acids like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which are attractive commercial nutritional oils. High lipid content is particularly enhanced in thraustochytrids which have been observed amassing

up to 80% of cell mass as lipids and as much as 50% of that oil comprised of DHA in specialized cultures (Raghukumar, 2008). These highly productive cultures are especially important considering microalgae production – photoautotrophic or heterotrophic – is still plagued with concerns of prohibitive costs (Chisti, 2013; Liang, 2013).

In addition to biofuels and nutritional oils as attractive applications for heterotrophic microalgae, there also exists prospects for co-production of select pigments such as photoprotective carotenoids and xanthophylls. Despite the early conclusions that heterotrophic cultures would be devoid of pigment, due to the absence of light during growth, contemporary research has demonstrated that some heterotrophic microalgae retain significant pigment composition (Perez-Garcia et al., 2011; Sun et al., 2008). Although pigment concentration within the cells may not match or exceed that of photoautotrophic cultures, the higher biomass productivity from heterotrophic conditions corresponds to substantial pigment yield. To date, the most suitable heterotrophic microalgae for astaxanthin production is *Chlorella zofingiensis*. One research team demonstrated that a fed-batch heterotrophic culture of *C. zofingiensis* reached cell concentrations of 51.8 g L^{-1} and produced 32.4 mg L^{-1} of astaxanthin as compared to 15.1 mg L^{-1} when produced photoautotrophically (Ip and Chen, 2005; Sun et al., 2008).

Though heterotrophic cultures do require advanced equipment for effective monitoring and control of cultures, much of this technology is well established from fermentation and biotechnology industries. Even more, when nutritional applications are targeted and production requires strict manufacturing control, heterotrophic fermentations are far superior to enclosed photobioreactors required for photoautotrophic cultures. The elimination of the requirement for efficient light penetration throughout the vessel greatly simplifies the engineering and energy constraints of production vessels. This allows for large-volume stirred-tank reactors rather than expansive arrays small diameter transparent tubes. These heterotrophic fermentors are also advantageous because of their multipurpose utility (in-vessel production and extraction), scalability, vertical arrangement and correspondingly low areal requirement (Bumbak et al., 2011). Additionally, the facility siting requirement for maximum solar input is removed with heterotrophic cultures and greater flexibility, in terms of climate and land type, are gained.

Table 2.4 Select obligate heterotrophic microalgae that have been identified as favorable commercial production species and their corresponding attributes. Source: Adapted from Bumbak et al., 2011; Ren et al., 2010; Ganuza et al., 2008.

Heterotrophic Microalgae	**Product**	**Features**	**Growth Conditions**	**Biomass Productivity ($g\ L^{-1}\ d^{-1}$)**	**Product Yield ($g\ L^{1}\ d^{-1}$)**
Chlorella protothecoides	Lipids	High lipid content, robust, good growth rates	Fed-batch, 2.5 days	48.0	19.2 (Lipids)
Chlorella pyrenoidosa	Biomass	Robust, good growth rates, nutritional marketability, well-researched	Fed-batch, 5 days	23.5	Biomass
Chlorella regularis	Biomass	Robust, good growth rates, nutritional marketability, well-researched	Fed-batch, 3 days	67.2	Biomass
Chlorella vulgaris	Biomass, lipids	Robust, good growth rates, nutritional marketability, well-researched	Fed-batch, 3 days	87.9	Biomass
Chlorella zofingiensis	Astaxanthin	Good growth rates, high pigment concentration in dark	Fed-batch, 15 days	3.5	0.002 (Astaxanthin)
Schizochytrium sp.	Lipids, DHA	Excellent growth rates, carbon source flexibility, high PUFA content	Fed-batch, 5.5 days	30.9	2.9 (DHA)
Thraustochytrium sp.	Lipids, DHA	Excellent growth rates, carbon source flexibility, high PUFA content	Flask, 3 days	10.3	2.3 (DHA)

The concept of differing culture modes typically pertains to the availability of nutrients in the bioreactor during fermentation (Armenta and Valentine, 2013; Shuler and Kargi, 2002). In experimental research, batch mode is typically employed at smaller reactor volumes (i.e. flask scale) and fed-batch or continuous are more common in larger volume

cultures. Batch cultures are those in which all nutrients and media are provided at the start of the fermentation, and the cells are allowed to grow until exhausting the resources. These conditions exhibit the classic microbial growth kinetics with an initial lag phase, followed by exponential growth and finishing with a stationary and die off phase (Shuler and Kargi, 2002). Batch mode is common in laboratory work and can result in substantial accumulation of intracellular products due to the resource exhaustion at the end of the growth phase (Armenta and Valentine, 2013). However, the logistical challenge of non-continuous product harvest as well as the potential for innovative two-stage regimes supports investigation into the alternative culture modes.

Fed-batch cultures differ from batch cultures in that at some point during the growth phase, additional nutrients are added to prevent resource limitation. The fed-batch mode allows for high concentrations of biomass via maintaining the availability of growth limiting resources (Ratledge and Kristiansen, 2006). Due to the advantages of fed-batch conditions, it is widely used in biotechnology applications and considered an ideal culture mode for thraustochytrids at commercial scales (Raghukumar, 2008). Continuous cultures, alternatively, are characterized by a steady influx of nutrient medium with a simultaneous separation and harvest of cell biomass. These conditions, although simplifying the labor and maintenance costs, introduce potential challenges with contamination and slow formation of mutant attributes in the culture (Ratledge and Kristiansen, 2006). While each of these growth regimes receives research attention, it appears the highest productivities are obtained in fed-batch cultures which also possess the best characteristics of batch and continuous cultures (Bumbak et al., 2011; Li et al., 2007).

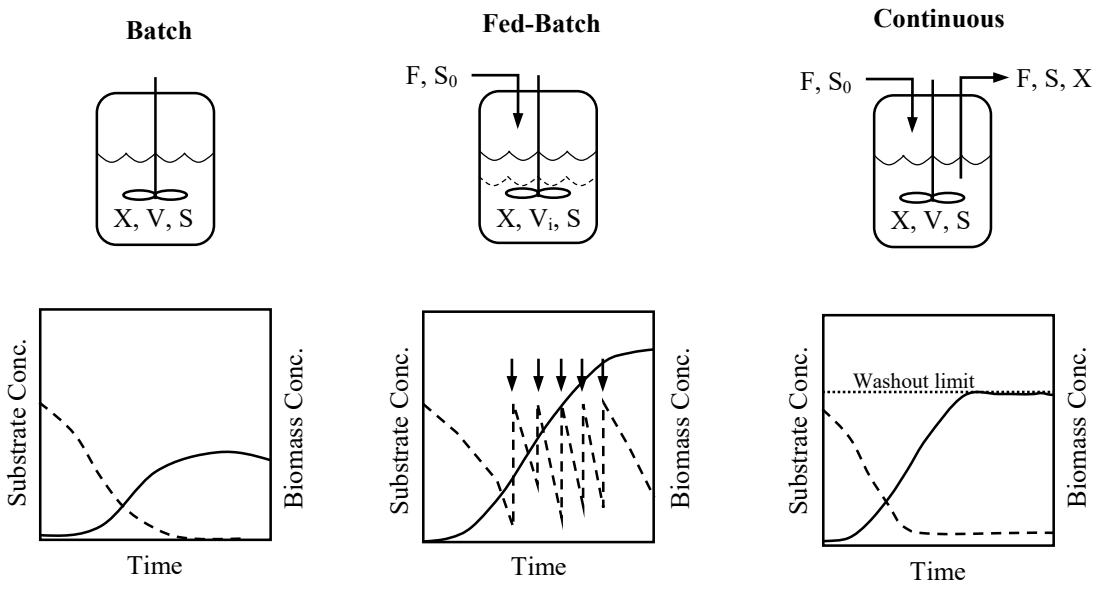

Figure 2.6 Generalized schematic of batch, fed-batch and continuous culture regimes and their corresponding growth kinetics. F = flow rate, S_0 = substrate in feed, S = substrate in medium, V = culture volume, V_i = initial volume, X = cell concentration. Solid lines indicate biomass concentration, dashed lines indicate substrate concentration, dotted lines indicate washout limits and arrows indicate isolated substrate additions. Source: Graverholt and Erikson, 2007; Li et al., 2007; Pandey et al., 2013.

2.6 THRAUSTOCHYTRIDS

Thraustochytrids are a lesser-known group of heterotrophic microalgae with a definitive role in ecosystem cycling of waste materials in highly brackish waters (Bongiorni, 2012; Raghukumar, 2008). Due to their distinct capacity for ultra-high productivity and considerable lipid accumulation, some specialized thraustochytrids are receiving attention as potential species for commercial production of biofuels and bioproducts (Chang et al., 2012; Lee Chang et al., 2014). As evidenced in Table 2.4, these species exhibit some of the highest productivities reported to date making them important for further investigation and optimization, and the focal species for this thesis.

2.6.1 CLASSIFICATION

The taxonomy of thraustochytrids was the subject of some uncertainty in recent years (Bongiorni et al., 2005a). Contemporary consensus based upon modern DNA analysis concludes they belong to the phylum Heterokonta and the class Labyrinthulomycota (Armenta and Valentine, 2013; Chang et al., 2012; Fan and Chen, 2011). The current genera groupings include: *Althornia, Diplophyhrys, Elina, Japonochytrium, Schizochytrium, Thraustochytrium* and *Ulkenia* (Fan and Chen, 2011). They are extremely common marine microorganisms with an essential role in the decomposition and remineralization of sedimentary organic material in coastal ecosystems (Bongiorni, 2012; Raghukumar, 1992). In addition, thraustochytrids play an important role as a food source for many microorganisms, which partly explains their capacity to upgrade organic material into energy rich lipids. Some of the most commercially attractive thraustochytrids include *Schizochytrium* sp. and genetically modified variants of *Thraustochytrium* sp. due to their ability to accumulate large concentrations of specific fatty acids (Armenta and Valentine, 2013; Chang et al., 2012). In addition to the aforementioned species, Lee Chang et al. (2014) included *Aurantiochytrium* sp. and *Ulkenia* sp. as thraustochytrids with great commercial potential.

There exist a large variety of naturally occurring thraustochytrids from which to select for commercial production. Selection criteria for production includes the ability to reach high biomass and lipid concentrations, and further, a proclivity for high value fatty acids is especially attractive. Considerable research attention has been focused on thraustochytrids isolated from mangroves due to their advanced ability to tolerate varying environmental conditions (e.g. salinity and temperature) as well as their ecological role as decomposers (Bongiorni, 2012; Fan and Chen, 2011; Raghukumar, 1992). Some recent studies investigate the most opportune species, classifying them based upon the aforementioned metrics. Among the organisms of interest include *Thraustochytrium aureum, T. roseum, T. aggregatum, Schizochytrium limacinum,* and *S. aggregatum* (Huang et al., 2003). One existing commercial example is *Schizochytrium limacinum* (SR21) which is particularly attractive due to its accumulation of large concentrations of fatty acids, in particular DHA (Burja et al., 2006). Another organism of emerging interest is T18, isolated

in a survey of Atlantic Canada (Burja et al., 2012, 2006), and that has demonstrated exceptional growth and accumulation of DHA.

2.6.2 METABOLISM

The mechanisms for nutrient and carbon intake by thraustochytrids are not extensively researched, however it is known that they are incredibly versatile in the sources they can use. They are unicellular, non-photosynthetic, heterotrophic protists (Kimura and Naganuma, 2001). Typically thraustochytrids are saprophytes, meaning they survive off consuming organic material and correspondingly are most commonly found in marine sediments (Armenta and Valentine, 2013; Burja et al., 2006). Raghukumar suggested that beyond the ability to synthesize extracellular enzymes for the degradation of organic molecules, proteins, lipids, and amino acids; thraustochytrids are also capable of phagotrophic uptake and remineralization of bacterial cells (Raghukumar, 1992). This versatile capability enables thraustochytrids to metabolize many substrates in diverse environments. An in depth investigation into the potential of thraustochytrids to synthesize specific enzymes that are catered toward metabolism of certain alternative substrates (e.g. spent biomass) is a research area of tremendous potential for further work. Although some preliminary studies have been done on this topic, it would be important to assess the production outcomes and the corresponding drawbacks.

The essential ecological role that the thraustochytrids play in marine environments lends an explanation to their unique versatility of nutrition source. In deriving sustenance from dissolved organic material, the cells can effectively meet their needs for carbon, nitrogen, microelements and vitamins. They employ a key advantage of enzymatic excretion to assist in the degradation of a wide array of organic substrates. One study by Bongiorni et al. (Bongiorni et al., 2005b) explored the production of 19 different enzymes in 11 thraustochytrid strains and found impressive widespread enzyme excretions. Enzymes produced include cellulase, amylase and lipase, among many others (Bongiorni et al., 2005b; Nagano et al., 2011). Production of such enzymes expands the organic substrates possible for cultivation to include plant detritus and other refractory organic material (Nagano et al., 2011).

The mechanism for the enzymatic secretion and corresponding degradation of organic material in their surrounding substrate has been associated with production of an ectoplasmic net (Bongiorni et al., 2005a; Perveen et al., 2006). Traditionally referred to as a "rhizoid", "holdfast" or "slime track", this structure is collectively now called the ectoplasmic net (Perkins, 1972). This intricate "slime" can increase the surface area and contribute to catalyzing the breakdown and uptake of various organic materials (Honda et al., 1998). Originating from the sagenogenetosome, just beneath the cell surface, the ectoplasmic net elements are an extension of the plasma membrane (Bongiorni et al., 2005a; Perkins, 1972). The occurrence can be observed by the production of extracellular polysaccharides (EPS), elevated enzyme activities and the visible presence of the ectocellular ultrastructures (Fig. 2.7). The exact composition of these structures is varied but tends to contain predominantly polysaccharides with smaller concentrations of proteins, nucleic acids and lipids (Jain et al., 2005; Lee Chang et al., 2014). Further, the synthesis of these structures is triggered by exposure to complex organic substrates and the resultant enzyme assortment aids in their metabolism (Honda et al., 1998). This distinct metabolic capability allows thraustochytrids to be productive in both optimized growth media as well as diverse eutrophic substrates.

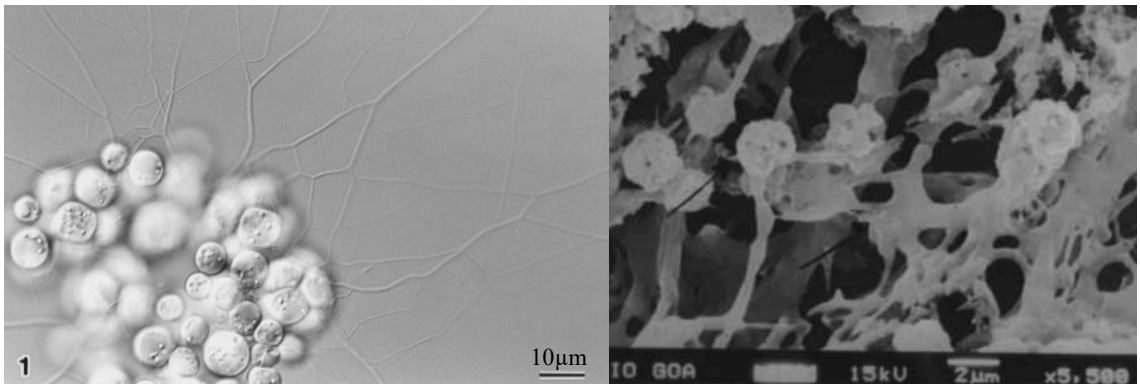

Figure 2.7 Microscopic observations of the ectoplasmic net structures in thraustochytrids. Light micrographs of *Schizochytrium limacinum* cells exhibiting ectoplasmic net elements (left) and scanning electron micrographs of *Schizochytrium* sp. CW1 displaying complex extracellular polysaccharide formations. Sources: (Honda et al., 1998; Jain et al., 2005).

2.6.3 PHYSICAL CHARACTERISTICS

Thraustochytrids are single-celled organisms known to be sized 5-20 μm in diameter (Kimura and Naganuma, 2001). Their role as primary decomposers of organic material makes them present in large concentrations in marine environments, and therefore they serve as a key food source for many organisms. Two key characteristics of thraustochytrids are their prolific growth rates and tendency to accumulate large concentrations of nutritionally important polyunsaturated fatty acids (Armenta and Valentine, 2013; Kimura and Naganuma, 2001). As seen in Fig. 2.8, this accumulation is so considerable that upon microscopic examination, oil globules can often easily be observed within and around the cell structure (Fan and Chen, 2011; Raghukumar, 2008). This oil production can be attractive for generating biofuels as well as omega-3 fatty acids. The most attractive of the fatty acids produced are DHA and EPA, with one or the other commonly occurring in significant amounts (Armenta and Valentine, 2013). Table 2.5 summarizes some key thraustochytrids and their reported culture densities, lipid contents and DHA accumulation. It is apparent that *Schizochytrium* sp. (SR21) and T18 exhibit very attractive maximum biomass concentrations as well as very favorable quantities of DHA, especially at the larger scale 5-liter bioreactor for T18.

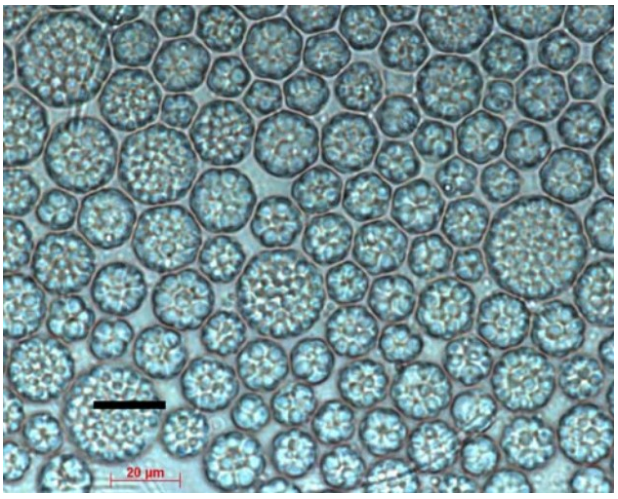

Figure 2.8 Cells of a *Schizochytrium* isolate showing visible lipid globules. Source: Raghukumar 2008

Some thraustochytrids have been reported to produce valuable carotenoids such as β-carotene, astaxanthin and zeaxanthin as well as potentially useful enzymes (Bongiorni et al., 2005b; Burja et al., 2006). These products can also be harvested as valuable nutraceutical compounds if they are found in sufficient concentrations in the produced biomass. However, in comparison to pigment-targeted microorganisms such as *Botryococcus braunii* or *Chlorella zofingiensis* (Ip and Chen, 2005), harvesting these compounds from thraustochytrids may be relegated to a second priority product (Burja et al., 2006). For example, astaxanthin concentrations in *C. zofingiensis* have been reported to reach 1,010 μg g^{-1} of cell biomass while the concentrations in T18 were approximately 1 μg g^{-1} of cell biomass (Armenta et al., 2006; Ip and Chen, 2005). However, the array of other pigments such as β-carotene may be more attractive as compounds derived from thraustochytrid biomass since they occur in larger quantities (17 to 20 μg g^{-1}) (Armenta et al., 2006; Burja et al., 2006).

Table 2.5 summarizes some reported outcomes of thraustochytrid production over the last couple decades. The most important columns to notice are the productivity columns that express the biomass, total lipid, and DHA productivities expressed in units per day. These productivities, although not representative of actual instantaneous productivity during exponential growth, suggest that the two microorganisms SR21 and T18 seem to exhibit the most desirable growth and product accumulation characteristics. It is also worth noting that most of these entries are over 15 years old, suggesting that some improvements may have been attained and not yet published due to proprietary reasons.

Table 2.5 Biomass and DHA production of various thraustochytrids. Adapted from: Lewis et al. 1999

Organism	Culture Conditions			Biomass ($g\ L^{-1}$)	Biomass Prod. ($g\ L^{-1}d^{-1}$)	Total Lipid (% wt)	Lipid Prod. ($g\ L^{-1}d^{-1}$)	DHA Production (% Total Fatty Acid)	DHA Prod. ($mg\ L^{-1}d^{-1}$)
	Age (d)	Temp (°C)	Vessel						
Schizochytrium sp. (SR21)	4		Fermentor (300 rpm)	48	12.00	77	9.24	36	3325
Schizochytrium sp. (SR21)	2.5	28	Fermentor	21	8.40	50	4.20	35	1880
Thraustochytrium sp. (T18)[b]	7	25	Fermentor (5 L)	28	4.00	81.7	3.27	31.4	1,026
Thraustochytrium sp. (T18)[a]	5	25	Flask (250 rpm)	31.7	6.34	36.9	2.34	37.8	882
S. limacinum (SR21)	5	25	Flask	38	7.60	37	--	33	840
T. roseum (ATCC 28210)	12	25	Flask (200 rpm)	17.1	1.43	25	2.81	49	175
T. roseum (ATCC 28210)	5	25	Flask (250 rpm)	7.6	1.52	18.2	--	50	130
T. aureum (ATCC 34304)	6	25	Flask (300 rpm)	3.8	0.63	16.5	0.36	49	45
Thraustochytrium sp. (ATCC 20892)	4	25	Flask (200 rpm)	2.7	0.78	7.3	--	35	17
T. aureum (ATCC 28211)	6	25	Flask (200 rpm)	0.8	0.13	--	0.28	3.7	0.67
S. aggregatum (ATCC 28209)	10	25	Flask (200 rpm)	0.9	0.09	--	--	1.7	0.04
T. aureum (ATCC 34304)	2.5	25	Flask	5.7	2.28	8.1	0.10	40	--
Thraustochytrium sp. (ATCC 26185)	6	28	Flask (120 rpm)	7.5	1.25	32	--	25	--

[a] Source: Scott et al., 2011

[b] Source: Burja et al., 2006

In terms of the physicochemical composition of the thraustochytrid biomass, which is important for assessing its value for non-lipid applications, Table 2.6 provides some information that was focused on using the biomass for fish feed (Carter et al., 2003). The raw thraustochytrid biomass (*Thraustochytrium* sp. ACEM 6063) that was examined in that study had almost 60% of its cell mass comprised of lipids, making it a highly oleaginous strain, with DHA comprising 15.7% of the total fatty acids in the biomass. That characteristic makes the biomass particularly attractive for fish feed due to the high energy content and large contribution of omega-3 fatty acids to the fish diet. The next most prevalent component to the biomass was protein, which made up 20% of the cell mass. This fraction is important because it suggests that after lipid extraction, up to 50% of the remaining spent biomass could be protein based, which may be of value for secondary applications.

Table 2.6 Chemical composition of non-extracted thraustochytrid biomass. Source: Carter et al., 2003.

Nutrient (g kg^{-1})		Essential Amino Acids (g kg^{-1})		Essential Fatty Acids (g kg^{-1})	
Dry Matter	979	Arginine	14.6	C18:2 n-6	0.14
Crude Protein	207	Histidine	5.3	C18:3 n-3	0.23
Total Lipid	589	Isoleucine	9.1	C20:4 n-6	0.53
Ash	85	Leucine	16.9	C20:5 n-3	2.63
Energy (MJ kg^{-1})	20.7	Lysine	13	C22:6 n-3	92.21
		Methionine	5.5		
		Phenylalanine	10.4		
		Threonine	10.9		
		Tryptophan	2.6		
		Valine	20.9		

2.6.4 PRODUCTION CONDITIONS

Commercial production of thraustochytrids for fatty acids is still developing with many research questions still unanswered. Due to the diversity of thraustochytrid strains, optimized conditions vary greatly depending on the origin of the isolate, the culture mode (batch, fed-batch, or continuous) and target products. For maximization of biomass and oil

content, production conditions usually involve optimized environmental conditions (e.g. temperature, agitation, dissolved oxygen, salinity, pH) and specialized growth medium. Table 2.5 includes select culture conditions that were employed to achieve certain biomass and DHA levels.

2.6.4.1 Culture Mode and Fermentation Length

Typically the fermentation process for accumulation of fatty acids in thraustochytrids ranges from 2 to 7 days to reach maximum outputs (see Table 2.5) (Armenta and Valentine, 2013; Fan and Chen, 2011; Raghukumar, 2008). In batch conditions, growth kinetics follow a typical sigmoidal pattern with a rapid exponential phase followed by a stationary phase induced by competition or resource limitation (Raghukumar, 2008; Shuler and Kargi, 2002). During this resource limitation cells have a tendency to store larger quantities of high energy lipids, including the high-value fatty acids for commercial production (Bumbak et al., 2011; Perez-Garcia et al., 2011). This dynamic supports the development of novel two-stage fermentations where a phase of mass cell propagation is followed by deliberate suboptimal conditions, in which the accumulated cells will store available carbon as lipids (Armenta and Valentine, 2013; Hallenbeck, 2012). Two common stressors are nitrogen deprivation and temperature reduction, although these are not the only options (Armenta and Valentine, 2013; Fan and Chen, 2011).

2.6.4.2 Carbon Sources

Carbon is the most important and often limiting ingredient in heterotrophic microalgae cultures. While photoautotrophic microalgae can obtain carbon via carbon dioxide sequestration, heterotrophs such as thraustochytrids must use organic carbon in the surrounding environment. Considering the predominance of carbon in the stoichiometric makeup of microalgae cells – classically reported as $C_{106}N_{16}P_1$ as the Redfield ratio – it is logical that during metabolism carbon would be required in great quantities (Bumbak et al., 2011; Graham and Wilcox, 2000; Redfield, 1934). That stoichiometry was even further specified for heterotrophic *Chlorella vulgaris* as $C_{3.96}H_{7.9}O_{1.875}N_{0.685}P_{0.0539}K_{0.036}Mg_{0.012}$, further elucidating the importance of carbon provided in the media (Bumbak et al., 2011;

Sansawa and Endo, 2004). The oleaginous nature of thraustochytrids even further magnifies the carbon content of the cells, thereby increasing the nutritional demand accordingly. Carbon, specifically in the form of glucose, is the most expensive medium component due to the large concentrations that are required for optimal growth of thraustochytrid cultures (Yan et al., 2011). Although glucose is energetically favorable, many other organic carbon sources are viable to support heterotrophic growth (Yang et al., 2000). However, in commercial applications it is imperative to not trade the high productivity yielded in glucose cultures by employing another organic carbon source.

Some alternative carbon sources that have been explored include acetate, fructose and glycerol with results suggesting they can be effective replacements for glucose, however results vary depending upon the microorganism (Nagano et al., 2009; Raghukumar, 2008; Scott et al., 2011; Yokochi et al., 1998). In fact, using reclaimed waste glycerol from fish oil processing Scott et al. (2011) demonstrated improved growth and DHA accumulation in *Thraustochytrium* sp. T18 as compared to a traditional glucose medium. These findings are supported by a previous study that used *Schizochytrium limacinum* SR21 and determined that glucose, fructose, glycerol and oleic acid were all viable carbon sources in terms of both biomass accumulated and total fatty acid content. Even more, glycerol yielded the largest concentration of DHA at approximately 1 g L^{-1} (Yokochi et al., 1998).

In addition to pure carbon sources, select microalgae can metabolize organic carbon compounds present in complex matrices such as wastewater. Some agricultural, domestic and industrial wastewaters contain an assortment of organic compounds including acetate, propionic acid, ethanol, glycerol, methanol, saturated fatty acids, monosaccharides (e.g. xylose and arabinose) and disaccharides (e.g. lactose and sucrose) that can be potentially bioavailable to thraustochytrids (Lowrey et al., 2014; Morales-Sánchez et al., 2014; Perez-Garcia et al., 2011). However, many of these compounds can be toxic in excessive concentrations and additionally, wastewaters typically contain a variety of potentially inhibiting substances (Lowrey et al., 2015). To ensure maximum productivity of microalgae cultures in the interest of minimizing costs, introducing complex substrates may cause more challenges than benefits.

2.6.4.3 Nitrogen Sources

Nitrogen alternatives for thraustochytrid production have been investigated similarly to carbon due to the significant contribution to medium cost as well as the elemental importance of nitrogen. However, nitrogen sources investigated are usually limited to complex nitrogen and mineral nitrogen (Chen et al., 2010; Yokochi et al., 1998). Although nitrogen is the second most expensive component of nutrient media, by-weight nitrogen sources are much more expensive than carbon. For example, microbiological grade yeast extract may cost 300-400 USD per kg as compared to 40-70 USD per kg for glucose (Sigma Aldrich Co., USA). In similar microbial settings such as microalgae production, alternative nitrogen sources have included plant and animal wastes as well as industrial and municipal wastewaters (Brennan and Owende, 2010). These techniques could be applied to thraustochytrid production but may introduce health and regulatory concerns when the biomass or fatty acids harvested are intended for human consumption (Armenta and Valentine, 2013).

When comparing the experimental results of multiple nitrogen sources used for cell culture, it is important to evaluate both the effects upon biomass yield as well as the target cell products (e.g. lipids, DHA, etc.). One study exploring the importance of nitrogen source upon the accumulation of squalene in *Schizochytrium* sp. used ammonium sulfate, corn steep liquor, monosodium glutamate, peptone, sodium nitrate, tryptone, urea and yeast extract as nitrogen sources (Chen et al., 2010). The results determined that the highest biomass achieved in 36 hours was from monosodium glutamate (9.82 g cells L^{-1}), peptone (8.81 g cells L^{-1}) and yeast extract (8.23 g cells L^{-1}). The target metabolite, squalene, was found to accumulate in much larger concentrations in media with complex nitrogen (corn steep liquor, peptone, yeast extract and tryptone) as compared to simple defined and organic sources (ammonium sulfate, monosodium glutamate, sodium nitrate and urea) (Chen et al., 2010). This conclusion is supported by similar results from *S. limacinum* (SR21) that observed maximal DHA yield from corn steep liquor, although some mineral nitrogen sources (ammonium acetate and ammonium nitrate) were very close in yield (Yokochi et al., 1998). However, it is worth noting that although the biomass concentrations in this study were much higher at 15-25 g L^{-1}, the growth period extended to 5 days (Yokochi et al., 1998). These findings support the assertion that the additional

minerals, metals and trace nutrients available in complex nitrogen sources have an important and positive influence on the yield of high-value products from thraustochytrid biomass.

In order to increase the efficiency of thraustochytrid production via cost reduction, nitrogen sources derived from waste sources must be investigated. Specific wastes may be sufficiently rich in complex nitrogen and carbon, but also introduce challenges due to product inconsistency and compositional uncertainty. *Schizochytrium* sp. (KH105) was shown to grow competently in distillery wastewater from the production of Schochu, a rice and barley-based Japanese alcoholic beverage (Yamasaki et al., 2006). The wastewater, rich in nitrogen, protein and amino acids, was supplemented with varying levels of glucose and pH treated prior to experimental fermentations. Optimal growth resulted in biomass concentrations of almost 30 g L^{-1} and DHA concentrations of 4-5 g L^{-1} in 4 days of fermentation (Yamasaki et al., 2006). The analysis conducted on the distillery wastewater before and after the fermentation also demonstrates a considerable reduction in protein and amino acids during fermentation (Table 2.7), thereby supporting the theory that thraustochytrids can metabolize many organic forms of nitrogen. Leveraging this capability of highly-productive thraustochytrids can be instrumental in producing a more cost-effective microalgae biomass.

Table 2.7 Changes in concentration of nitrogen, protein, formol nitrogen (alpha amino acids and ammonia) and amino acids during cultivation of *Schizochytrium* sp. Source: Adapted from Yamasaki et al., 2006.

	Cultivation Period (d)			
Parameter	0	2	4	% Change
Total Nitrogen (% w/v)	0.16	0.11	0.05	-68.8%
Crude Protein (% w/v)	0.99	0.67	0.32	-67.7%
Formol Nitrogen (% w/v)	0.11	0.06	0.03	-72.7%
Amino Acids (mg $100mL^{-1}$)	451.8	211.8	68.5	-84.8%

2.7 NUTRIENT RECYCLING

Another possible avenue for improving the economics of microalgae production is by improving the efficiency of raw material consumption. An assessment of the anticipated resource demands of large-scale microalgae production for biofuels in the United States concluded that the effective management of water and nutrients is an important consideration for the economic and resource efficiency of microalgae production (Pate et al., 2011). Even in high-density heterotrophic cultures operating in optimized conditions, a significant quantity of water is required for microalgae production, where high density cultures (150 to 200 g L^{-1} dry weight) correspond to 80 to 85% water. This water is typically laden with dissolved compounds sourced from the original culture medium, excreted from the cells, or released during harvest or extraction steps (Bohutskyi et al., 2014b; Discart et al., 2014; Fon Sing et al., 2014; Hadj-Romdhane et al., 2012). Upon removal of the target compounds from the microalgae cells, water is typically considered as a waste stream and the effluent wastewater must be treated or discharged. When extending these considerations to commercial-scale production, this large volume of water and potential residual nutrition represents a considerable loss of material and potential regulatory concern for the producer (Passell et al., 2013; Rocha et al., 2014; Rösch et al., 2012; Yang et al., 2011).

Alternatively, the effluent water from microalgae production can be recycled internally and used as a secondary culture media with additional nutrients added as needed (Biller et al., 2012; Bohutskyi et al., 2014b; Du et al., 2012; González-López et al., 2013; Hadj-Romdhane et al., 2012; Rodolfi et al., 2003). At multiple junctures in the production process there exist opportunities for capturing and recirculating media and water (Fig. 2.9). If successfully conducted, an appropriate nutrient recycling regime can translate to significant savings of raw material inputs and avoided discharge treatment and disposal costs. Nutrient recycling is equally as attractive in heterotrophic cultures as conventional photoautotrophic cultures, with the added potential of cells metabolizing dissolved organic compounds generated during cell lysis and product extraction (González et al., 2008; Ratledge et al., 2001; Zheng et al., 2012a). The following sections explore the concept of nutrient recycling of the various potential waste streams, their likely composition, results from prior work, and the prospective associated challenges.

2.7.1 MATERIALS FLOW AND PROCESS SCHEMATIC

The composition of the material leaving the various stages of microalgae production and processing system is highly dependent upon the specific production parameters used (Biller et al., 2012; Rösch et al., 2012). These parameters include the metabolic state of the microalgae (heterotrophic, photoautotrophic or mixotrophic), species selection, initial medium composition, feeding strategy, and product extraction techniques. As such, a materials balance could approximate the characteristics of the effluent waste material if the physicochemical makeup of the inputs, losses and harvested products was known. The following sections will provide general information regarding the anticipated waste materials and their composition and usefulness for nutrient recycling.

Waste products that maintain potential value exit the microalgae production schematic at multiple points in the process. Figure 2.9 illustrates a simplified schematic of processing microalgae biomass to lipid products and the corresponding stages at which waste materials are generated. Included in the outgoing waste material are two generalized liquid streams, denoted *spent media* and *hydrolysate*, which will make up considerably greater quantities than the solid waste (*spent biomass*) leaving the system. However, depending upon the separation technique and process involved, the post-extraction solid and liquid waste materials may be ineffectively separated and treated instead as a waste slurry. The conventional standard for lipid extraction is mechanically-assisted solvent extraction, which is typically conducted on dehydrated microalgae biomass (Mercer and Armenta, 2011; Pragya et al., 2013). Those extraction methods would eliminate the *extraction liquid* recycling option, as that material would be primarily solvent, and the majority of water would be *spent media*. After lipid collection, the *spent biomass* could be reprocessed to use any remaining carbohydrates and protein (Rashid et al., 2013). In many operations it will be beneficial to remove the majority of the *spent media* prior to product extraction to increase the process efficiency or reduce transportation costs (Uduman et al., 2010). Doing so will also promote a larger proportion of waste leaving the system to be as *spent media* rather than *extraction liquid*, which is advantageous for nutrient recycling of unaltered media constituents and results in a reduced potential for waste slurry production. Consequently, this *spent media* can be returned and supplemented for use as a secondary

fermentation medium while the post-extraction wastes are reprocessed or disposed of externally (Rösch et al., 2012).

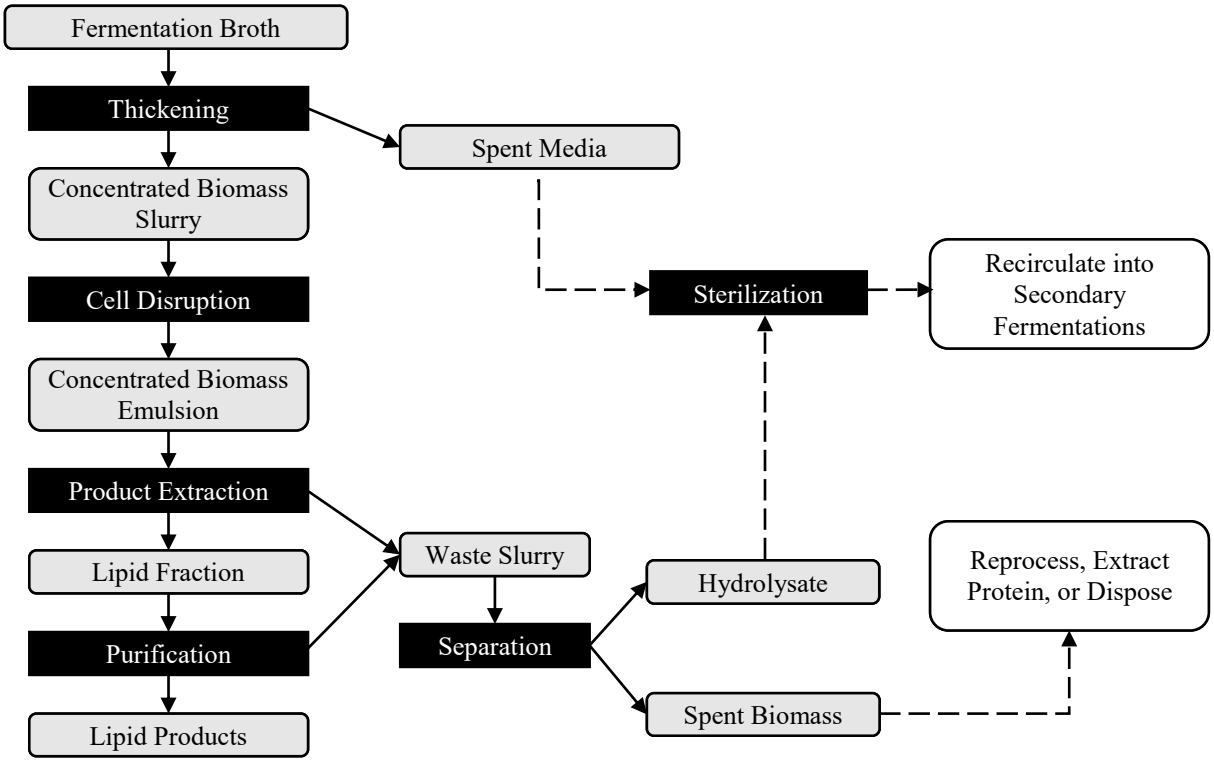

Figure 2.9 Generalized process schematic for microalgae production and product extraction steps (black boxes) with an emphasis on the products and generated waste streams (grey boxes).

2.7.2 Liquid Waste: Spent Media and Extraction Liquid

The principal difference between the liquid streams identified here is that *extraction liquid* includes solubilized cell material and any solvents, catalysts or enzymes used in the cell lysis and product extraction steps. Generically, it can be expected that the liquid waste streams possess the unused or residual components of the original fermentation medium, hence the term *spent media* (Rösch et al., 2012). The relative purity of the *spent media* is attractive for recycling into secondary fermentations and can readily be supplemented to desirable concentrations of nutrients without introducing any unknown compounds formed during product extraction (González-López et al., 2013; Rodolfi et al., 2003). These residual nutrients include varying concentrations of residual organic carbon (sugars,

carbohydrates, etc.), nitrogen (inorganic and complex organic nitrogen), phosphate and essential minerals that were not completely depleted during the primary fermentation (Discart et al., 2014; Rösch et al., 2012).

Any available literature exploring nutrient recycling to date is solely focused on photoautotrophic or mixotrophic growth conditions – however, these results can provide justification for exploration in heterotrophic cultures. One experiment attempting nutrient recycling of *spent media* used multiple recycles of LC Oligo nutrient media for cultivation of photoautotrophic *Scenedesmus quadricauda*. Treatments that reintroduced nutrients into the culture media exhibited comparable growth rates on the second and third use to that of the fresh medium (0.43 d^{-1}, 0.42 d^{-1}, 0.41 d^{-1}, respectively) (Rocha et al., 2014). Additional studies employing *spent media* for secondary microalgae cultures indicate mixed results with biomass production rarely reaching parity with a virgin media control (Table 2.8). Photoautotrophic *Nannochloropsis gaditana* was able to exceed the production of its respective control treatment but it is worth noting that the total biomass productivity attained was only 0.8 g L^{-1} d^{-1}. While this research was targeted at biomass production for an aquaculture application, productivities of this level are not likely sufficient for biofuel markets (González-López et al., 2013).

The *extraction liquid* is a much more variable and challenging material for nutrient recycling. In the *extraction liquid*, beyond possessing the components of the *spent media,* there may exist cellular debris (cell husks, carbohydrate, protein and minerals) depending upon the degree of cell lysis during product extraction (Biller et al., 2012). If a hydrolysis step is used for product extraction, there is potential for solubilisation of cell carbohydrates and proteins, resulting in a presence of organic carbon and amino acids in the *extraction liquid* (Bilad et al., 2014; Discart et al., 2014; Zheng et al., 2012a). The presence of the aforementioned compounds in solution offers potential for metabolism by microalgae during a secondary growth, especially in the case of heterotrophic cultures. The *extraction liquid* may present logistical challenges for optical analysis of the broth due to increased turbidity, viscosity and color formation and may also introduce mechanical impediments for pumping, mixing, or filtration. Additionally, any chemicals required throughout certain extraction processes (e.g., hexane, chloroform, etc.) may reside in the liquid and pose hazards for secondary fermentations. Ultimately, the increased complexity of the substrate

matrix of the *extraction liquid* can also present challenges for unknown reactions and potential inhibition due to accumulation of compounds or their intermediaries (Biller et al., 2012; Discart et al., 2014; Rösch et al., 2012; Zhu et al., 2013). Accordingly, it may be more favorable to concentrate this waste stream and export the remaining material as a cell extract for another industry as opposed to recycling within microalgae production.

Multiple studies have investigated using an *extraction liquid* derived from hydrothermal liquefaction of microalgae biomass as a growth substrate for secondary cultures of photoautotrophic/mixotrophic microalgae (Table 2.8). In these studies, obtaining comparable growth to that of the experimental control was possible, especially in cases where the *extraction liquid* was diluted sufficiently. Biller et al. (2012) suggested that the improvement in the diluted treatments was indicative of reducing the concentration of inhibitory compounds formed during the hydrothermal liquefaction process. However, this dilution strategy must be balanced with maintaining the concentration of valuable nutrients and reducing water consumption. Additionally, less intensive extraction conditions than employed in those studies may mitigate the formation of these inhibitors as will be discussed later.

2.7.3 Solid Waste: Spent Biomass

While the lipid fraction is removed from microalgae biomass produced for oil products, it is well known that the remaining components of microalgae biomass are primarily carbohydrates, protein and minerals (Gao et al., 2012; Rashid et al., 2013; Romero García et al., 2012). On an elemental scale this biomass is comprised primarily of carbon, oxygen, hydrogen and nitrogen (Bumbak et al., 2011; Williams and Laurens, 2010). These remaining materials in the *spent biomass* – sometimes called lipid-extracted algae – and the potential for repurposing them within the microalgae production system is the focus of an emerging research area (Rashid et al., 2013; Zheng et al., 2012a). There are many possible applications for this material including biogas production, animal feed supplements, fertilizers and even possible use as a chemical sorbent for wastewater treatment (Rashid et al., 2013). Ideally an application for this material would require minimal processing to avoid additional costs. Recycling the nutritional value of *spent biomass* internally in microalgae production may be less attractive than external

applications since the proteins and carbohydrates would need to be solubilized to bioavailable forms which requires some degree of reprocessing.

While reprocessing the *spent biomass* for internal nutrient recycling could be accomplished via conventional physical or chemical treatment by high heat or acidic conditions, new research explores using specialized enzyme cocktails to solubilize the cells. Zheng et al. (2012a) conducted experiments using lipid-extracted *Chlorella vulgaris* biomass and a two-step cellulase, alcalase, neutrase mixture to produce a hydrolysate for secondary microalgal culture. In doing so they produced a nutritional hydrolysate that was inoculated with a second culture of *C. vulgaris* and cultivated in an illuminated bubble column photobioreactor. The results demonstrated that the microalgae could effectively grow (albeit slowly over 10 days) to a maximum concentration of 3.28 g L^{-1} with a lipid content of 35% while depleting all available sugar and amino acid resources in the hydrolysate. These outcomes are very promising for demonstrating the conceptual possibility of *spent biomass* reprocessing and nutrient recycling. However, these results must be considered in comparison to the productivity of fresh media as well as factoring in the *spent biomass* processing costs to draw larger-scale conclusions. Additionally, the aforementioned results employ a mixotroph *C. vulgaris* which could leverage both organic carbon in the recycled media and light for cell synthesis. It is important to also validate these results in purely heterotrophic conditions.

