Western Graduate&PostdoctoralStudies

Western University
Scholarship@Western

Electronic Thesis and Dissertation Repository

8-12-2016 12:00 AM

Direct Conversion of Wet Microalgae and Oleaginous Yeast to Biodiesel Using Ionic Liquids

Valerie Orr
The University of Western Ontario

Supervisor
Dr. Lars Rehmann
The University of Western Ontario

Graduate Program in Chemical and Biochemical Engineering
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
© Valerie Orr 2016

Follow this and additional works at: https://ir.lib.uwo.ca/etd

 Part of the Biochemical and Biomolecular Engineering Commons

Recommended Citation

Orr, Valerie, "Direct Conversion of Wet Microalgae and Oleaginous Yeast to Biodiesel Using Ionic Liquids" (2016). *Electronic Thesis and Dissertation Repository*. 3950.
https://ir.lib.uwo.ca/etd/3950

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlswadmin@uwo.ca.

Abstract

This thesis examined the development of an oleaginous yeast or microalgae based biorefinery process. First, improvements were made to the Nile Red assay, a high-throughput method for monitoring lipid accumulation in oleaginous organisms resulting in a significantly more reproducible and accurate assay. This assay was then used to optimize lipid production during heterotrophic cultivation of microalgae on glucose using response surface methodology resulting in microalgae with high lipid content (37.6% wt.). In order to improve the renewability of heterotrophic lipid production, both oleaginous yeast and microalgae were cultivated using pyrolytic sugars, produced via fast pyrolysis of pinewood waste. The effects of inhibitors on glucose consumption and lipid accumulation as well as the quality of the produced fatty acid methyl esters (FAME) were examined.

Upon the establishment of cultivation processes for these two organisms, the overall objective of this work shifted towards the development of a fractionation process for producing and recovering multiple end products. Over 20 ionic liquids were screened for the ability to disruption microalgal cell structure. This was the first report of non-imidazolium ionic liquids assessed for algal bioprocessing applications and the first examining room temperature ionic liquids. The leading candidate ionic liquid was further studied for its ability to disrupt fresh microalgal cultures which were dewatered containing up to 82% water content. This was the first in depth report of the effect of process parameters on the use of ionic liquds for algae disruption. The resulting lipid extraction process was minimized to a simple 1.5 h process conducted at ambient temperature with wet algae.

This was further extended to include a catalyst in order to directly convert intracellular lipid to biodiesel from whole yeast biomass. The effects of the reagent ratios, reaction temperatures, and reaction time were studied in depth using response surface methodology. Recovery of the ionic liquid and catalyst for reuse was quantified