2.7.4 Outcomes in Photoautotrophic Cultures

To date, no literature exists on nutrient recycling of waste products generated in purely heterotrophic microalgae cultures. However, there are multiple studies that have incorporated nutrient recycling into their production systems or experiments using photoautotrophic, or mixotrophic microalgae. These studies are typically not specifically looking at nutrient recycling as a primary objective, but rather attempting to demonstrate the usefulness of the waste products that are obtained from intensive extraction procedures (e.g., hydrothermal liquefaction). Observing the results of these similar experiments, such as those explained in Table 2.8, we can infer about the potential of a purely heterotrophic nutrient recycling regime.

The results in Table 2.8 suggest that multiple nutrient recycling experiments have been successful, with some important caveats. The success is demonstrated by favorable comparison to the control (fresh media), which is shown to be close or equal in performance for both liquid waste streams. The results recycling the spent biomass did not report a control for comparison, but did yield fairly high biomass concentrations in a moderate growth period, thereby indicating the recycled material does support a secondary culture. Those outcomes suggest that equal growth can be attained using recycled waste materials to fresh media, strongly suggesting that similar outcomes could be obtained in heterotrophic culture. However, the considerations with those results have important implications for implementation of nutrient recycling in heterotrophic cultures. First, all of the biomass concentrations reported are significantly lower than those obtained in heterotrophic cultures which may result in a reduced concentration of any unwanted inhibitory byproducts produced during product extraction. Even still, those results recycling an extraction liquid from hydrothermal liquefaction required dilution (50-400 fold) to avoid any inhibition caused by the waste material. This heavy dilution means much of the waste material would not be recycled.

Table 2.8 Summarized growth results and experimental parameters for secondary cultivation of non-heterotrophic microalgae in waste materials derived from virgin-media microalgae production. Metabolic states: P = photoautotrophic, M = mixotrophic.

Microalgae	Metabolic State	Waste Material	Extraction Technique	Secondary Growth Result			Notes	Reference
				Max. Biomass	Ratio to Control	Duration (d)		
Chlorella vulgaris	P/M	Spent biomass hydrolysate	Two-step enzymatic	3.28 g L^{-1}	--	10	3% CO_2 sparging	(Zheng et al., 2012a)
Chlorella vulgaris	P/M	Spent biomass hydrolysate	Two-step enzymatic	3.76 g L^{-1}	--	10	5% CO_2 sparging	(Zheng et al., 2012b)
Chlorella vulgaris	P/M	Spent biomass hydrolysate	Two-step enzymatic	3.83 g L^{-1}	--	10	0.5 vvm air sparging	(Zheng et al., 2012b)
Nannochloropsis oculata	P/M	Extraction liquid	Hydrothermal liquefaction	0.8 g L^{-1}	1.00	6.5	80 x diluted substrate	(Levine et al., 2013)
Chlorella vulgaris	P/M	Extraction liquid	Hydrothermal liquefaction	0.88 g L^{-1}	0.86	11	100 x diluted substrate	(Biller et al., 2012)
Scenedesmus dimorphous	P/M	Extraction liquid	Hydrothermal liquefaction	0.05 g L^{-1}	0.41	12	400 x diluted substrate	(Biller et al., 2012)
Spirulina platensis	P/M	Extraction liquid	Hydrothermal liquefaction	0.66 g L^{-1}	0.93	11	400 x diluted substrate	(Biller et al., 2012)
Chlorella vulgaris	P/M	Extraction liquid	Hydrothermal liquefaction	0.79 g L^{-1}	4.39[a]	4	50 x diluted substrate	(Du et al., 2012)
Nannochloropsis gaditana B-3	P	Spent media	N/A	0.49 g L^{-1}	0.90	7	Ozone sterilized	(González-López et al., 2013)

Microalgae	Metabolic State	Waste Material	Extraction Technique	Secondary Growth Result			Notes	Reference
Nannochloropsis gaditana B-3	P	Spent media - supplemented	N/A	0.57 g L^{-1}	1.04	7	Ozone sterilized	(González-López et al., 2013)
Nannochloropsis sp.	P	Spent media w/particulates - supplemented	N/A	1.97 g L^{-1}	0.41	25	Supplemented fully	(Rodolfi et al., 2003)
Nannochloropsis sp.	P	Spent media w/o particulates - supplemented	N/A	2.39 g L^{-1}	0.50	25	Supplemented fully	(Rodolfi et al., 2003)
Nannochloropsis sp.	P	Spent media w/o particulates - supplemented	N/A	1.88 g L^{-1}	0.39	25	Supplemented nitrate and phosphate only	(Rodolfi et al., 2003)

[a] Control did not exhibit any growth

2.7.5 CARBON RECYCLING

For heterotrophic microalgae cultures the most essential element of the growth substrate is carbon – in part due to the essential biochemical role as well as the cost of providing a carbon source in the media (Bumbak et al., 2011; Perez-Garcia et al., 2011; Richmond, 2008). Culture media is typically formulated with the stoichiometric composition of the produced microalgal biomass in mind; which although varied for each species and metabolic condition, is predominantly organic carbon (Bumbak et al., 2011). In optimized non-continuous cultures it is favorable to harvest cells upon carbon and nitrogen depletion of the substrate, resulting in minimal concentrations of those nutrients residing in the effluent *spent media*. The sequestered carbon has been converted by the microalgae cells from simple organic carbon into complex cellular material (e.g., lipids, carbohydrates, etc.) via multiple metabolic pathways (Perez-Garcia et al., 2011; Yang et al., 2000). After biomass harvesting and lipid extraction there still remains considerable quantities of cellular carbon locked into carbohydrates that is nutritionally unavailable for further uptake by microalgae, yet a potentially valuable leftover material (Gao et al., 2012; Rashid et al., 2013; Romero García et al., 2012). For successful nutrient recycling to be possible, in both the liquid and solid waste material, it is essential to identify methods for and the feasibility of solubilizing this cell material into its bioavailable constituents.

Some product extraction techniques that employ harsh conditions result in hydrolyzing the carbonaceous material into a variety of organic acids and alcohols (Biller et al., 2012; Levine et al., 2013). Hydrothermal liquefaction is one product extraction technique that relies upon high heat and elevated pressure to convert microalgal biomass slurries into a valuable biocrude oil and a corresponding *extraction liquid.* This effluent wastewater contains a plethora of organic carbon and nitrogen compounds derived from the decomposing biomass, some of which are potentially valuable for cell metabolism (Biller et al., 2012; Jena et al., 2011). Select heterotrophic microalgae have been shown to tolerate and metabolize substrates with high concentrations of organic acids like acetic acid (Lowrey et al., 2014; Perez-Garcia et al., 2011). Simultaneously during the decomposition of *spent biomass*, enhanced concentrations of potentially inhibitory compounds can be formed which may have adverse effects upon secondary microalgae cultures. Some inhibitory compounds to microalgae formed during hydrothermal liquefaction include

heavy metals, phenols, fatty acids, and ammonia (Biller et al., 2012; Jena et al., 2011; Levine et al., 2013). This challenge with inhibition has been mitigated by dilution of the *extraction liquid* to concentrations tolerable for the secondary culture, while still providing ample nutrition and recycling of water.

Targeted enzymatic treatment of the carbohydrate-containing *spent biomass* can also result in improved bioavailability of carbon, making it more suitable for secondary cultures (Zheng et al., 2012a). This technique has the distinct advantage of precise control of the decomposition reactions being catalyzed by the enzymes, thereby avoiding unknown reactions and formation of unwanted compounds. However, enzyme treatment is limited by costs and the availability of specialized enzymes for hydrolysis of target compounds (Mercer and Armenta, 2011; Pragya et al., 2013). In the studies by Zheng et al., an enzyme mixture was designed for targeted hydrolysis of cellular compounds of *Chlorella vulgaris* to bioavailable forms for secondary microalgae cultures. Initially, the lipids were extracted using a cellulase, thereby targeting the cellulose-containing cell walls and lysing the cells. The *spent biomass* decomposition was executed by the addition of cellulase, neutrase and alcalase to sequentially target hydrolysis of carbohydrates and proteins to sugars and amino acids, respectively. Of the sugars detected after decomposition of the *spent biomass*, glucose was most prevalent followed by arabinose and xylose; all of which appeared to be depleted during secondary culture (Zheng et al., 2012a, 2012b). Employing similar enzymatic strategies can provide a "clean" method for converting *spent biomass* as well as *spent media* and *extraction liquid* into bioavailable compounds for use in secondary microalgae cultures without the formation of potentially inhibitory compounds.

2.7.6 NITROGEN RECYCLING

Nitrogen is an essential nutrient for microalgae metabolism as it is an integral element for cell structure, energy carriers and protein synthesis (Barsanti and Gualtieri, 2006; Graham and Wilcox, 2000). Nitrogen is available to microalgae in the forms of ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), urea ($(NH_2)_2CO$), complex nitrogen (cell extracts) and amino acids (Anderson, 2005; Barsanti and Gualtieri, 2006; Perez-Garcia et al., 2011; Richmond, 2008). Typically nitrogen is provided in the media in complex organic forms such as protein-containing soy peptone and yeast extract or inorganic forms like nitrate and

ammonium (Anderson, 2005). While required in lesser concentrations than carbon, complex nitrogen sources are significantly more expensive, making it imperative that they are used with great efficiency (Cheng et al., 2009; Davis et al., 2011). Theoretically, optimized cultures are commonly finished with a period of nitrogen deficiency which triggers intracellular accumulation of lipids, therefore making a more valuable biomass product (Bumbak et al., 2011). In this case the *spent media* obtained from cell harvest prior to any cell disruption will likely be largely nitrogen deplete. However, the *extraction liquid* and *spent biomass* will contain cellular nitrogen in the form of protein and amino acids, varying in concentration corresponding to the degree of cell lysis during product extraction (Biller et al., 2012). This is significant because the protein content of microalgae constitutes a considerable portion of the biomass, comprising up to of 70% of lipid-extracted biomass (Singh et al., 2011). Greater cell degradation during product extraction will favor protein breaking into its constituent peptides and amino acids which will reside in the *extraction liquid* as opposed to the protein-containing *spent biomass*. In these soluble derivative forms, the nitrogen may be more bioavailable for a secondary microalgae culture.

Enzymatic treatment is an attractive method for selectively hydrolyzing the carbohydrates and proteins in microalgae *spent biomass.* It has been shown that using specific enzyme mixtures can release amino acids into solution either for nutrient recycling or collection (Romero García et al., 2012; Zheng et al., 2012a). For example, an alcalase (protease) and flavourzyme (peptidase) cocktail was employed for solubilization of *Scenedesmus almeriensis* protein into amino acids. In doing so, a degree of hydrolysis ranging from 51% to 66% was reported with a wide array of essential amino acids produced (Romero García et al., 2012). After solubilization of the proteins in microalgae *spent biomass* the collected amino acids can be recycled as an organic nitrogen source for a secondary culture. The aforementioned studies by Zheng et al. (Zheng et al., 2012a) using a two-stage enzymatic hydrolysis of microalgae *spent biomass* to support a secondary culture demonstrated the value of solubilized protein in the substrate. The original *C. vulgaris* biomass consisted of 34.1 % (wt.) protein which was targeted by the neutrase and alcalase during hydrolysis. The catalyzed decomposition of the protein produced 478.1 mg L^{-1} of amino acids which were fully depleted within 9 days of secondary growth (Zheng et al., 2012a). While it has been previously theorized that organic nitrogen (e.g., amino acids)

can support microalgae growth, these results provide evidence that *C. vulgaris* can indeed utilize an array of essential amino acids (Levine et al., 2013). This capability encourages the prospect of successful nitrogen recycling by hydrolyzing *spent biomass* into soluble amino acids which can in turn be metabolized during secondary cultures.

Alternative methods for product extraction and hydrolysis of protein-containing waste material exist, however, the nitrogen cycling is rarely a primary concern during those experiments. For example, the hydrothermal liquefaction methods previously discussed for production of biocrude and *extraction liquid* induce extreme conditions which can favor the hydrolysis of cell protein. However, this protein was not quantified and is likely reacted with other compounds in solution – such as reducing sugars – in conditions of high temperature (Nursten, 2005). Among these complex reactions the formation of inhibitory compounds can occur, as evidenced by the poor secondary growth results in undiluted *extraction liquids* formed by hydrothermal liquefaction. In contrast, when those *extraction liquids,* and any present inhibitors were diluted, secondary growth increased (Biller et al., 2012; Levine et al., 2013).

The formation of inhibitory compounds during exposure to high temperatures due to complex Maillard reactions is the primary concern for successful nitrogen recycling of waste materials in microalgae production. This concept, although well known to be related to reactions between amine groups (proteins, peptides, amino acids) and carbonyl groups (reducing sugars), is highly complex and variable depending upon the reaction conditions and the presence and concentration of the reactants (Nursten, 2005). As both reactants are present in the *spent media* and *extraction liquid* it is disadvantageous to expose them to heat which will result in conversion of the valuable carbon and nitrogen to potentially inhibiting compounds. Unfortunately, heat is the most practical means for sterilization of growth media prior to inoculation. Two possible means for avoiding unwanted Maillard reactions due to nitrogen recycling are i) diverting the *spent media* by separating it prior to product extraction, thereby avoiding increasing the concentrations of solubilized protein in the *spent media*, and ii) employing non-heat dependent sterilization methods for the waste streams.

2.7.7 MICRONUTRIENTS RECYCLING AND ACCUMULATION

Beside carbon and nitrogen, there are many essential nutrients and minerals required for optimized microalgae growth. These elements of the media are added initially and may not be consumed completely during the growth phase, leaving them present in substantial quantities in the effluent waste materials (Biller et al., 2012; Jena et al., 2011). As with carbon and nitrogen, the specific concentrations of these additional nutrients will vary depending upon the growth conditions, species and metabolic state; thereby making broad characterizations of their abundance difficult and entirely case-specific. However, accumulation of some of these elements may be of concern due to known interactions in the substrate. For instance, iron is known to bind with phosphates during sterilization in the autoclave due to elevated pH, resulting in irreversible precipitation and subsequent loss of nutrients (Anderson, 2005). While this can be avoided by addition of a chemical chelator or sterilization of the reactants separately, the latter solution is not possible when recycling the media if concentrations are still present in the waste liquids. The formation of an iron-phosphate precipitate can be avoided by introduction of the chelator upon recycling, or careful management of the primary culture to ensure depletion of phosphates. Additionally, maintaining separate solutions of supplemented media during sterilization to later be combined aseptically may mitigate precipitation of iron phosphates. This will result in minimized losses of nutrients by avoiding precipitation of any fresh nutrients added for the second culture and restricting any precipitation to occurring in the separate waste material.

Accumulation of minerals or salts is another important consideration for a successful nutrient recycling regime. As stated previously, nutrient media recipes are typically formulated based upon the expected stoichiometric equation of microalgae cells in order to provide the required elements for effective cell synthesis (Barsanti and Gualtieri, 2006; Bumbak et al., 2011; Graham and Wilcox, 2000; Richmond, 2008). This concept is well suited for single-use substrates as it provides sufficient resources to sustain microalgae growth. However, the reality is that the composition – and corresponding stoichiometric equation – of microalgae cells varies during growth, depending upon whether the cells are focused on rapid reproduction in nutrient replete environments or energy storage in deprived environments (Laurens et al., 2014). Accordingly, the exact consumption of substrate components by microalgae cells is complicated and formulating an ultra-efficient

recipe without sacrificing productivity would be challenging. The outcome is a likely excess of nutrition in the media, and a corresponding presence of some nutrients in the effluent wastes. During a 5-day culture of photoautotrophic *Chlorella protothecoides* 65 to 91% of Cu, Mg, N, Fe, Mn, and S and > 95 % of the other tested elements remained in the substrate at the end of a modest initial growth. However, a second heterotrophic growth phase demonstrated rapid nitrogen depletion, along with 87 to 99% removal of micronutrients within 20 hours; this corresponded to an accumulation of up to 121 g L^{-1} of cell biomass by 120 hours. The most abundant of the residual micronutrients included boron, sulfur and calcium with all others (Zn, Cu, Co, Fe, Mo, Mn, Mg) remaining at some level (Bohutskyi et al., 2014b). These results illustrate that photoautotrophic cultures are especially prone to residual nutrients existing in the waste streams, however, even an optimized heterotrophic culture producing considerable biomass still leaves unused micronutrients and minerals in the substrate.

In highly recycled media it is anticipated that certain medium components are most likely to accumulate. Among these compounds are ions like chloride derived from nutrient complexes in macronutrients, salts and trace metal solutions, thereby having important implications for vessel corrosion (discussed later) (Anderson, 2005). A study conducted by Hadj-Romdhane et al. (2012) specifically explored trying to synthesize an optimized culture media for a continuously cultivated photoautotrophic *Chlorella vulgaris* to ensure maximum uptake of all elements. In the process the ionic concentration was monitored of a conventional Sueoka medium during multiple harvests and medium recycles. The results depicted increasing concentration of the total ions in solution from an initial concentration of 1,400 mg L^{-1} to 4,800 mg L^{-1} during 16 recycles over 8 weeks of continuous culture. The most prominent ions observed increasing in solution throughout this period were sodium and chloride, from 400 mg L^{-1} up to 1,600 mg L^{-1} and from 600 mg L^{-1} up to 2,600 mg L^{-1} respectively (Hadj-Romdhane et al., 2012). These increasing concentrations were predictable in culture where sodium bicarbonate (Na_2HCO_3) and ammonium chloride (NH_4Cl) would result in conversion to bicarbonate and the metabolism of ammonium as a nitrogen source, leaving behind ions of sodium (Na^+) and chloride (Cl^-). In repeated recycles of the *spent media*, these ions gradually accumulate in the broth.

If a nutrient recycling regime was used with multiple recycles, these excess nutrients and minerals can translate to an accumulation of certain elements in the substrate, possibly exceeding the tolerance levels of the microalgae and having adverse impacts upon the secondary growth. Increasing salt concentrations are of particularly important concern, due to anticipated carryover from previous cultures and known salinity sensitivities of many microalgae (Fon Sing et al., 2014; Ghezelbash et al., 2008). While this is not a concern for freshwater species, there is an incentive to pursue marine microalgae to mitigate the consumption of freshwater and the corresponding resource intensity of production. One study explored the effect of salt accumulation on an outdoor culture of *Tetraselmis* sp. over the course of a 127-day continuous culture using a nutrient recycling regime after electroflocculation to harvest the biomass. The experiment observed an increase from 5% to 12% of salt content of the medium, while productivity was maintained between 23.8 and 37.5 g m^{-2} d^{-1}, as compared to 14.3 and 17.0 g m^{-2} d^{-1} in the control (Fon Sing et al., 2014). These results are indicative of a successful nutrient recycling regime for a relatively low density, halotolerant microalgae but may not entirely translate to other high-productivity species. Additionally, alternative cultivation strategies like fed-batch heterotrophic systems may have very different outcomes as well as infrastructural considerations with corrosion of stainless steel production equipment.

An important concern for nutrient recycling in optimized heterotrophic cultures is the impact that culture media would have on the fermentation equipment. Considering industrial-scale heterotrophic cultures of microalgae will likely be conducted in stainless steel fermentors, a major concern that arises is the prospect of corrosion by chloride ions originating from salts and trace elements in the culture media. Stainless steel is particularly susceptible to corrosion by chloride ions which are able to penetrate the surface and cause pitting, eventually deteriorating the integrity and sterility of the material (Craig and Anderson, 1994). This damage represents a significant cost and liability to commercial-scale fermentation operations which introduces a considerable risk. Many microalgae medium recipes include compounds such as ammonium chloride (NH_4Cl), calcium chloride ($CaCl_2$), potassium chloride (KCl), ferric chloride ($FeCl_3$), manganese chloride ($MnCl_2$), cobalt chloride ($CoCl_2$) and zinc chloride ($ZnCl_2$) (Anderson, 2005). While these are essential components of the media, they are not always depleted in the primary culture

and may exist in sufficient concentrations to accumulate from nutrient recycling. Furthermore, as the metabolically important elements are taken up, the remaining ions of chloride can reside in the *spent media* which can accumulate to detrimental levels after multiple nutrient recycles (Hadj-Romdhane et al., 2012).

The challenge of chloride corrosion remains largely unexplored in current research, but some possible mitigation strategies can be suggested. In some instances, nutrients can be provided in compounds where they are complexed to more benign ions. For example, ammonium sulfate could be a viable alternative to ammonium chloride, therefore eliminating a large contributor of chloride ions in the *spent media* (Yaguchi et al., 1997). In marine cultures employing nutrient recycling, the salinity of the recycled media should be monitored and subsequent additions of salt (NaCl) may be unnecessary. The contribution of chloride ions from trace metals may not be avoidable, but considering those medium components are added in substantially lesser concentrations the impact may be minor (Anderson, 2005). Ultimately, in adherence to technical specifications, there may be a maximum recycle rate corresponding to a maximum chloride concentration that limits the potential corrosion damage to stainless steel equipment.

2.7.8 Practical Constraints and the Sterilization Challenge

Nutrient recycling offers the potential to increase the resource usage efficiency of microalgae production by decreasing water consumption and more effectively allocating nutrients into the growth substrate, but there are also some practical limitations that have been eluded to in the preceding sections. The most challenging issue with successful nutrient recycling is the possible presence of inhibitory compounds in the recovered waste products. Accordingly, a major concern is heat-sterilization of these waste products, which, due to their anticipated composition will likely result in formation of unwanted compounds. Most notably are the Maillard reactions which result from reactions at elevated temperatures between protein and protein derivatives with reducing sugars present in the heterotrophic growth media (Nursten, 2005). While currently it is uncertain that the Maillard products will be harmful to a secondary microalgae culture, there are many potential reactants in typical growth media (i.e., glucose, protein, amino acids, ammonium) which can result in degradation of the intended media components and formation of

unknown products. This concern is especially pronounced in heterotrophic cultures due to the presence of organic carbon in the media recipe which would not be added in photoautotrophic cultures.

One potential avenue for avoiding the formation of heat-induced inhibitors is to employ alternative sterilization techniques such as filtration. A study by González-López et al. (2013) explored a variety of accepted sterilization techniques for recycling nutrient media sourced from outdoor photobioreactors producing biomass for aquaculture feed. The results suggested that filtration and ozonation were the most effective methods for sterilizing recycled media for production of *Nannochloropsis gaditana* biomass (0.8 g L^{-1} each) in a secondary culture. Heat-sterilization resulted in diminished growth (0.45 g L^{-1}) and was postulated to be likely associated with formation of undesirable inhibitors, although this assertion was unverified (González-López et al., 2013). Filtration is commonly used for sterilization of laboratory-scale micronutrients and vitamins due to their heat sensitivity (Anderson, 2005). However, these components are added in considerably smaller volumes than the rest of the media and contain no particulates that may block the small (0.2 μm) filter pores. For nutrient recycling scenarios, filtration would be significantly more challenging due to the probable presence of small particulates and cell debris which could result in rapid blockage of the relatively expensive membrane. However, after successful separation from the harvested cells, a *spent media* stream may present a more viable solution for filter sterilization as it should not contain suspended solids or cell debris.

Since heat-sterilization is an accepted industrial technique for sterilizing culture media it is important to consider strategies to successfully execute nutrient recycling without requiring a separate filter sterilization apparatus. A practical solution is to maintain the highest degree of separation of the recycled materials from virgin nutrient solutions during sterilization. Although some reactions may occur within the waste liquid, they would not cause detrimental reactions within virgin nutrient solutions. This restricts any possible adverse reactions to the recycled materials, and due to their depleted concentrations, minimizes the abundance of inhibitors formed. In a system producing a large volume of *spent media* this translates to sterilizing this solution separately from any additional nutrients being supplemented, possibly by preparing concentrates of the virgin

nutrients to reach targeted concentrations with a reduced volume. In regard to *extraction liquid* it may be necessary to dilute the material prior to secondary culture, which could also be achieved by a concentrated solution of virgin nutrients.

2.7.9 TOXICITY AND AUTOINHIBITION

The previous sections illustrate how nutrient recycling introduces the challenge of accumulating a variety of compounds in the growth substrate, possibly to the point of exceeding the toxicity thresholds of the microalgae being cultured. In addition to accumulating elements that are added in the culture media formulation, there is a potential concern of accumulating metabolites synthesized and released by the cells themselves. These autoinhibitory compounds and the mechanisms of their synthesis are poorly understood but have been recognized in some high-density cultures for decades (Borowitzka and Moheimani, 2012; Rodolfi et al., 2003). As research progressed in algal biotechnology, many proposed inhibition theories were left unproven or dismissed as a deficiency in the culture medium composition or light availability instead (Richmond, 2008). Nonetheless, some research still suggests specific cases of autoinhibition occurring, mostly in photoautotrophic cultures (Richmond, 2008). It has been hypothesized that inhibition occurs upon nutrient depletion at high cell densities of *Nannochloropsis* sp*.,* a problem that was abated by replacement of the media (Zou et al., 2000). This terminal-phase secretion of undesirable compounds would have direct implications for recycling the growth media from optimized cultures for secondary cultures. However, no specific pathways or have been identified and this trend has only been observed in select species. This phenomenon has obvious implications for a nutrient recycling regime and would need to be addressed on a case-by-case basis to determine if the microalgae being produced secretes an inhibitory substance (Borowitzka and Moheimani, 2012; Richmond, 2008).

CHAPTER 3 : PRODUCTION AND CHARACTERIZATION OF WASTE MATERIAL

3.1 INTRODUCTION

As this research focuses on the recycling of fermentation waste, it is important to characterize the waste materials of interest. Therefore, to provide a basis for further experiments, this chapter investigates the typical composition of these materials. This assessment incorporates traditional proximate analysis (moisture, protein, carbohydrate, lipid and minerals), typical wastewater monitoring parameters, and application-specific assays. These collective parameters provide a basis of understanding to interpret the nutrient availability, substrate habitability, as well as the secondary fermentation growth outcomes and resource usage efficiency.

This chapter begins with a detailed description of the production of the original waste material that would be derived from standard fermentation processing (i.e. from the primary fermentation and subsequent biomass processing and enzymatic lipid extraction procedures), followed by the characterization methodologies used. Results from the primary fermentation are then presented as evidence that typical fermentation outcomes and waste materials were produced and the composition of raw biomass is reported. In accordance with thesis objectives (i) and (ii), the compositions of the waste products are determined and their suitability as a nutrient source for secondary fermentations is discussed. The waste materials described in this chapter are then used in Chapter 4 for recycling experiments.

3.2 METHODS AND MATERIALS

3.2.1 30-LITER PRIMARY FERMENTATION (2015-30L-18)

The intent of the primary fermentation was that it be representative of an optimized, glucose-based fed-batch fermentation, to yield results typical of fermentations run at Mara Renewables Corporation laboratories. Accordingly, the primary fermentation was conducted with the assistance of experienced fermentation scientists Kevin Berryman and Mercia Valentine who oversaw the planning, setup and operation of the fermentation. In order to produce sufficient material for complete analysis and secondary fermentations

(Chapter 4), a 30-liter fermentor was used. At the end of the primary fermentation, a sample of "raw biomass" was used for compositional analysis. The remaining liquid and solid materials from the primary fermentation were retained for further biomass processing and enzymatic lipid hydrolysis.

3.2.1.1 Seed Preparation

The first step in any fermentation is the preparation of the seed for inoculation of the culture vessel. In accordance with protocol, 1.5 liters of seed (5.0% v/v) was the volume used for inoculation of the 30-liter fermentor. Therefore, four 2-liter baffled flasks were filled with 500 mL of prepared Windust Lite medium (described in Table 3.1) and sterilized in the Getinge Model733LS autoclave (Göteborg, Sweden). After cooling, each flask was inoculated with a loop-full of plate-cultured T18 and placed on an orbital shaker for 3 days at 200 rpm and 28ºC. Upon completion of the seed growth, each culture was inspected under a Nikon Eclipse 600 phase contrast microscope (Tokyo, Japan) to detect any contamination and assess cell health.

3.2.1.2 Fermentor Setup and Inoculation

The process for setting up and beginning a fermentation in the Sartorius Biostat C Plus 30-liter fermentors (Goettingen, Germany) requires preparation of the fermentation medium and feed stock solution as well as sterilization of all equipment and media components. During a fed-batch fermentation, the starting volume is less than the target final volume as a large volume of concentrated carbon is typically added throughout the growth period. This disparity between initial and final volume required preparation of the medium in higher concentrations than a fixed-volume fermentation. Table 3.2 describes the medium composition for the primary fermentation. Sterile stock solutions of trace elements and vitamins were in the concentrations depicted in Table 3.1.

Table 3.1 Windust Lite recipe for seed preparation in addition to stock solutions for trace metals and vitamins.

Medium Recipe		**Trace Metals Stock Solution**	
Reagent	**Amount per 1 L**	**Reagent**	**Concentration**
Yeast Extract	1 g	Copper sulfate	2 mg mL^{-1}
Soy Peptone	4 g	Sodium molybdate	1 mg mL^{-1}
Glucose	60 g	Zinc sulfate	2 mg mL^{-1}
Mg heptahydrate sulfate	4 g	Cobalt (II) chloride	1 mg mL^{-1}
Sodium chloride	2 g	Manganese chloride	1 mg mL^{-1}
Ferric chloride	0.5 mL of 0.01 g mL^{-1} solution	Nickel sulfate	1 mg mL^{-1}
Trace element solution	1.5 mL	**Vitamin Stock Solution**	
*Potassium phosphate (monobasic)	10 mL of 0.2 g mL^{-1} solution	**Reagent**	**Concentration**
		Vitamin B12	0.01 g L^{-1}
*Ammonium sulfate	17 mL of 0.4 g mL^{-1} solution	Biotin	0.01 g L^{-1}
*Vitamin stock solution	2 mL	Thiamin hydrochloride	2 g L^{-1}
*Calcium chloride dihydrate	500 µL of 0.2 g mL^{-1} solution		

* Added aseptically after autoclaved

Once the medium was prepared, the procedure for setting up the 30-liter fermentor was as follows. First, all fermentor components and accessories were assembled, calibrated (probes) and installed. 15 liters (less the separately sterilized part) of medium components were then added to the fermentor, followed by initiating the sterilization program which employed steam sterilization at 15 psi and 121ºC. The separately sterilized 1.5 liter buffer solution (containing ammonium sulfate and phosphates) was pumped via an aseptic peristaltic pump into the fermentor. Prior to feeding the seed solution into the fermentor, the vessel was cooled to 28ºC. Acid, base, and feed bottles were then attached and tubing connected to the automated pumping system. Once all connections were made, fermentation conditions were set to pH 5.75, 28ºC, 1.0 vvm aeration with 750 rpm mixing. At this point the fermentation was started, along with a monitoring computer and data collector, and sampling initiated.

Table 3.2 Medium recipe for 30-liter fed-batch primary fermentation with initial volume of 15 liters.

Medium Recipe

Reagent	Amount per 1 liter	Amount for Final Volume
Glucose (cerelose)	165 g	2,475 g
Soy Peptone	2 g	30 g
Mg heptahydrate sulfate	4 g	60 g
Sodium chloride	1.65 g	26.4 g
Ferric chloride	0.5 mL of 0.01 g mL^{-1} solution	7.5 mL
Trace element solution	1.5 mL	22.5 mL
Antifoam (DC 1500)	0.1 mL	1.5 mL
*Potassium phosphate (monobasic)	2.2 g	33 g
*Potassium phosphate (dibasic)	2.4 g	36 g
*Ammonium sulfate	20 g	300 g
*Vitamin stock solution	3 mL	45 mL
*Calcium chloride dihydrate	500 µL of 0.2 g mL^{-1} solution	7.5 mL
Base (5M NaOH)	N/A	As needed
Acid (25% H_3PO_4)	N/A	As needed
Carbon Feed (75% w/v Glucose)	N/A	15 L

* Added aseptically after steam sterilization

3.2.1.3 Sampling Program

The fermentation was monitored by continuous data logging of fermentation parameters (pH, temperature, dissolved oxygen, exhaust carbon dioxide, stir rate, base feed volume, acid feed volume, and glucose fed) as well as manual quantification of biomass concentration, total fatty acid content and substrate glucose concentration. This suite of parameters was used to effectively manage the fermentation and assess the quality of the biomass produced.

The following sampling and processing protocol was used to obtain the manually quantified parameters. First, the valve for steam sterilization of the fermentor sample port was opened to allow steam to flow through for at least 30 seconds. Once sterilized, 50 mL of fermentation broth was collected in a 50-mL sterile centrifuge tube. For duplicates, 20 mL aliquots of the homogenized sample were transferred into two new, pre-weighed 50-

mL centrifuge tubes. After recording the wet weight of the sample in the pre-weighed tube, the samples were centrifuged at 2000 rpm, 2ºC, for 20 minutes. The supernatant was separated and collected for substrate analysis (glucose, ammonium, etc.) while the cell pellet was re-suspended with distilled water, vortexed thoroughly, and centrifuged again using the same conditions. The washing steps were repeated twice before the cell pellet was freeze dried to a constant weight. This pellet, termed "raw biomass" was used for quantification of biomass concentration as well as total fatty acid content. Biomass concentration was calculated using Equation 3.1.

$$\text{Biomass concentration, g } L^{-1} = \frac{(\text{dry wt. - tube wt.})}{(\text{wet wt. -tube wt.})} \times 1000 \qquad \text{(Eq. 3.1)}$$

3.2.1.4 Oil Removal Procedure for Raw Biomass Prior to Proximate Analysis

Prior to completing the proximate analysis of the biomass, it was essential for protein analysis to remove all oil from the sample. To achieve the best oil extraction possible, the conventional solvent extraction technique originating from Bligh and Dyer (1959) was most favorable. The adapted protocol for hexane lipid extraction includes the following steps. Thawed finished fermentation broth (cells and media) was centrifuged at 4,600 rpm at 20ºC for 20 minutes to separate the cells from the media. The lighter, oil-bearing cell layer was punctured to pour off the spent media and the biomass cake was freeze dried to remove all remaining water. Two hundred gram aliquots of the dried biomass were added to a 2-liter beaker and topped off with 1.2 liters of hexane. The biomass/hexane mixture was then exposed to high shear force using the VWR PT6100 Polytron (Radnor, U.S.A.) at 6,000 rpm for 10 minutes. After homogenization, samples were returned to the centrifuge at 4,600 rpm at 20ºC for 20 minutes to collect the oil/hexane supernatant while leaving behind lipid-extracted cells. The oil/hexane solution was then transferred to a pre-weighed 500-milliliter round-bottom flask which was mounted to a Buchi R-215 Rotary Evaporator (Flawil, Switzerland) and evaporated at 60 rpm with a 40ºC water bath, under vacuum, until no visible hexane condensate was evident on the condenser coil. The recovered oil was then weighed and repeated identical hexane extractions were conducted until the oil recovery was negligible. Upon complete extraction of cell oil, the remaining cell cake was evaporated overnight to remove hexane and then freeze dried.

3.2.2 Waste Production from Biomass Processing and Enzymatic Lipid Extraction

At the end of the primary fermentation, it was important to differentiate between two possible liquid waste streams being produced during lipid extraction: the *spent media* obtained from dewatering prior to cell lysis and the *hydrolysate* produced during the cell destruction process. These two waste streams have considerably different characteristics, mostly attributable to the presence or absence of solubilized cell material. The *spent media* is an optional waste stream that is dependent upon the inclusion of a dewatering step in the product recovery schematic (Fig. 2.9). In this study, both waste streams were minimally processed to maintain commercially representative composition as well as to eliminate any possible treatment steps.

3.2.2.1 Preparation and Separation of Waste Streams

To produce both liquid waste streams from the finished 30-liter primary fermentation it was necessary to split the batch into equal parts: one being removed for dewatering prior to lipid extraction and the other remaining in the fermentor for the subsequent enzymatic extraction. The removed portion was dewatered by centrifugation at 4,600 rpm, at 20ºC for 20 minutes using Thermo Scientific Sorvall LegendXTR (Waltham, U.S.A.). The lighter lipid-containing cell cake was gently punctured to pour off the heavier *spent media* which was collected and stored in 1-liter glass bottles at 4ºC until needed for analysis or secondary fermentations (Chapter 4).

The portion of the finished fermentation broth remaining in the 30-liter fermentor was then prepared for *in situ* hydrolysis to remove the lipid products based on Mara's patented methodology (Dennis and Armenta, 2015). To do so, fermentor conditions were adjusted to 55ºC, pH 8.0 and 175 rpm agitation. After conditions stabilized, 90 mL (0.5% v/v) Alcalase (Novozymes, Denmark) was aseptically injected through the septum to commence protein-targeted cell lysis. A 24 hour hydrolysis was conducted to ensure complete lysis of all cells. After hydrolysis, the resultant broth was transferred to centrifuge bottles for centrifugation at 4,600 rpm, at 20ºC, for 20 minutes. The separation resulted in a distinct hydrolysate layer beneath a spent biomass and oil emulsion. Following centrifugation, the bottles were refrigerated to solidify the spent biomass/oil emulsion to

improve removal of the hydrolysate. Once the emulsion hardened sufficiently to remain in the tube, the hydrolysate was poured through a cheesecloth into glass bottles for freezing at -18ºC until needed for analysis or secondary fermentations (Chapter 4).

3.2.2.2 Oil Removal Procedure for Enzymatically-Treated Biomass Prior to Proximate Analysis

To separate the oil from the spent biomass, the solidified spent biomass/oil emulsion remaining after separation of the hydrolysate was suspended in hexane and shaken at 250 rpm, 55ºC for 30 minutes. After, the solution was centrifuged at 4,600 rpm at 20ºC for 20 minutes. Due to incomplete separation of the oil and biomass, samples were then transferred to smaller 50-mL centrifuge tubes to improve layer formation and centrifuged again at a higher temperature (40ºC) to improve oil solubility. Again, poor separation necessitated increasing the temperature to 60ºC which successfully solubilized the oil for removal. The oil-removed samples were then left to evaporate residual hexane overnight and the following day the small volume of heavy spent media was removed with a syringe. The isolated spent media cake was then freeze dried using a Labconco FreeZone 18 freeze dryer (Kansas City, U.S.A.). Upon complete drying of the samples it was evident that they still contained oil, therefore a hexane extraction (following the protocol in Section 3.2.1.4) was then conducted on the spent biomass to remove any remaining oil prior to analysis.

3.2.3 Characterization of the Raw Biomass and Waste Materials

At the end of the primary fermentation, apart from the raw biomass, three distinct waste materials were collected and separated: two liquid – the spent media and the hydrolysate, and one solid – the enzymatically hydrolyzed spent biomass (see Fig. 2.9). Prior to any experiments processing or recycling these waste materials it was necessary to determine their physicochemical composition. Obtaining this information would provide insight into potential nutrient deficiencies or excesses, and indicate possible challenges associated with successful recycling. The parameters of interest were selected based on standard analyses used in agricultural/food analysis and wastewater treatment, and the intent was to fully describe the suitability of the material for recycling as a growth substrate as well as identify

potentially concerning characteristics that would have consequences for environmental discharge restrictions. This assessment incorporated traditional proximate analysis (moisture, protein, carbohydrate, lipid and minerals), typical wastewater monitoring parameters, and application-specific assays. The following methods are organized in sequence starting with solids determination (total solids, total suspended solids, volatile solids) followed by nitrogen (total nitrogen, protein, free amino acids, ammonium), carbon (total organic carbon, glucose, glycerol), lipids (total fatty acids) and additional discharge metrics (BOD and phosphates).

3.2.3.1 Total Solids

Total solids (TS) is a benchmark laboratory method used in water and wastewater quality to quantify the total amount of matter in a water. More specifically, TS is the amount of remaining matter after evaporation of the water sample (APHA, 1998). The American Public Health Association protocol (APHA 2540B) was used for the quantification of TS as follows. Aluminum or ceramic dishes, were first pre-treated by ignition at 550ºC in a muffle furnace for 1 hour. After ignition, dishes were cooled to room temperature in a desiccator and pre-weighed using an analytical balance. A known volume of homogenous sample (enough to yield 2.5 to 200 mg) was pipetted into a labelled dish for drying at 103 to 105ºC for 24 hours, or until completely dry. Samples were then cooled to a constant weight in the desiccator and weighed on the analytical balance. TS was then calculated using Equation 3.2.

$$\text{Total solids, mg L}^{-1} = \frac{\text{dry weight-dish weight, mg}}{\text{sample volume, mL}} \qquad \text{(Eq. 3.2)}$$

3.2.3.2 Total Suspended Solids

Total suspended solids (TSS) is a more specific version of total solids that requires a filtration step to selectively target matter in excess of a certain size and effectively differentiate between dissolved and suspended particulates. TSS is a continuation of the protocol for TS with the following added steps (APHA 2540D). Glass fiber filters were washed by mounting onto vacuum filtration apparatus and rinsing with three successive 20-mL portions of distilled water. For tare weights, filters were transferred to aluminum

weighing dishes and ignited at 550ºC in a muffle furnace for 15 minutes. Once cooled in a desiccator, the filters and weigh dishes were weighed on an analytical balance. After mounting the filter on a vacuum filtration apparatus a known volume of sample was pipetted onto the filter with the vacuum applied, with care taken not to block the filter with excess volume. The vacuum was continued until all sample liquid was passed through the filter. The filter and sample were returned to the same weigh dish it was pre-weighed in and placed in an oven at 103 to 105ºC for 24 hours. After cooling in a desiccator and samples were weighed using an analytical balance, yielding the sample weight. TSS was then calculated using Equation 3.3.

$$\text{Total suspended solids, mg L}^{-1} = \frac{\text{filter and residue weight-filter weight, mg} \times 1000}{\text{sample volume, mL}} \quad \text{(Eq. 3.3)}$$

3.2.3.3 Volatile Solids

Volatile suspended solids (VS) is another variation of solids analysis that is specifically intended to quantify the volatile, or organic content of the solids. It is particularly useful because in addition to the organic content, the opposing non-volatile part is the mineral portion of the solids. As with TS and TSS, this method is a further adaptation of those two methods (APHA, 1998). This technique is particularly important to the mineral component of proximate analysis of algal biomass using the adaptations from the National Renewable Energy Laboratory procedure (Wychen and Laurens, 2013), as follows. After completion of the TS method, sample residues in the weigh dish were placed in a muffle furnace at 575 ± 25ºC for 24 hours. After ignition, samples were removed from the furnace and placed in the desiccator to cool to room temperature. Once cooled, the samples were weighed using an analytical balance to determine ash weight. Equations 3.4 and 3.5 were used to calculate VS and ash, respectively.

$$\text{Volatile solids, mg L}^{-1} = \frac{\text{dry residue weight-ash residue weight, mg}}{\text{sample volume, mL}} \times 1000 \quad \text{(Eq. 3.4)}$$

$$\text{Ash, \%} = \frac{\text{ash weight-dish weight, mg}}{\text{TS dry weight, mg}} \times 100 \quad \text{(Eq. 3.5)}$$

3.2.3.4 Total Nitrogen

The total nitrogen content of a sample is typically measured elementally using a specialized elemental analyzer or by total Kjeldahl nitrogen (TKN). TKN is often used because it measures organic nitrogen (proteins, peptides, amino acids, etc.) as well as ammonia. These measures are particularly useful to quantify the nitrogen component of biomass samples. Additionally, TKN is commonly used for approximation of the protein content of biomass using a common conversion factor. However, TKN does not measure inorganic nitrogen (nitrate, nitrite, etc.) that may be present in growth media (APHA, 1998). For the purpose of analyzing the nitrogen content of the liquid waste samples, which should only contain the originally provided nitrogenous materials in addition to organic nitrogen released during cell hydrolysis, TKN is appropriate to quantify all forms of nitrogen present in the sample. Considering no inorganic nitrogen is present in the medium formulation it would be unnecessary to monitor those forms of nitrogen. Similarly, in the solid spent biomass, TKN would effectively measure the organically bound nitrogen.

Due to limitations in analytical equipment, TKN analysis was contracted out to RPC Laboratories (Fredericton, Canada). Frozen liquid samples and freeze dried solid samples were shipped overnight for rapid analysis and maximal sample stability. RPC followed the APHA 4500 organic nitrogen by phenate colorimetry method (APHA, 1998).

3.2.3.5 Protein

Quantification of protein in complex sample matrices proved to be a challenging task requiring external laboratory analysis and indirect measurements. In the food industry, protein can be measured using a variety of colorimetric techniques, or, at a greater expense, acid hydrolysis followed by amino acid analysis. After exploration of two common colorimetric protein methods (Lowry and BCA), it was determined that sample interferences made the results unreliable. The most common mutually interfering compounds in these protein methods are amino acids and ammonium, which were both expected to be present in the samples. Accordingly, it was desirable to use a combination of external laboratory analysis and amino acid analysis to quantify the protein of the waste materials.

Industry standards and considerable research suggest that estimation of protein concentration by a conversion factor from Kjeldahl nitrogen is acceptable (González López et al., 2010; Lourenço et al., 2002, 2004), although the exact factor value is debatable. However, this technique is used for solid biomass (algal or food) rather than liquids, which are likely to have different concentrations of soluble organic nitrogen. Therefore, for the liquid waste samples, a single sample was contracted for protein analysis to develop an appropriate N-protein conversion factor for these materials. The selected sample was the hydrolysate, which was frozen, stored on dry ice, and shipped to the SPARC BioCentre (Toronto, Canada) for total amino acid by acid hydrolysis and Pico-tag chromatography. In conjunction with the previously determined TKN values for these materials, an appropriate N-protein conversion factor of 3.44 was developed:

$$\text{N-protein conversion factor} = \frac{\text{total amino acids, g L}^{-1}}{\text{total kjeldahl nitrogen, g L}^{-1}} \quad \text{(Eq. 3.5)}$$

For the solid spent biomass, it was acceptable to use an established N-protein conversion factor of 4.78 for algal biomass. This factor was derived from a comprehensive average of multiple microalgae species, across phylogenic groups, at all stages of growth (exponential through stationary) (Lourenço et al., 2004). Additionally this N-protein conversion factor is also accepted by the U.S. National Renewable Energy Laboratory as a standard for protein estimation (Laurens, 2013). While it was expected that the T18 biomass likely had a unique conversion factor, in view of the variability associated with growing conditions and resultant changes in biomass composition, a cumulative average provided a reasonable estimation.

3.2.3.6 Free Amino Acids

Free amino acids (FAA) are the soluble amino acids that are present in the fermentation broth, either originating from the nutrient medium or released from the degradation of the cellular protein during hydrolysis. These amino acids are an organic nitrogen source that has been shown to be used by microalgae during growth (Zheng et al., 2012a). One of the most common analytical methods for quantification of free amino acids is by reverse-phase

high performance liquid chromatography (HPLC). However, due to complex sample matrices, sample preparation and cleanup are required prior to injection to eliminate interfering compounds and degradation of the column. One major interfering compound known to cause considerable challenges for amino acid determination is ammonium, which commonly reacts similarly to amino acids with derivatization reagents.

Sample preparation for free amino acid analysis required prior protein precipitation with solid phase extraction. In this procedure, an aliquot of homogenized sample was added to 3-mL Captiva ND Lipids cartridges (Agilent A5300635) with 3:1 methanol:sample to remove protein and lipid interferences. A rapid precipitate formed with the free amino acids flowing through the cartridge packing under vacuum. The filtrate was collected in acid-washed 15-mL glass tubes and evaporated at 50ºC under a constant stream of nitrogen then restored to the original sample volume in 0.1M HCl. Finally samples were transferred to 2-mL chromatography vials for analysis.

Amino acid analyses were performed on an Agilent 1100 series HPLC system. Separation was performed on an Agilent Poroshell HPH-C18 column (4.6 x 150mm, 2.7µm) at 40°C. Pre-column derivatization was performed using o-phthalaldehyde (OPA) and d 9- fluorenylmethyl chloroformate (FMOC) reagents purchased through Agilent (Mississauga, Canada). The gradient elutionmethod and injection program were performed according to Agilent Application Note (Long, 2015) modified to accommodate the 150 mm column.

Figure 3.1 is a chromatogram of the amino acid standard solution that is used to calibrate the HPLC output for amino acid analysis. As shown, there are 17 amino acids that are detected using this method. Peak shape (symmetry) and elution resolution are demonstrated to be acceptable with little overlap, even between closely eluting amino acids like phenylalanine and isoleucine. The expected interference caused by ammonium was evaluated by including an ammonium sulfate standard, prepared at the maximum concentration seen in the Windust Lite medium. The chromatogram illustrates the challenge with the ammonium sulfate peaks closely eluting to methionine and proline. For methionine, this can be mitigated by careful management of the integration however, in cases of low proline concentrations and high ammonium sulfate, the proline peak is lost.

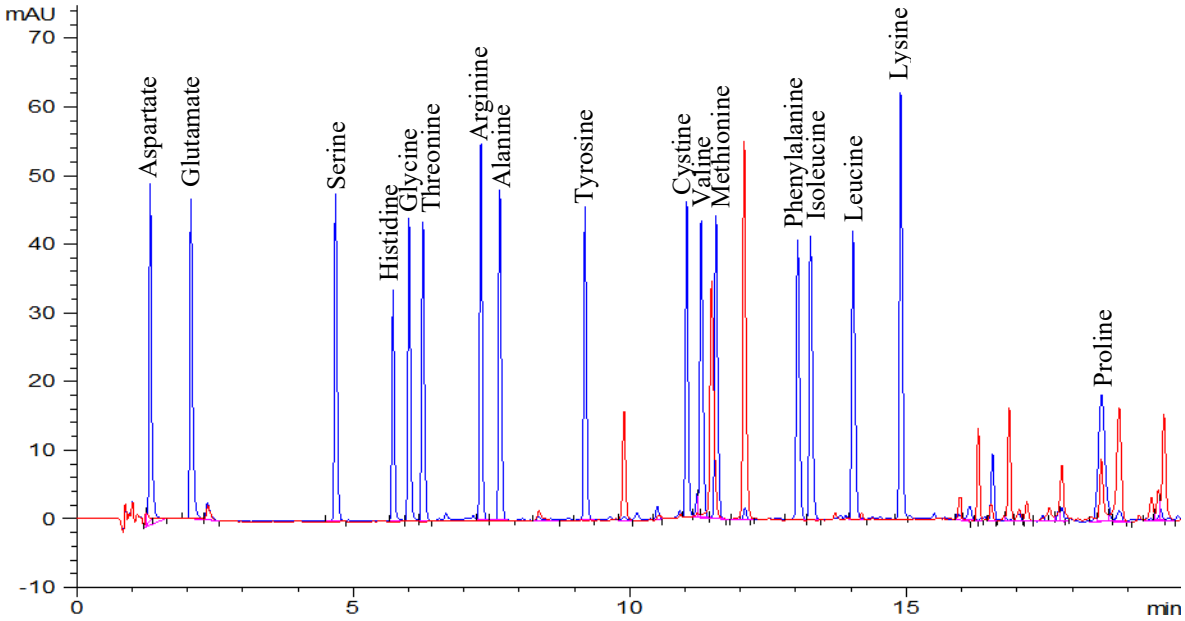

Figure 3.1 HPLC chromatogram of standards for the 17 detected amino acids (blue line) and the known interfering ammonium sulfate standard (red line).

3.2.3.7 Ammonium

The principal, and only inorganic source of nitrogen in the Windust Lite medium is provided by ammonium sulfate ($(NH_4)_2SO_4$). As the fermentation proceeds, nitrogen is consumed steadily for cell synthesis. Upon depletion of nitrogen, microalgae are known to start accumulating cellular lipids resulting in enhanced concentrations, making nitrogen depletion an important condition for effective management of fermentations for maximum lipid production (Perez-Garcia et al., 2011). Ammonium was measured in the fermentation broth by colorimetric determination using a phenol-hypochlorite reaction adapted from Weatherburn (1967). Samples were reacted with the phenol-hypochlorite reagent at room temperature for 30 minutes prior to measuring absorbance at 630 nm on an Ultrospec 2100 pro UV/Vis spectrophotometer (GE Healthcare, Little Chalfont, U.K.).

3.2.3.8 Total Organic Carbon

Considering the importance of organic carbon to heterotrophic microalgae growth it was useful to conduct an assessment of the total concentration of organic carbon (TOC) in the

liquid waste samples. Although glucose is the only organic carbon source specifically added for metabolism, there are potentially important trace sources of carbon derived from the complex nitrogen (yeast extract and soy peptone) as well as the cell hydrolysis. Samples were submitted for external analyses to Agat Laboratories at the same time as the samples for BOD analyses. The method was executed according to USEPA Standard Method 5310B – TOC by High Temperature Combustion.

3.2.3.9 Glucose

For fermentations of heterotrophic microalgae, the carbon source is the single most important constituent of the medium due to both the high cost and the large quantity that is required (Bumbak et al., 2011). In T18 fermentations at Mara, glucose ($C_6H_{12}O_6$) is widely used, thereby making effective quantitation of glucose important. Accordingly, an HPLC-based analytical method was developed for measuring glucose and other important saccharide concentrations that may be present in complex fermentation media.

Glucose concentration in the fermentation broth was analyzed using Mara's in-house saccharides method after filtration using 0.45 µm hydrophilic syringe filters. Saccharides analyses were performed on an Agilent 1200 series HPLC system. Separation was conducted on an Agilent HiPlex H+ column (200 x 6.5mm, 8µm) with H^+ and CO_3^{2-} de-ashing guard cartridges from Bio-Rad (p/n 125-0118, Hercules, U.S.A.) to assist in the removal of salt interferences. Isocratic mobile phase was 1mL per 2L acetonitrile in MilliQ H_2O flowing at 0.75mL per minute, with a column temperature of 55°C. Detection was by refractive index detector (RID) set at 45°C.

The HPLC output for glucose is depicted in Figure 3.2 where glucose elutes prior to other detected organic carbon sources including fructose, xylose, xylitol and glycerol. Although both peak shape and resolution were good, there was a minor overlap in peaks from glucose and the fructose/xylose peaks. Since those two compounds elute similarly using this method, it was difficult to distinguish between them. As shown here, both glucose and glycerol were quantified using these chromatography outputs.

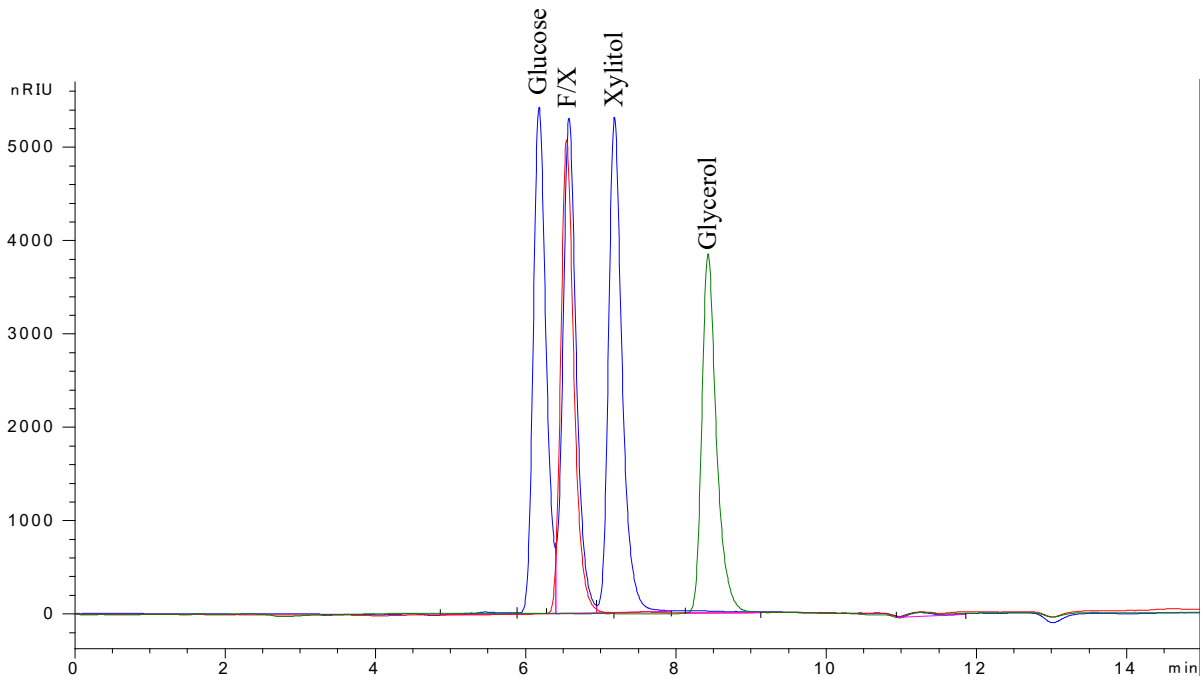

Figure 3.2 HPLC chromatogram showing standards for glucose, fructose/xylose (F/X), xylitol and glycerol from the saccharides method.

3.2.3.10 Glycerol

The presence of glycerol ($C_3H_8O_3$) in the fermentation media would be considered unusual in the standard Windust Lite formulation, however it is a major component of the enzyme broth and therefore present in the hydrolysate. Additionally, it has been confirmed that T18 can metabolize glycerol, making it a potentially important carbon source to monitor throughout the secondary fermentations (Scott et al., 2011). Glycerol was also included in the HPLC-based saccharides method used for glucose measurements, making it possible to monitor glucose and glycerol simultaneously. All sample preparation and analysis was done in concert with that of the glucose assay.

3.2.3.11 Lipid Analysis by Gas Chromatography

The standard protocol from Mara for the quantification of lipids in T18 cells was used, based on fatty acid methyl ester (FAME) determination by one-step transesterification (Armenta et al., 2009). 950 µL of a 1 mg mL-1 C23:0 standard in toluene was added to 25-50 mg of dried cells as an internal standard. Direct transesterification of microalgae biomass was conducted by adding 6 mL of 12.5% (v/v) acetyl chloride in methanol with 6

mL of toluene to the sample solution. The mixed sample was then incubated at 80ºC for 120 minutes and 10 mL of 6% w/v sodium carbonate added after cooling to room temperature. Samples were then centrifuged at 1100 rpm at 4ºC for 15 minutes to separate the aqueous and toluene layers. The organic layer was passed through a silica SPE cartridge to remove interferences prior to analysis by gas chromatography (GC).

FAME analyses were performed on an Agilent 6890 GC with FID. The column program was set to 190°C increasing by 5 °C per minute to 240 °C with a 3 minute hold. Carrier gas used was H_2 flowing at 2 mL per minute through a FAMEWAX column (30m x 0.32mm, 0.25µm df) obtained through Mandel Scientific (p/n RSK-12498 Guelph, Canada). The resultant fatty acid concentrations are reported as milligrams per gram of cell mass in which the summation of all fatty acids was considered the lipid content.

3.2.3.12 Biochemical Oxygen Demand

To assess the overall organic material load in the liquid waste materials it was useful to employ the common wastewater quality metric of biochemical oxygen demand (BOD), which is an indicator of water quality in most discharge regulations (APHA, 1998). Due to the need for specialized laboratory supplies and equipment for measurement of BOD, samples were sent to an external laboratory for analyses. The frozen samples of spent media and hydrolysate were sent to Agat Laboratories (Dartmouth, Canada) where the analyses were conducted the analyses in accordance with the United States Environmental Protection Agency (USEPA) Standard Method 5210B – 5 Day BOD.

3.2.3.13 Phosphate

Phosphate is present in the Windust Lite medium by the addition of monobasic and dibasic potassium phosphate (KH_2PO_4 and K_2HPO_4, respectively). The role of phosphate, more specifically phosphorus, is essential to the synthesis of the energy carrier adenosine triphosphate (ATP), cellular DNA, phospholipids and ribosomes (Graham and Wilcox, 2000). Additionally, as a non-renewable mineral, phosphate is important to use efficiently in any plant culture system (Pate et al., 2011). To quantify phosphate concentrations in the waste materials a simple colorimetric method for phosphate determination was used. Phosphate was determined by reduction of phosphomolybdate to molybdene blue

proportionate to the concentration of phosphate in solution as adapted from Lanzetta et al. (1979). After reagent addition, samples were reacted at room temperature for 30 minutes prior to measuring absorbance at 750 nm.

3.3 Results and Discussion

3.3.1 Fermentation Results

The performance of the primary fermentation was on par with the anticipated results in terms of biomass productivity, total fatty acid content, and fermentation time required. As shown in Fig. 3.3, biomass concentration reached 164.22 g L^{-1} by 91.6 hours while accumulating 79.9% (w/w) of oil. Further, the growth curve exhibited a trend typical for a fed-batch fermentation with a declining growth rate as the substrate approached nutrient depletion. The depletion of carbon (cerelose) was evident by a precipitous decline in the carbon consumption rate at 91.4 hours, which was estimated by monitoring the carbon dioxide exhausted from the fermentor using a Magellan TandemPro Gas Analyzer (Hertfordshire, U.K.).

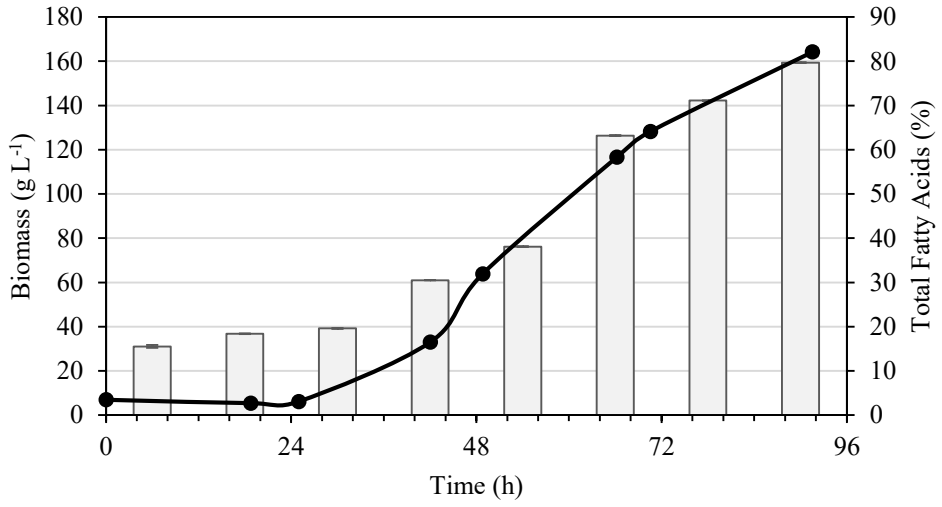

Figure 3.3 30-liter primary fermentation biomass accumulation (line) and total fatty acid content (bars) for the duration of the fermentation. Error bars indicate ± standard deviation (n=2).

3.3.2 Composition of Raw Biomass from Primary Fermentation

Because the waste materials evaluated for recycling potential are derived from the hydrolysis of the 'raw biomass' from the primary fermentation, it is very useful to understand the composition of that initial biomass prior to hydrolysis. As stated previously, the primary fermentation biomass accumulated 164.22 g L^{-1} of cells by 91.6 hours. Within that biomass the vast majority of cell mass was comprised of the lipid fraction, which was 79.9% (w/w) by the conclusion of the fermentation. Additionally, a typical proximate analysis completed on the biomass yielded the other major contents of the biomass as depicted in Fig. 3.4.

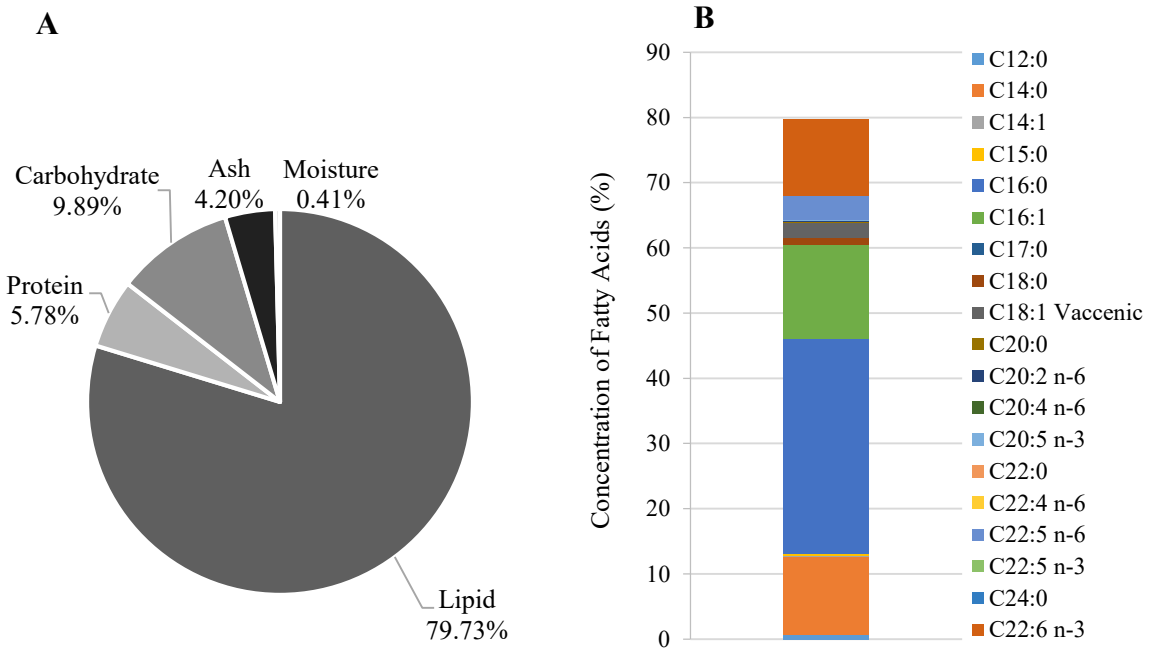

Figure 3.4 (A) Proximate analysis of the primary fermentation T18 biomass and (B) the fatty acid profile of the lipid fraction.

The proximate analysis was indicative of the uniqueness of this microorganism – amassing almost 80% of the cell mass as lipids. While this characteristic is highly favorable for oil-based applications, it is the remaining cell contents that are most important to the potential for waste recycling. The carbohydrate and protein fractions predominate the remaining cell composition, registering 9.89% and 5.78% respectively. This general breakdown of thraustochytrid biomass, being mostly lipid with protein comprising almost

half of the remaining biomass, is consistent with other reported analyses (Carter et al., 2003). Considering that the lipid extraction method used at Mara utilizes a proteolytic enzyme (Dennis and Armenta, 2015), it is expected that the liquid waste materials contain large quantities of this protein fraction in various states of decomposition. The carbohydrate material, partially originating from the sulphated polysaccharide cell wall, is bound in complex carbohydrates that can be potentially degraded into simpler mono and di-saccharides with additional processing (Bongiorni et al., 2005a). However, in the existing process, this carbohydrate fraction may remain in an unfavorable form for secondary fermentation metabolism.

3.3.3 Composition of the Spent Media and Hydrolysate

The composition of the three waste materials was expected to be consistent with the degree of processing and cell lysis that was subjected to each. Upon visual inspection of the liquid waste materials (Fig. 3.5), the hydrolysate was considerably more opaque than the spent media, largely due to the significantly higher amount of cell debris and other suspended solids in solution. However, in each case the liquid waste material was considerably less transparent than the original fresh medium. The visible darkening of the hydrolysate was likely Maillard reactions due to prolonged exposure to elevated temperature (55ºC) during the enzymatic lipid-extraction process where residual sugar likely reacted with amino acids in solution, as was explained in Section 2.7.6. The summary of all physicochemical parameters observed for the spent media and hydrolysate is presented in Table 3.3. The difference in pH of the two liquids is attributable to the adjustment made by addition of NaOH prior to enzyme addition where the optimal pH for hydrolysis was 8.0. In contrast, the pH of the spent media is close to the maintained 30-liter fermentor condition of pH 5.75.

The increase in solids in the hydrolysate as compared to the spent media was consistent with the TS and TSS values observed in Table 3.3. The TS of the spent media was 36.18 g L^{-1} while that of the hydrolysate was almost double at 63.61 g L^{-1}. During the proteolytic activity undertaken by the enzyme, much of the remaining cell material remained in the liquid resulting in the high solids load shown. Notably, the vast majority of these solids were very small since the TSS – accounting for particles greater than 1.2

μm – comprised only 3.7% of the spent media and 8.4% of the hydrolysate. This observation suggested a high degree of cell lysis during enzymatic hydrolysis, as much of the 5-20 μm diameter T18 cells have been degraded into considerably smaller debris (Kimura and Naganuma, 2001).

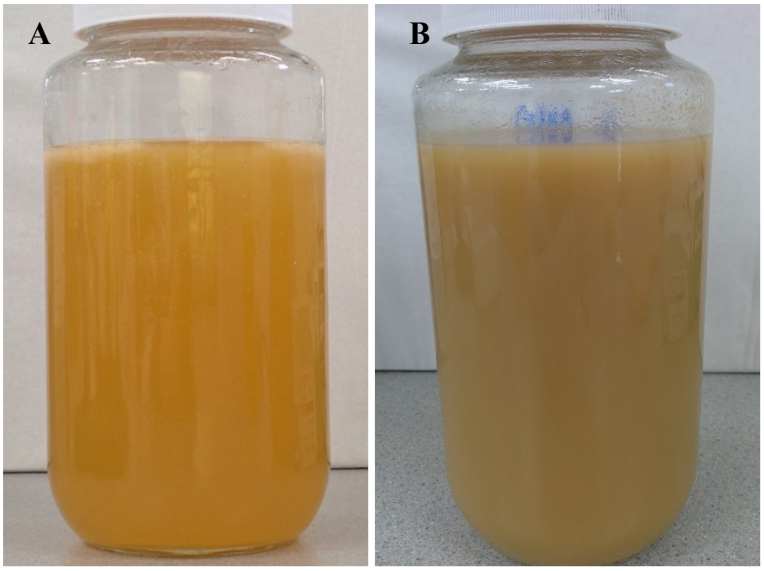

Figure 3.5 (A) Untreated spent media and (B) hydrolysate derived from the primary fermentation dewatering, and enzymatic hydrolysis, respectively.

Closer inspection of the chemical parameters of the waste materials was intended to illustrate their suitability for secondary fermentation substrate. Since the enzymatic hydrolysis would target cellular protein, these materials were expected to have an elevated nitrogen content. TKN, which can be contributed to by the organic nitrogen sources in the original medium (soy peptone and yeast extract), as well as the degradation of primary fermentation cells was correspondingly elevated to 2.80 g L^{-1} in the hydrolysate in comparison to the 0.40 g L^{-1} in the spent media. Looking more specifically at nitrogenous compounds, the protein and free amino acid contents were 9.63 g L^{-1} and 2.38 g L^{-1} in the hydrolysate, respectively, considerably more than the 1.38 g L^{-1} and 1.40 g L^{-1} seen in the spent media.

The broader organic content of the liquid wastes was described by BOD and TOC. The TOC can be potentially useful in explaining the biomass production of heterotrophic

microalgae cells, which can potentially consume an array of organic carbon compounds other than glucose (Nagano et al., 2009; Raghukumar, 2008; Scott et al., 2011; Yokochi et al., 1998). Potential sources of this carbon could be the result of complex organic nitrogen sources (e.g. proteins) or potentially cell debris produced throughout the primary fermentation. As seen with the other physicochemical parameters, the TOC was greater with 20.6 g L^{-1} in the hydrolysate compared to 8.83 g L^{-1} in the spent media. The BOD exhibited a similar trend with both values in excess of many wastewater discharge restrictions, possibly requiring treatment for regulatory compliance dependent upon locality (Hoornbeek, 2012). For example, BOD discharge limits for wastewater in the municipality of Halifax, Nova Scotia, requires a BOD no greater than 0.300 g L^{-1} (Halifax Regional Water Commission, 2011) which is considerably lower than the 14.43 g L^{-1} in the spent media and 30.77 g L^{-1} in the hydrolysate (see Table 3.3). Both TOC and BOD are important when evaluating the efficacy of the nutrient recycling as it pertains to an added environmental benefit (i.e. fewer discharged organics).

Glucose and glycerol were organic carbon sources specifically monitored to assess the latent metabolic potential of these wastes for heterotrophic microalgae. The 30-liter primary fermentation was intended to run until all carbon (i.e. glucose) was sequestered from the medium by the cells. The residual concentrations of 0.49 g L^{-1} in the spent media and 0.70 g L^{-1} in the hydrolysate confirm that carbon depletion was reached without excessively starving the culture. Glycerol, however, was not provided in the original Windust Lite medium rather it was introduced in the substrate for the enzyme during lipid extraction. Accordingly, the concentration is very minor with 0.49 g L^{-1} in the spent media as compared to the 3.90 g L^{-1} found in the hydrolysate.

Finally, concentrations of inorganic nitrogen and phosphate were measured to further indicate the ability of the waste materials to support a secondary fermentation. As with carbon, the optimized fed-batch fermentations are conducted with the intent to deplete nitrogen and trigger the anticipated boost in cellular lipid content. As expected, the ammonium concentration was extremely low with 0.01g L^{-1} of ammonium in both the spent media and hydrolysate. Phosphate is less managed during the fermentation and provided in excess as indicated by the remaining concentrations of 2.88 g L^{-1} in the spent media and 1.21 g L^{-1} in the hydrolysate.

Table 3.3 Physicochemical composition of spent media and the enzymatic hydrolysate derived from biomass processing and enzymatic lipid extraction after the primary fermentation.

Parameter	Spent Media	Hydrolysate
pH	[a]5.97 ± 0.02	[a]8.27 ± 0.03
Total Solids	36.18 ± 0.36	63.61 ± 0.14
Total Suspended Solids	1.35 ± 0.01	5.31 ± 0.95
Total Nitrogen	0.40 ± 0.52	2.80 ± 0.07
Protein	[b]1.38 ± N/A	9.63 ± 1.09
Free Amino Acids	1.40 ± 0.15	2.38 ± 0.02
Biochemical Oxygen Demand	14.43 ± 0.12	30.77 ± 7.62
Total Organic Carbon	8.83 ± 0.45	20.6 ± 0.17
Glucose	0.58 ± 0.04	0.70 ± 0.25
Glycerol	0.49 ± 0.05	3.90 ± 0.08
Ammonium	0.01 ± 0.00	0.01 ± 0.00
Phosphate	2.88 ± 0.02	1.21 ± 0.02

[a] All units g L^{-1}, except pH

[b] Protein content estimated by Kjeldahl conversion using a factor of 3.44

3.3.4 Composition of the Enzymatically Hydrolyzed Spent Biomass

In an effort to recycle all waste streams generated during the lipid-extraction of the microalgae biomass, the solid spent biomass was evaluated in addition to the liquid wastes. In compliance with industry standards, the biomass was assessed using proximate analysis to establish the major categories of material breakdown (Laurens, 2013). Initially, a visual comparison of the raw T18 biomass compared to the enzymatically extracted T18 biomass illustrated a darkening of color and significant reduction in mass (Fig. 3.6). The color change may be explained by the removal of the largest component of the biomass – the heavily pigmented lipids. As the biomass oil is removed, the remaining fractions become more concentrated making the darker minerals become more prominent.

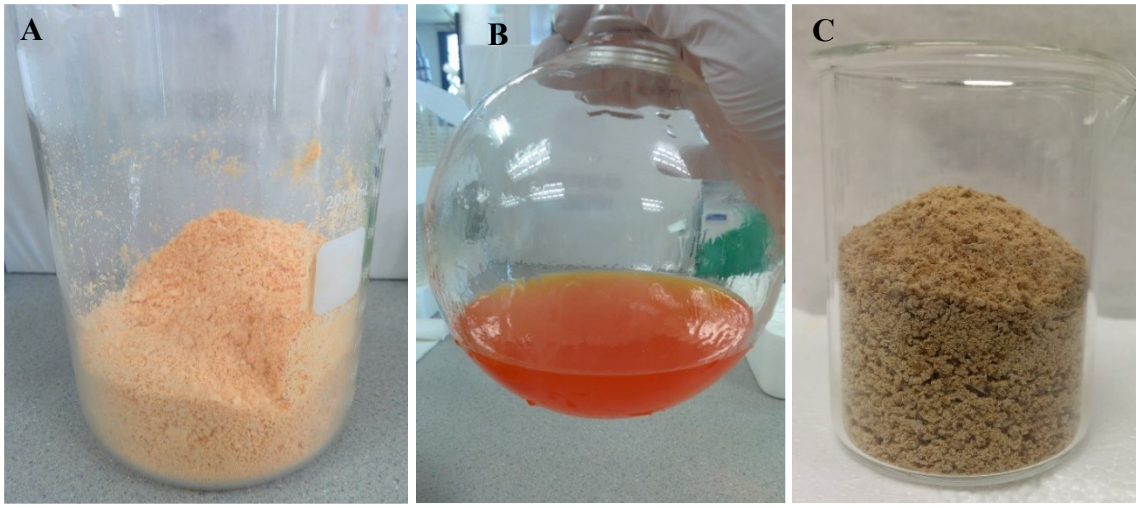

Figure 3.6 (A) Freeze-dried T18 cell biomass obtained from the 30-liter primary fermentation, (B) recovered lipids from oil-extraction and (C) freeze-dried enzymatically hydrolyzed biomass.

The proximate analysis was conducted identically to that of the raw biomass from the 30-liter primary fermentation. The most noticeable feature of the enzymatically-extracted biomass is the considerable decrease in the lipid content (See Fig. 3.4 and 3.8) as is expected from the lipid extraction process. However, the remaining lipid content of 9.6% is indicative of some residual oil that is not fully extracted. While that percentage remaining may seem significant, it is important to consider that during enzymatic extraction the amount of biomass is reduced significantly by the proteolytic activity of the enzyme.

The biomass degradation was specifically investigated for another fermentation by Mara's scientist, Paula Mercer, providing some insight into the movement of material during lipid-extraction (Fig. 3.7). The reduction in biomass due to protein lysis is illustrated by a 53.9% reduction in the solids content of the liquid seen during enzymatic extraction. This reduction is mostly attributable to the removal of the lipid fraction. However, despite the retention of solid material in the hydrolysate, the solids are significantly reduced in size as evidenced by the predominantly dissolved solid portion that is described by the difference between TS and TSS in Table 3.3. The majority of that solubilized cell mass enters the liquid waste which contained 88.5% of the original non-oil solids as compared to the 7.8% of non-oil mass that resided in the oil/spent biomass emulsion.

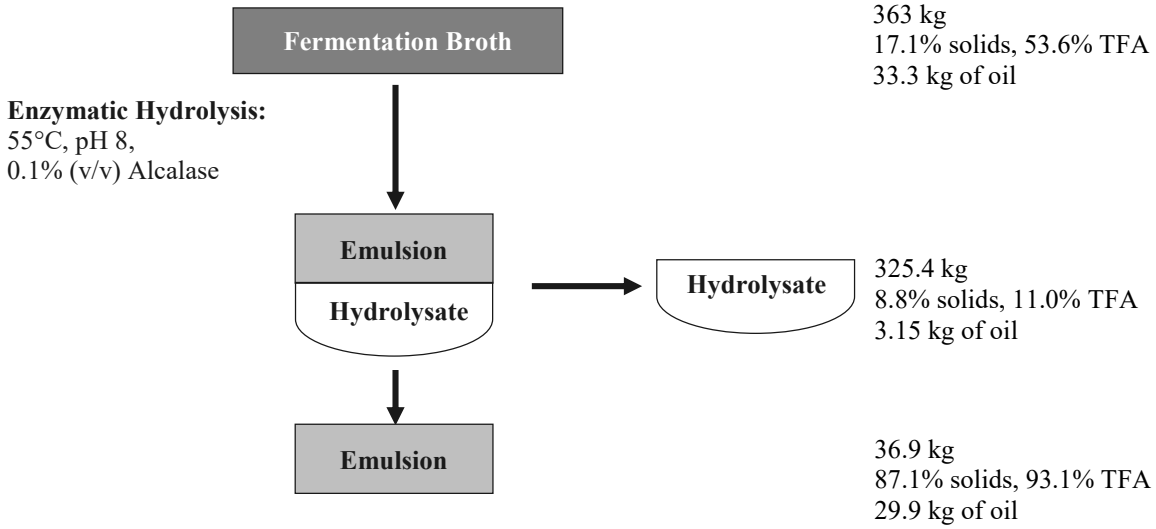

Figure 3.7 Mass balance on enzymatic extraction process of T18 biomass from a different batch conducted by Mara's scientist Paula Mercer, based on Mara's patented method (Dennis and Armenta, 2015).

In concert with the reduction in lipid content of the spent biomass, the protein is also a smaller proportion of the remaining biomass. The protein represented 28.5% (w/w) of the defatted raw biomass while it only represented 16.1% (w/w) of the enzymatically-extracted spent biomass. This is readily explained by the action of the enzyme during lipid extraction. As a protease, the cellular protein was broadly cleaved resulting in the degradation of complex cell protein into smaller soluble peptides and amino acids. While these compounds still contained nitrogen, they were partially released into the hydrolysate resulting in the elevated levels of protein and amino acids seen in Table 3.3.

The carbohydrate and mineral (ash) portion of the biomass were both left largely intact during lipid-extraction, resulting in much greater fractions remaining in the spent biomass (Fig. 3.8). Here, the carbohydrate increased from 9.89% to 38.40% while the mineral fraction jumped from 4.20% to 35.90%. While this spent biomass may be valuable for recycling the protein, carbohydrate, or mineral fractions, it will likely require some pretreatment to solubilize those elements for metabolism in a secondary culture.

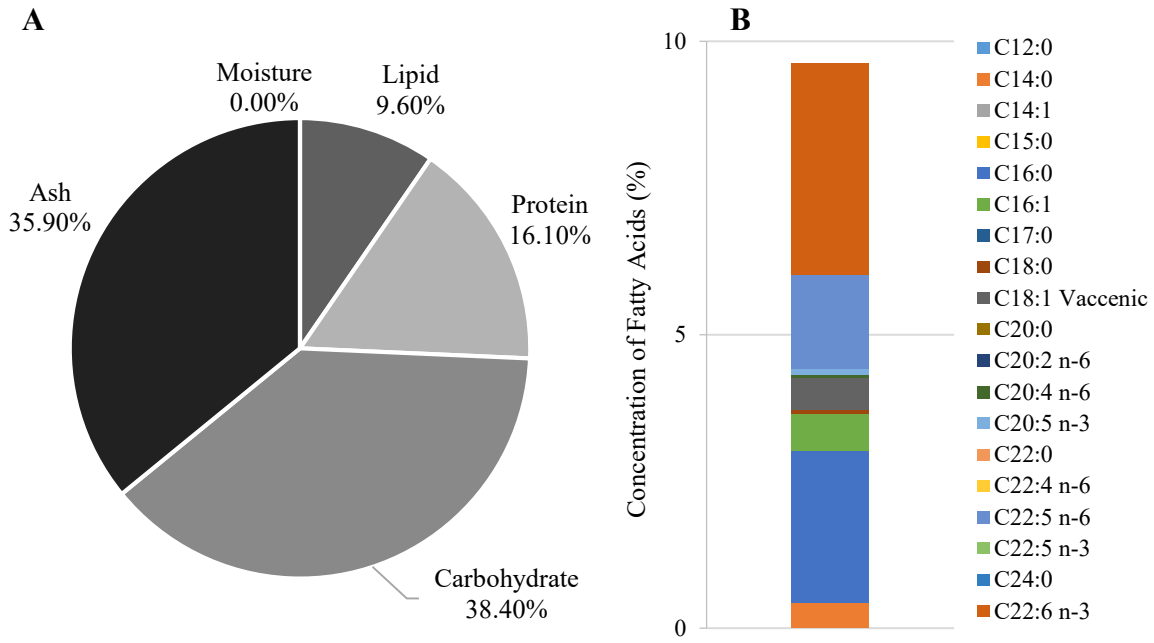

Figure 3.8 (A) Proximate analysis of the freeze-dried enzymatically extracted T18 biomass produced from the 30-liter primary fermentation and (B) the fatty acid profile from that biomass.

3.4 Conclusion

The physicochemical analysis of the waste materials provided some insight into the fate of nutrients and the prospects of recycling them with secondary fermentations. The vast majority of the material resided in the liquid wastes, which is reasonable considering the quantity of water used and the degree of cell lysis during lipid-extraction. In light of that fact, the liquid waste streams carry a greater significance for recycling nutrients and mitigating environmental impacts. This was further supported by the high concentration of organic material present in the spent media and hydrolysate – potentially representing an opportunity as a substrate for heterotrophic metabolism – but with certain challenges in terms of regulatory discharge limits.

The proteolytic enzyme lipid extraction has apparent value for degrading the nitrogenous protein into a soluble, and potentially bioavailable form of amino acids. With these free amino acids present in the hydrolysate there is an opportunity to provide some of the nitrogen required by the secondary culture without adding fresh ingredients. Further, the actions of the enzyme and the extraction conditions do not produce an unsuitable environment from addition of harsh chemicals or solvents. This opportunity for nitrogen

recycling was evaluated in the secondary fermentation experiments described in the next chapters.

In summary, the waste liquids produced during lipid-extraction of the microalgae biomass have some potential for recycling of valuable nutrients. At this point it was unclear whether their high organic content would be metabolically favorable for the secondary *Thraustochytrium sp.* (T18) culture, however it can be inferred that based upon prior work, at least the soluble organic nitrogen should be used by the cells. Considering the majority of waste material resided in the liquid waste streams rather than as solid waste, Chapter 4 and 5 will focus solely on secondary fermentations recycling the waste liquids.

CHAPTER 4 : FLASK-SCALE SECONDARY FERMENTATIONS

Sections of this chapter are derived from the published manuscripts:

Lowrey, J., Brooks M. S., Armenta, R. E. (2016) 'Nutrient Recycling of Lipid-Extracted Waste in the Production of an Oleaginous Thraustochytrid' Applied Microbiology and Biotechnology. doi: 10.1007/s00253-016-7463-2.

Lowrey, J., Armenta R. E., Brooks M. S. *Recycling of Lipid-Extracted Hydrolysate as Nitrogen Supplementation for Production of Thraustochytrid Biomass* published in Industrial Microbiology and Biotechnology on. Accepted.

4.1 INTRODUCTION

The concept of using waste liquids from microalgae production and lipid extraction for recycling into secondary cultures has received minimal research attention until recently despite awareness of the resource consumption intensity of microalgae production (Fon Sing et al., 2014; Pate et al., 2011; Rodolfi et al., 2003; Zhu et al., 2013). Although the concept is logical and has been advocated, increasing productivity and decreasing cost has taken priority. When considering commercial large scale production however, the waste management issue is of high importance. This chapter investigates the feasibility of recycling the liquid waste streams generated from the current production and extraction techniques at Mara in secondary fermentations at the flask scale. This flask step is an essential step toward exploring alternative production conditions prior to expanding to higher-volume fermentors. In relation to specific objective (iii) of this thesis, recycling of the liquid wastes was explored with a focus on carbon and nitrogen supplementation and the effect upon biomass and lipid productivity. Here, the spent media and hydrolysate liquid wastes that originated from the 30-liter primary fermentation described in Chapter 3 were used.

This chapter begins with a general materials and methods section, relevant to all the fermentations in this section. The procedure, results and conclusions from the three main experiments then follow. Firstly, Secondary Fermentation 1 (2015-500ML-01) is described, which was conducted as a feasibility assessment to compare recycled hydrolysate to fresh fermentation media, where the waste material was sterilized together

with fresh media components. Secondary Fermentation 2 (2015-500ML-02) follows, where the sterilization procedure was improved, otherwise the same conditions as Secondary Fermentation 1 were used. Finally, Secondary Fermentation 3 (2015-500ML-03) is described, which explored reducing or removing nitrogenous media elements to optimize the supplementation recipe. Conclusions from this chapter were then summarized and rationale for the work conducted in Chapter 5 was explained, including scale up from flasks to 2-Liter fermentors.

4.2 Materials and Methods

4.2.1 Microalgae and Enzyme

The microalgae strain used in primary and secondary fermentations was *Thraustochytrium sp.* T18, isolated from coastal waters of Nova Scotia, Canada (Burja et al., 2012, 2006). The hydrolyzing enzyme Alcalase 2.4 L FG was obtained from Novozymes (Bagsvaerd, Denmark).

4.2.2 Windust Lite Media

Throughout the secondary fermentations Windust Lite media was used as the regular fermentation medium for all seeds and experimental treatments, unless otherwise indicated. The recipe for Windust Lite is described in Table 3.1.

4.2.3 Secondary Fermentation Conditions and Sampling Protocol

All secondary fermentations were executed using the same growth conditions and sampling regime. Prepared and inoculated sample flasks were placed on a New Brunswick Scientific Innova 4430 orbital shaker (Edison, U.S.A.) at 200 rpm and 25ºC to be maintained throughout the fermentations. The first sample was collected after a few minutes of mixing to ensure sample homogeneity and daily samples were taken thereafter. The sampling began with weighing labelled 15-mL centrifuge tubes on an analytical balance prior to placing the tubes under ultraviolet (UV) light in the laminar flow hood. After 20 minutes of UV light exposure, sample flasks were placed in the hood and a 13 mL aliquot was aseptically taken from each and placed in the corresponding 15 mL tube. The wet weight of each sample-filled 15-mL tube was determined using an analytical balance followed by

centrifugation at 2,000 rpm at 2ºC for 20 minutes. The supernatant was collected and placed in new, labelled 15-mL centrifuge tubes while the cell pellet was re-suspended in distilled water and vortexed. Washed cells were then centrifuged again at 2,000 rpm at 2ºC for 20 minutes. After a second washing following the preceding steps the remaining cell pellet was then frozen at -80ºC and freeze dried for measurement of constant dry weight. The recovered substrate from the first centrifugation was frozen for analysis of pH, glucose, glycerol, ammonium, and phosphate.

4.2.4 Estimation of Biomass

The freeze dried biomass obtained from the cell washing protocol was dried to a constant weight and weighed on a Sartorius Quintix 223-1S analytical balance (Goettingen, Germany). Biomass dry weight was reported as grams of dried cells per liter of sampled fermentation broth.

4.2.5 Substrate Analyses

Substrate analyses for glucose, glycerol, ammonium and phosphate were conducted using the same analytical methods described in Chapter 3 for the physicochemical characterization of the waste material. Prior to submission for HPLC analysis of glucose and glycerol, samples were filtered by hydrophilic 0.20 μm disk filters.

4.2.6 Lipid Analysis of Secondary Fermentation Biomass

All lipid contents were determined by fatty acid methyl ester (FAME) determination by one-step transesterification and quantitation by gas chromatography (Armenta et al., 2009), as described in Chapter 3.

4.2.7 Analysis of Amino Acids

Samples analyzed for free amino acids were first prepared by protein precipitation and solid phase extraction using the Captive ND cartridges. The collected filtrate was evaporated under a constant stream of nitrogen and then restored to the original sample volume in 0.1M HCl. Amino acid analyses were performed on an Agilent 1100 series HPLC system using the OPA and FMOC derivatization protocol described in Chapter 3.

4.3 SECONDARY FERMENTATION 1 (2015-500ML-01): FEASIBILITY ASSESSMENT

The first experiment in secondary fermentation with the spent media and hydrolysate was designed to investigate the possibility of using both raw and Windust Lite (WDL)-supplemented wastes. Minimal manipulation of the materials was desired to ensure good representation of their composition, regardless of the scale. Additionally, the same microorganism and growing conditions were maintained to ensure comparability of the results to typical fermentations. The experimental design in Table 4.1 was employed, where each observation was from an individual flask and each treatment (1-6) received triplicate observations. The raw treatments received no added nutrients and the supplemented ones had all Windust Lite components added. Treatment 3 was the negative control which was only water and seed while Treatment 6 was the positive control with the standard Windust Lite formulation.

Table 4.1 Experimental design for Secondary Fermentation 1 (2015-500ML-01). Factors were Supplementation Level and Media Type and all treatments were conducted in triplicate.

		Media Type			
		Spent Media	Hydrolysate	Water	
Supplementation Level	Raw	1	2	3	n = 3
	WDL-Supplemented	4	5	6	N = 18

4.3.1 MATERIALS AND METHODS

The materials and methods used in this experiment are described below and in Section 4.2.

4.3.1.1 Seed Preparation

For the 18 flasks used, 432 mL of total seed was required to inoculate all of them using 24 mL each. This required preparing two 2-liter seed flasks (for redundancy) with a volume of 500 mL of Windust Lite medium each. The seed medium recipe was the same as for the 30-liter fermentation, described in Table 3.1. Once the seed medium was prepared and autoclaved, each seed flask was aseptically inoculated with one full loop of plated T18

biomass grown on standard fermentation media agar in petri dishes that had been refrigerated for no more than 8 weeks. Seed flasks were then placed on a New Brunswick Scientific Excella E25 (Edison, U.S.A.) orbital shaker at 200 rpm and 25ºC. The seed was grown for three days prior to evaluation for further use.

After the growth period, each flask was aseptically sampled to assess biomass accumulation and culture health. This assessment was conducted by optically measuring the absorbance at 600 nm of a 1:10 diluted sample, where an absorbance value of approximately 1.8 would indicate sufficient biomass accumulation without reaching starvation conditions. Additionally, samples were examined with a Nikon Eclipse 600 phase contrast microscope (Tokyo, Japan) at 1000x magnification to inspect cell health and identify any contamination. Based upon the outcome of the seed check in Table 4.2, it was apparent that both seeds may have been slightly overgrown and potentially starved but acceptably healthy (in terms of cell size, evidence of active cell division and contaminant free). Seed 2 was selected because it was likely to be less overgrown based upon the lower optical density value.

Table 4.2 Observations from the seed quality check for Secondary Fermentation 1 (2015-500ML-01).

	Optical Density (A_{600}) 1:10	**Microscopy**
Seed 1	2.231	Healthy cell size, no contamination
Seed 2	2.195	Healthy cell size, no contamination

4.3.1.2 Experimental Media Preparation

Prior to inoculating the seed, the experimental media required preparation according to the design outlined in Table 4.1. The Windust Lite recipe was the same as for the seed (Table 3.1). However, supplemented waste materials were to be prepared by using the liquids in place of the water to which all recipe elements were added. No media additions were made to the raw treatments. All treatments were prepared in 500-mL Erlenmeyer flasks and autoclaved prior to addition of the buffer solution (to avoid precipitation). After

sterilization and buffer addition, 24 mL of seed was added to each flask for a starting volume of 240 mL.

Table 4.3 Flask preparation recipe for the experimental treatments in Secondary Fermentation 1 (2015-500ML-01).

Treatment	Description	Abbreviation	Flask Recipe
1	Spent Media	SM	216 mL spent media + 24 mL seed
2	Hydrolysate	H	216 mL raw hydrolysate + 24 mL seed
3	Negative Control	C-	216 mL water + 24 mL seed
4	Supp. Spent Media	SSM	208 mL supp. spent media + 8 mL buffer + 24 mL seed
5	Supp. Hydrolysate	SH	208 mL supp. hydrolysate + 8 mL buffer + 24 mL seed
6	Positive Control	WDL	208 mL Windust Lite + 8 mL buffer + 24 mL seed

4.3.2 Results and Discussion

4.3.2.1 Biomass

During the preparation of the fermentation media, after sterilization of the waste materials there was apparent precipitation and color change, most notably in the supplemented treatments. The precipitate was more obvious in the spent media than the hydrolysate which may have been due to higher concentration of phosphate – leading to iron phosphate precipitates – or maybe simply more easily observed in the less opaque spent media. Such precipitation is well-documented in algal growth media when iron and phosphates are autoclaved without a chelator present (Anderson, 2005). The color change was likely an indication of some caramelization or Maillard-type reaction having occurred (Nursten, 2005). Figure 4.1 shows these flasks after exposure to the extreme conditions in the autoclave. These visible changes were not seen in the negative or positive control.

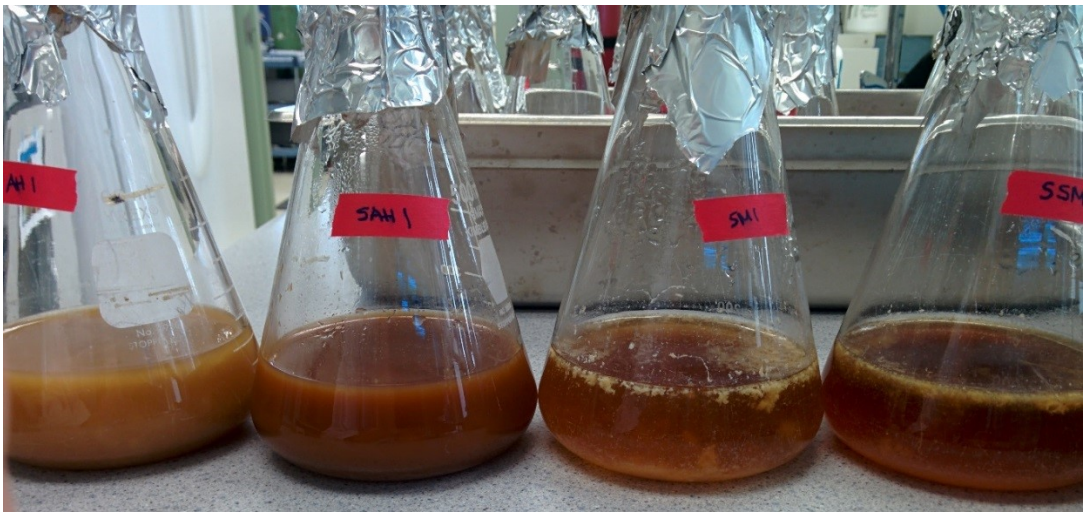

Figure 4.1 Observation of the color change and precipitate formation in the experimental flasks for 2015-500ML-01 of the raw hydrolysate, supplemented hydrolysate, spent media, and supplemented spent media (left to right).

Despite concerns regarding precipitation in the spent media, the experiment was continued. After 4 days of fermentation, biomass accumulation was evident in most treatments except for the negative control (Fig. 4.2). Not surprisingly, the positive control attained the highest final biomass concentration with 9.93 g L^{-1} after 96 hours. However, this was considerably less than in other experiments which achieved almost 25 g L^{-1} of T18 by 96 hours (Scott et al., 2011). This reduced biomass production may be attributable to differences in agitation and oxygen transfer, or may be indicative of suboptimal seed health as was evidenced by the lag phase. The supplemented spent media was the second best growing treatment which reached 7.76 g L^{-1} in the same time. Although there was evidently growth in the supplemented hydrolysate and the unsupplemented treatments, they appeared to plateau after 48 hours. This trend was understandable in the unsupplemented spent media and hydrolysate as the cells used all remaining nutrition (principally carbon) that was available and then ceased reproduction (Fig. 4.3A and C). However, the poor growth demonstrated in the supplemented hydrolysate, reaching a maximum of 4.42 g L^{-1} at 96 hours, was surprising. Notwithstanding those flasks having a full supplement of Windust Lite nutrients the cells in the supplemented hydrolysate were still not growing.

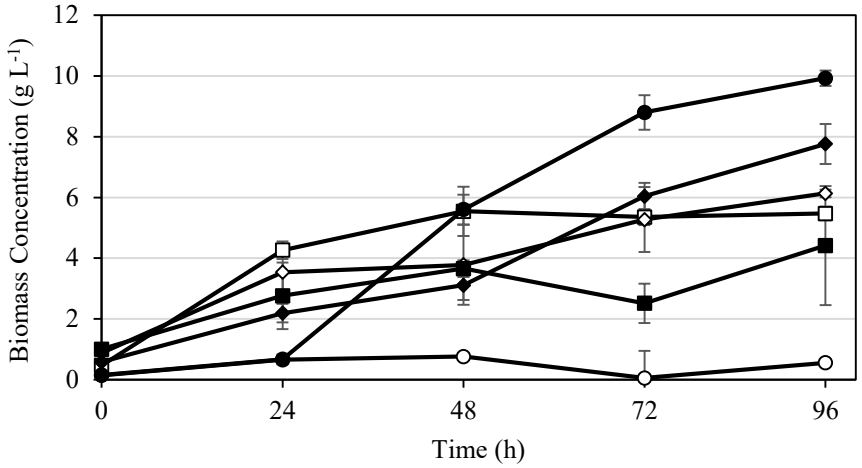

Figure 4.2 2015-500ML-01 growth curves of T18 in raw spent media (◊), raw hydrolysate (□), negative control (○), supplemented spent media (♦), supplemented hydrolysate (■) and positive control (●). Error bars indicate ± standard deviation (n=3).

4.3.2.2 Substrate Usage

To better understand the results of the fermentation, the concentrations of essential nutrients in the substrate were observed throughout. These data are illustrated in Fig. 4.3 with each experimental treatment having a dedicated panel showing the biomass accumulation in conjunction with the nutrient consumption. The positive control (Fig. 4.3F) clearly depicts the accumulation of cell biomass with the steady decrease in nutrient concentration. All of the unsupplemented treatments showed the eventual limitation of most nutrients as the scarce resources were depleted. It was clear that glucose consumption was an excellent indicator of cell growth as the relationship is almost always strongly inverse, especially in non-limited conditions (r = -0.95 for all supplemented treatments). Therefore, examining glucose consumption rates also provided an indicator of culture health and metabolic activity. Accordingly, the glucose consumption rate (GCR) of the positive control averaged 7.46 g L^{-1} d^{-1} while the supplemented spent media was 4.43 g L^{-1} d^{-1} and the supplemented hydrolysate only 1.44 g L^{-1} d^{-1}.

In addition to observing the carbon uptake by the GCR, it is important to quantify the yield ($Y_{X/S}$) of T18 cells corresponding to the amount of glucose consumed. Considering a mass and energy balance, it was not possible to yield exactly the amount of

cells as the amount of carbon consumed, therefore these values are always less than 1. The value can vary depending on the specific growth conditions and metabolic pathways of the species, but previous results using a *Thraustochytrium sp.* exhibited cell yields of approximately 0.40 in glucose (Lee Chang et al., 2014; Scott et al., 2011). The yield observed in the positive control was 0.33, indicating that for every 1 gram of glucose consumed, there was 0.33 grams of cells produced. Interestingly the yield in the supplemented spent media was 0.41 and the supplemented hydrolysate was 0.42. It is important to note that there was glycerol in the hydrolysate that was readily metabolized as a carbon source by T18, therefore that yield factors in glycerol consumption in addition to glucose (Scott et al., 2011). The higher yields in the waste materials was indicative of additional organic carbon being metabolized by the cells that is not being quantified.

The cell growth in supplemented spent media and positive control was closely associated with declines in all observed nutrients, indicating healthy, normal metabolism. As previously noted in Fig. 4.2, the supplemented hydrolysate did not support significant cell growth. That is also reinforced by the slow uptake of glucose. However, the slow growth as opposed to no growth, was suggestive of some inhibitory condition in the substrate that slowed growth. Considering the only difference between these treatments was the addition of the recycled waste material, it can be concluded that some factor associated with the hydrolysate was inhibiting the secondary fermentation in that material. Further, this inhibition did not appear to occur in the unsupplemented hydrolysate as the growth was approximately equal to that of the unsupplemented spent media. Knowing that the spent media and hydrolysate contained large amounts of organic nitrogen (amino acids) and glucose and they were being exposed to high heat during sterilization, there was a possibility of Maillard reactions taking place and forming a variety of unknown compounds (Nursten, 2005). This theory was also supported by the visible darkening of the material after autoclaving (Fig. 4.1). These Maillard intermediaries could potentially have inhibited the secondary fermentation as has been seen with 5-hydroxymethylfurfural (HMF) and levulinic acid (Larsson et al., 1999).

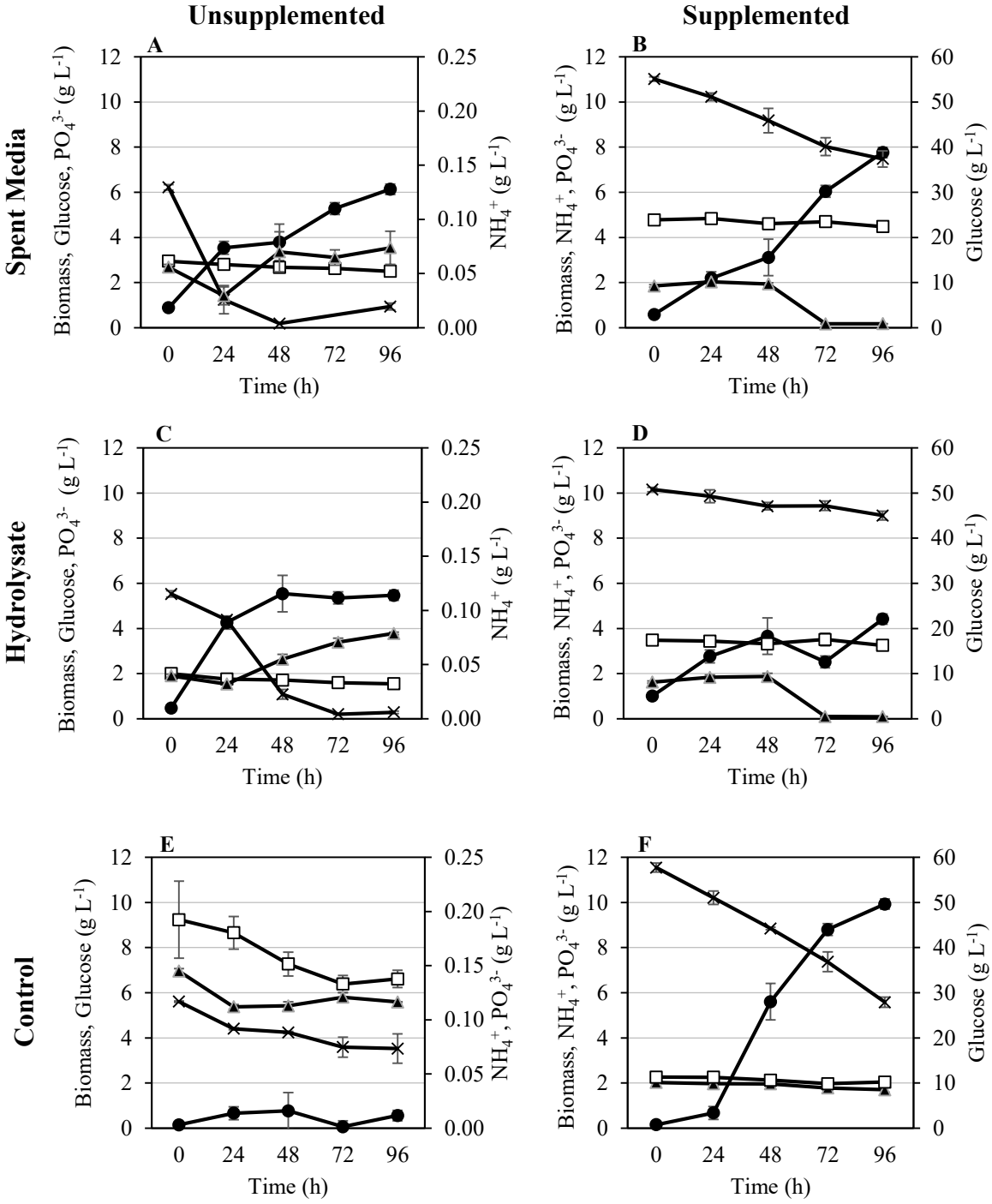

Figure 4.3 Cell growth and nutrient uptake curves in 2015-500ML-01 for in raw spent media (A), supplemented spent media (B), raw hydrolysate (C), supplemented hydrolysate (D), negative control (E) and positive control (F). Symbols denote cell biomass (●), glucose concentration (x), ammonium (▲) and phosphate (□). Error bars indicate ± standard deviation (n=3).

4.3.3 CONCLUSION

The first secondary fermentation experiment yielded some positive outcomes as well as potential challenges for the prospect of recycling the liquid waste materials originating from enzymatic lipid-extraction. While the recycled waste materials appeared to support secondary fermentation there was a reduced growth rate. The supplemented spent media exhibited adequate growth during the secondary fermentation, although it was less than the positive control in fresh media. Further, the supplemented hydrolysate did not show much growth and was actually outperformed by the unsupplemented hydrolysate. Considering these recycled treatments were supplemented with the full Windust Lite recipe, as was the positive control, they should have theoretically achieved similar growth. The discrepancy was suggestive of inhibition occurring in those materials, likely caused by the heat sterilization process which is partially responsible for the darkening of the waste materials.

4.4 SECONDARY FERMENTATION 2 (2015-500ML-02): SEPARATE STERILIZATION

In prior work, mitigating inhibition in recycled media has been achieved either by filter sterilization or simply diluting the waste material prior to secondary culture (Biller et al., 2012; Du et al., 2012; González-López et al., 2013). However, another conventional method for avoiding unwanted reactions during autoclaving is to keep the reactants separate during exposure to the extreme sterilization conditions and then combine the separate fractions in an aseptic manner. This has been regularly done in preparation of microalgae growth media with regards to preparing and sterilizing the phosphates separately from the ferric compounds to avoid formation of precipitates (Anderson, 2005). The technique was employed in this research with the goal to mitigate any Maillard reactions from taking place in the medium during autoclaving. It was accomplished by preparing the supplementing solution (the Windust media) separately in a concentrated solution to later be diluted to the desired concentration with recycled waste liquid. This would minimize any reactions from taking place between the recycled material and the fresh media.

The objective of this experiment was to explore the efficacy of the separated sterilization technique, therefore all other factors were maintained from the prior experiment (2015-500ML-01). The inevitable difference was that the waste materials were

diluted to 50% of their original concentration due to the addition of separately prepared media. The experimental design used for this secondary fermentation was identical to that used in the previous experiment (Table 4.1) with both the spent media and hydrolysate being compared to a control. Each waste material was used raw, and also supplemented with the Windust Lite recipe (Table 3.1). Also, the negative control used the standard media recipe with the organic carbon omitted to ensure equivalent concentrations of nitrogen in the media during sterilization. The growth period was also extended to 120 hours.

4.4.1 Materials and Methods

The materials and methods used in this experiment are described below and in Section 4.2.

4.4.1.1 Seed Preparation

For the 18 flasks, 378 mL of total seed was required to inoculate all of them using 21 mL each. This required preparing two 2-liter seed flasks (for redundancy) with a volume of 500 mL of Windust Lite medium each. Once the seed medium was prepared and autoclaved, each seed flask was aseptically inoculated with plated T18 biomass from cultures grown on standard fermentation media agar in petri dishes that had been refrigerated for no more than 8 weeks. Seeds were placed on an orbital shaker agitated at 200 rpm and 25ºC. The seed was grown for three days prior to evaluation for further use. After the incubation period the seed was evaluated to ensure that acceptable growth occurred and no morphological issues or contamination existed (Table 4.4). Seed 2 was selected due to slightly lower optical density indicating a smaller chance of the culture being overgrown and experiencing starving conditions.

Table 4.4 Observations from the seed quality check for Secondary Fermentation 2 (2015-500ML-02).

	Optical Density (A_{600}) 1:10	**Microscopy**
Seed 1	1.855	Healthy cells, no contamination
Seed 2	1.825	Healthy cells, no contamination

4.4.1.2 Experimental Media Preparation

Secondary fermentations were conducted in triplicate using 500-mL Erlenmeyer flasks containing 192 mL of media and a 21 mL (10% v/v) inoculation of seed. As illustrated in Table 4.5, six experimental treatments were used, with three not receiving media supplements (negative control, raw spent media, raw hydrolysate) and three being supplemented with the standard fermentation media (positive control, supplemented spent media, supplemented hydrolysate). The prepared medium composition was identical to that of the seed with a 50 % (v/v) addition (i.e. 96 mL) of hydrolysate or spent media obtained from the primary fermentation after oil-extraction. The negative control was the normal fermentation media without glucose. During the secondary fermentation the flasks were maintained at 25ºC on an orbital shaker at 200 rpm for 6 days.

Table 4.5 Flask preparation recipe for the experimental treatments in Secondary Fermentation 2 (2015-500ML-02). Starting volume was 213 mL with a 10% v/v seed inoculation. CWDL = concentrated Windust Lite.

Treatment	Abbreviation	Flask Recipe
Hydrolysate	H	96 mL raw hydrolysate + 96 mL water + 21 mL seed
Supp. Hydrolysate	SH	96 mL hydrolysate + 89 mL CWDL + 7 mL buffer + 21 mL seed
Spent Media	SM	96 mL raw spent media + 96 mL water + 21 mL seed
Supp. Spent Media	SSM	96 mL spent media + 89 mL CWDL + 7 mL buffer + 21 mL seed
Negative Control	C-	185 mL Windust Lite (no glucose) + 7 mL buffer + 21 mL seed
Positive Control	WDL	185 mL Windust Lite + 7 mL buffer + 21 mL seed

4.4.1.3 Scanning Electron Microscopy

Select samples were inspected by scanning electron microscopy (SEM) to detect morphological differences in response to the different growth conditions. For this work, a Hitachi S-4700 Field Emission Scanning Electron Microscope (Tokyo, Japan) from the Institute of Materials Research (IRM) at Dalhousie University was used. To prepare the samples for SEM, the cells were separated from the media by gravity settling to avoid inducing physically harsh conditions that may be imposed by centrifugation. After separation, cells underwent primary fixation using 2.5% glutaraldehyde followed by a distilled water wash. Secondary fixation followed using 2% osmium tetroxide followed by

washing. The samples were dehydrated using an ethanol gradient followed by critical point drying, then attached to a specimen stub and coated with a gold/palladium alloy for viewing.

4.4.2 Results and Discussion

4.4.2.1 Biomass

The initial comparison to make between the first (2015-500ML-01) and second (2015-500ML-02) secondary fermentations was the appearance of the waste materials after being autoclaved. As was described in the previous experiment and shown in Fig. 4.1, there was noticeable darkening of both the spent media and hydrolysate after exposure to the autoclave. In comparison, there was minimal darkening of those materials after autoclaving separately from the Windust Lite media elements (Fig. 4.4). However, it is important to note that to be consistent across all treatments, these materials were diluted to 50% concentration which would contribute to the lightening of their appearance. Regardless, there was considerably less evidence of unwanted reactions (Maillard or precipitate formation) in 2015-500ML-02 as compared to 2015-500ML-01.

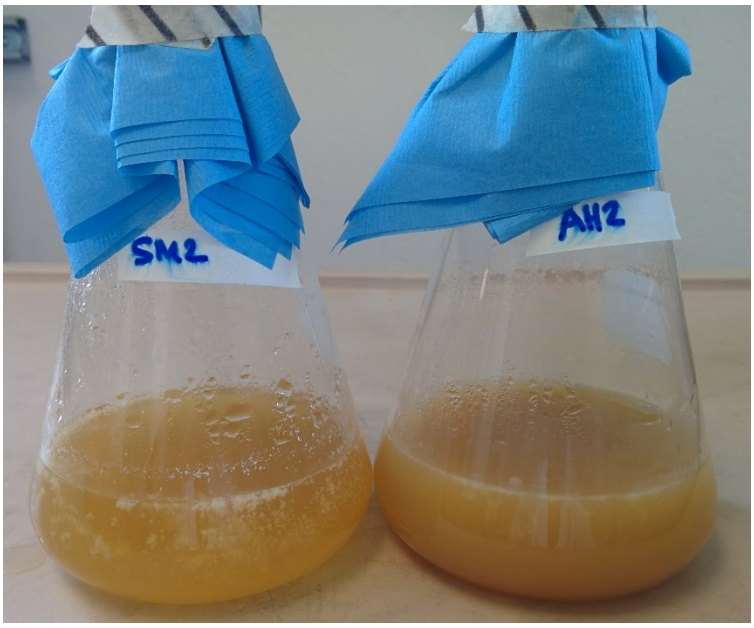

Figure 4.4 Observation of the color change and precipitate formation in the experimental flasks of the 50% diluted raw spent media and the raw hydrolysate in 2015-500ML-02 (left to right).

In order to improve the interpretation of the secondary fermentation results the growth period was extended to 120 hours. This was intended to allow more time for nutrient depletion and improved resolution of the different treatments at the end of the fermentation. As shown in Fig. 4.5, the supplemented hydrolysate rapidly produced biomass and maintained a greater cell concentration than all other treatments throughout the duration of the secondary fermentation. By 120 hours, the supplemented hydrolysate reached 20.48 g L^{-1} of T18 biomass as compared to 13.01 g L^{-1} in the supplemented spent media and 13.63 g L^{-1} in the positive control. The corresponding biomass productivities were 6.30 g $L^{-1}d^{-1}$, 3.45 g $L^{-1}d^{-1}$ and 3.33 g $L^{-1}d^{-1}$ for the supplemented hydrolysate, supplemented spent media and control, respectively.

The performance of the raw spent media and raw hydrolysate depicted a modest accumulation of biomass by 48 hours followed by a levelling off for the remainder of the fermentation. This outcome was consistent with the previous experiment (Fig. 4.2) and indicative of a rapid consumption of the few remaining carbon sources in the waste materials prior to a halt in growth. This was further supported by the slightly higher maximum biomass concentration attained in the hydrolysate compared to the spent media or the negative control (with no carbon available).

The considerable growth in the supplemented hydrolysate as compared to the positive control was significant considering the carbon budget was almost identical for those two treatments. This point is further emphasized by a cell yield (grams of cell biomass per gram carbon consumed, $Y_{X/S}$) of 0.48 for the supplemented hydrolysate as compared to 0.36 in the supplemented spent media and 0.28 in the positive control. The substantial improvement in productivity of the supplemented hydrolysate in comparison to the supplemented spent media and positive control may be attributable to the elevated concentrations of solubilized organic carbon and nitrogen in the recycled media, providing additional nutrients for cell metabolism. Specifically, compounds present in the hydrolysate such as glycerol, may have been preferentially metabolized by T18, thereby providing a boost during the initial growth stages. For example, the glycerol uptake by T18 was previously observed by Scott et al. (2011) to produce algal oil. Additionally, this organic carbon may have originated from degraded cell carbohydrates and protein from the primary fermentation (Zheng et al., 2012a), which will be discussed with the SEM analysis.

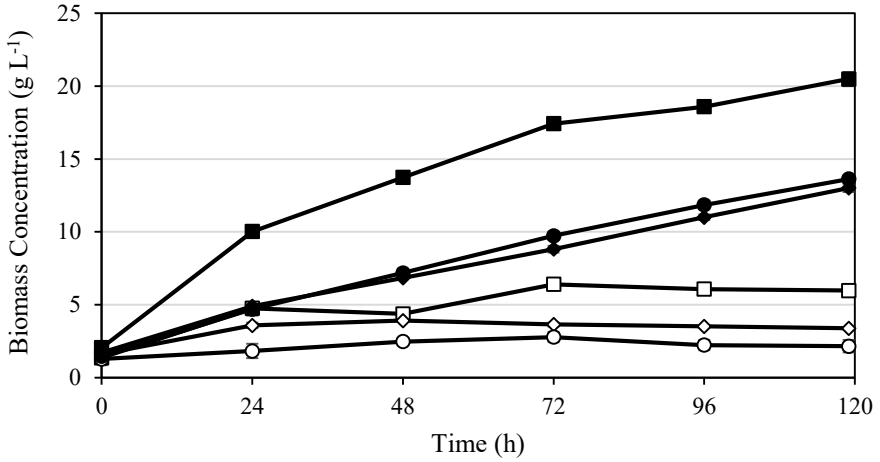

Figure 4.5 2015-500ML-02 growth curves of T18 in raw spent media (◊), raw hydrolysate (□), negative control (○), supplemented spent media (♦), supplemented hydrolysate (■) and positive control (●). Error bars indicate ± standard deviation (n=3).

4.4.2.2 Production of Ectoplasmic Net Structure

An interesting observation from these secondary fermentations was the emergence of a less compact pellet obtained from the supplemented hydrolysate after centrifugation during the biomass washing procedure. Compared to the other treatments, the pellet formed in the supplemented hydrolysate possessed a more gelatinous structure (Fig. 4.6). Even after repeated washing to remove all media, the pellet remained poorly compacted. This observation was not made in any other experimental treatments. Upon examination with SEM it was observed that the difference in the pellets from these treatments was likely attributable to a distinct morphological change. Shown in Fig. 4.7, T18 cells in Windust Lite fermentation media exhibited a smoother surface with minimal connective material. In contrast, the cells observed in the supplemented hydrolysate showcased a considerable web-like structure surrounding the cells as well as dispersed throughout the background. The structure and shape of the cells was similar between both conditions and no visible contaminants were observed.

This morphological phenomenon can be explained by the tendency of thraustochytrids to secrete extracellular polysaccharides in the form of an ectoplasmic net, which is used to improve surface area and facilitate the breakdown and metabolism of

organic matter (Bongiorni et al., 2005a; Jain et al., 2005; Lee Chang et al., 2014; Liu et al., 2013). In the complex recycled hydrolysate, the elevated levels of organic matter can trigger this mechanism. Further, the synthesis of this cell ultrastructure can also explain the elevated biomass values observed and even the gelatinous pellet consistency. The polysaccharide content of this ectoplasmic net clarifies the pellet composition since creating a density gradient in the centrifuged samples would introduce separation challenges.

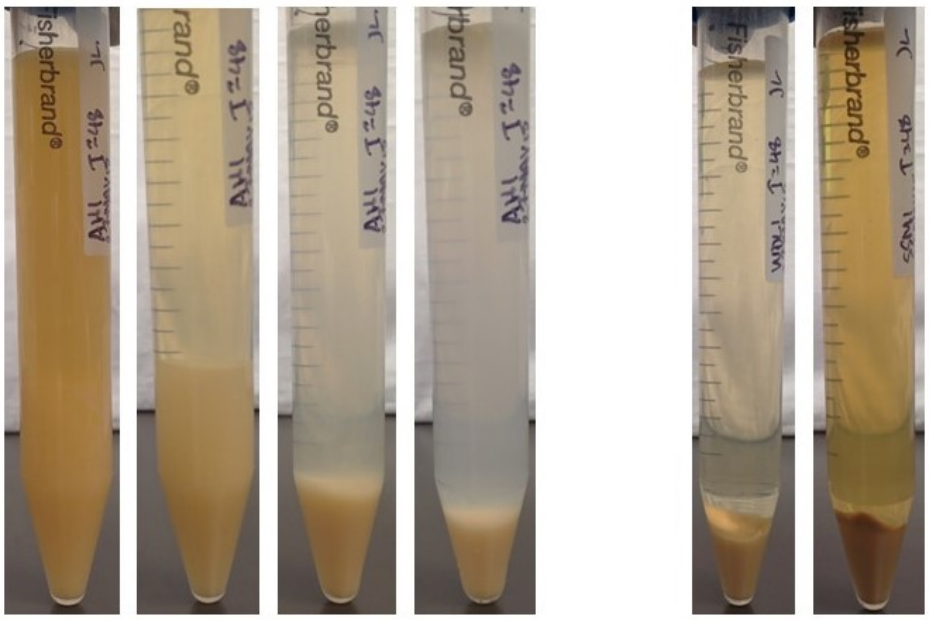

Figure 4.6 Cell pellet comparison after centrifugation between hydrolysate-containing treatments as opposed to the control and supplemented spent media.

The production of extracellular polysaccharides is also commonly associated with secretion of enzymes to aid in decomposition of organic substrates (Bongiorni et al., 2005b; Jain et al., 2005; Nagano et al., 2011). This occurrence indicates that T18 may be capable of using organic carbon and nitrogen compounds in the hydrolysate, which would have contributed to an increased availability of nutrients in those experimental treatments. Accordingly, the higher cell yield ($Y_{X/S}$) and reduced glucose consumption witnessed in

the supplemented hydrolysate supported this assessment. However, any advantage of the enhanced biomass production in the supplemented hydrolysate was dependent on the impact that these morphological changes had upon lipid synthesis and the fatty acid profile of the biomass.

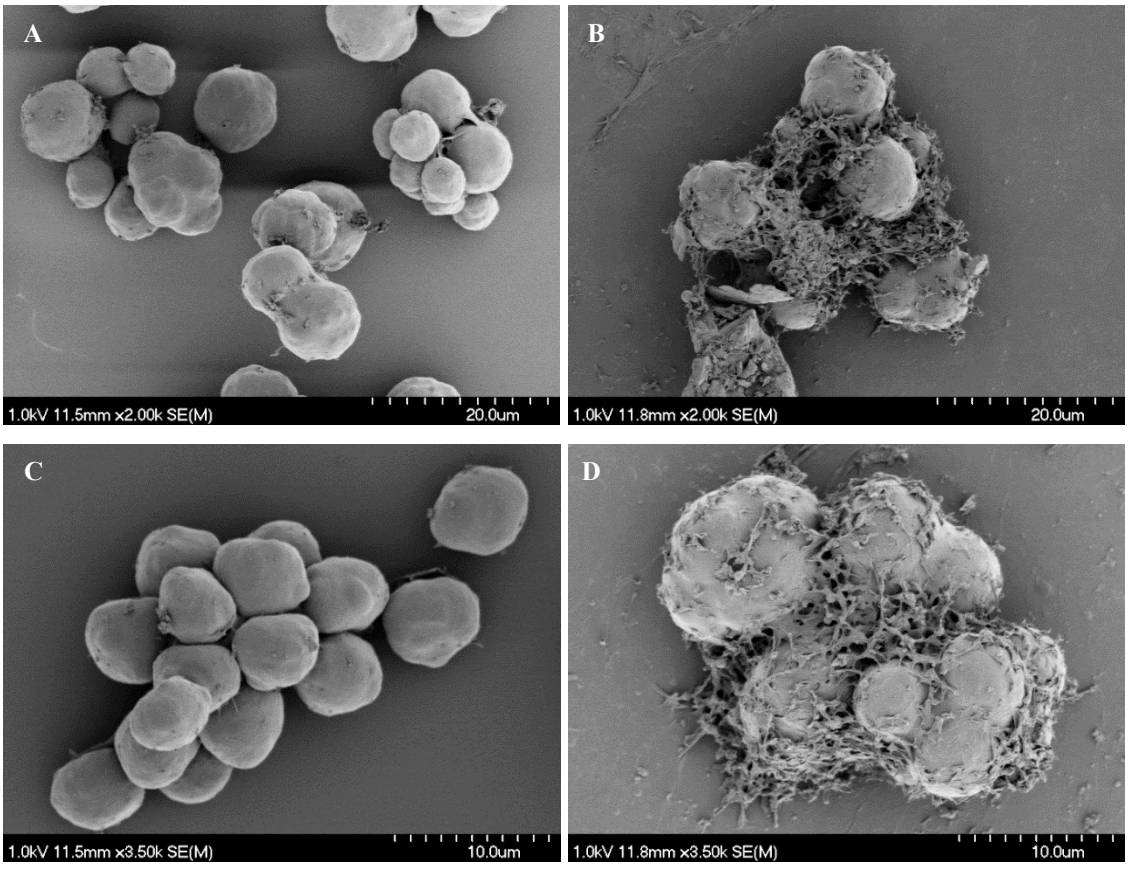

Figure 4.7 Scanning electron micrographs of T18 cultured in regular fermentation media (A and C) as opposed to supplemented hydrolysate (B and D). Micrographs were captured at 2,000x and 3,500x magnification.

4.4.2.3 Substrate Usage

The nutrient analysis of the secondary fermentation (2015-500ML-02) was also consistent with the results observed in 2015-500ML-01 where a steady decline in glucose concentration was apparent through the course of the fermentation. In the positive control, the glucose consumption rate (GCR) was 8.67 g L^{-1} d^{-1} which was slightly higher than seen in 2015-500ML-01 (7.46 g L^{-1} d^{-1}). In contrast, the GCR for the supplemented spent media

(Fig. 4.8) was 6.26 g L^{-1} d^{-1} and the supplemented hydrolysate was 7.33 g L^{-1} d^{-1} (Fig. 4.9) which was much higher than seen in 2015-500ML-01 (4.43 g L^{-1} d^{-1} and 1.44 g L^{-1} d^{-1}, respectively). This difference is evidence that T18 was not inhibited by the substrate and the metabolic rate was similar to the positive control. The glucose uptake in the raw spent media and raw hydrolysate showed an immediate depletion of carbon within 24 hours, consistent with the tapering growth seen in Fig. 4.5.

An important potential carbon source, glycerol, was present in the supplemented hydrolysate at a concentration of 1.85 ± 0.07 g L^{-1}, originating from the Alcalase enzyme solution added for lipid hydrolysis in the primary fermentation. Within the first 24 hours, the glycerol concentration was reduced by 88% and remained approximately at that depleted concentration for the remainder of the secondary fermentation. Uptake of glycerol by microalgae cells, and specifically T18, has been reported in the heterotrophic microalgae literature (Cerón Garcí et al., 2000; Pyle et al., 2008; Scott et al., 2011) and was observed in our results. This additional carbon consumption by T18 cells would provide additional organic carbon for metabolism in the glycerol-containing hydrolysate, thereby adjusting the yields reported above.

Ammonium and phosphate consumption rates exhibited modest reductions over the course of the fermentation. Ammonium concentration, starting at 1.46 g L^{-1} in the supplemented spent media and 1.81 g L^{-1} for the supplemented hydrolysate, declined by only 5.8% and 3.8% respectively. This, when compared to an 18.3% reduction in the positive control, represents a much higher rate of nitrogen consumption. The disproportionate ammonium consumption in the control may be explained by the presence and uptake of organic nitrogen along with the ammonium in both the supplemented waste materials. Phosphate uptake was similarly minimal with concentrations in the supplemented spent media and the supplemented hydrolysate declining by 27.5% and 10.0% from 3.57 g L^{-1} and 2.51 g L^{-1}, respectively. The abundant concentrations of ammonium and phosphate at the beginning and end of the secondary fermentation were indicative of excessive dosing, suggesting that for secondary fermentations in recycled waste it is advisable to reduce medium provisions of those components.

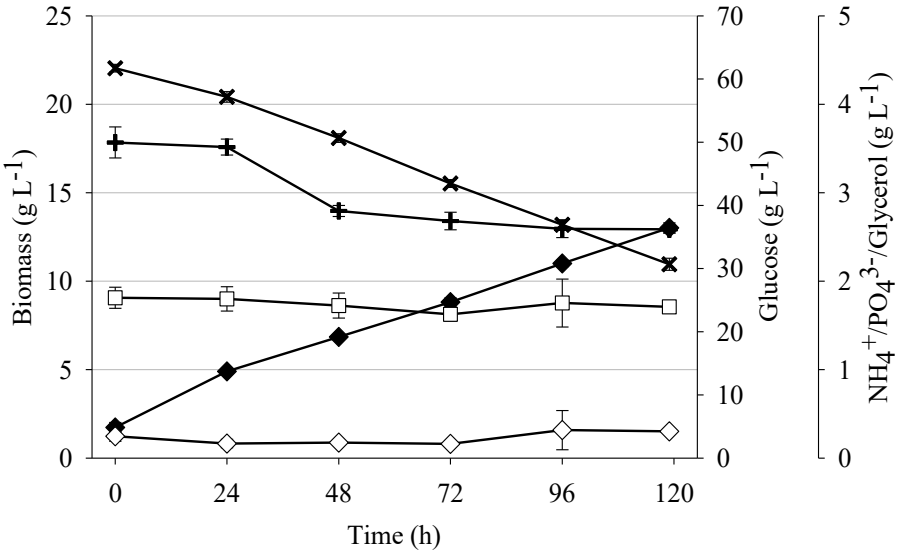

Figure 4.8 Growth curve of T18 during 2015-500ML-02 in supplemented spent media and nutrient depletion of the corresponding fermentation broth. Biomass (♦), glucose (x), phosphate (+), ammonium (□), and glycerol (◊). Error bars indicate ± standard deviation (n=3).

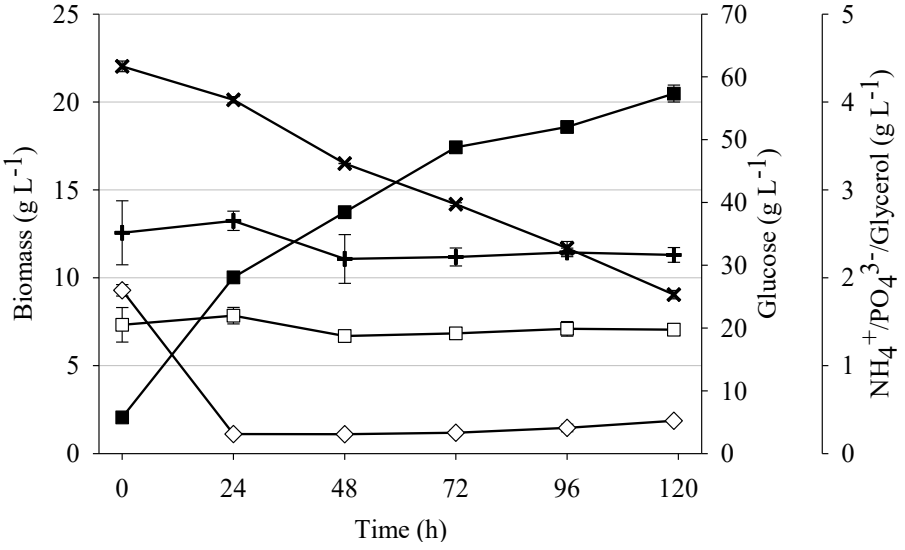

Figure 4.9 Growth curve of T18 during 2015-500ML-02 in supplemented hydrolysate and nutrient depletion of the corresponding fermentation broth. Biomass (■), glucose (x), phosphate (+), ammonium (□), and glycerol (◊). Error bars indicate ± standard deviation (n=3).

Considering that cell lysis for lipid extraction was executed using a protein-targeting enzyme, it is important to observe the content and possible uptake of free amino acids in the recycled materials. The only treatment that should contain elevated free amino acid concentrations attributable to the enzymatic activity was the hydrolysate-containing secondary fermentations. The origin of the amino acids in the fresh media and the spent media was the complex nitrogen containing medium element soy peptone, which is a complex nitrogen-containing medium component. As seen in Fig. 4.10 there were elevated concentrations of free amino acids in the supplemented spent media (1011.6 $\mu g\ mL^{-1}$) and supplemented hydrolysate (1426.2 $\mu g\ mL^{-1}$) in comparison to the positive control (592.2 $\mu g\ mL^{-1}$).

Both the supplemented spent media and positive control (regular fermentation media) demonstrated that the amino acids were metabolized during the secondary fermentation, reaching maximum uptake rates of 404.2 $\mu g\ mL^{-1}\ d^{-1}$ and 317.6 $\mu g\ mL^{-1}\ d^{-1}$, respectively (Fig. 4.10). By the conclusion of the secondary fermentation, approximately 90% of those free amino acids were removed from the substrate. As is shown in Fig. 4.11, the uptake of all free amino acids was ubiquitous except for arginine and lysine which remained throughout the secondary fermentations. Glutamate, the most prevalent amino acid measured, was rapidly consumed in all treatments. This preference for glutamate was consistent with many reports of microalgae consuming it readily as an essential metabolic product of inorganic nitrogen assimilation (Chen et al., 2010; Perez-Garcia et al., 2011). The less-preferred amino acids were shown to be metabolized in the nitrogen-limited raw wastes, where up to 98% of amino acids were removed from the substrate (unpublished data). These results do support prior literature demonstrating amino acid metabolism by microalgae, and further suggest the usefulness of nutrient recycling (Zheng et al., 2012a).

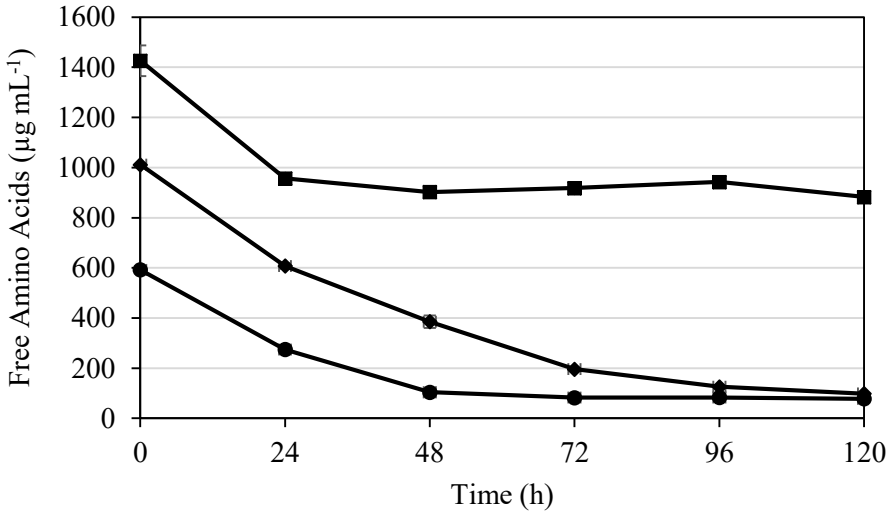

Figure 4.10 Free amino acid (µg amino acids per mL substrate) depletion during the secondary fermentation (2015-500ML-02) of the supplemented hydrolysate (■), supplemented spent media (♦) and positive control (●). Error bars indicate ± standard deviation (n=3).

In contrast to the supplemented spent media and positive control, the supplemented hydrolysate treatment showed an early decrease in free amino acids; however, the concentration levels off by the end of the fermentation, despite ample biomass accumulation (Fig. 4.10). Throughout the secondary fermentation, concentrations of free amino acids, as well as the specific amino acids barely changed (Fig. 4.12). The difference in uptake between the supplemented hydrolysate as opposed to the supplemented spent media and positive control may be explained by the greater concentration of organic nitrogen in that substrate (shown in Table 3.3), wherein some more preferred sources of nitrogen may have been available. This organic nitrogen can exist in a variety of protein and peptide structures in the hydrolysate which were not monitored during this experiment, but were observed in the raw material. Furthermore, the gradual uptake of amino acids suggests that it would be useful to further investigate nitrogen-depleted medium formulations to promote the uptake of recycled organic nitrogen rather than fresh inorganic sources.

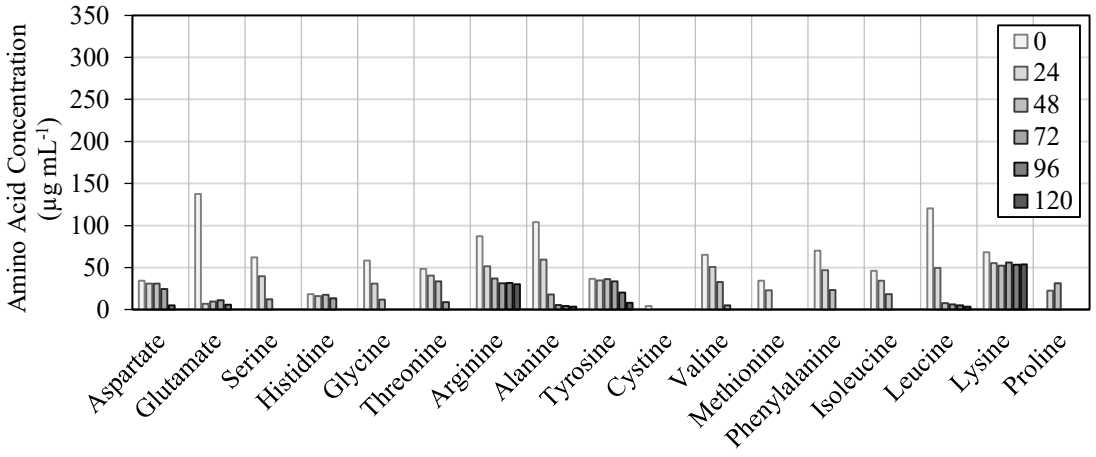

Figure 4.11 Concentration of amino acids in the supplemented spent media throughout the duration of the secondary fermentation (2015-500ML-02). Bars represent the time of six sampling points (in hours).

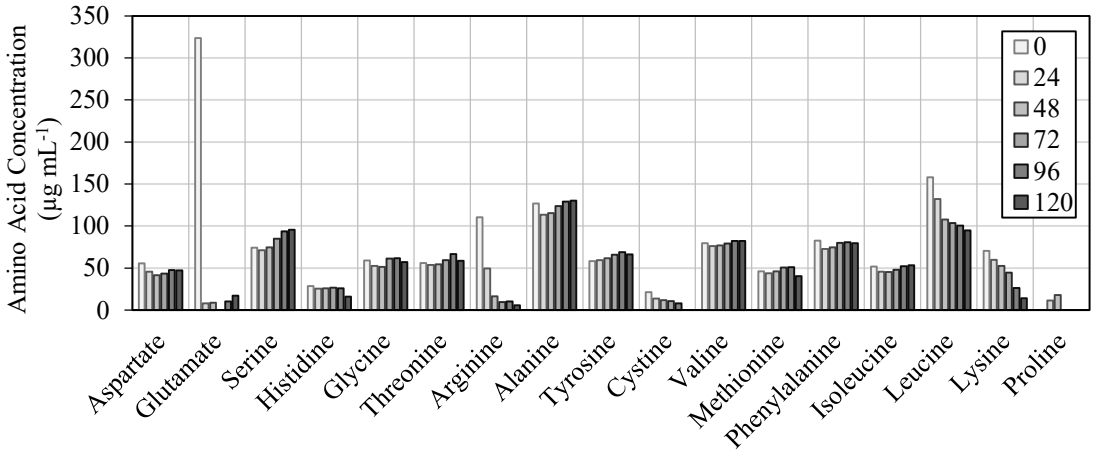

Figure 4.12 Concentration of amino acids in the supplemented hydrolysate throughout the duration of the secondary fermentation (2015-500ML-02). Bars represent the time of six sampling points (in hours).

4.4.2.4 Lipid Analysis

In addition to monitoring the substrate throughout the secondary fermentation, it was important to quantify the lipid content of the biomass produced. The lipid content, as measured by FAME concentration, was measured in the biomass produced in each of the treatments by quantification of the total fatty acid concentration. In order to assess this

product formation, the lipid content relative to the biomass accumulated (i.e. the lipid concentration) was reported. The results suggest that the highest production of lipids was observed in the supplemented hydrolysate with a lipid concentration of 8.37 g L^{-1} as compared to the control at 7.45 g L^{-1}. The higher lipid concentration in the supplemented hydrolysate treatments was attributable to the enhanced biomass content, despite the reduced cellular lipid content. Table 4.6 indicates that the positive control had approximately 57% oil content as opposed to 43% and 41% in the supplemented spent media and supplemented hydrolysate, respectively. The lipid content demonstrated in each of the treatments was on the higher end of most reported values expected for microalgae (ranging from 10 to 55% w/v), which was surprising in a nutrient replete environment and minimal culture manipulation to induce stress (Demirbas and Demirbas, 2011; Singh et al., 2011).

In addition to the total fatty acid content (TFA) of the biomass produced during the secondary fermentations, the fatty acid profile was indicative of the oil quality (Table 4.6). In all treatments, the produced biomass exhibited fatty acid profiles consistent with reported values for thraustochytrids (Chang et al., 2012; Huang et al., 2003). Amongst the commonly prevalent fatty acids were docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Both fatty acid profiles of the supplemented waste materials were relatively similar to the control, with some slight deviations in the C14:0, C16:0 and DHA content. The DHA fraction of the oil profile increased from the control at 28.9%, to the spent media with 32.0%, and the hydrolysate with 31.6%. These changes were counteracted by corresponding decreases in C14:0 from 11.6% in the control, to 9.8% in the spent media and 10.5% in the hydrolysate as well as decreases in C16:0 from 41.7% in the control to 36.7% in the spent media and 37.8% in the hydrolysate.

Such changes in fatty acid profile are commonly seen corresponding to changes in the medium composition and metabolic state of the microalgae, with higher density cultures tending to accumulate higher concentration of these fatty acids (Scott et al., 2011). The declining total fatty acid content of the cells from the control to the supplemented spent media and supplemented hydrolysate was indicative of greater residual nutrient concentrations in the fermentation broth. However, changes in relative percentage of

specific fatty acids did not exceed 3% except for C16:0 and DHA (described above), suggesting similar fatty acid profiles in each treatment.

Table 4.6 Fatty acid profile of biomass produced from secondary fermentations in supplemented spent media and supplemented hydrolysate as compared to the positive control (n=2).

Fatty Acid	Concentration (mg per gram dry cells)		
	Supp. Spent Media	Supp. Hydrolysate	Positive Control
C12:0	2.42 ± 0.05	2.81 ± 0.03	5.16 ± 0.03
C14:0	41.92 ± 0.17	42.92 ± 0.04	66.21 ± 0.10
C15:0	10.09 ± 0.05	6.66 ± 0.01	7.97 ± 0.01
C16:0	156.05 ± 0.66	154.36 ± 0.11	237.29 ± 0.48
C16:1	6.94 ± 0.04	9.09 ± 0.08	4.46 ± 0.03
C17:0	3.50 ± 0.03	2.02 ± 0.12	2.00 ± 0.01
C18:0	8.72 ± 0.02	6.74 ± 0.04	10.57 ± 0.03
C18:1 (Oleic)	1.68 ± 0.02	0.88 ± 0.01	1.94 ± 0.03
C18:1 (Vaccenic)	3.35 ± 0.02	4.77 ± 0.02	4.74 ± 0.02
C20:0	1.64 ± 0.05	1.23 ± 0.03	2.30 ± 0.02
C20:4 n-6	1.78 ± 0.03	1.66 ± 0.09	1.58 ± 0.03
C20:4 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:5 n-3 (EPA)	5.83 ± 0.04	5.60 ± 0.05	4.76 ± 0.01
C22:0	0.39 ± 0.01	0.32 ± 0.05	0.58 ± 0.02
C22:4 n-6	0.45 ± 0.03	0.32 ± 0.02	0.31 ± 0.02
C22:5 n-6 (DPA)	40.06 ± 0.22	38.31 ± 0.06	51.60 ± 0.24
C22:5 n-3 (DPA)	2.52 ± 0.04	1.21 ± 0.03	1.41 ± 0.02
C24:0	0.75 ± 0.04	0.55 ± 0.03	1.05 ± 0.03
C22:6 n-3 (DHA)	136.22 ± 0.95	129.22 ± 0.16	164.25 ± 0.74
C24:1 n-9	1.38 ± 0.66	0.00 ± 0.00	0.19 ± 0.23
Total	425.70 ± 2.01	408.79 ± 0.47	568.38 ± 1.50

4.4.3 CONCLUSION

Results of this secondary fermentation were both supportive of the feasibility of recycling the two waste materials as well as indicative of a need to sterilize them separately from the media. By comparison to the positive control, both the supplemented spent media and the

supplemented hydrolysate can attain similar growth outcomes. Even more, the recycled hydrolysate exhibited greater biomass production that the positive control, possibly suggesting an improved productivity associated with greater concentration of organic nutrients in that substrate. This conclusion encourages further exploration into the specific composition of this organic material as well as the possibility of excluding, or reducing other medium elements during recycling.

This study provided a unique perspective for nutrient recycling using thraustochytrids, where the morphological response to the recycled hydrolysate was especially indicative of the ability of thraustochytrids to metabolize complex substrates, making them an ideal candidate for nutrient recycling. The results from the supplemented hydrolysate also indicated a surge in biomass concentration, which was likely a product of the secretion of extracellular polysaccharides to aid in metabolism of the substrate. Despite this anomaly, the reduction in cellular lipid content was still offset by the enhanced production of biomass. Furthermore, minimal compromising effects on oil quality (i.e. fatty acids, specifically omega-3 fatty acids) were observed. Another noteworthy observation from this experiment was the reduction in the lipid content of the T18 cells produced in the recycled waste as opposed to the positive control. This reduction of oil content may be attributed to the abundance of nutrients in the recycled wastes preventing nitrogen limitation – a known condition to trigger oil accumulation. If so, it may be advantageous to extend the secondary fermentation for improved nitrogen limitation, or possibly reduce the concentration of nitrogenous elements in the supplementing medium.

4.5 Secondary Fermentation 3 (2015-500M-03): Nitrogen Reduction

Based upon the findings of the prior flask-scale secondary fermentations it was desirable to investigate the potential for adjusting the medium recipe to reduce the concentration of the nitrogen sources provided in the medium. Considering the proteolytic activity of the Alcalase added during lipid extraction, it was expected that the spent media and hydrolysate would contain an appreciable amount of organic nitrogen in solution. This was verified in Chapter 3 by the total nitrogen and amino acid analysis of those wastes. However, during the secondary fermentation (2015-500ML-02) only a portion of the free amino acids were taken up by T18 (Fig. 4.11 and 4.12), despite sizable accumulation of

biomass. Furthermore, only a fraction of the available ammonium available in the fermentation substrate was consumed which was indicative of an excess of nitrogen in the media. Therefore, it was pertinent to explore the potential for reducing or removing the nitrogen sources added to the secondary fermentation and depending solely on the organic nitrogen available in those substrates.

To appropriately investigate this, it was necessary to narrow the scope of the experiment. Accordingly, only the hydrolysate was employed for this secondary fermentation. The justification was that the hydrolysate would have a much greater organic nitrogen content in comparison to the spent media, due to the former being present during the enzymatic hydrolysis. Additionally, the hydrolysate was produced in large quantities at Mara. The sources of nitrogen present in the Windust Lite fermentation media were the organic nitrogen (soy peptone and yeast extract) and ammonium sulfate. As shown in Table 4.7, the experimental design consisted of removing the soy peptone/yeast extract or the ammonium sulfate that was added to the hydrolysate.

Table 4.7 Nitrogen ingredient inclusion (+) or omission (-) in the media for the experimental treatments during the Secondary Fermentation 3 (2015-500ML-03). All treatments were conducted in triplicate.

Nitrogen Source	Control	Hydrolysate-Containing		
		SH	AS-	N-
Yeast Extract	+	+	+	-
Soy Peptone	+	+	+	-
Ammonium Sulfate	+	+	-	-

4.5.1 Materials and Methods

The materials and methods used in this experiment are described below and in Section 4.2.

4.5.1.1 Seed Preparation

For the 12 flasks in the experiment 315 mL of total seed was required to inoculate all of them using 21 mL each. This required preparing two 2-liter seed flasks (for redundancy)

with a volume of 500 mL of Windust Lite medium each. Once the seed medium was prepared and autoclaved, each seed flask was aseptically inoculated with plated axenic T18 biomass grown on standard fermentation media agar on petri dishes for 4 weeks. Prepared seeds were then placed on the orbital shaker at 200 rpm and 25ºC. The seed was grown for three days. After the incubation period the seed was evaluated prior to use to ensure that acceptable growth occurred and no morphological issues or contamination existed (Table 4.8). Seed 1 was selected for use in the secondary fermentation due to a lower optical density.

Table 4.8 Observations from the seed quality check for Secondary Fermentation 3 (2015-500ML-03).

	Optical Density (A_{600}) 1:10	**Microscopy**
Seed 1	1.867	Healthy cells, no contamination
Seed 2	1.943	Healthy cells, no contamination

4.5.1.2 Experimental Media Preparation

Prior to inoculating the seed for the secondary fermentation, the media was prepared according to the experimental design. The standard Windust Lite formulation was used (Table 3.1) except that only 20 g L^{-1} of glucose was provided as opposed to 60 g L^{-1}. This adjustment was to ensure that all the available carbon in the treatments would be depleted within the prescribed growth period. Secondary fermentations were conducted in triplicate using 500-mL Erlenmeyer flasks containing 192 mL of prepared media and a 10% (v/v) inoculation of seed. Four experimental treatments were used, consisting of a control, fully supplemented hydrolysate (SH), supplemented hydrolysate without ammonium sulfate (AS-) and supplemented hydrolysate without any added nitrogen (N-). All supplementation of medium ingredients followed the same recipe as the control and the seed. The experimental differences between the treatments were the inclusion of nitrogenous ingredients as described in Table 4.7. Media and hydrolysate ingredients were separately autoclaved and combined aseptically in a laminar flow hood prior to inoculation. Hydrolysate-containing treatments were prepared using 96 mL (50% v/v) raw hydrolysate with 96 mL of either water or a double-concentrated media solution to reach equivalent

concentrations. Once all treatments were prepared and received buffer addition, 21 mL of seed was added to each flask for a starting volume of 213 mL (Table 4.9).

Table 4.9 Flask preparation recipe for the experimental treatments in Secondary Fermentation 3 (2015-500ML-03). Starting volume was 213 mL with 10% (v/v) seed inoculation. CWDL = concentrated Windust Lite, YE = yeast extract, AS = ammonium sulfate.

Treatment	Abbreviation	Flask Recipe
Full Nitrogen	SH	96 mL hydrolysate + 89 mL CWDL + 7 mL buffer + 21 mL seed
No Ammonium Sulfate	AS-	96 mL hydrolysate + 89 mL CWDL + 7 mL buffer (no AS) + 21 mL seed
No Nitrogen	N-	96 mL hydrolysate + 89 mL CWDL (no SP/YE) + 7 mL buffer (no AS) + 21 mL seed
Positive Control	WDL	185 mL Windust Lite + 7 mL buffer + 21 mL seed

4.5.1.3 Total Organic Carbon

Total organic carbon (TOC) of select samples was measured by submission to Agat Laboratories (Dartmouth, Canada) for analysis. TOC analysis was executed according to USEPA Standard Method 5310B – TOC by High Temperature Combustion. All values reported represent the concentration of elemental organic carbon in the samples.

4.5.2 Results and Discussion

4.5.2.1 Biomass

During the secondary fermentation (2015-500ML-03) all treatments exhibited ample growth within the 141-hour growth period. Fig. 4.13 illustrates the characteristic growth curves and the plateauing when the cells reached carbon limitation. Each of the experimental treatments in the hydrolysate accumulated equal or greater biomass to that of the control. The fully supplemented hydrolysate also exhibited the same rapid amassing of biomass within the first 24 hours that was observed in the previous secondary fermentation (Fig. 4.5). The other hydrolysate treatments (i.e. without ammonium sulfate and without added nitrogen) closely followed the growth curve of the control initially, but the AS- and N- treatments continued to accumulate additional biomass after the control plateaued

around 48 hours. The maximum biomass attained was in SH at 15.68 g L^{-1} followed by AS- and N- with 15.44 g L^{-1} and 15.16 g L^{-1}, respectively. In comparison the control grew to 9.78 g L^{-1}.

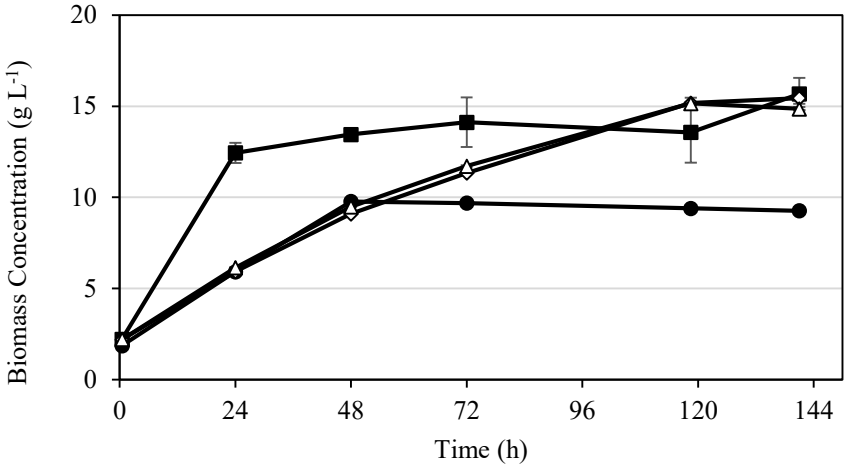

Figure 4.13 2015-500ML-03 growth curves of T18 in supplemented hydrolysate without ammonium sulfate (◊), without any added nitrogen (Δ), fully supplemented hydrolysate (■) and positive control (●). Error bars indicate ± standard deviation (n=3).

The continued cell growth after carbon limitation in the hydrolysate-based treatments produced a notably higher cell yield in those treatments. The control had a cell yield ($Y_{X/S}$) of 0.33 which is consistent with prior secondary fermentations (2015-500ML-01 and 2015-500ML-02) as well as yields observed for another promising thraustochytrid, *Schizochytrium limacinum* SR21 (Patil and Gogate, 2015). The SH yielded an impressive 0.60, which was slightly higher than previously while the AS- and N- yielded 0.59 and 0.54, respectively. This higher cell yield and continued growth beyond carbon limitation was suggestive of T18 using other sources of organic carbon present in the media, as has been described in the literature for other thraustochytrids (Bongiorni, 2012; Bongiorni et al., 2005b; Raghukumar, 2008). Also, an improved cell yield suggests improved efficiency of carbon use, which is the most expensive component of heterotrophic cultures (Li et al., 2007).

4.5.2.2 Substrate Usage

Glucose consumption rates were also similar to those observed in prior secondary fermentations, especially in the hydrolysate-containing treatments. The control exhibited a higher average GCR at 11.20 g L^{-1} d^{-1} as compared to 8.67 g L^{-1} d^{-1} (2015-500ML-02) and 7.46 g L^{-1} d^{-1} (2015-500ML-01). The supplemented hydrolysate consumed glucose at 8.06 g L^{-1} d^{-1} which was close to the 7.33 g L^{-1} d^{-1} observed in 2015-500ML-02. AS- and N-treatments also exhibited similar GCR with 7.42 g L^{-1} d^{-1} and 7.83 g L^{-1} d^{-1}, respectively. In addition to glucose consumption, glycerol is another major carbon source that has been demonstrated to support growth of thraustochytrids (Patil and Gogate, 2015; Raghukumar, 2008; Scott et al., 2011). Originating in the Alcalase broth, the hydrolysate-containing treatments all possessed small concentrations of glycerol which was rapidly consumed by T18. In all of those treatments, the initial concentration of glycerol of approximately 1.80 g L^{-1} was depleted within 24 hours.

To explore the uptake of organic carbon more thoroughly select samples were submitted for analysis of total organic carbon at the beginning and end of the secondary fermentation. The findings indicated a considerable uptake of total organic carbon in the control (9.52 g L^{-1}), the supplemented hydrolysate (11.55 g L^{-1}) and the supplemented hydrolysate without added nitrogen (12.46 g L^{-1}). In addition to glucose, this carbon could have been from a variety of bioavailable organic compounds (e.g. glycerol, acetic acid, fatty acids, etc.) or metabolic intermediaries (e.g. galactose, succinic acid, etc.) that resided in the hydrolysate (Li et al., 2013; Zheng et al., 2012a). Such metabolic dexterity is well-known for thraustochytrids in a variety of artificial and natural substrates, such as food wastes (Fan et al., 2000). The decreasing concentration of organic carbon measured in this substrate can be attributed to both the direct uptake of some compounds, as well as via degradation of complex organic material into more bioavailable forms through enzymatic activity (Bongiorni et al., 2005b; Liu et al., 2013).

The amount of total organic carbon consumed corresponded closely with the biomass accumulated in those treatments ($r = 0.90$). This relationship suggested that the hydrolysate-containing treatments, which had much more organic carbon, had greater capacity to support T18 growth due to the presence of a variety bioavailable carbon forms. Using the known glucose uptake – as measured by HPLC – and the stoichiometric

conversion of glucose ($C_6H_{12}O_6$) to elemental carbon, the fraction of organic carbon consumed that was attributable to glucose metabolism was estimated. The resulting difference reported in Table 4.10 was the concentration of non-glucose carbon that was metabolized by T18. While the specific carbonaceous compounds were not identified, it can be concluded that they provided sustenance for the cells beyond the glucose provided in the media.

Table 4.10 Total organic carbon (TOC) content of the secondary fermentation substrate at the initial (T_0) and final (T_F) sampling points. Glucose uptake was calculated using the HPLC data and the stoichiometric conversion from glucose to elemental carbon (Glucose:Carbon = 2.5:1). All samples were in duplicate except for the control (single) and all units are g L^{-1}.

	Control	SD	SH	SD	N-	SD
Total Carbon (initial), T_0	11.20	N/A	22.65	0.21	21.60	0.14
Total Carbon (final), T_F	1.68	N/A	11.10	0.14	9.14	0.09
Uptake, T_0-T_F	9.52		11.55		12.46	
Glucose Uptake	8.87		8.28		8.51	
Non-Glucose Carbon Uptake	0.65		3.27		3.95	

Ammonium consumption rates exhibited modest reductions over the course of the fermentation followed by slight increases after carbon limitation (Fig. 4.14). Ammonium concentration, starting at 1.65 g L^{-1} in the control and 1.52 g L^{-1} for the supplemented hydrolysate, declined by only 11.9% and 9.6%, respectively. These modest reductions were indicative of an excess supply of ammonium provided in the substrate for those treatments. In fact, similar experiments using thraustochytrids cultivated in flask conditions employed only peptone and yeast extract (0.20 g L^{-1} each) as the primary nitrogen source, with no additional ammonium. The biomass production was comparable, further indicating a possible excess of nitrogen in the control and SH (Lee Chang et al., 2014). The starting concentrations and corresponding reductions were minimal in the AS- and N- treatments – due to a negligible initial concentration of ammonium. The higher ammonium consumption in the control may be explained by the presence and uptake of organic nitrogen along with the ammonium in both the supplemented waste materials. These nitrogen uptake rates were similar to those witnessed in 2015-500ML-02 with the key difference being the medium

recipe providing 60 g L^{-1} of glucose as opposed to 20 g L^{-1} in this experiment. This is important because ammonium uptake ceased upon the cultures reaching carbon limitation around 48 to 72 hours.

Considering the known concentration of organic nitrogen (protein and amino acids) present in the hydrolysate, the free amino acid concentration was determined in the fermentation broth throughout the secondary fermentation. Shown in Fig. 4.15, the free amino acid concentration of each of the treatments declined during the secondary fermentation. Most notably, the positive control, AS- and N- treatments ended the growth period with almost all (98%, 94%, and 93% respectively) of the free amino acids taken up. In comparison, the supplemented hydrolysate treatment removed 55% of free amino acids from media. The maximum free amino acid uptake rates for all treatments except AS- varied between 200 to 400 µg mL^{-1} d^{-1}, similar to those observed in 2015-500ML-02. The maximum free amino acid uptake rate for AS- was 638.6 µg mL^{-1} d^{-1}, demonstrated by a rapid uptake of organic nitrogen after 24 hours.

Secondary cultures grown in an enzymatic hydrolysate containing amino acids has previously been demonstrated with a mixotrophic *Chlorella vulgaris* absorbing all available amino acids (Zheng et al., 2012a). However, those cultures were grown in the hydrolysate alone, without additional nutrients added for improved productivity. The high biomass yields of T18 in supplemented hydrolysates that were observed in this study were due to balancing the productivity of the secondary culture with resource efficiency. Efficient uptake of amino acids was best observed in the treatments with reduced nitrogen additions. Specifically, the most extreme treatment containing supplemented hydrolysate with no added nitrogen showed excellent accumulation of biomass (in excess of the control) and efficient uptake of amino acids with 93.1% of all monitored amino acids removed from the recycled substrate.

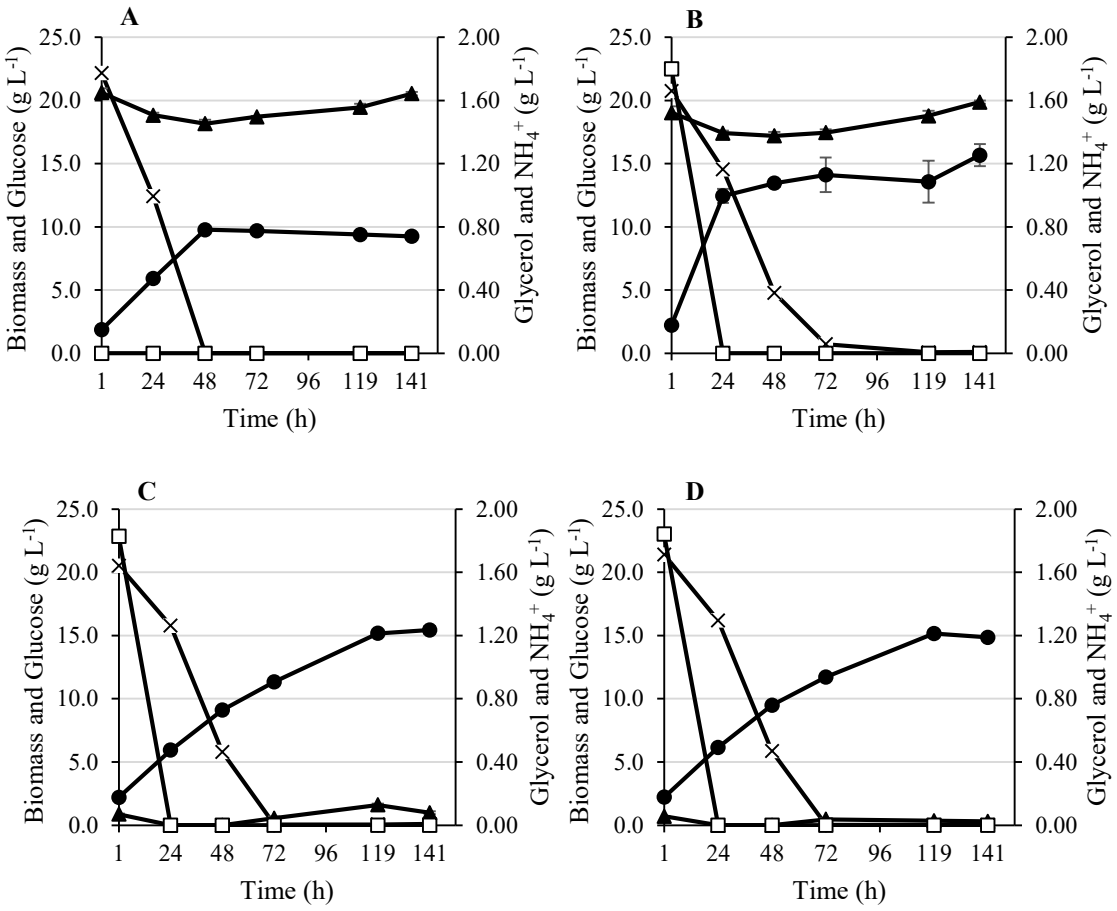

Figure 4.14 Cell growth and nutrient uptake curves in 2015-500ML-03 for in the positive control (A), supplemented hydrolysate (B), supplemented hydrolysate without ammonium sulfate (C), supplemented hydrolysate without added nitrogen (D). Symbols denote cell biomass (●), glucose concentration (x), ammonium (▲) and glycerol (□). Error bars indicate ± standard deviation (n=3).

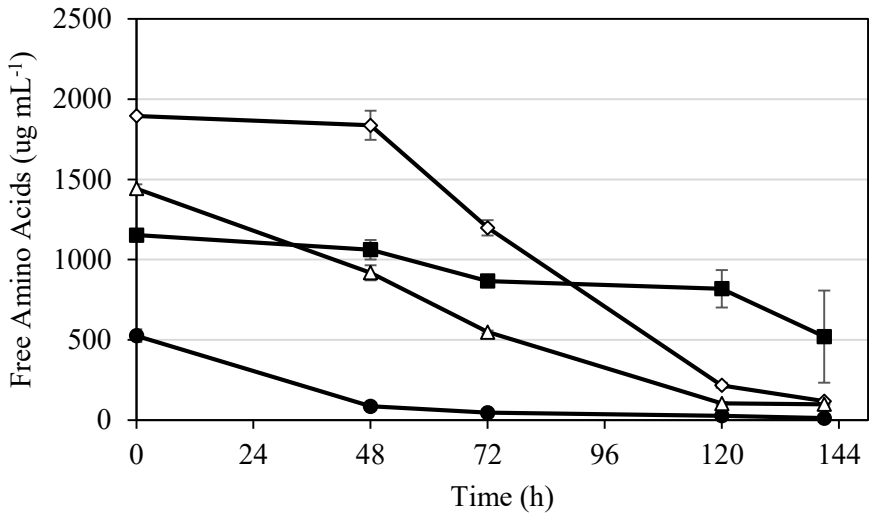

Figure 4.15 Free amino acid (µg amino acids per mL substrate) depletion during the secondary fermentation (2015-500ML-03) of the positive control (●), supplemented hydrolysate (■), without ammonium sulfate (◊), without any added nitrogen (Δ). Error bars indicate ± standard deviation (n=3).

The uptake of specific free amino acids during the secondary fermentations in the various treatments is illustrated in Fig. 4.16-4.18. In accord with the results in 2015-500ML-02, glutamate was present in the largest concentrations and rapidly consumed by T18 in each of the hydrolysate-containing treatments. Both the prevalence in the hydrolysate (and pre-hydrolyzed biomass) as well as the preferential uptake of glutamate are attributable to the essential role the amino acid plays in metabolic processes (Perez-Garcia et al., 2011). The only treatment receiving an ammonium sulfate supplement (SH, Fig. 4.16) contained residual concentrations of most amino acids after 141 hours, likely due to a preferential uptake of ammonium instead of the amino acids. Specifically, the only amino acids to be completely removed from the substrate were arginine, cystine, and lysine with glutamate and leucine also being decreased significantly (95.6% and 96.0%, respectively). Additionally, the secretion of extracellular polysaccharides with the corresponding enzymes degrading organic matter could contribute to increasing concentrations of amino acids in solution, therefore complicating the flux analysis (Bongiorni et al., 2005b; Taoka et al., 2009).

In contrast, the two ammonium sulfate deficient treatments (Fig. 4.17-4.18) each had much less residual amino acids present at the end of the secondary fermentation. One exception was alanine, which although reducing considerably in concentration, was still present at the end of those secondary fermentations. This trend was also witnessed in the previous experiment (2015-500ML-02) as shown in Fig. 4.12 where alanine barely changed throughout the growth period. For these treatments 100% of each of the amino acids measured was removed except for aspartate, glutamate, and alanine (82.6%, 98.3%, and 55.9% respectively). The ubiquitous uptake of all measured amino acids was consistent with other studies observing amino acid metabolism by microalgae (Patil and Gogate, 2015; Zheng et al., 2012a). The complexity of examining the flux of specific amino acids (i.e. alanine) is enhanced by reports of amino acid accumulation or intracellular protein degradation during exposure to fermentation stress (Li et al., 2013). For example, *Schizochytrium* sp. was observed increasing and decreasing intracellular alanine content during a 140 hour fermentation, which the authors explained as being due to the formation of intermediate metabolites of pyruvate in the glycolytic pathway (Qu et al., 2013). These poorly understood stress protection mechanisms may be critical to explaining the amino acid flux during fermentations of T18, and the subsequent recycling of the enzymatic hydrolysates.

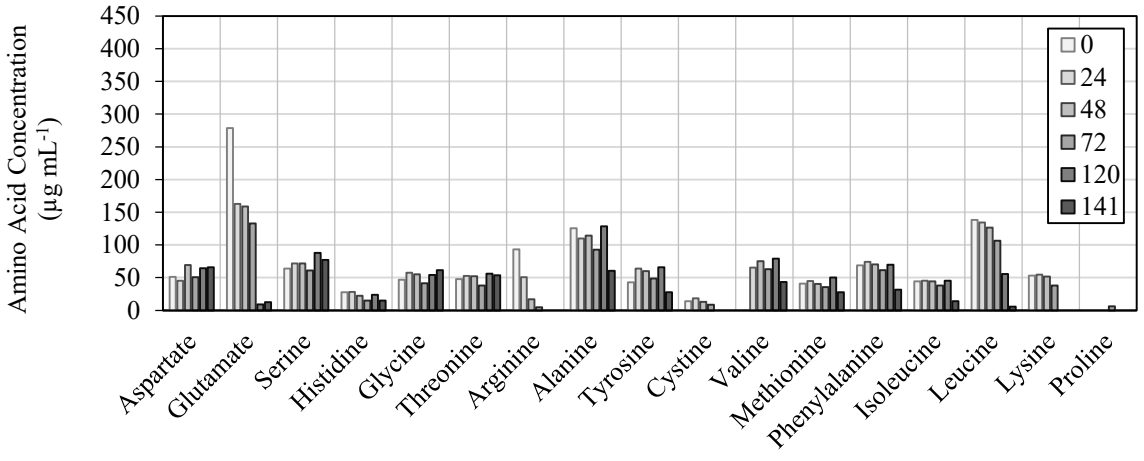

Figure 4.16 Concentration of amino acids in the supplemented hydrolysate throughout the duration of the secondary fermentation (2015-500ML-03). Bars represent the time of six sampling points (in hours).

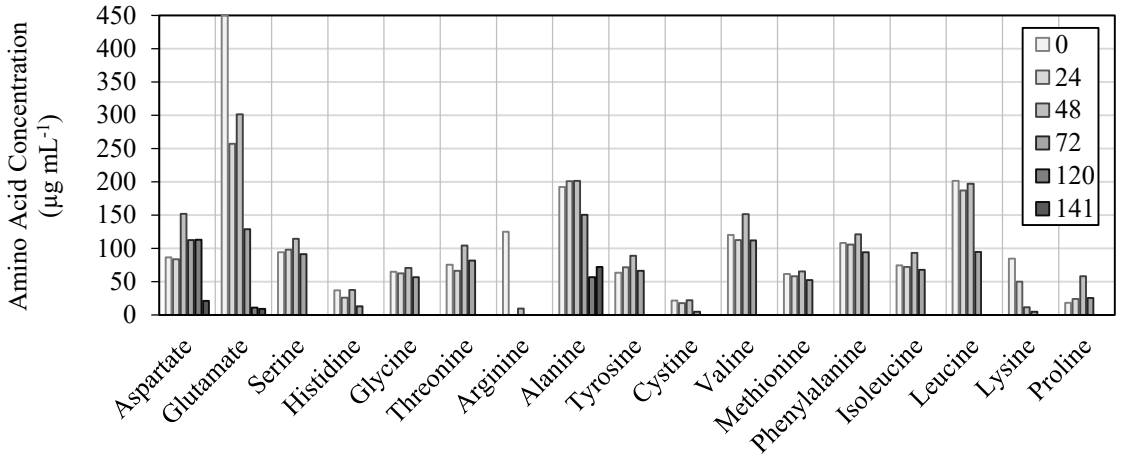

Figure 4.17 Concentration of amino acids in the supplemented hydrolysate without ammonium sulfate throughout the duration of the secondary fermentation (2015-500ML-03). Bars represent the time of six sampling points (in hours).

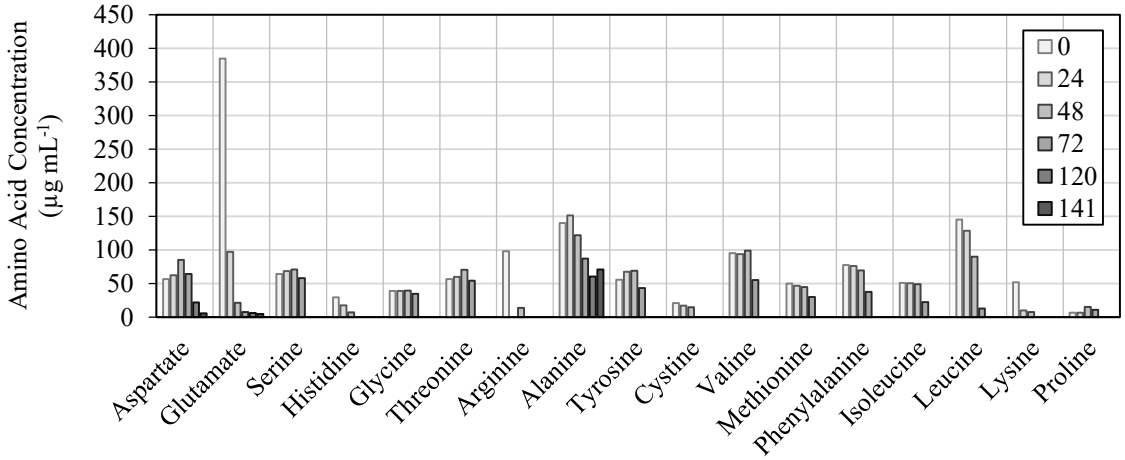

Figure 4.18 Concentration of amino acids in the supplemented hydrolysate without added nitrogen throughout the duration of the secondary fermentation (2015-500ML-03). Bars represent the time of six sampling points (in hours).

4.5.2.3 Lipid Analysis

Fig. 4.19 shows the relative concentration of total fatty acids produced in each of the experimental treatments as well as the corresponding percentage of the cell biomass. The control exhibited typical biomass and total fatty acid yield of 46.9% considering the

short growth period and limited glucose provided (Lowrey et al., 2016; Scott et al., 2011). The supplemented hydrolysate was unusual because despite large biomass production (15.68 g L^{-1}) by 90 hours, only 21.5% of the cells were fatty acids. This may be explained by the lower carbon to nitrogen ratio, resulting in a lessened capacity to reach high cell density and induce nitrogen limitation. The study by Lee Chang et al. (2014) also illustrates this, where the cultivation of four thraustochytrids in two concentrations of glucose had samples with the greater concentration of glucose exhibiting higher concentrations of biomass and total fatty acids.

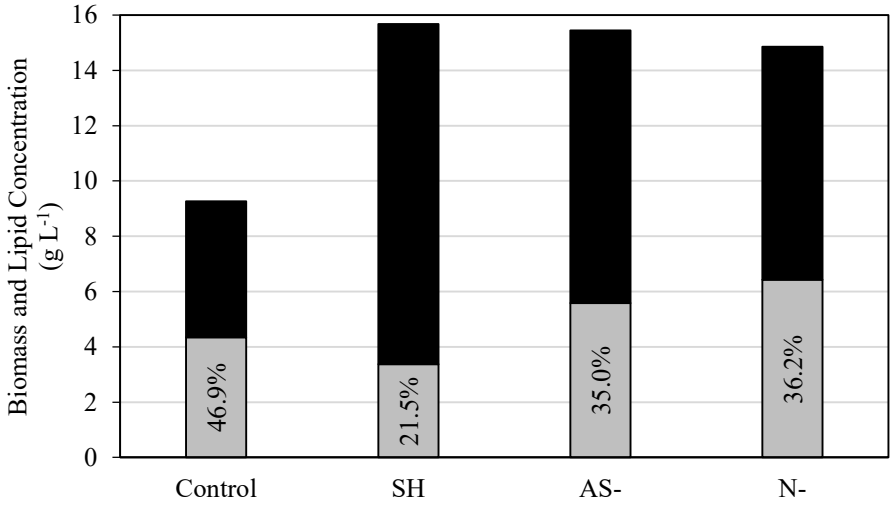

Figure 4.19 Final biomass (combined) and lipid concentrations (grey) for each of the flask treatments and the corresponding cellular lipid content (percentage). SH = supplemented hydrolysate, AS- = supplemented hydrolysate without ammonium sulfate and N- = supplemented hydrolysate without added nitrogen.

The other supplemented hydrolysate treatments were also lower in total fatty acids than the control but appeared to increase with decreasing available nitrogen, further supporting the importance of limiting nitrogen in the secondary fermentation. The supplemented hydrolysate without ammonium sulfate (AS-) and the supplemented hydrolysate without any added nitrogen (N-) contained 36.2% and 43.2% total fatty acids respectively. Considering the greater biomass yield in both of those conditions, the actual lipid concentration in the culture was greater (5.59 g L^{-1} and 6.43 g L^{-1}, respectively) than

that of the control (4.35 g L^{-1}). This observed trend between fatty acid accumulation and the availability of nitrogen in the substrate is consistent with the widely accepted influence of stress-induced lipid accumulation in microalgae (Perez-Garcia et al., 2011; Rodolfi et al., 2009). Managing nitrogen concentrations is critical when recycling a hydrolysate derived from a proteolytic enzyme, which results in high organic nitrogen in solution.

In terms of assessing the oil quality, it is useful to examine the fatty acid profile for abundance of desirable fatty acids. Shown in Table 4.11, the specific fatty acids that were most prevalent across all of the treatments were C22:6 n-3 (docosahexaenoic acid, DHA), C16:0, C22:5 n-6 and C14:0 as was observed in prior experiments with T18 (Lowrey et al., 2016; Scott et al., 2011). The control contained 35.2% (of the total fatty acids) of DHA while SH, AS- and N- contained 37.2%, 34.1% and 32.3% respectively. Although the concentrations of specific fatty acids varied slightly, none deviated by more than 3.7% (of the total) from the value observed in the control. This profile is comparable to that of healthy cells growing in similar environments, thereby suggesting the recycled hydrolysate was not detrimental to the fatty acid profile of thraustochytrids (Burja et al., 2006; Lee Chang et al., 2014; Perveen et al., 2006).

Table 4.11 Fatty acid profile of the final biomass produced in the secondary fermentations in the control, the supplemented hydrolysate (SH), supplemented hydrolysate without ammonium sulfate (AS-) and the supplemented hydrolysate with no added nitrogen (N-). All samples are analyzed in duplicate (n=2) in addition to duplicate injections into the gas chromatograph.

Fatty Acid	Concentration (mg per gram dry cells)			
	Control	SH	AS-	N-
C12:0	2.77 ± 0.02	1.26 ± 0.06	2.31 ± 0.09	3.18 ± 0.03
C14:0	48.54 ± 0.51	17.51 ± 0.09	34.23 ± 0.53	45.81 ± 0.39
C14:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C15:0	5.30 ± 0.04	2.71 ± 0.04	6.22 ± 0.08	8.75 ± 0.08
C16:0	161.61 ± 1.23	66.06 ± 0.43	121.01 ± 1.73	144.32 ± 1.35
C16:1	1.94 ± 0.04	3.83 ± 0.10	6.71 ± 0.10	11.39 ± 0.11
C17:0	1.07 ± 0.01	0.88 ± 0.11	1.77 ± 0.08	2.01 ± 0.01
C18:0	6.51 ± 0.06	2.66 ± 0.04	5.07 ± 0.07	6.05 ± 0.09
C18:1 Oleic	0.86 ± 0.06	0.25 ± 0.27	0.61 ± 0.02	0.68 ± 0.02
C18:1 Vaccenic	1.58 ± 0.05	2.24 ± 0.02	5.16 ± 0.07	7.92 ± 0.07
C18:2 n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:3 n-6	1.23 ± 0.10	0.26 ± 0.26	0.92 ± 0.02	1.15 ± 0.02
C18:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:4	1.12 ± 0.03	0.57 ± 0.02	0.76 ± 0.03	0.83 ± 0.03
C20:0	1.92 ± 0.02	0.27 ± 0.29	0.92 ± 0.05	1.28 ± 0.03
C20:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:3 n-6	2.21 ± 0.01	1.50 ± 0.19	1.58 ± 0.03	1.65 ± 0.03
C20:4 n-6	2.21 ± 0.08	1.50 ± 0.04	1.58 ± 0.08	1.65 ± 0.08
C20:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:4 n-3	2.43 ± 0.08	1.00 ± 0.05	1.79 ± 0.02	2.04 ± 0.04
C20:5 n-3 (EPA)	5.61 ± 0.10	5.22 ± 0.03	4.60 ± 0.09	4.70 ± 0.05
C22:5 n-6 (DPA)	57.08 ± 0.39	27.94 ± 0.17	43.23 ± 0.55	49.34 ± 0.41
C22:5 n-3 (DPA)	1.53 ± 0.03	0.96 ± 0.06	1.00 ± 0.02	1.12 ± 0.05
C22:6 n-3 (DHA)	165.27 ± 1.59	80.05 ± 0.37	123.20 ± 1.67	139.52 ± 1.02
C24:1 n-9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total	469.49 ± 3.95	215.37 ± 0.96	361.73 ± 5.11	432.48 ± 3.66

4.5.3 CONCLUSION

The outcomes of this flask experiment (2015-500ML-03) reiterated that waste recycling was possible without any detriment to the culture health or productivity. This is depicted by the comparison of biomass accumulated in the treatments containing hydrolysate versus the standard fermentation media which showed equal or greater growth in the recycled treatments (Fig. 4.13). In addition, this experiment went further to demonstrate that upon recycling the media, addition of fresh nutrients can be reduced and possibly eliminated while still reaching acceptable yields of biomass and comparable levels of fatty acids. In fact, the yield of biomass per unit of carbon consumed illustrated that recycling the hydrolysate enabled T18 to more efficiently synthesize cells. This improved efficiency may be a result of the capability of the microorganism to metabolize alternative forms of organic carbon and nitrogen by secretion of extracellular enzymes. However, verifying this metabolic activity would require specific experimentation designed at tracking the fate of a wider array of organic compounds in the substrate.

The focus of this experiment was on optimization of nitrogen additions into the secondary fermentation which was dependent upon the analysis of amino acids in the fermentation broth. The results demonstrated a high capacity of T18 to consume free amino acids as a nitrogen source in lieu of inorganic nitrogen. Efficient uptake of amino acids was best observed in the treatments with reduced nitrogen additions. Specifically, the most nitrogen lacking treatment, containing supplemented hydrolysate with no added nitrogen, showed excellent accumulation of biomass (in excess of the positive control) and efficient uptake of amino acids with 93.1% of all monitored amino acids removed from the recycled substrate. Also, an improved cell yield (0.54 as opposed to 0.33 in the positive control) demonstrated improved efficiency of carbon uptake, which is the most expensive component of heterotrophic cultures. Even more, the fatty acid concentration taking into account the enhanced biomass produced, was higher than the control. These outcomes are indicative of an opportunity for improved process economics by reducing the nitrogen requirements of secondary fermentations in recycled media.

4.6 CHAPTER CONCLUSIONS

The combined interpretation of the three flask experiments designed at exploring nutrient recycling with enzymatic hydrolysate and thraustochytrids suggested that the opportunity does exist, with some considerations. First, the importance of managing medium elements during sterilization was clearly illustrated by the inhibited outcomes in 2015-500ML-01. In that initial experiment, the supplemented hydrolysate and the supplemented spent media, to a lesser extent, were negatively affected by the sterilization process. The following experiment demonstrated that by maintaining separate sterilization of the waste material from the supplementing medium elements, those inhibitory affects were removed. Further, it can be concluded that the active inhibitory compounds were some form of Maillard intermediaries. This is evident by the darkening of the materials after autoclaving and the known presence of sugar and amino acids. An alternative sterilization technique (e.g. filter sterilization) may be another option to accomplish this outcome, however the added processing time and cost is a deterrent.

The second flask experiment (2015-500ML-02) employed the separated sterilization technique and investigated the ability to recycle the spent media and hydrolysate waste streams. The outcome demonstrated that both materials could support a secondary fermentation with proper reallocation of nutrients. The cleaner spent media is an attractive material for recycling, mostly because of the lower content of dissolved organic materials. Accordingly, the secondary fermentation using supplemented spent media showed excellent biomass growth almost exactly on par with the positive control. While this material possesses a reduced content of solubilized cellular protein, it is also closer to the initial fermentation media, thereby minimizing uncertainties with composition and accumulation of inhibitory compounds (e.g. Maillard intermediaries). However, one detracting aspect of the secondary fermentation in supplemented spent media was that the produced biomass contained a lower lipid content than the positive control. This observation may be attributed to the residual nutrients available in that substrate resulting in less stressful conditions at the end of the secondary fermentation, and correspondingly less lipids accumulated.

In comparison to the spent media, the hydrolysate exhibited similar outcomes in the secondary fermentation. Interestingly, the biomass rapidly exceeded any other

treatment and maintained elevated levels until the end of the secondary fermentation. The final biomass concentration was 50.3% greater than the positive control. This occurrence was attributable to the production of an ectoplasmic net which is likely a morphological response to the high organic load of the hydrolysate. However, as was observed with the supplemented spent media, the lipid content of this biomass was diminished (40.9%) compared to the positive control (56.8%). Despite these differences, the larger cell biomass concentration more than makes up the difference with a 12.3% increase in total lipid concentration when compared to the positive control. Furthermore, the nitrogen optimization experiment (2015-500ML-03) suggested that when recycling the hydrolysate, it is possible to eliminate the nitrogen-containing media ingredients without any detrimental effect to the fermentation outcome. This was explained by confirming the ability of T18 to metabolize free amino acids that originated from the primary fermentation enzymatic hydrolysis.

The collective conclusion of these flask experiments confirms the opportunity to successfully recycle cell protein when pairing thraustochytrid production with an enzymatic lipid extraction process. However, some considerations emerged during these secondary fermentations that may be important to the prospect of implementing this nutrient recycling strategy. First, the opacity of the hydrolysate introduced some practical challenges with handling the material as well as employing traditional analytical techniques for fermentation monitoring. For example, when centrifuging to separate the biomass from the substrate, recovering a definitive cell pellet was much more challenging in the hydrolysate as compared to the fermentation media. Due to the turbidity of the hydrolysate, careful effort was required to effectively differentiate between the substrate and the cell biomass. A second important concern for nutrient recycling is expanding these conclusions to a more representative scale for commercial production; that is, conducting the secondary fermentations in highly controlled fermentors. Expanding to fermentor-scale will verify the comparison between growth rates of the fermentation media and the recycled waste as well as reveal any limitation that may be present in optimized conditions. Furthermore, the rapid growth rates in fermentors will better test the ability of the hydrolysate to provide organic nitrogen from amino acids in in place of the nitrogenous media elements.

CHAPTER 5 : SCALED-UP 2-LITER SECONDARY FERMENTATIONS

Sections of this chapter are derived from the published manuscripts:
Lowrey, J., Brooks M. S., Armenta, R. E. (2016) 'Sequential Recycling of Enzymatic Lipid-Extracted Hydrolysate in Fermentations with a Thraustochytrid' Bioresource Technology. 209:333-342. doi: 10.1016/j.biortech.2016.03.030

5.1 INTRODUCTION

Validation of experimental concepts is frequently hindered by discrepancies between the conditions of the laboratory and the commercial environment. In microalgae research, this is illustrated by the differences between flask scale experiments and the experimental outcomes of highly controlled commercially representative vessels. Flask experiments are important to explore a variety of treatments and establish a statistically meaningful result. The narrowed scope of experiments – after thorough exploration in flasks – is then applied to the more optimized and replication-limited fermentors. In this work, the experiments were further narrowed to focus solely on the hydrolysate as the major waste product generated at Mara. This logical framework was used to justify the need to expand the previous nutrient recycling results into the 2-liter fermentors.

Results from the flask experiments in Chapter 4 indicated that recycling of the spent media and hydrolysate was possible with little negative impact on productivity. During these secondary fermentations carbon was consumed and biomass produced at rates that were consistent with healthy fermentations. However, nitrogen in the substrate, as measured by ammonium, did not decrease appreciably throughout the growth period. Additionally, the amino acids available in the recycled waste materials provided added nitrogen to the cells; more so in the hydrolysate than the spent media. At the conclusion of the secondary fermentations, the resultant biomass produced did not exhibit as high a concentration of lipids within the cells, as was evident in the standard fermentation media. This minimal nitrogen uptake and the reduced lipid content may be causally related, and moreover may be attributable to the flask productivity being insufficient to deplete the provided nitrogen and induce lipid accumulation. Expanding the nutrient recycling experiments to higher-productivity fermentors should better prompt complete nitrogen

uptake and illuminate the true differences in lipid accumulation between the recycled waste and standard fermentation media.

Another critical challenge to successful nutrient recycling is the potential for accumulation of micronutrients, salts or inhibitory compounds in the substrate after repeated recycles of media (Discart et al., 2014; Hadj-Romdhane et al., 2012; Lowrey et al., 2015). As was discussed in Chapter 2, compounds of specific concern include NaCl and Maillard intermediaries such as hydroxymethylfurfural (HMF). To explore this possibility, at least two sequential secondary fermentations should be conducted using the same recycled materials, while adding all medium ingredients prior to each recycle. Employing the monitoring capabilities of the fermentors and an in-vessel enzymatic hydrolysis, this concept can be investigated.

The aim of this chapter is to validate the experimental outcomes of the flask experiments using commercially representative 2-liter fermentors. In relation to specific objectives (iv) and (v) of this thesis, this involved developing a scaled-up protocol for commercially representative production vessels using recycled waste materials in the secondary fermentations, and investigating the effect of multiple sequential recycles. The work involved pushing the capability of T18 to elevated levels of production to allow comparison of productivity outcomes between the recycled hydrolysate and standard fermentation media, and also after multiple sequential recycles of the same media, as an indication of inhibition. As a result, differences in biomass yields and substrate usage efficiency may offer some insight into the viability of a more extensive application of nutrient recycling for thraustochytrids in enzymatically hydrolyzed waste.

This chapter will begin with a general materials and methods section which applies to both of the subsequent secondary fermentations. Within this section a description of the primary fermentation, enzymatic lipid extraction and hydrolysate recovery was included. After this, the next section focuses on the first recycle (2015-2L-13) of hydrolysate with relevant materials and methods, as well as results from the secondary fermentation. This first recycle was accompanied with an enzymatic lipid extraction and hydrolysate collection procedure to obtain the waste material for the subsequent secondary fermentation. The following section pertains to the second recycle (2015-2L-14) of the previously recovered hydrolysate and includes all relevant materials and methods as well

as results. The chapter concludes with a comparison of both sequential secondary fermentations and then the cumulative conclusion for the chapter.

5.2 Materials and Methods

5.2.1 Primary Fermentation (2015-500L-009) and Hydrolysate Production

To meet the volume requirements for multiple 2-liter secondary fermentations an additional source of hydrolysate was required. To best match the conditions of the 30-liter primary fermentation in Chapter 3 (2015-30L-18) an optimized 500-liter glucose-fed batch T18 fermentation (2015-500L-009) was used as the hydrolysate source. At the end of the 187-hour fermentation, enzymatic lipid extraction commenced using 0.1% (v/v) Alcalase. Hydrolysis conditions as described by Dennis and Armenta (2015), were 55ºC and pH 8.0 with 100 rpm mixing for 12 hours resulting in an 87.7% extraction efficiency. The resultant emulsion was then separated using a Westfalia ASD-2 continuous disc centrifuge (Rheda-Wiedenbrück, Germany) operating at 10,000 rpm. The heavy-phase waste product, henceforth designated as the hydrolysate, was then collected and frozen for subsequent recycling experiments.

5.2.2 2-Liter Fermentors

Three Sartorius Biostat B-DCU 2-liter fermentors (Goettingen, Germany) were used for the experiments in this chapter, operated in fed-batch mode. Sartorius MFCS/DA 3.0 software was used to continuously measure the pH, temperature, dissolved oxygen (DO), agitation rate and the volume acid and base additions. The fermentors consisted of a high-strength jacketed borosilicate glass vessel and a stainless steel lid and impeller frame (Fig. 5.1). Temperature was controlled by pumping heated or chilled water from the VWR International 1197P Programmable Temperature Controller (Radnor, U.S.A.) through the double-walled vessel. Aeration was provided through a flow meter into two 0.20 µm disc filters and through the stainless steel air sparger at the bottom of the fermentor vessel. All probes were threaded into the lid and sterilized during autoclaving. Inflowing or exiting liquids were stored in sterilized glass bottles (Fig. 5.1) and the liquids pumped via peristaltic pump through the lid into the vessel. Each fermentor was connected to an

individual control tower (Part: 8847843) and a central user interface computer (Part: 8847002).

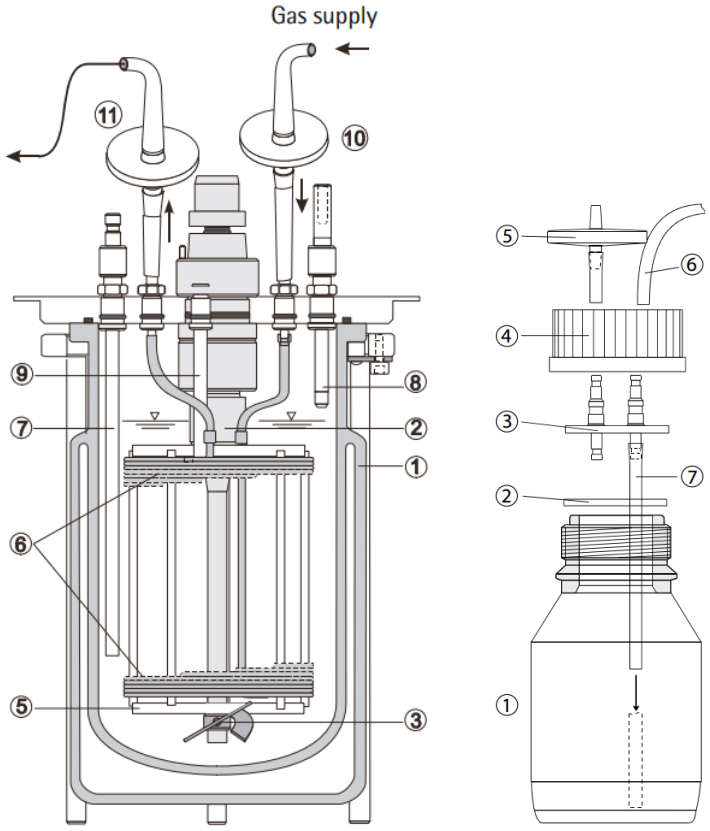

Figure 5.1 Sartorius Biostat B-DCU 2-L fermentor (left) with (1) double-walled glass jacket, (2) stirrer shaft, (3) bladed segment impeller, (5) holder, (6) aeration membrane, (7) sample tube, (8) probe, (9) aeration basket mount, (10) 0.2 µm inlet gas disc filter, (11) exhaust filter. Accompanying sterile bottle (right) for acid and base additions including (1) Pyrex glass media bottle, (2) silicone gasket, (3) stainless steel headpiece, (4) screw cap, (6) silicon tubing, (7) submerged tubing (Sartorius, 2012).

5.2.3 Fermentor Setup

Proper setup of the 2-liter fermentors required installation and calibration of all the required accessories for complete functionality. The first step was checking impeller spacing to guarantee they were submerged in the expected fermentation volume and ensuring equivalence in each fermentor. To seal the vessel, vacuum grease was applied to the glass and stainless steel lid interface prior to screwing the lid on. Once sealed, a dissolved oxygen

(DO) probe, temperature probe and calibrated pH electrode were installed into available ports. To prepare full functionality of the control features, tubing was attached to the various ports. The sample port was attached including sufficient silicon tubing for passage through the pump head. Chemically resistant neoprene tubing was attached to two of the quad-port ports with sufficient length for passage through the pump head and into the corresponding acid and base bottles, and silicon tubing was attached to the third quad-port (closing the fourth) with enough length for passage through the pump head and connection to the glucose feed. For sterile oxygenation, two 0.20 μm disc filters were connected with sufficient silicon tubing to the aeration port. Once all the tubing was installed, the fermentor was filled with the prescribed volume of the media and the final port was closed by attaching the condenser.

Outside of the fermentor it was necessary to prepare one 1-L media bottle (exhaust), one 500-mL media bottle (base), and one 250-mL media bottle (acid) with two-port caps, sufficient tubing and one 0.2 μm disc filter for air exchange (Fig. 5.1). The exhaust bottle was then connected to the fermentor and 800 mL of hydrolysate and 600 mL of 70% glucose were added to separate 1-L media bottles each with a two-port cap, sufficient tubing and a 0.2 μm disc filter. All bottles and the fermentor were then autoclaved at 121ºC and 15 psi for at least 30 minutes. After cooling, all bottles, probes and water lines were connected to the fermentor. In sequence via a peristaltic pump, the buffer solution (ammonium sulfate), hydrolysate, and seed were added to the fermentor. Once all ingredients were aseptically added, temperature control (25ºC), agitation (600 rpm) and pH control (4.50) were initiated along with the automated monitoring program.

5.2.4 SAMPLING PROTOCOL

During the 2-liter fermentations 30 mL samples were regularly collected to quantify the concentration of biomass and observe the consumption of glucose, glycerol, ammonium and amino acids in the fermentation substrate. Carbon was measured by YSI for rapid determination in order to ensure proper feeding according to the concentration in the substrate. Additional samples were collected as needed to quantify the concentration of glucose to determine the required feeding interval. Whenever a glucose feeding was conducted it was bookended by samples to establish an accurate glucose consumption rate.

Toward the end of the fermentation, the biomass pellet obtained after centrifugation was examined by near infrared spectroscopy (NIR) for total fatty acid and docosahexaenoic acid (DHA) concentration. The sample collection and processing techniques closely followed those described in Chapter 4 with the exception of the sample collection being done from the fermentor via the sampling port, rather than from flasks in the laminar flow hood.

5.2.5 YSI Fermentation Substrate Analysis

Throughout the 2-liter fermentations a YSI 2900 Series Biochemistry Analyzer (Yellow Springs, U.S.A.) was used for rapid monitoring of the glucose and ammonium concentrations in the fermentation broth. The procedure for using this instrument required preparation of samples according to the sampling protocol (below). Upon obtaining the supernatant after centrifugation, samples were diluted if necessary and 300 µL was pipetted into a 96-well plate. The accompanying program automatically proceeded with the prescribed analyses.

Glucose concentration via YSI was estimated by glucose-oxidase reaction, similar to established enzymatic blood glucose methods. This method has a 0.5 to 25.0 g L^{-1} detection range with precision of 2% or 0.2 g L^{-1} based upon an internal calibration program and proprietary reagents. Ammonium (YSI probe 2974) and potassium (YSI probe 2975) were simultaneously measured by an ion selective electrode. To avoid interference, both parameters were measured and automatically cross-referenced to ensure accuracy; sodium may interfere with quantification of these ions, although the amount is typically less than 1%. Detection ranges for these parameters are 10 to 500 mg L^{-1} for ammonium and 20 to 1,000 mg L^{-1} for potassium with precision of 5% for both (YSI, 2015).

5.2.6 Near Infrared Spectroscopy (NIR)

A Thermo Scientific Antaris II FT-NIR (Waltham, U.S.A.) with a custom model was employed for rapid estimation of the total fatty acid and docosahexaenoic acid (DHA) in the fermentation biomass. This method required concentration of T18 biomass by centrifugation and subsequent washing according to the sampling protocol outlined in

Chapter 4. Washed cells were transferred by pipet into a 19 x 51 mm glass vial and measured by NIR. The reported values were averages of triplicate samples.

5.2.7 Measurement of Algal Biomass

Cell growth during the secondary fermentations was quantified by determination of dry weight. Cell pellets were collected from the centrifuged samples and were washed by adding Milli-Q water, centrifuging, discarding the supernatant and repeating the process to obtain the twice-washed pellets. Washed cell pellets were then freeze-dried using a Labconco FreeZone18 (Kansas City, U.S.A.) in pre-weighed centrifuge tubes until constant weight to determine the dry weight of the biomass.

5.2.8 Analysis of Glycerol and Amino acids

Substrate analysis was conducted using the same analytical methods described in Chapter 3 for the physicochemical characterization of the waste material. Prior to submission for HPLC analysis for amino acids, glucose and glycerol, samples were filtered by hydrophilic 0.20 μm disk filters.

5.2.9 Lipid Analysis of Secondary Fermentation Biomass

The lipid content of the samples was determined by fatty acid methyl ester (FAME) determination by one-step transesterification and quantitation by gas chromatography, as described in Chapter 3. Additionally, near infrared spectroscopy (NIR) was employed for rapid estimation of total fatty acid and DHA content.

5.2.10 Experimental Layout of the Sequential Secondary Fermentations

The logical framework for the sequential secondary fermentations is outlined in Fig. 5.2. In the first fermentor, the control fermentations during the first (2015-2L-13) and second recycle (2015-2L-14) contained only fresh media with no recycled hydrolysate. The replicates of the supplemented hydrolysate in the first recycle were denoted AH1-1 and AH1-2 and employed the other two fermentors. The subsequent replicates of the supplemented hydrolysate in the second recycle were denoted AH2-1 and AH2-2.

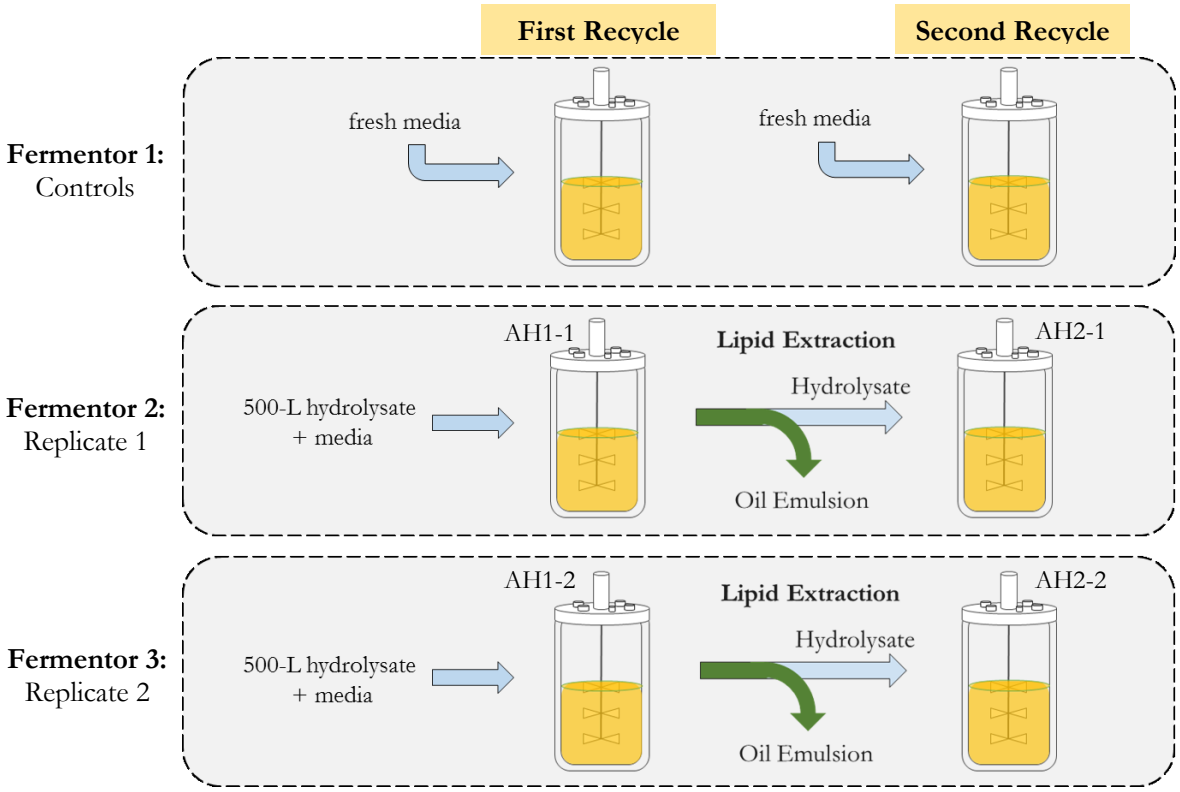

Figure 5.2 Experimental layout of the sequential secondary fermentations utilizing three 2-liter fermentors for the first (2015-2L-13) and second recycle (2015-2L-14).

5.3 Secondary Fermentation: 2-Liter First Recycle (2015-2L-13)

The first 2-liter fermentation conducted was the first recycling of the enzymatically-derived hydrolysate from a prior T18 fermentation. This hydrolysate was sourced from an optimized glucose-fed batch (2015-500L-009) using the standard enzymatic hydrolysis protocol. Similar to the flask experiments in Chapter 4 (2015-500ML-02 and 2015-500ML-03), the hydrolysate was blended to 50% with double-concentrated fermentation media to reach equivalence to the control. The control was prepared according to the recipe in Table 5.1. The secondary fermentation was planned to run for an undefined duration with the goal of conducting at least two carbon feedings and reaching carbon and nitrogen depletion prior to termination. In response to glucose depletion, 120 mL of 70% (w/v) glucose was added after 44 and 66 hours.

Table 5.1 Fermentation media recipe for 2-liter fermentations using recycled hydrolysate prepared to a total volume of 1.6 L. Acid and base additions are based upon the pH control and added as needed throughout the fermentation.

Ingredient	Amount (L^{-1})	Total
Glucose	60 g	96 g
Soy Peptone	2 g	3.2 g
$MgSO_4.7H_2O$	4 g	6.4 g
NaCl	9 g	14.4 g
$FeCl_3.6H_2O$ (0.01g mL^{-1} stock)	0.5 mL	0.8 mL
Trace Solution (stock)	1.5 mL	2.4 mL
Antifoam	0.1 mL	0.16 mL
KH_2PO_4	2.2 g	3.52 g
K_2HPO_4	2.4 g	3.84 g
$(NH_4)_2SO_4$	10 g	16 g
Vitamin solution (stock)	3 mL	4.8 mL
$CaCl_2.2H_2O$ (0.2g mL^{-1} stock)	0.5 mL	0.8 mL
200 g L^{-1} NaOH solution	80 mL	128 mL
250 g L^{-1} H_3PO_4 solution	30 mL	48 mL

5.3.1 Materials and Methods

5.3.1.1 Seed Preparation

For the three 2-liter fermentors in the experiment 300 mL total of seed was required to inoculate all of them using 100 mL (6.25% v/v) each. Inoculum was obtained from refrigerated cultures of T18 grown on standard fermentation media agar on petri dishes for 4 weeks. The cells were used to inoculate 2-liter Erlenmeyer flasks with 500 mL of the Windust Lite medium. Once the seed medium was prepared and autoclaved, the seed flask was aseptically inoculated with one full loop of plated T18 biomass and placed on the New Brunswick Scientific Excella E25 orbital shaker (Edison, U.S.A.) at 200 rpm and 25ºC. The seed was allowed to grow for three days prior to evaluation for further use. After the incubation period the seed was evaluated prior to use to ensure that acceptable growth occurred and no morphological issues or contamination existed (Table 5.2). Seed 1 was selected for use in the secondary fermentation due to a lower optical density.

Table 5.2 Observations from the seed quality check for the 2-liter secondary fermentation (2015-2L-13).

	Optical Density (A_{600}) 1:10	Microscopy
Seed 1	1.671	Healthy cells, no contamination, fewer cells
Seed 2	1.754	Healthy cells, no contamination, mixed cell sizes

5.3.1.2 Experimental Design

Using three 2-liter vessels, one was designated as a control and the other two were duplicates of the supplemented hydrolysate (AH-1 and AH-2). All vessels were setup identically and conditions across all three were set to the same values. To match the conditions in the flask fermentations, the hydrolysate was diluted by adding 800 mL (50% v/v) into 600 mL of fermentation media (prepared to 1.6 L recipe amounts as shown in Table 5.1). In the fermentor 100 mL of buffer and 100 mL of seed were introduced, bringing the total secondary fermentation starting volume to 1.6 L.

5.3.1.3 Growth Conditions

The fermentor-scale secondary fermentations were intended to promote conditions of high productivity to show differences between the recycled hydrolysate and a standard fermentation media that may not have been evident at a smaller scale. Accordingly, the conditions in the fermentors were managed to maximize growth of T18 according to Mara's proprietary methods (Burja et al., 2014; Sun et al., 2015). Vessel temperature was maintained at 25ºC with 600 rpm of mixing, 0.6 slpm of aeration and a pH maintained at 4.50. Temperature was maintained by circulation of chilled water through the fermentation computer thermostat and the vessel water jacket. The pH was maintained at a level known to inhibit contamination without any harm to T18 and managed by gradual addition of the acid or base (shown in Table 5.1). During fed-batch operation, a calculated volume of the 70% glucose solution was added to restore the concentration of glucose to approximately 60 g L^{-1}. For equivalence, both the control and the supplemented hydrolysate duplicates were fed at the same time. No additions of nitrogen were made.

5.3.1.4 Enzymatic Hydrolysis

After completion of the secondary fermentation, the resultant biomass was hydrolyzed using an Alcalase enzyme according to the protocol described by Dennis and Armenta (2015) in Chapter 3. This hydrolysis was conducted in-vessel by adjusting the conditions to those required by the enzyme (50ºC, pH 8.0, with 400 rpm agitation). Upon reaching those conditions 0.1% (v/v) of Alcalase was injected into the fermentor with a sterile syringe through the septum. After 24 hours of hydrolysis, the broth was transferred to 500-mL centrifuge bottles and centrifuged at 4,600 rpm at 4ºC for 20 minutes. The heavy-phase hydrolysate was collected after passing through a cheesecloth and stored in 1-liter glass bottles for subsequent recycling.

5.3.2 RESULTS AND DISCUSSION

5.3.2.1 Biomass

The accumulation of biomass throughout the secondary fermentation was the most important parameter for comparison between the fermentation with the recycled waste and the control. Fig. 5.3 depicts the growth curves of the three cultures for the 90 hour growth period. Both supplemented hydrolysate treatments (AH1-1 and AH1-2) followed the growth rate of the control very closely. In fact, the average productivity of the control 22.69 g $L^{-1}d^{-1}$ compared to 22.89 g $L^{-1}d^{-1}$ and 22.99 g $L^{-1}d^{-1}$ for the first (AH1-1) and second supplemented hydrolysate (AH1-2), respectively. These productivities were lower than some maximums reported in the literature such as for *Chlorella vulgaris,* which reached 87.9 g $L^{-1}d^{-1}$ in fed-batch conditions (Doucha and Lívanský, 2012). Furthermore, it is informative to note that the consistency of these productivities demonstrates that T18 is growing equally well in the recycled material as the fresh media. The final biomass concentration, upon depletion of all carbon and nitrogen, was 58.59 g L^{-1}, 62.65 g L^{-1} and 64.71 g L^{-1} for the control, AH1-1 and AH1-2. The parity between fresh media (control) and supplemented hydrolysate was impressive compared to most other experiments conducting nutrient recycling, where secondary cultures typically underperformed in comparison to their respective controls (Biller et al., 2012; Du et al., 2012; González-López et al., 2013; Lowrey et al., 2015).

Another important metric for the health and outcome of a fermentation is the cell yield ($Y_{X/S}$, grams of cells per gram of carbon consumed). All treatments exhibited acceptable cell yields that were 0.33, 0.35 and 0.35 for the control, AH1-1 and AH1-2, respectively. In comparison to prior flask experiments in Chapter 4 cultivating T18 in the same waste material, the cell yield in the control was very similar. However, flask treatments employing the supplemented hydrolysate exhibited much higher cell yields of (0.55 to 0.60) which was likely attributable to the following two factors. Firstly, the higher biomass concentration in the fermentors demanded greater glucose consumption, therefore reducing the fraction of unidentified organic carbon that contributed to cell synthesis, bringing the cell yield closer to the control. Secondly, the higher shear (i.e. greater degree of agitation) in the fermentors could damage extracellular polysaccharide structures which may inhibit the metabolism of complex organic compounds in the hydrolysate.

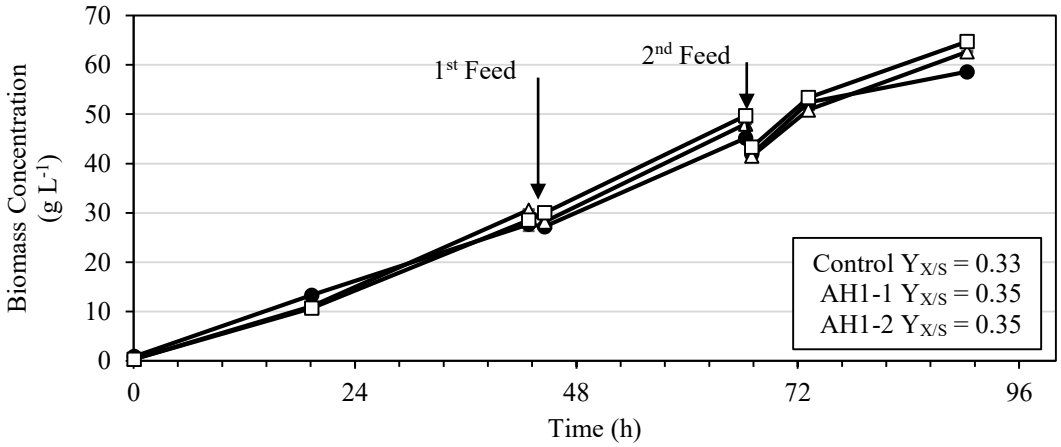

Figure 5.3 Biomass concentration of T18 during the first recycle of the hydrolysate in the 2-liter fermentors (2015-2L-13) in standard fermentation media (●), and the first (Δ) and second (□) duplicate of supplemented hydrolysate. Cell yields ($Y_{X/S}$) are indicated as grams of cells per gram of carbon. Error bars indicate ± standard deviation (n=2).

5.3.2.2 Substrate Usage

In the 2-L fermentations it was important to monitor the concentration of nutrients available in the substrate throughout the secondary fermentation. Glucose was particularly important due to the need to feed the fermentations, which was done by injecting additional

glucose during the growth period. Multiple samples were taken daily to observe the glucose uptake rate and ensure proper feeding intervals to avoid excessive periods of starvation. As is shown in Fig. 5.3, there were two glucose feedings which were intended to restore the substrate glucose to approximately the starting concentration, 60 g L^{-1}. Fig. 5.4-5.6A portrays the uptake of each nutrient, including glucose, for each treatment individually. In each treatment the glucose was rapidly consumed and depleted by about 44 hours after which it spikes, verifying the feeding.

In addition to monitoring the glucose concentration by sampling, an indirect indicator of metabolic activity (i.e. glucose availability) in the fermentation was attained by observing the dissolved oxygen concentration (DO) in the culture (Bumbak et al., 2011; Doucha and Lívanský, 2012). This indicator works as T18 undergoes respiration during growth, and removal of oxygen from the substrate. As glucose becomes limited, the metabolism of T18 slows, and in the presence of continuous agitation and aeration, dissolved oxygen climbs back to saturation. This oxygen saturation dynamic was thoroughly investigated and verified by Chi et al., (2008) using another highly productive thraustochytrid, *Schizochytrium limacinum* SR21.

During 2015-2L-13, the control exhibited continuous growth, correlating to a steady uptake of glucose and ammonium (Fig. 5.4). In fact, both glucose and ammonium were depleted by approximately 44 hours. The feed only introduces supplemental carbon, so the ammonium concentration remained depleted while the glucose concentration returned to 63.7 g L^{-1}. This condition was desirable as T18 continued to amass cell biomass in the presence of limited nitrogen; even more, the low nitrogen concentration is desirable for inducing lipid accumulation (Doucha and Lívanský, 2012; Perez-Garcia et al., 2011).

It is important to note that glucose monitoring was limited to sampling frequency, unlike the in-vessel continuous monitoring of dissolved oxygen. This was significant because based upon the dissolved oxygen concentration in the control (Fig. 5.4B), the fermentation was glucose limited much longer than intended. Specifically, the dissolved oxygen spiked around 25, 53 and 73 hours while the feeding was done at 44 and 66 hours. This resulted in a 19 hour and another 13 hour glucose deprivation period in this fermentation. Based upon the dissolved oxygen data, the glucose consumption rates for the control were 2.20 g L^{-1} h^{-1} prior to the first feed, 7.08 g L^{-1} h^{-1} between the first and second

feeding, and 7.67 g L^{-1} h^{-1} after the second feed. This condition was due to the objective of maintaining consistency between the three fermentations, where it was decided to conduct the feeding at the same time for each vessel.

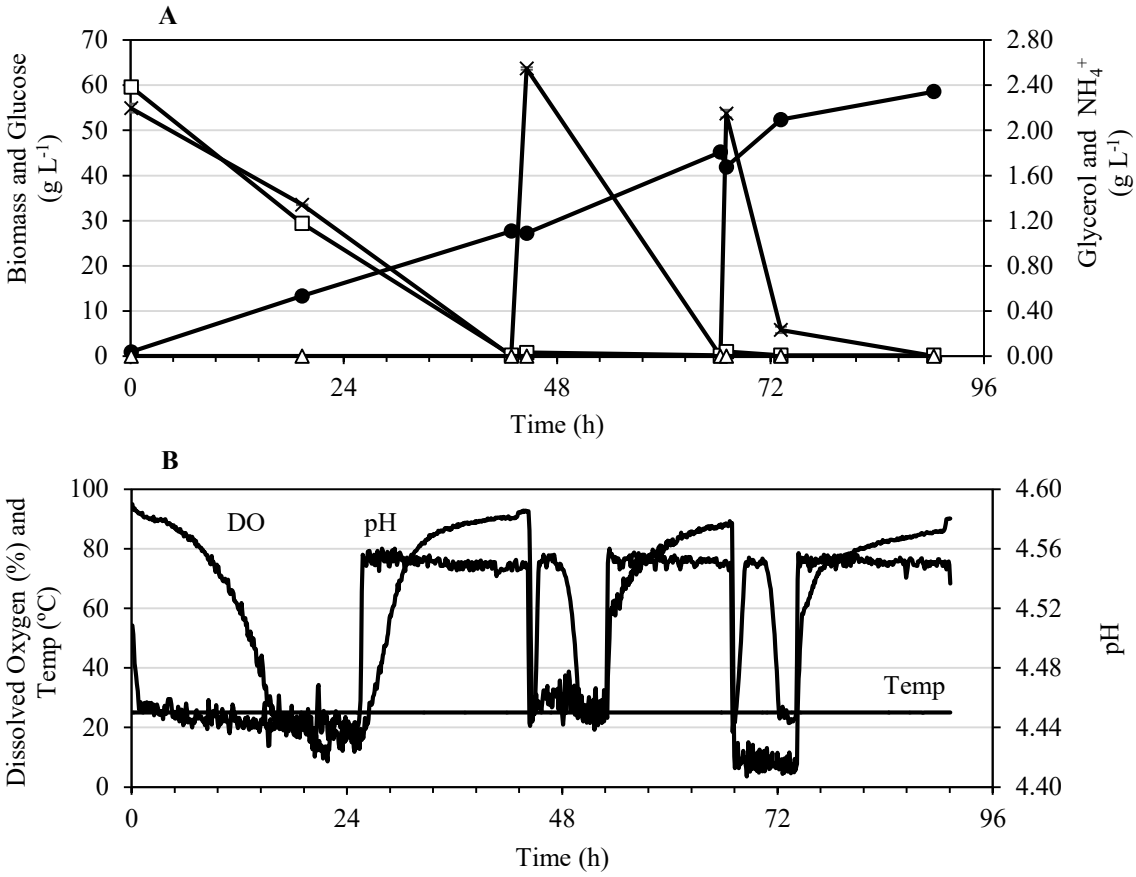

Figure 5.4 Growth curve and nutrient uptake (A) as well as the automated monitoring data for dissolved oxygen, temperature, and pH (B) for the control in the first recycle (2015-2L-13). Symbols denote cell biomass (●), glucose (x), ammonium (□) and glycerol (Δ). Error bars indicate ± standard deviation (n=2).

In the first fermentor replicate (AH1-1), recycling of the supplemented hydrolysate resulted in very similar rates of biomass accumulation and final concentration to that of the control. Accordingly, the glucose and ammonium available in the substrate were readily consumed and depleted by midway through the secondary fermentation. In addition, the 0.68 g L^{-1} of glycerol observed in the hydrolysate was rapidly consumed within 19 hours

(Fig. 5.5A). The dissolved oxygen concentration spikes in this treatment (Fig. 5.5B) indicated the occurrence of carbon limitation occurred at approximately 41 hours and again at 65 hours. This indicated that glucose uptake in AH1-1 was slower than was observed in the control, despite similar biomass growth. The dissolved oxygen spikes in AH1-1 were also employed as an indicator to begin feeding, thereby minimizing the culture's exposure to carbon limitation. Using the dissolved oxygen data, the glucose consumption rate for AH1-1 was 1.36 g L^{-1} h^{-1} prior to the first feed, 3.21 g L^{-1} h^{-1} between the first and second feeding, and 3.82 g L^{-1} h^{-1} after the second feed. The final feed of glucose was consumed by 82 hours, ensuring the secondary fermentation was deplete of nitrogen and carbon at the end of the growth period. Despite being almost half of the glucose consumption rate observed in the control, the biomass growth was still comparable. This trend was consistent with the results that were obtained in the preceding flask experiments where biomass was produced at a higher yield per unit carbon consumed. The enhanced carbon efficiency was attributed to the ability of thraustochytrids to secrete extracellular enzymes for degradation and eventual uptake of organic compounds in the substrate (Bongiorni et al., 2005b; Li et al., 2013; Liu et al., 2013; Zheng et al., 2012a).

In the second fermentor replicate (AH1-2), the culture supplied with supplemented hydrolysate displayed a very similar growth rate and nutrient uptake to that observed in the first replicate (AH1-1). Much like AH1-1, AH1-2 exhibited a steady growth rate accompanied by uptake of the glucose and ammonium available in the substrate. Also, the initial 0.66 g L^{-1} of glycerol in the culture was absorbed within 19 hours. Due to computer error, the monitoring data for this secondary fermentation was not observed until 22 hours (Fig. 5.6B). However, the trends were almost identical in the remainder of the secondary fermentation and the missing data likely follows that seen in Fig. 5.5B. The glucose uptake rates observed using dissolved oxygen values were 1.44 g L^{-1} h^{-1} prior to the first feed, 3.33 g L^{-1} h^{-1} between the first and second feeding, and 3.81 g L^{-1} h^{-1} after the second feed. All of the glucose was consumed by about 82 hours, ensuring the secondary fermentation was deplete of carbon at the end of the growth period.

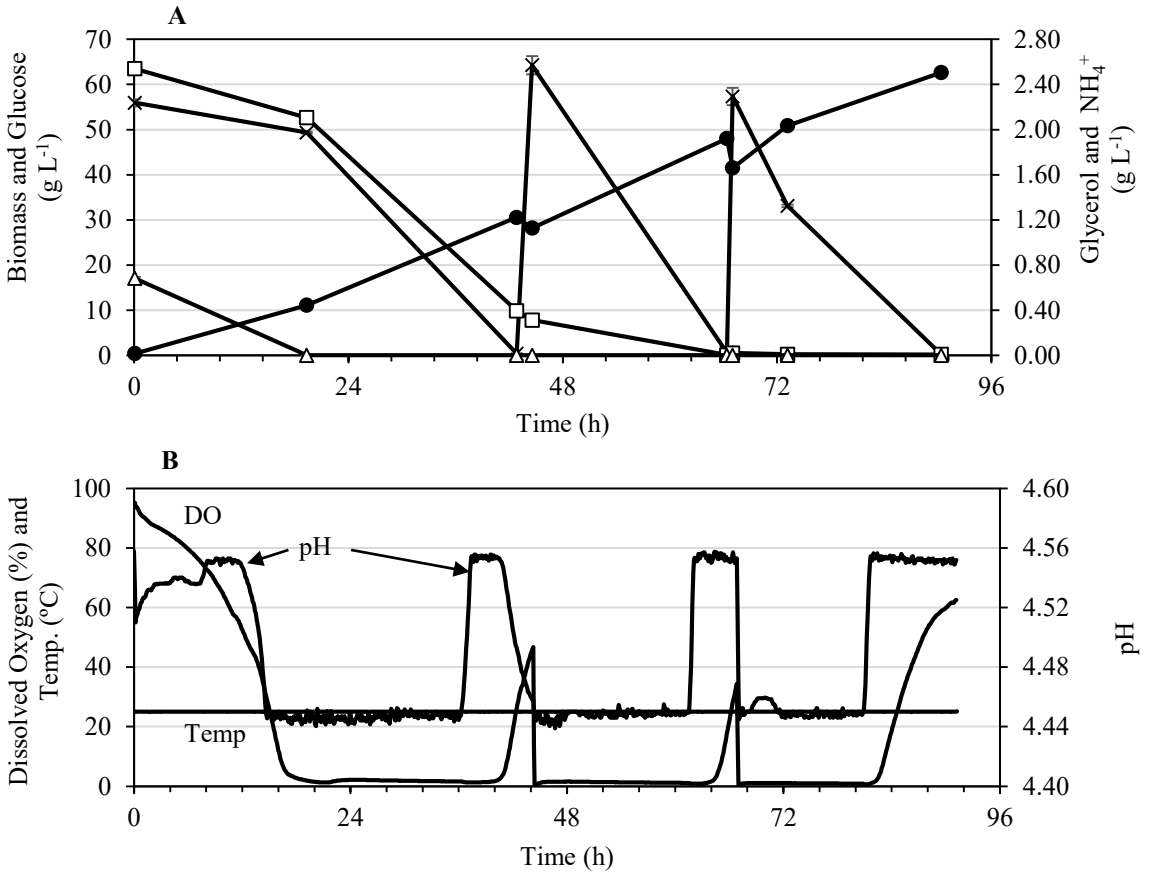

Figure 5.5 Growth curve and nutrient uptake (A) as well as the automated monitoring data for dissolved oxygen, temperature, and pH (B) for the first fermentor with supplemented hydrolysate (AH1-1) in the first recycle (2015-2L-13). Symbols denote cell biomass (●), glucose (x), ammonium (□) and glycerol (Δ). Error bars indicate ± standard deviation (n=2).

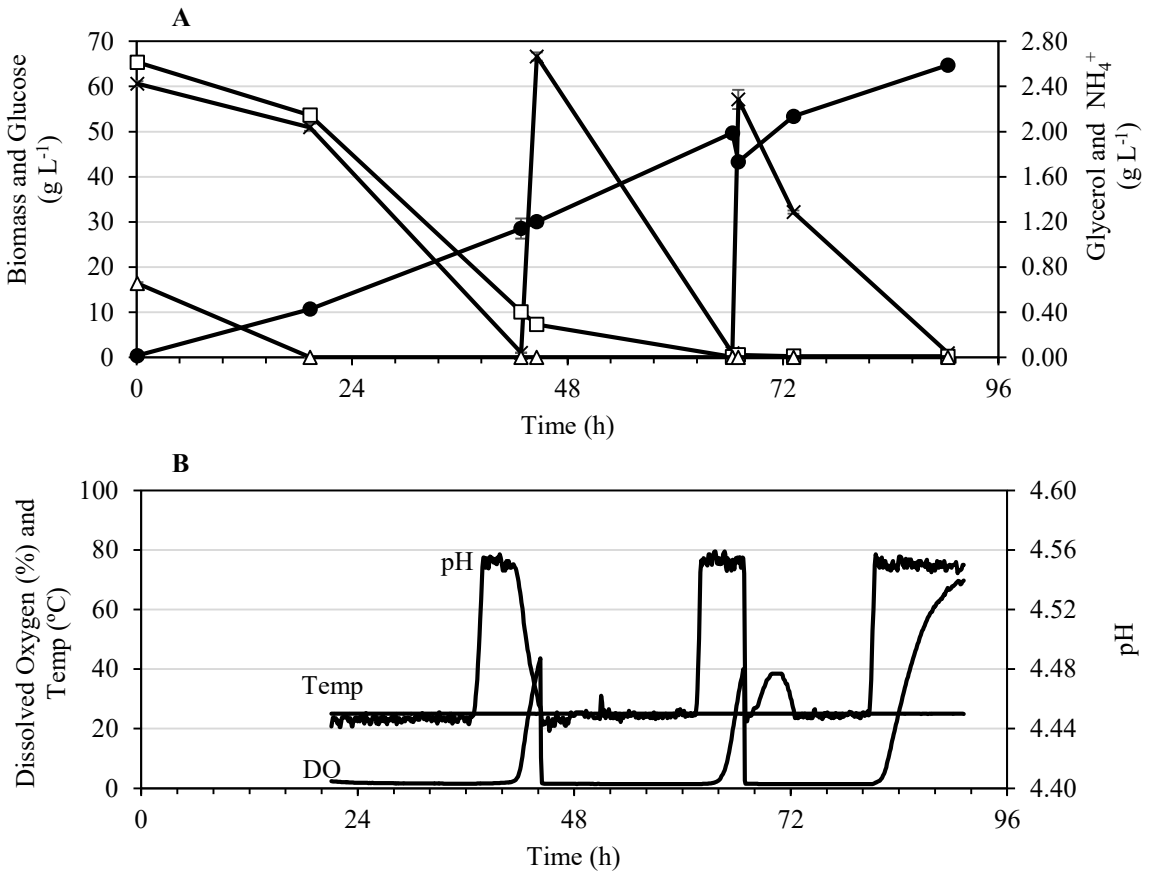

Figure 5.6 Growth curve and nutrient uptake (A) as well as the automated monitoring data for dissolved oxygen, temperature, and pH (B) for the second fermentor with supplemented hydrolysate (AH1-2) in the first recycle (2015-2L-13). Symbols denote cell biomass (●), glucose (x), ammonium (□) and glycerol (Δ). Error bars indicate ± standard deviation (n=2).

5.3.2.3 Amino Acid Analysis

Further investigation into substrate usage in the secondary fermentations required analysis of the organic nitrogen in solution throughout the growth period. Figure 5.7 shows the summative free amino acid (FAA) content of each of the treatments during 2015-2L-13. In each of the treatments, the majority of the free amino acids are rapidly absorbed by T18. Both fermentors containing supplemented hydrolysate exhibited the initial rapid decline in amino acids followed by a plateau at 100 to 200 μg mL^{-1} for the next 46 hours. Around 67 to 73 hours all three treatments showed a slight increase in free amino acid content, which was then followed by another decline to their plateaued levels. The overall decreases in

free amino acids for the control, AH1-1 and AH1-2 were 96.8%, 78.1% and 80.4% respectively.

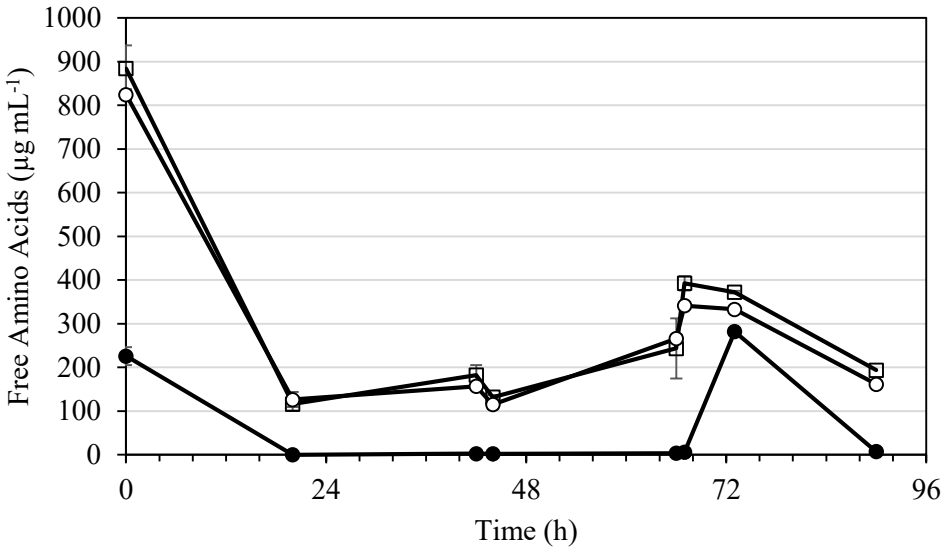

Figure 5.7 Free amino acid concentration in the substrate of the control (●), the first fermentor with supplemented hydrolysate (AH1-1, □) and the second fermentor with supplemented hydrolysate (AH1-2, ○) during the first recycle (2015-2L-13). Error bars indicate ± standard deviation (n=2).

Detailed inspection of the specific amino acids and their concentrations during the secondary fermentations revealed similar uptake trends to those observed in the flask experiments (2015-500ML-002 and 2015-500ML-003). In particular, all amino acids quantified were readily absorbed by T18 (Fig. 5.8-5.10). Concentrations of specific amino acids and their propensity for being consumed also matched prior results. Most notably, glutamate, which was the predominant amino acid in the supplemented hydrolysate, was rapidly consumed. Glutamate has a proven essential role in metabolic processes (i.e. nitrogen assimilation) for thraustochytrids and is readily consumed, as well as accumulated in cells as a metabolic intermediary from uptake of other nitrogen sources (Chen et al., 2010).

An interesting anomaly observed in this experiment was the elevated amino acid concentrations in all treatments around 67 to 73 hours (Fig. 5.8-5.10). While many of the

amino acids were detected in larger concentrations at this time, the most prevalent were alanine in the control and glutamate and proline in the supplemented hydrolysate. There are multiple possible explanations for this occurrence, none of which can be conclusively substantiated with the available data. A probable reason is that upon nitrogen limitation – a known stressor to cell health – metabolic mechanisms initiated endogenous enzyme secretion to catabolize and eventually consume organic nitrogen sources in the substrate. Proline and glutamate are recognized as commonly synthesized by plant cells during exposure to environmental stress, particularly for osmoregulatory function (Ahmad and Hellebust, 1988; Szabados and Savouré, 2010). This process could result in increased amino acids being detected in the substrate, originating from the enzymes themselves as well as products from degradation of proteins in the substrate. This hypothesis also explains why the increase of free amino acids was greater in the supplemented hydrolysate replicates as well as the ensuing reduction of those amino acids at later sampling points.

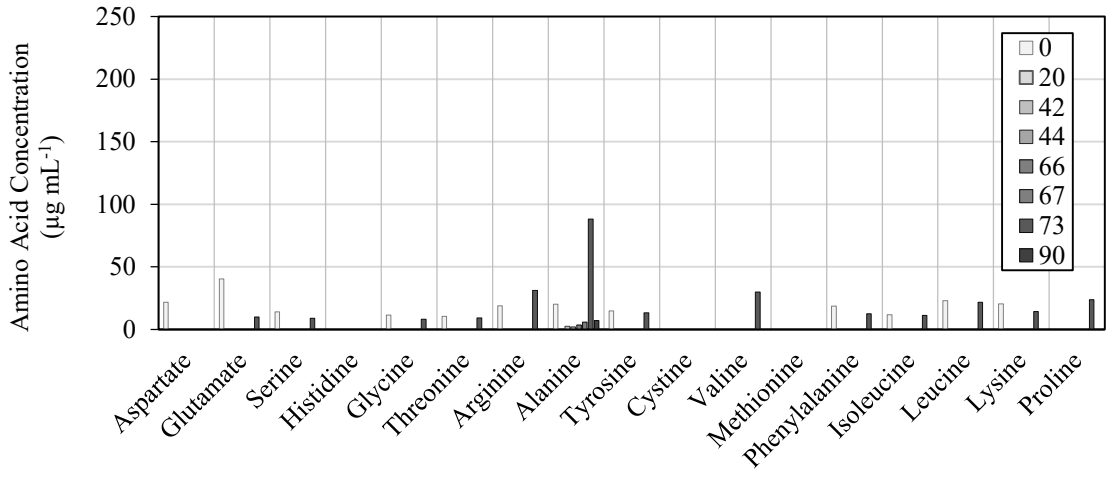

Figure 5.8 Free amino acid concentration in the control during the first recycle of the sequential secondary fermentation (2015-2L-13). Sample time points (in hours) are represented by the colored bars.

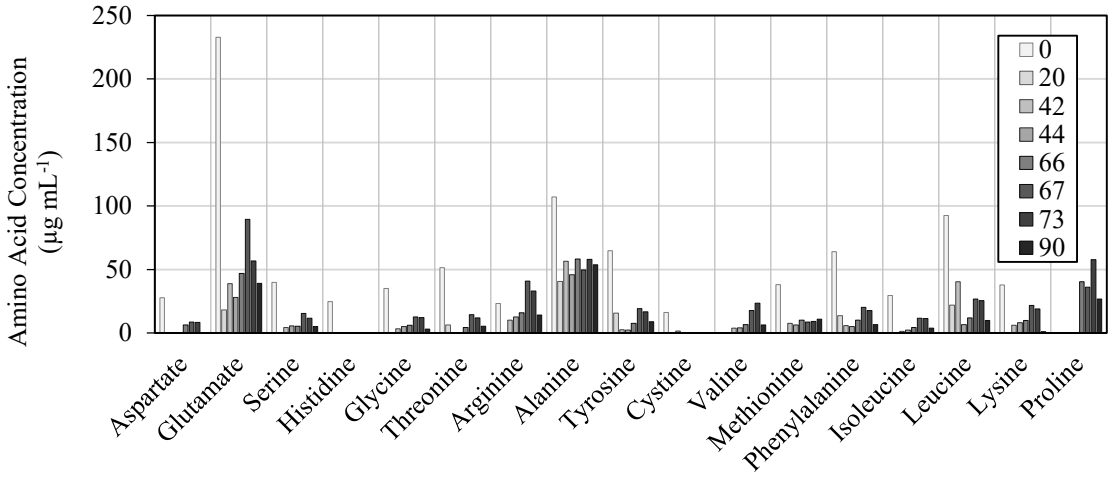

Figure 5.9 Free amino acid concentration in the first fermentor with supplemented hydrolysate (AH1-1) during the first recycle of the sequential secondary fermentation (2015-2L-13). Sample time points (in hours) are represented by the colored bars.

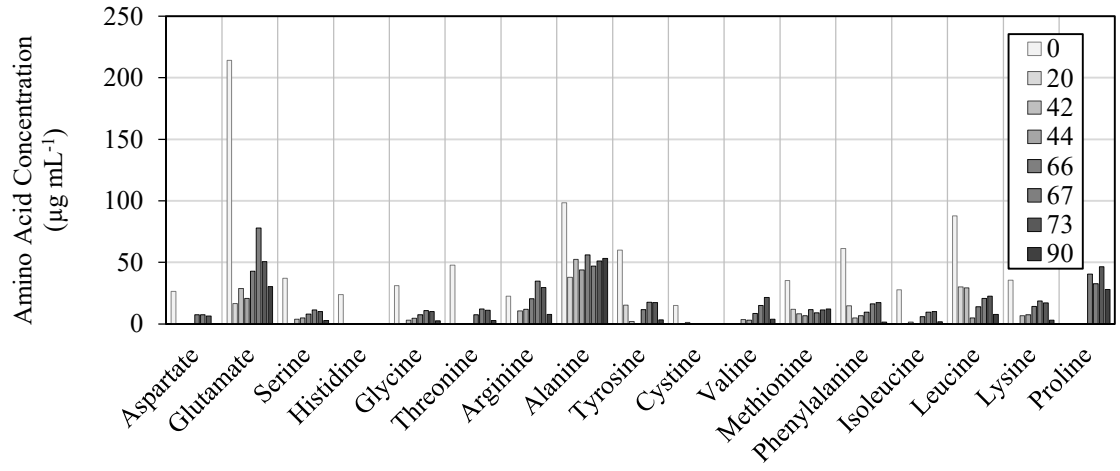

Figure 5.10 Free amino acid concentration in the second fermentor with supplemented hydrolysate (AH1-2) during the first recycle of the sequential secondary fermentation (2015-2L-13). Sample time points (in hours) are represented by the colored bars.

5.3.2.4 Lipid Analysis

Due to the optimized conditions of the 2-L fermentors it was expected that the biomass concentrations and lipid content would be enhanced as compared to those outcomes observed at the flask-scale. As previously shown, the biomass was definitely

much higher with a considerably greater productivity (Fig. 4.2, 4.5 and 4.13). The lipid content, as measured by total fatty acids, was also quantified throughout the secondary fermentation. Due to the time requirements and material expense of the GC-based fatty acid methyl ester (FAME) method, a rapid near infrared (NIR) technique was used to estimate total fatty acids (TFA) and DHA content during the secondary fermentations. The final sample was analyzed by GC for FAME to obtain the most accurate measurement and a full fatty acid profile.

Throughout the secondary fermentation, samples were prepared for NIR for the majority of the latter sampling points when growth was adequate to obtain sufficient biomass. The control demonstrated that as biomass accumulates and the secondary fermentation reached nitrogen-limitation, the total fatty acid content increased (Fig. 5.11). By 66-hours, the biomass in the control contained 61.22% total fatty acids of which DHA made up 20.2%. After reaching this level of TFA, the subsequent samples varied slightly while DHA content dropped slightly. The final amounts of TFA and DHA for the control were estimated to be 63.96% and 18.1% respectively.

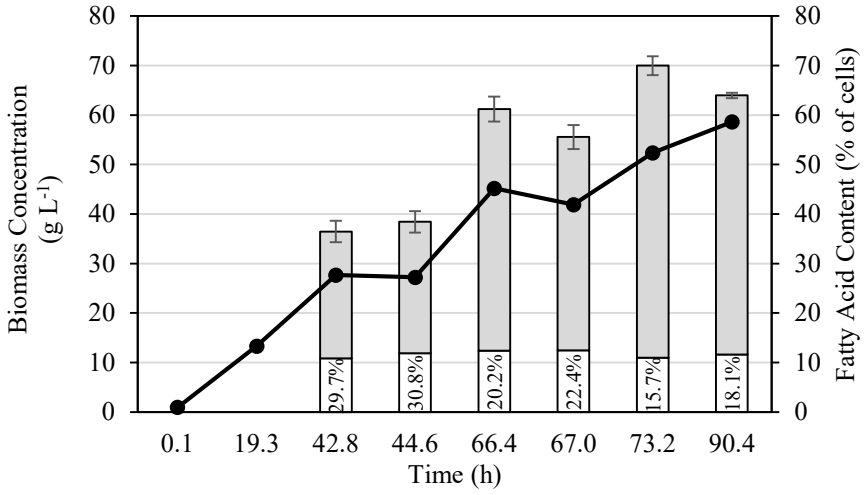

Figure 5.11 Total fatty acids and docosahexaenoic acid (DHA) content of the T18 biomass produced during the secondary fermentations in the control of the first recycle (2015-2L-13). The white bar labels correspond to the percentage DHA of the total fatty acids. Note that the horizontal axis is spaced evenly, as opposed to proportionate to the temporal distance between those samples. Error bars indicate ± standard deviation (n=3).

The NIR analysis of the biomass produced during secondary fermentation in supplemented hydrolysate showed some deviations from that of the control (Fig. 5.12). It is important to note that the NIR model was developed using T18 biomass that was grown in the standard fermentation media, identical to the control in this experiment. Using this rapid determination technique for the biomass produced in optically variant recycled hydrolysate may not yield as reliable results as for the control. The most apparent distinction between the control and the replicates of the supplemented hydrolysate was that the rate of TFA accumulation was slower and the final samples did not contain as high TFA as was observed in the control. The abundance of bioavailable nitrogen sources in the supplemented hydrolysate prompted a shortened nitrogen deprivation period, a known stressor for triggering enhanced lipid synthesis (Bumbak et al., 2011; Perez-Garcia et al., 2011; Rodolfi et al., 2009). The TFA content appeared to fluctuate around 50% after 66-hours, reaching a maximum of 51.85% and 52.37% in the first and second replicate respectively. Additionally, the DHA content of the fatty acid profile was elevated in the supplemented hydrolysate replicates; constituting up to 28.6% at 67-hours.

To ensure a more accurate and detailed determination of the fatty acid profile of the secondary fermentation biomass, the benchmark gas chromatography-based FAME method was conducted on the final sampling points. The comprehensive fatty acid profile of those three samples is presented in Table 5.3. These GC-FAME results did not have the limitations the NIR data did for the supplemented hydrolysate treatments and should provide accurate results, regardless of the biomass source. Based upon the GC-FAME data, the TFA content of the three treatments showed reduced oil content in the recycled hydrolysate as compared to the control, and accumulation of 68.2% total fatty acid in the control as compared to 49.9% in AH1-1 and 51.6% in AH1-2. The fatty acid profile was predominantly C16:0, C22:6 n-3 (DHA), C14:0 and C22:5 n-6 (DPA) as had been observed in multiple other thraustochytrids (Lee Chang et al., 2014). However, the difference between the control and the supplemented hydrolysate replicates showed a movement of saturated fatty acids in the control into DHA in AH1-1 and AH1-2. The DHA content of the control was 14.0% of TFA while it was 25.2% and 24.7% in AH1-1 and AH1-2, respectively. The combined concentrations of C14:0 and C16:0 in the control were 11.7% and 11.3% higher than AH1-1 and AH1-2, was almost the inverse of the difference in DHA

content. Whether this trend of DHA accumulation was induced by exposure to the hydrolysate or rather a response to the elevated nitrogenous organic matter present in the substrate is yet undetermined.

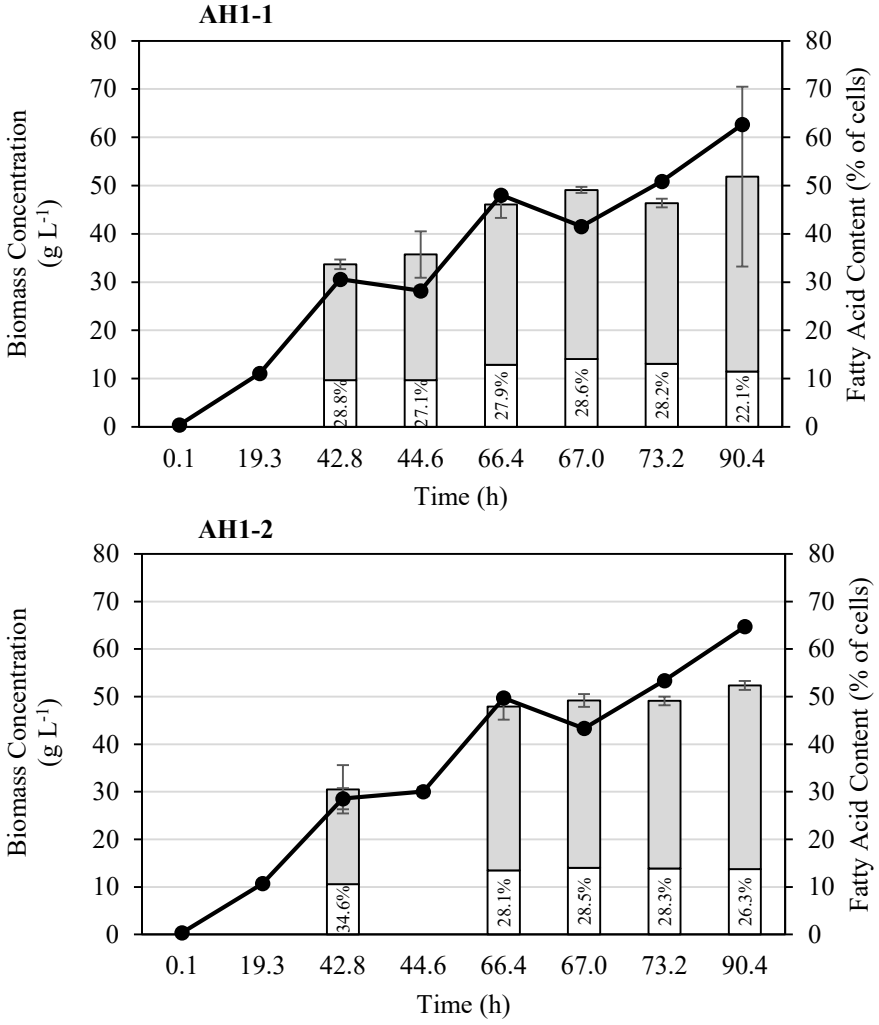

Figure 5.12 Total fatty acids and docosahexaenoic acid (DHA) content of the T18 biomass produced during the secondary fermentations in the first (AH1-1) and second (AH1-2) fermentors of supplemented hydrolysate in the first recycle (2015-2L-13). The white bar labels correspond to the percentage DHA of the total fatty acids. Note that the horizontal axis is spaced evenly, as opposed to proportionate to the temporal distance between those samples. Error bars indicate ± standard deviation (n=3).

Table 5.3 Fatty acid profile of the final samples of the control and the first (AH1-1) and second (AH1-2) replicate of the supplemented hydrolysate for the first recycle (2015-2L-13). All values were duplicate samples with duplicate injections.

Fatty Acid	Concentration (mg per gram dry cells)		
	Control	Supp. Hydrolysate 1	Supp. Hydrolysate 2
C12:0	10.05 ± 0.03	6.59 ± 0.13	7.06 ± 0.14
C14:0	157.30 ± 0.43	96.30 ± 2.32	101.95 ± 1.31
C14:1	0.32 ± 0.02	0.19 ± 0.13	0.23 ± 0.17
C15:0	2.13 ± 0.05	4.99 ± 0.10	5.39 ± 0.05
C16:0	337.25 ± 1.30	207.12 ± 5.27	214.22 ± 2.60
C16:1	35.27 ± 0.20	8.88 ± 0.14	9.55 ± 0.09
C17:0	0.30 ± 0.01	0.81 ± 0.02	0.78 ± 0.04
C18:0	8.66 ± 0.03	6.59 ± 0.15	6.76 ± 0.08
C18:1 Oleic	0.00 ± 0.00	0.26 ± 0.16	0.27 ± 0.16
C18:1 Vaccenic	3.75 ± 0.09	1.98 ± 0.05	2.24 ± 0.03
C18:2 n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:3 n-6	0.51 ± 0.02	0.51 ± 0.02	0.55 ± 0.01
C18:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:4	0.42 ± 0.04	0.66 ± 0.02	0.70 ± 0.01
C20:0	2.37 ± 0.03	2.08 ± 0.06	2.13 ± 0.04
C20:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:3 n-6	0.68 ± 0.02	0.90 ± 0.16	0.88 ± 0.13
C20:4 n-6	0.68 ± 0.02	0.90 ± 0.09	0.88 ± 0.02
C20:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:4 n-3	1.07 ± 0.02	1.48 ± 0.06	1.51 ± 0.03
C20:5 n-3 (EPA)	2.14 ± 0.01	3.17 ± 0.06	3.18 ± 0.04
C22:5 n-6 (DPA)	23.40 ± 0.16	28.53 ± 0.75	29.23 ± 0.38
C22:5 n-3 (DPA)	0.75 ± 0.06	1.90 ± 0.13	1.88 ± 0.04
C22:6 n-3 (DHA)	95.29 ± 0.47	125.42 ± 2.92	127.41 ± 1.70
C24:1 n-9	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Total	681.92 ± 2.64	498.62 ± 11.79	516.18 ± 6.28

5.4 SECONDARY FERMENTATION: 2-LITER SECOND RECYCLE (2015-2L-14)

In addition to validating the scalability of the nutrient recycling findings, one of the major objectives of the 2-liter secondary fermentations was to investigate multiple recycles of the same waste material. As with 2015-2L-13, the original hydrolysate was obtained from lipid extraction of an optimized glucose-fed batch (2015-500L-009) using the patented enzymatic hydrolysis method (Dennis and Armenta, 2015). After production of that original hydrolysate, it was used as a growth substrate for 2015-2L-13. Completion of the secondary fermentation was followed by another enzymatic hydrolysis to extract the cellular lipids, yielding another hydrolysate. This hydrolysate was employed as the growth substrate for the second recycle of the hydrolysate (2015-2L-14).

To ensure comparability, the exact experimental design was followed from the first 2-liter recycle. Accordingly, three fermentors were prepared with a starting volume of 1.6 L: a control using standard fermentation media, and two replicates of the supplemented hydrolysate. The control media was prepared using the recipe disclosed in Table 5.1. The supplemented hydrolysate was a combination of 50% (v/v) raw hydrolysate with another 50% of double-concentrated fermentation media, as described in Chapter 4. All conditions during the fermentation were maintained equal to those in 2015-2L-13.

5.4.1 MATERIALS AND METHODS

5.4.1.1 Seed Preparation

Similar to all prior fermentations, it was necessary to produce a healthy seed for inoculation into the fermentors. Each fermentor would received an inoculating volume of 100 mL (6.25% v/v) which meant 300 mL of seed was required in total. Accordingly, two 2-L baffled Erlenmeyer flasks were prepared with standard seed media and inoculated with one loop-full of plated T18 biomass. Both seed flasks were placed on the orbital shaker at 200 rpm and 25ºC. The seed was allowed to grow for three days prior to a health assessment to ensure that acceptable growth occurred and no morphological issues or contamination existed (Table 5.4). Seed 1 was selected for use in the secondary fermentation due to a lower optical density and minimal presence of artifacts (cell debris).

Table 5.4 Observations from the seed quality check for the 2-liter secondary fermentation (2015-2L-14).

	Optical Density (A_{600}) 1:10	Microscopy
Seed 1	2.061	Healthy cells, no contamination
Seed 2	2.068	Mostly healthy cells, no contamination, some cell debris

5.4.1.2 Experimental Design

Following the experimental design of 2015-2L-13, three 2-liter vessels were used for this secondary fermentation. One was designated as the control, and the remaining two were the first and second replicates of supplemented hydrolysate, AH2-1 and AH2-2, respectively. Setup and operation of the vessels was maintained to be as uniform as possible to ensure identical conditions in each vessel. As with the prior 2-L fermentor and flask experiments, the separately-sterilized hydrolysate was diluted to 50% (v/v) by adding 800 mL into 600 mL of sterile fermentation media (prepared to recipe amounts shown in Table 5.1). In all fermentors, 100 mL of media buffer and 100 mL of seed were added into the fermentors, ensuring the starting volume of 1.6 L.

5.4.1.3 Growth Conditions

Fermentation conditions were intended to replicate those of the previous 2-L secondary fermentation as best as possible. Vessel temperature was set to 25ºC with 600 rpm of mixing, 0.6 slpm of aeration and a pH maintained at 4.50. Upon depletion of glucose during the secondary fermentation, 70% glucose solution was added as needed to restore the concentration of glucose to approximately 60 g L^{-1}. To maintain equivalence, both the control and the supplemented hydrolysate duplicates were fed at the same time. However, in contrast to 2015-2L-13, the feeding timing was dictated by the needs of the fastest growing vessel to avoid any periods of starvation. No additions of nitrogen were made.

5.4.2 Results and Discussion

5.4.2.1 Biomass

The biomass accumulation in the three treatments (control, AH2-1 and AH2-2) during this secondary fermentation is illustrated in Fig. 5.13. During this secondary fermentation, each of the treatments followed a similar growth curve, with the control slightly exceeding the supplemented hydrolysate replicates in growth rate. Correspondingly, the average productivities observed were 24.10 g L^{-1} d^{-1}, 17.88 g L^{-1} d^{-1} and 17.60 g L^{-1} d^{-1} for the control, AH2-1 and AH2-2, respectively. Compared to the first 2-L recycle (2015-2L-13), the control is higher and the supplemented hydrolysate replicates are lower. The differences were partly explained by the increased feeding rate, which resulted in improved growth potential by minimizing periods of glucose limitation, as was seen in the control. The suppressed productivity in the supplemented hydrolysate replicates may have been due to the accumulation of an inhibitory compound during multiple recycles of the waste material. Furthermore, the reduced productivities observed by T18 may also have been associated with accumulation of algogenic organic matter (AOM) that is suspected to cause culture deterioration during nutrient recycling with photoautotrophic species such as *Chlorella vulgaris* (Discart et al., 2014; Hadj-Romdhane et al., 2013). This observation contrasts the results that were observed in two recycles of growth media for a photoautotrophic *Scenedesmus quadricauda*, which saw negligible change in productivity in the subsequent batches (Rocha et al., 2014). However, many differences in strain metabolism (i.e. photoautotrophic vs. heterotrophic), media composition (i.e. freshwater vs. saltwater) and growth conditions factor into the discrepancy in results.

Despite the different growth rates in the second recycle, the final biomass concentration of each was very similar with 64.92 g L^{-1} in the control, 64.46 g L^{-1} in AH2-1 and 66.08 g L^{-1} in AH2-2. The cell yields observed throughout the secondary fermentation were also indicative of a healthy fermentation. The yield in the control was 0.37, AH2-1 was 0.37 and AH2-2 was 0.39, which were slightly elevated over the first recycle (2015-2L-13). As was observed previously, the similarity between these values in each treatment suggests that *Thraustochytrium sp.* (T18) can be equally productive in the recycled substrate as it is in the standard fermentation media. Extending these findings suggest that secondary fermentations using recycled hydrolysate will reach the same final

concentration of biomass given a fixed amount of carbon, in a slightly longer amount of time.

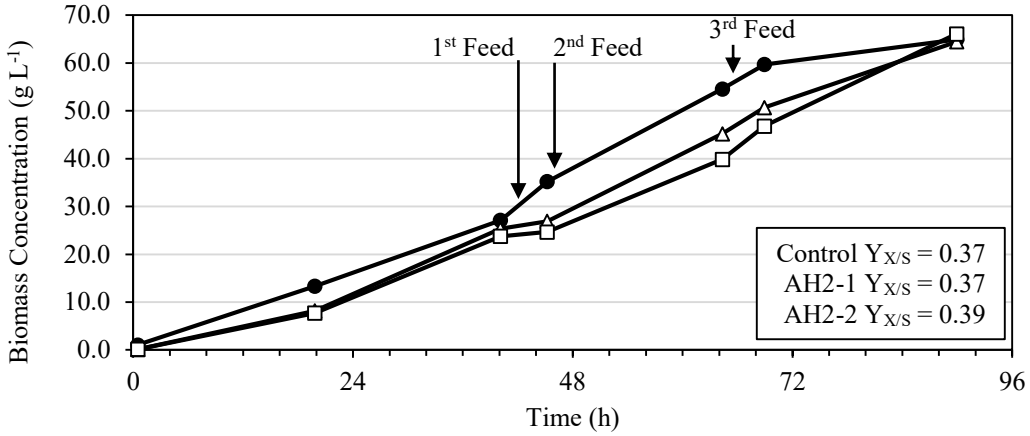

Figure 5.13 Biomass concentration of T18 during the second recycle of the hydrolysate in the 2-liter fermentors (2015-2L-14) in the control (●), and the first (AH2-1, Δ) and second (AH2-2, □) fermentors with supplemented hydrolysate. Cell yield ($Y_{X/S}$) is shown as grams of cells per gram of carbon. Error bars indicate ± standard deviation (n=2).

5.4.2.2 Substrate Usage

During the secondary fermentation the glucose concentration was restored by feeding as needed. All three fermentors were fed 70% (w/v) sterile glucose solution at the same time, based upon the need of the fastest growing treatment. Three glucose feedings were done at 42.08, 46.47 and 66.13 hours. As discussed in 2015-2L-13, the dissolved oxygen content was also continuously measured to approximate the metabolic activity, and corresponding glucose concentration of the cultures. Fig. 5.14 illustrates the growth curve, nutrient uptake and monitoring data for the control in 2015-2L-14. The three glucose feedings were evident by the spike in the sampling data for glucose concentration. Between feedings there were some periods of glucose starvation that the culture was exposed to. Before the first feeding there was approximately a 19 hour starvation, followed by another 13 hour period before the last feeding. While these conditions were avoided if possible, the high glucose consumption rates made it possible for the control to rapidly deplete the available carbon

prior to being fed. The highest glucose consumption rate observed was 8.14 g L^{-1} hr^{-1} which was comparable to that observed in 2015-2L-13 of 7.67 g L^{-1} hr^{-1}.

In addition to carbon availability, ammonium (NH_4^+) and glycerol concentration was measured throughout the secondary fermentation. As the control substrate was derived from pure chemicals, no glycerol was observed at any point in the secondary fermentation. However, the initial concentration of 2.50 g L^{-1} of ammonium was steadily consumed by T18. All detected ammonium was depleted by 45 hours thereby allowing a considerable period of nitrogen limitation for the end of the growth period.

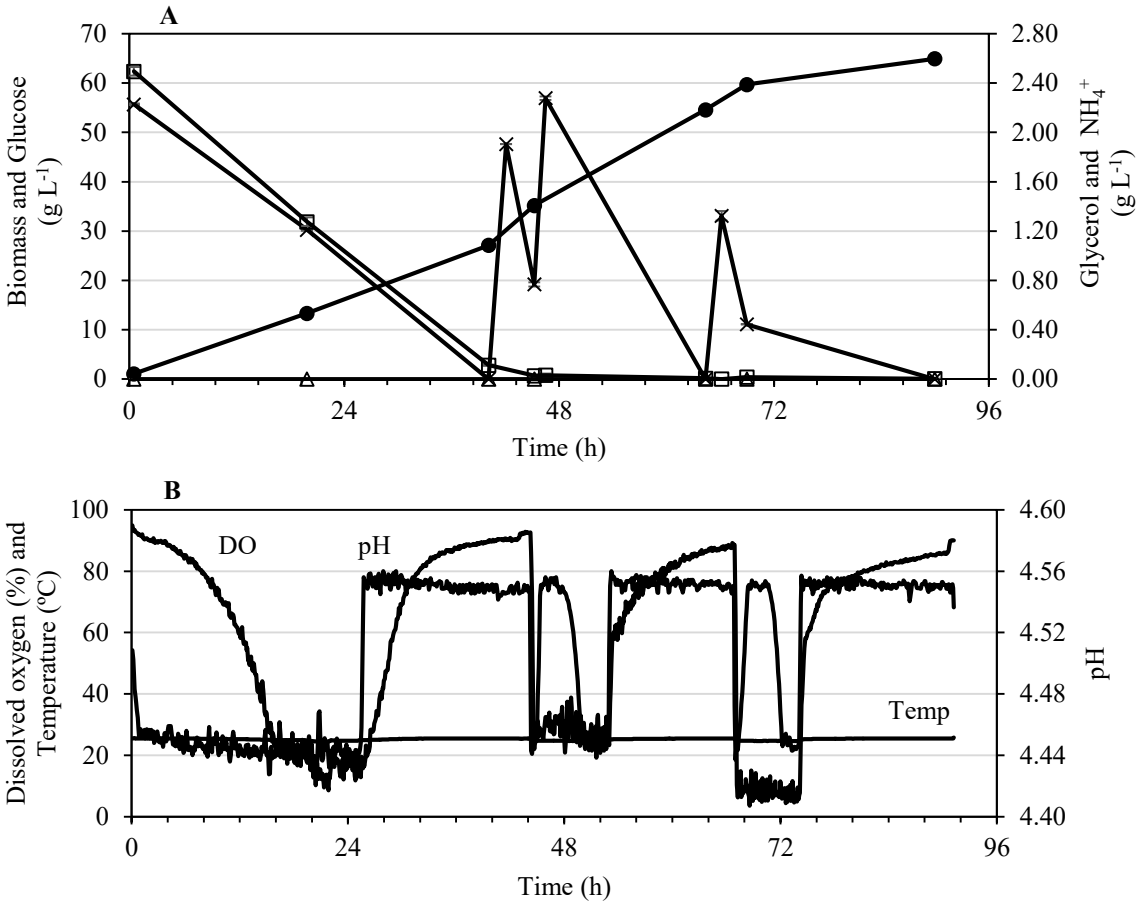

Figure 5.14 Growth curve and nutrient uptake (A) as well as the automated monitoring data for dissolved oxygen, temperature, and pH (B) for the control in the second recycle (2015-2L-14). Symbols denote cell biomass (●), glucose (x), ammonium (□) and glycerol (Δ). Error bars indicate ± standard deviation (n=2).

The first fermentor replicate of the supplemented hydrolysate (AH2-1) during 2015-2L-14 is described by the growth curve, nutrient depletion and fermentor conditions in Fig. 5.15. As was observed in the control, the glucose concentration in the substrate was rapidly consumed and the defined spikes in the sampling data were evidence of the three glucose feedings. In contrast to the control, however, the periods of starvation were significantly lessened where a 3 hour period was shown prior to the first feeding as well as another 3 hour period before the last feeding. This observation was reinforced by a slower glucose consumption rate relative to the control which was a maximum of 3.98 g L^{-1} hr^{-1}. This rate was also similar to the 3.82 g L^{-1} hr^{-1} seen in 2015-2L-13. All remaining glucose in the substrate was consumed by 83 hours, ensuring glucose-depletion at the conclusion of the secondary fermentation.

The 0.5 g L^{-1} of glycerol present in the hydrolysate was also rapidly consumed by the first sample point at 20 hours. This same trend was consistently observed in all prior secondary fermentations using the supplemented hydrolysate. An initial concentration of ammonium of 2.54 g L^{-1} was depleted by 64 hours, taking considerably longer than in the control. This difference in ammonium consumption rate was mirrored in the previous 2-L secondary fermentation (2015-2L-13) and may be indicative of the presence and uptake of alternative forms of nitrogen (i.e. amino acids and peptides) in the supplemented hydrolysate. Furthermore, the prolonged presence of nitrogen in the substrate may have implications for the mechanism of lipid synthesis in the T18 cells.

As Fig. 5.16 indicates, the second fermentor replicate with the supplemented hydrolysate (AH2-2) closely followed all of the trends of biomass accumulation and nutrient uptake as those observed in the first replicate (Fig. 5.15). The maximum glucose uptake rate was 4.32 g L^{-1} hr^{-1} which although slightly higher than the first replicate, was consistent with the prior secondary fermentation 2015-2L-13 (3.81 g L^{-1} hr^{-1}). In addition, both glucose and ammonium were depleted by the end of the fermentation. The missing data in the first 22 hours of monitoring data was caused by a computer error but did not exclude any of the important changes in dissolved oxygen or pH.

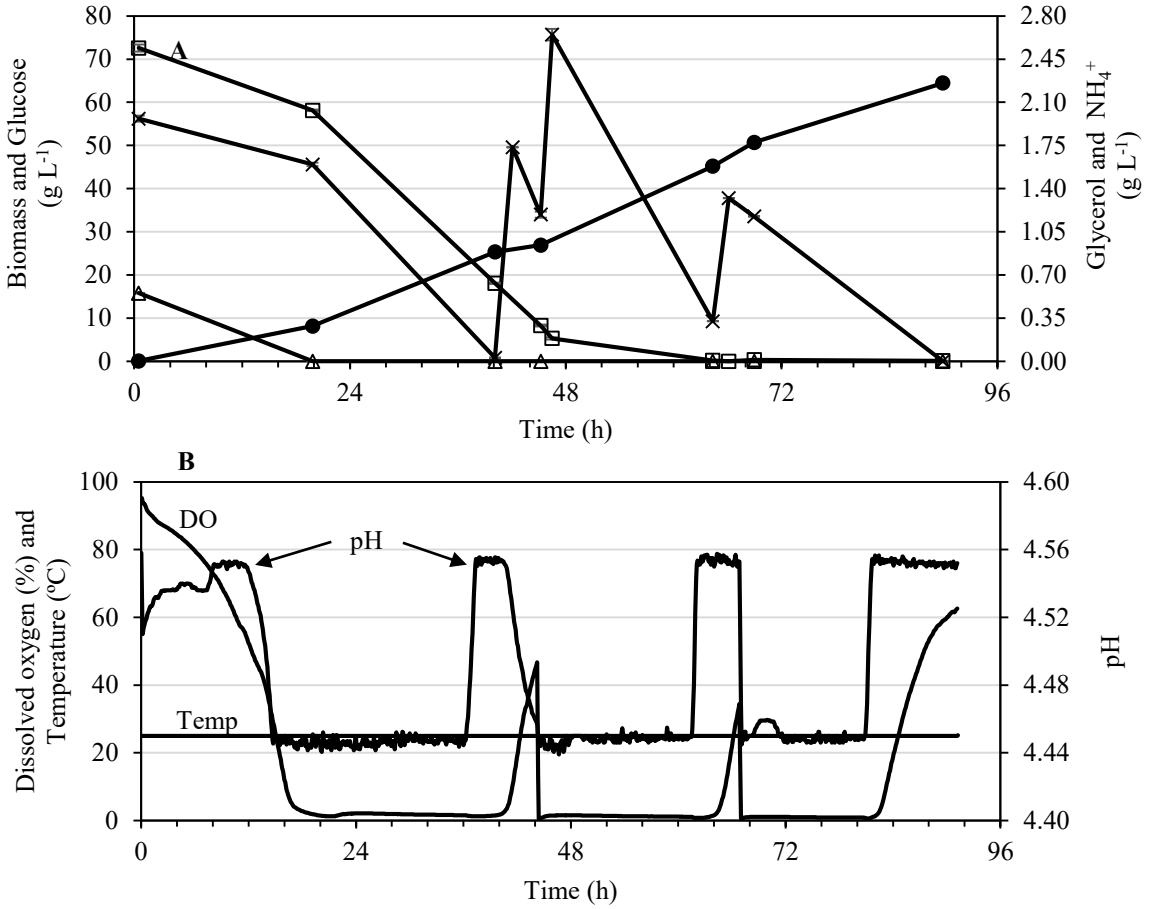

Figure 5.15 Growth curve and nutrient uptake (A) as well as the automated monitoring data for dissolved oxygen, temperature, and pH (B) for the first fermentor with supplemented hydrolysate (AH2-1) in the second recycle (2015-2L-14). Symbols denote cell biomass (●), glucose (x), ammonium (□) and glycerol (Δ). Error bars indicate ± standard deviation (n=2).

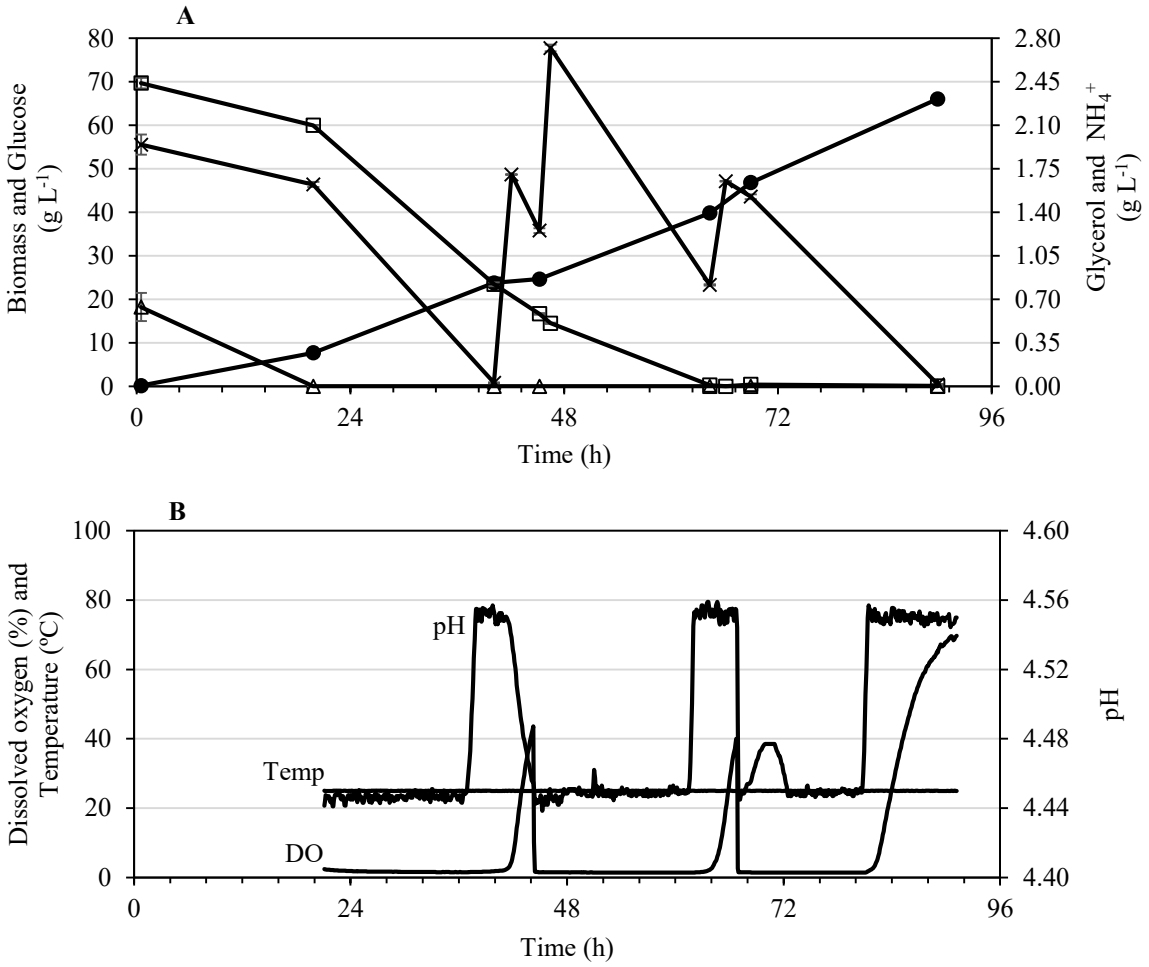

Figure 5.16 Growth curve and nutrient uptake (A) as well as the automated monitoring data for dissolved oxygen, temperature, and pH (B) for the second fermentor with supplemented hydrolysate (AH2-2) in the second recycle (2015-2L-14). Symbols denote cell biomass (●), glucose (x), ammonium (□) and glycerol (Δ). Error bars indicate ± standard deviation (n=2).

5.4.2.3 Amino Acid Analysis

To assess usage of waste organic nitrogen in the hydrolysate, amino acid concentrations in each treatment were measured. The total free amino acid content is depicted in Fig. 5.17 which followed a similar, although more exaggerated trend as the first recycle of the hydrolysate (2015-2L-13). The initial rapid decline in total free amino acids was consistent across all treatments with a removal of 98.6% in the control, 82.4% in AH2-1 and 78.7% in AH2-2 by 20 hours. However, instead of an extended period of stable values for the replicates of the supplemented hydrolysate, the concentration steadily rose until it peaked

at around 64 hours. By the end of the secondary fermentation the amount of free amino acids consumed in the control, AH2-1 and AH2-2 was 96.1%, 67.1% and 61.9% respectively.

It is important to note that initial uptake of free amino acids in the first 20 hours was then followed by a period of amino acid synthesis. This trend may have been indicative of increased environmental stress acting upon T18 as was suggested with 2015-2L-13. One potential stressor in this secondary fermentation that exceeded that of the first recycle is the enhanced ionic strength of the substrate, as a result of further addition of salts in the supplementation recipe. Various physiological responses have been recognized for osmoregulation by microalgae in environments with increasing salinity, including increased synthesis of proline (Ahmad and Hellebust, 1988).

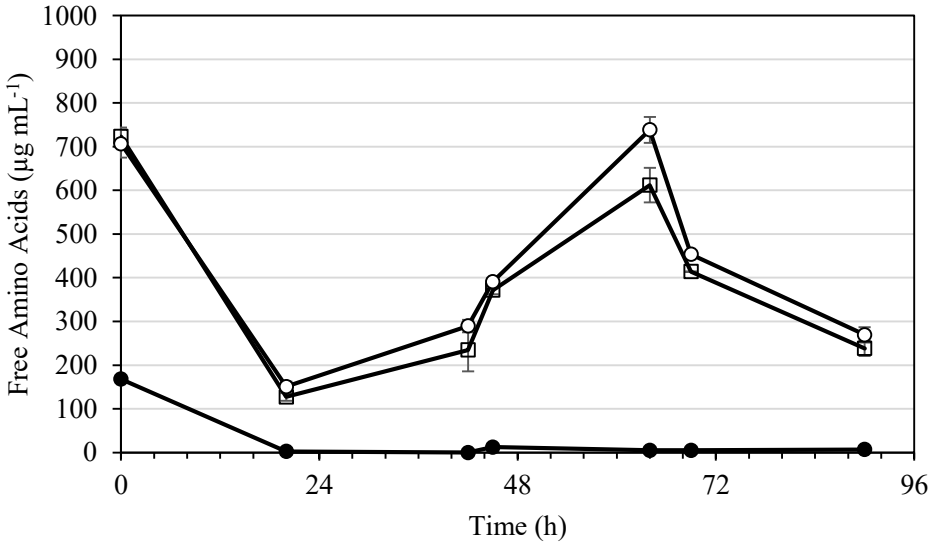

Figure 5.17 Free amino acid concentration in the substrate of the control (●), the first fermentor of the supplemented hydrolysate (AH2-1, □) and the second fermentor of supplemented hydrolysate (AH2-2, ○) during the second recycle (2015-2L-14). Error bars indicate ± standard deviation (n=2).

Another better substantiated stress source is nitrogen limitation in the substrate. As described in more detail later in Section 5.4.2.4, nitrogen limitation was reached much sooner in 2015-2L-14 at around 50 to 60 hours than for 2015-2L-13, which was approximately 80 hours. This incidence of nitrogen limitation may trigger a metabolic

response in T18 to secrete enzymes to catabolize and absorb organic nitrogen sources (proteins and peptides) in the surrounding environment, resulting in increased amino acids detected (Bongiorni et al., 2005b; Kanchana et al., 2011; Nagano et al., 2011; Taoka et al., 2009). The successive increase and decrease in the presence of those amino acids in solution can be explained by the degradation of complex nitrogen followed by uptake by the cells.

Fig. 5.18-5.20 illustrate the specific amino acid concentrations in each of the treatments throughout the secondary fermentation. As has been previously observed, almost all amino acids were metabolized with varying degrees of preference by T18. To further address the increasing amino acids concentration between 20 and 64 hours it was useful to determine which amino acids were being synthesized. Much like 2015-2L-13, glutamate and proline show the two largest increases, with proline emerging from undetected to about 170 µg mL^{-1} at 64 hours (Fig. 5.18-5.20). After 64 hours, all amino acid concentrations began to decrease again until the end of the secondary fermentation. The most prevalent amino acid residing in the final substrate of the supplemented hydrolysate was alanine, which never significantly decreased from the starting concentration.

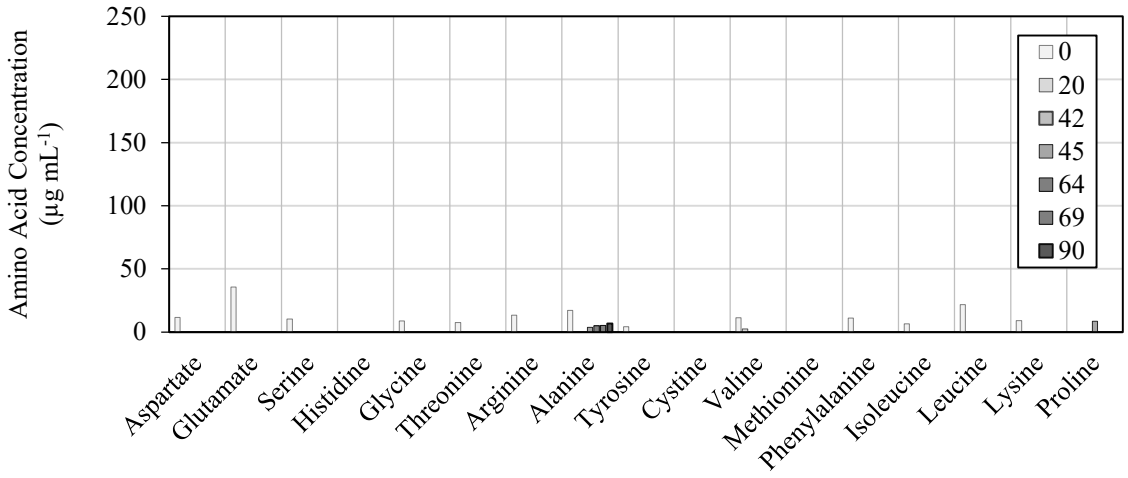

Figure 5.18 Free amino acid concentration in the control during the second recycle of the sequential secondary fermentation (2015-2L-14). Sample time points (in hours) are represented by the colored bars.

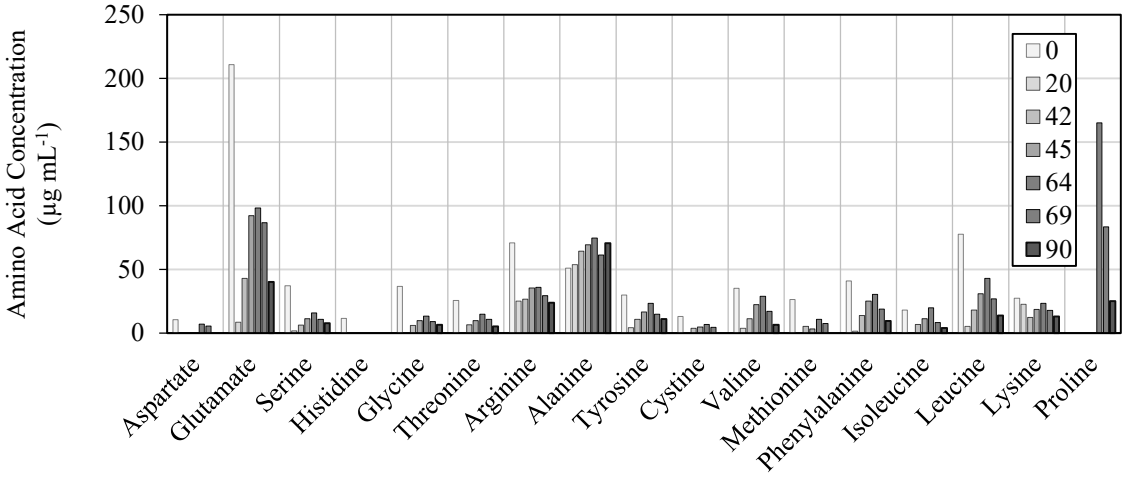

Figure 5.19 Free amino acid concentration in the first fermentor with supplemented hydrolysate (AH2-2) during the second recycle of the sequential secondary fermentation (2015-2L-14). Sample time points (in hours) are represented by the colored bars.

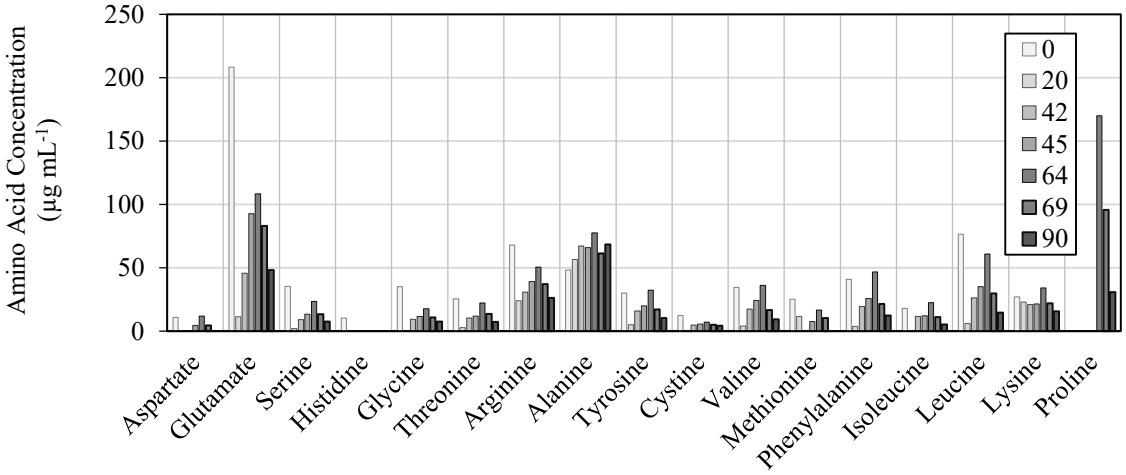

Figure 5.20 Free amino acid concentration in the second fermentor with supplemented hydrolysate (AH2-2) during the second recycle of the sequential secondary fermentation (2015-2L-14). Sample time points (in hours) are represented by the colored bars.

5.4.2.4 Lipid Analysis

The lipid quantity and quality in the final samples from the secondary fermentations of 2015-2L-14 were analyzed by GC-FAME. The total fatty acids of the three treatments were very similar with the control amassing 69.4% TFA, AH2-1 having 62.4% TFA and AH2-

2 containing 62.0% TFA (Table 5.5). In comparison to the previous 2-L secondary fermentation, the control had a very similar amount of TFA (68.2%, previously). However, the supplemented hydrolysate replicates contained significantly greater amounts of fatty acids than 2015-2L-13 (49.9% and 51.6% for AH1-1 and AH1-2, respectively).

The reason for the elevated fatty acid accumulation in the second recycle (2015-2L-14) may be attributable to a more rapid depletion of the available nitrogen in the substrate as compared to the first recycle (2015-2L-13), resulting in extended periods of stress and correspondingly greater fatty acid accumulation. This occurrence was supported by inspecting the automated monitoring data collected by the fermentation software which tracks the addition of all acid and base into the system. The fermentation team at Mara uses the cessation of base addition to the system as a reliable indicator of nitrogen limitation. This relationship is partly explained by the use of ammonium sulfate as a nitrogen source, which, upon metabolism, contributes to a lowering of pH. To maintain a stable pH in the media, the fermentation software adds base to restore the pH.

Fig. 5.21 illustrates the cumulative base additions in each of the treatments for both 2015-2L-13 and 2015-2L-14. The differing amounts of base added across the treatments was unimportant and attributable to the calibration of the vessel's computer. More importantly, the rate of addition was telling about the metabolic activity of the fermentation. This data reveals that the base additions plateaued earlier in each of the treatments in the latter secondary fermentation, therefore indicating a longer period of nitrogen limitation. The approximate time of nitrogen depletion in 2015-2L-13 for the control, AH1-1 and AH1-2 for 2015-2L-13 was 25 hours, 80 hours and 78 hours, respectively. In 2015-2L-14 nitrogen depletion occurred in the control, AH2-1 and AH2-2 at 26, 51, and 64 hours respectively. In comparison to the nutrient depletion sampling data presented in Fig. 5.4-5.6, 5.11-5.13, all of these estimated nitrogen depletion points correspond to the ammonium data.

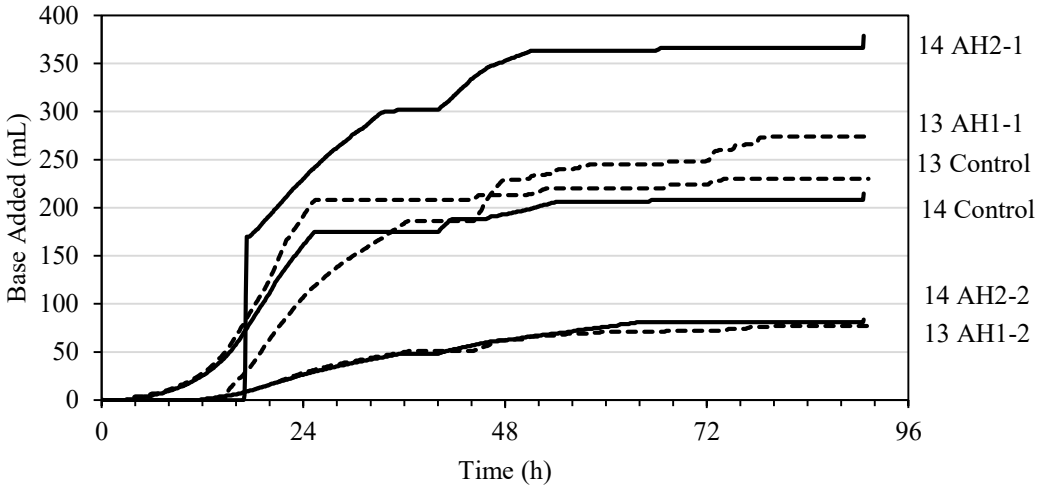

Figure 5.21 Cumulative volume of base (10M NaOH) added throughout the secondary fermentations of 2015-2L-13 (dotted lines) and 2015-2L-14 (solid lines) in each of the treatments. Data was collected by the fermentation management software and the corresponding pumps associated with the base tubing.

Returning to the fatty acid profile of the final biomass produced in the secondary fermentation, it was apparent that the oil quality is similar to all previously observed samples (Table 5.5). Consistent with 2015-2L-13 (Table 5.3), the predominant fatty acids present in these samples are C16:0, C22:6 n-3 (DHA), C14:0 and C22:5 n-6 (DPA). Additionally, the relative concentrations of these fatty acids between the control and the supplemented hydrolysate replicates exhibited a similar trend to what was observed in Table 5.3. The most notable differences are the shift from saturated fatty acids (C14:0 and C16:0) to DPA and DHA from the control to the recycled hydrolysate. This shift was large enough that the DHA content of the fatty acids increased from 14.0% in the control to 27.2% in AH2-1, and 30.0% in AH2-2. An important difference between the oil profiles of 2015-2L-13 and 2015-2L-14 is that the DHA content of the cell biomass increased in the latter secondary fermentations using the supplemented hydrolysate. The control in each had 9.52% and 9.73% DHA, respectively. In contrast, AH1-1 and AH2-1 had 12.54% and 17.01% DHA while AH1-2 and AH2-2 had 12.74% and 18.65% DHA, respectively. If these increases are repeatable, they are potentially important for improving the quality of the produced oil.

Table 5.5 Fatty acid profile of the final samples of the control and the first (AH2-1) and second (AH2-2) fermentors with supplemented hydrolysate for the second recycle (2015-2L-14). All values are duplicate samples with duplicate injections.

Fatty Acid	Concentration (mg per gram dry cells)		
	Control	Supp. Hydrolysate 1	Supp. Hydrolysate 2
C12:0	10.84 ± 0.03	8.94 ± 0.25	7.99 ± 0.06
C14:0	164.75 ± 0.77	122.24 ± 2.36	108.79 ± 0.35
C14:1	0.31 ± 0.03	0.26 ± 0.01	0.11 ± 0.12
C15:0	2.22 ± 0.02	6.25 ± 0.11	6.68 ± 0.03
C16:0	333.81 ± 1.21	250.09 ± 4.14	239.38 ± 0.67
C16:1	38.53 ± 0.20	7.04 ± 0.22	5.91 ± 0.03
C17:0	0.32 ± 0.07	0.98 ± 0.09	1.09 ± 0.01
C18:0	8.83 ± 0.10	7.48 ± 0.09	7.28 ± 0.03
C18:1 Oleic	0.00 ± 0.00	0.41 ± 0.02	0.74 ± 0.01
C18:1 Vaccenic	4.72 ± 0.05	2.23 ± 0.03	2.20 ± 0.02
C18:2 n-6	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:3 n-6	0.58 ± 0.03	0.78 ± 0.05	0.84 ± 0.07
C18:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C18:4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:0	2.27 ± 0.01	2.19 ± 0.05	1.80 ± 0.03
C20:1	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:3 n-6	0.75 ± 0.05	0.93 ± 0.03	0.91 ± 0.01
C20:4 n-6	0.75 ± 0.05	0.93 ± 0.04	0.91 ± 0.09
C20:3 n-3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:4 n-3	1.11 ± 0.03	1.97 ± 0.04	2.27 ± 0.02
C20:5 n-3 (EPA)	1.78 ± 0.08	3.24 ± 0.03	3.44 ± 0.04
C22:5 n-6 (DPA)	24.88 ± 0.11	37.28 ± 0.53	41.13 ± 0.09
C22:5 n-3 (DPA)	0.60 ± 0.03	1.60 ± 0.05	1.67 ± 0.10
C22:6 n-3 (DHA)	97.29 ± 0.44	170.14 ± 2.90	186.48 ± 0.36
C24:1 n-9	0.00 ± 0.00	0.00 ± 0.00	1.04 ± 0.83
Total	693.87 ± 2.79	624.41 ± 10.68	620.21 ± 1.47

5.5 Comparison of Fermentations with First and Second Recycle

Comparing the sequential 2-liter fermentations side-by-side was helpful for drawing conclusions about the efficacy of multiple recycles of waste hydrolysate. Fig. 5.22 depicts the growth curves of the first and second recycle (2015-2L-13 and 2015-2L-14) plotted on the same chart to clearly illustrate the similarity of those secondary fermentations. As was previously stated, the biomass productivity for the supplemented hydrolysate replicates was marginally lower for the second recycle although this difference may be mostly attributable to a slight dip around 40 to 45 hours. Ultimately, the biomass concentration achieved was very similar by 90 hours of the secondary fermentations.

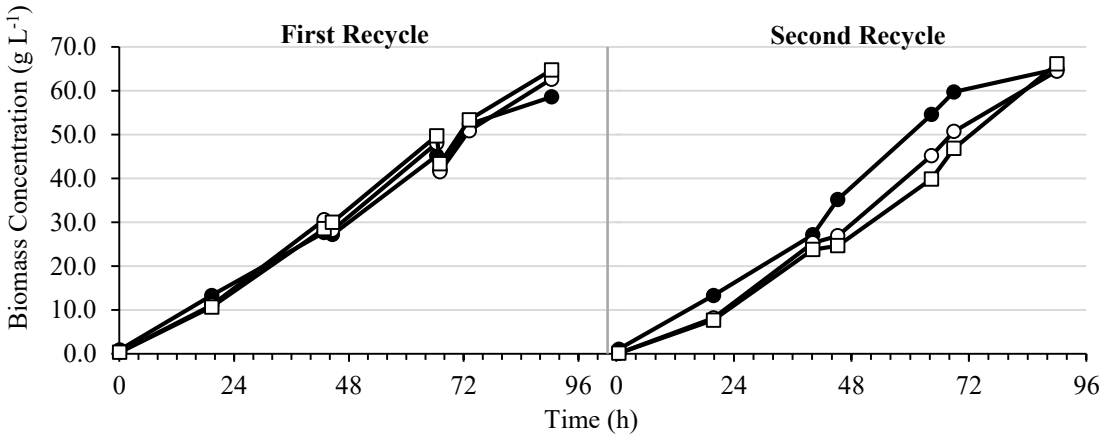

Figure 5.22 Sequential growth curves of T18 in the first (2015-2L-13) and second recycle (2015-2L-14). Symbols denote the control (●), the first replicate of supplemented hydrolysate (AH1-1, ○) and the second replicate of supplemented hydrolysate (AH1-2, □). Error bars indicate ± standard deviation (n=2).

The second major parameter of interest in assessing the success of the secondary fermentations was the fatty acid content and profile of the finished biomass. The FAME data is summarized in Fig. 5.23 showing the fatty acid profile of each treatment during the secondary fermentations. The findings demonstrate that considerable accumulation of fatty acids occurred in all treatments, especially the control. The consistency of the controls was indicative of good experimental repeatability as both were produced in identical conditions and media. While the replicates of each secondary fermentation using supplemented hydrolysate were consistent within the recycles, the second recycle generated

approximately 10% greater fatty acid content in the cells. In all treatments the fatty acid profile was predominantly composed of C14:0, C16:0, DPA and DHA.

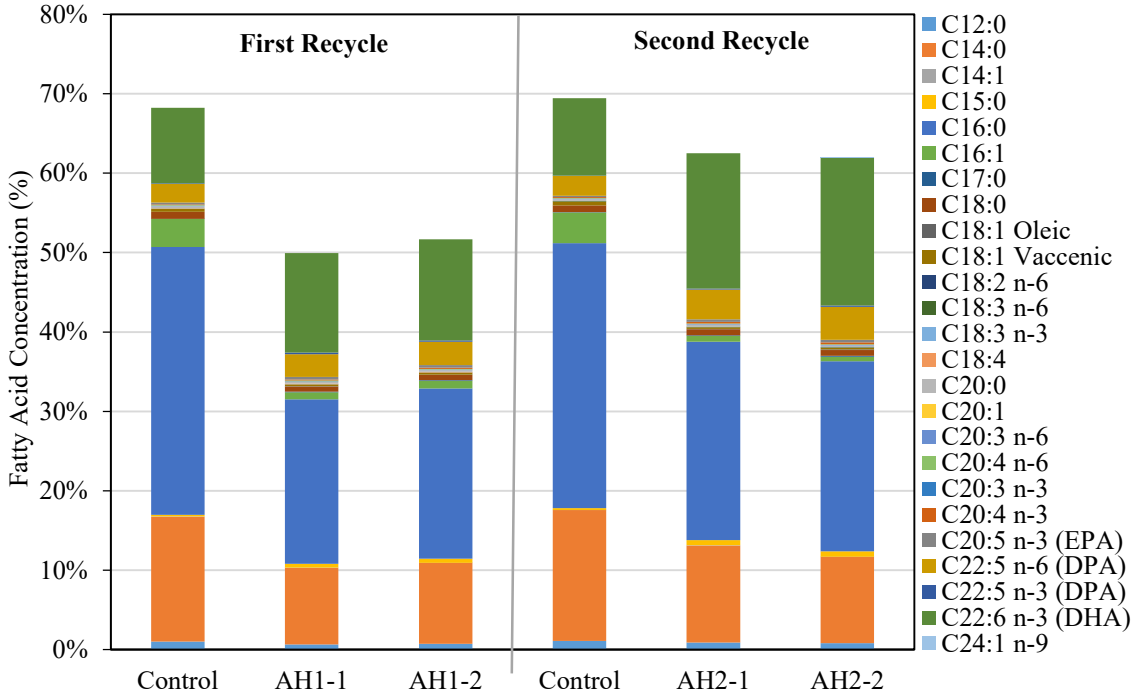

Figure 5.23 Fatty acid profiles of T18 biomass in the first (2015-2L-13) and second recycle (2015-2L-14). All fatty acid concentrations are reported as a percentage of the biomass (w/w).

5.6 Chapter Conclusion

The feasibility of conducting multiple recycles of the lipid-extraction hydrolysate was undeniably confirmed with the sequential 2-liter fermentations. The primary metrics for fermentation success – biomass and lipid production – were both shown to be largely unaffected by recycling the hydrolysate into a secondary fermentation in comparison to using fresh media. In addition, the second recycle of the same hydrolysate that was conducted in 2015-2L-14 proved to be successful on all measured levels. The biomass produced during that secondary fermentation was comparable to the amount produced in the control with the fresh media, suggesting no negative impact from multiple recycles of waste material. It is important to note that there was a slightly diminished growth rate in the recycled hydrolysate. Although this reduced productivity may have been indicative of

an emerging challenge by some inhibitory compound accumulating in the hydrolysate, that concern was partially mitigated by the similarity in final biomass concentrations. Furthermore, cell yields ($Y_{X/S}$) observed in all three treatments were very similar which suggests that T18 was efficiently using available carbon in the substrate for cell synthesis.

The total fatty acids accumulated within the T18 cells were slightly lower in the supplemented hydrolysate as compared to the control, albeit in the second recycle, they were much closer. The discrepancy was likely associated with differences in the substrate composition, with the cells in the supplemented hydrolysate taking longer to reach nitrogen limitation. However, the fatty acid profiles were very similar in both treatments with DHA, C14:0 and C16:0 dominating the profile. These observations further illustrate the potential industrial value that T18 has as a producer of high-value fatty acids with established commercial markets, such as DHA. In addition, C14:0, which is another abundant fatty acid, is another fatty acid with value as a possible feedstock for bio-based chemicals and surfactants (Hu et al., 2008; Metzger and Bornscheuer, 2006). Having such valuable products is important to ensuring a versatile and diversified product portfolio.

The increased abundance of organic nitrogen (i.e. amino acids) in the supplemented hydrolysate treatments contributed a confirmed source of nitrogen for cell metabolism. However, employing a nitrogen rich hydrolysate may require modification of the supplementing growth medium or extension of the secondary fermentation period to expose the culture to extended nitrogen starvation, resulting in the desired maximum cellular lipid content.

The conclusive evidence of successful multiple recycles of lipid-extraction hydrolysate provides an important motive for further exploration into the limits of this technique of nutrient recycling. With repeated additions of micronutrients, trace elements and salts, it is likely that some inhibition will emerge due to excessive concentrations of the aforementioned compounds. This limitation may not emerge until many more sequential recycles are conducted. Further exploration of those practical boundaries for nutrient recycling is important, however, exploring the opportunity to reduce or eliminate those nutrient elements is also an essential approach to reducing costs. Even with only one recycle employed using these techniques, water consumption would be reduced by 50%.

Such improvements in resource consumption in microalgae production could be invaluable for progressing toward a profitable technology.

CHAPTER 6: CONCLUSION

6.1 SUMMARY AND CONCLUSIONS

The overall goal of this project was to explore the potential of recycling the wastes remaining after enzymatic lipid extraction of thraustochytrid biomass produced in optimized conditions. As a review, the specific objectives outlined in **Chapter 1** involved three generalized stages: 1) determining the content of the wastes, 2) exploring the feasibility of recycling the material at laboratory scale, and 3) scaling up the recycling experiments to obtain commercially representative data. These objectives were framed to ensure relevance and value to the industry collaborator, Mara Renewables Corporation. In addition, this research contributed a valuable foundation of information to the scientific community on the subject of nutrient recycling in microalgae cultures, especially heterotrophic commercial applications.

In this thesis, **Chapter 2** began with an introduction to microalgae production, giving some insight into the history of the industry and the future direction. This discussion included the potential industries and applications for microalgae products with a focus on biofuels and nutritional products. The challenges for the commercialization of microalgae technology were then outlined, transitioning the focus toward highly productive microalgae (i.e. thraustochytrids) and the prospect of incorporating nutrient recycling into these production systems.

Chapter 3 then addressed the first and second specific objectives of producing and characterizing the composition of the waste material, which required a variety of laboratory methods to determine the important physical and chemical attributes. Most of the key nutritional properties (i.e. glucose, glycerol and ammonium) were predictable based upon the analytical observations of the primary fermentation. The high degree of degradation of the solid waste streams by the enzymatic hydrolysis prioritized the liquid waste streams for the subsequent experiments. However, the large dissolved solids content of the hydrolysate, specifically, was indicative of elevated concentrations of important effluent parameters like biochemical oxygen demand. These observations confirmed the prospective challenges for discharge or treatment of this material at commercial scale.

Further, understanding the origin of the material and the proteolytic focus of the enzyme, the primary interest of this project was directed at nitrogenous content, specifically free amino acids. As anticipated, there was a considerable content of such organic nitrogen in the resultant hydrolysate. Ultimately, the characterization of both the spent media and the hydrolysate was indicative of an opportunity to recycle this waste material as valuable nutrient source.

Chapter 4 was dedicated to addressing the third specific objective of investigating the feasibility and establishing protocols for nutrient recycling in this system. Initial exploratory experiments conducted small-scale recycling of the waste materials to determine any immediate challenges or deficiencies. These experiments quickly illustrated the importance of mitigating the production of inhibitors by further separation of media elements during sterilization. Once successful secondary fermentations were completed, two lessons were apparent. First, the recycled materials could support equally productive secondary fermentations as the fresh media control. Second, the secondary fermentations in the hydrolysate resulted in a physiological response from T18 that yielded enhanced biomass production and diminished oil content. These findings were further supported by investigation of reduced nitrogen supplementation in secondary fermentations, which yielded similar results in the most nutrient rich conditions.

Deeper investigation of this elevated biomass phenomenon revealed that the morphological changes in T18 were an evolutionary response to being exposed to complex organic carbon and nitrogen sources in the surrounding environment. The production of the ectoplasmic nets was clearly evident in the supplemented hydrolysate, which was also a well-documented trait of thraustochytrids (Bongiorni et al., 2005a, 2005b; Lee Chang et al., 2014; Perveen et al., 2006; Taoka et al., 2009). These extracellular structures are an essential tool for thraustochytrids to secrete enzymes and enhance cell surface area to better facilitate the degradation and uptake of complex nutrients in their environments. In these experiments, the elevated biomass, reduced intracellular lipid content, slower glucose uptake, uptake of alternative organic carbon sources, free amino acid flux and most importantly, the scanning electron micrographs, were all indicative of T18 adapting to the substrate and secreting the aforementioned ectoplasmic nets. This theory can be further validated by specially catered experiments to observe this response and quantify the

enzymatic activities of the substrate. Within the scope of this project, the observation of this anomaly was simply a useful tool in explaining the trends observed in secondary fermentations.

Chapter 5 covered the fourth and fifth specific objectives of expanding the nutrient recycling experiments to commercially representative production vessels and conducting sequential recycling of the waste material. This objective was to expand the findings of the initial experiments into a fermentor-scale study to illuminate the outcomes and challenges of nutrient recycling in commercially representative conditions. The results were highly successful, with biomass production very closely matching the fresh-media control. Furthermore, the same findings were observed in the second recycle of the same hydrolysate. The only important distinction between the control and the recycled hydrolysate was that accumulation of cellular lipids was slightly diminished in the hydrolysate. Observation of nutrient dynamics during these secondary fermentations revealed that changes in lipid content were most likely attributable to the enhanced nitrogen content of the substrate, which could be mitigated by reducing provided inorganic nitrogen or extending the fermentation for a prolonged exposure to nitrogen limited conditions.

The outcomes of the fermentor-scale sequential secondary fermentations presented some interesting possibilities for further experimentation. Most notably, the reduced nutrient uptake rates in the recycled hydrolysate, without any adverse impact on biomass accumulation, suggested the metabolism of organic carbon and nitrogen from the substrate. These observations present an opportunity for modification of the supplementing growth medium recipe to better utilize nutrients available and reduce material inputs. Simultaneously, this approach could help avoid accumulation of any potentially toxic compounds emerging in later sequential recycles of waste material. Additionally, it would be useful to expand the number of times that the hydrolysate was recycled to attempt to reach the threshold of inhibition. In doing so, some insight could be gained on the most important inhibitor compound.

In summary, this project determined that nutrient recycling of waste products in thraustochytrid production has great promise for potential commercial adoption. Minimal modifications to existing production protocols was required and the resultant product yields were comparable to existing conditions. These findings were extended to commercially

representative conditions with minimal negative impacts on culture health and productivity. Further, some interesting observations were made regarding the capacity of T18 to metabolize diverse organic compounds in the substrate. The results were indicative of considerable potential for reducing production costs as well as minimizing the environmental impact and resource usage intensity of thraustochytrid production at commercial scale.

6.2 Novel Contributions to Science

Various aspects of this project venture into novel research areas considering the microalgae production industry has, with few exceptions, not matured sufficiently to demand extensive exploration into resource use efficiency. Accordingly, the concept of nutrient recycling in microalgae cultures is, in itself, minimally researched. Furthermore, any existing literature concerns only photoautotrophic or low-productivity systems. The following specific outcomes from this study constitute novel contributions to the field:

- Hydrolysate from enzymatic lipid extraction was shown to have bioavailable amino acids and peptides present, as a result of proteolytic activity occurring during the lipid recovery method and catabolism of cellular proteins. This corresponding influence on the concentration of favorable nitrogen sources for secondary cultures contributed to its suitability for use in growth media.
- Sterilization protocols for mitigating the production of inhibitors in the preparation of recycled waste for secondary fermentation were successfully developed.
- The observed morphological response of T18 cells to the high-organic content substrate of recycled hydrolysate verified early literature observations of thraustochytrids producing ectoplasmic nets in their natural sedimentary ecosystems. This observation was important to confirming the dynamic metabolic capability of this highly specialized microalga which has implications for understanding the potential for diversification of production feedstocks.

- Equivalent biomass productivity, in spite of reduced glucose consumption (increased yields, $Y_{X/S}$) was established in the latter two flask studies (2015-500ML-02 and 2015-500M-03) and was indicative of potential for reduction of the supplemented nutrients into the secondary fermentations. This finding compounds the appeal of using a nutrient recycling strategy in similar production systems.
- Demonstration of secondary fermentations at the 2-L fermentor scale confirmed that recycling of the hydrolysate was possible in high productivity conditions without significantly compromising the critical outcomes (biomass and lipid productivity). While this has been demonstrated in minimally productive photoautotrophic raceway ponds, no known examples exist in fed-batch heterotrophic conditions.
- Proof of sequential secondary fermentations in the recycled hydrolysate combines the novelty of all aforementioned points with the application of realistic production conditions and the desire to maximize the recycling of water and nutrient resources. These results are suggestive of the possibility for a closed loop system with minimal waste discharge of water and excess nutrients.

6.3 Suggestions for Future Research

- The investigation into nitrogen supplementation conducted in Chapter 4 (2015-500ML-03) could be further expanded to all of the medium elements, thereby demonstrating possibilities for cost reduction. This would be particularly relevant for non-metabolic compounds such as NaCl as well as serving to potentially mitigate known concerns of accumulating pollutants (e.g. SO_4^{2-}, Mg^{2+})
- Further validation of the observed ectoplasmic nets in the secondary fermentations could be achieved by conducting enzymatic activity assays on the fermentation substrate throughout the growth period. This would also enhance the understanding of what specific enzymes are being secreted and elucidate which targeted organic compounds were being metabolized by the thraustochytrid cells. In addition, this information would be valuable to

monitoring the nutrient flux in the substrate and improve understanding of the biomass productivities and yields observed.

- Observing the activity of the aforementioned enzymes being secreted by T18 cells could be valuable for identifying potentially valuable co-products to further improve production costs.
- Detailed experiments could be conducted investigating and optimizing the production conditions of T18 in recycled hydrolysate in 2-L fermentors. These conditions include supplementation levels as well as growth conditions (e.g. agitation, pH). There is potential that higher levels of agitation may be detrimental to the production of ectoplasmic nets, which could obviate the observed advantages of employing recycled hydrolysate.
- Extending these experiments to include spent media (as opposed to hydrolysate) in the commercially representative production vessels would be valuable if the production schematic was altered to include a biomass dewatering step prior to lipid extraction. As demonstrated in 2015-500ML-01 and 2015-500ML-02, the spent media was an attractive waste material for recycling in secondary fermentations.

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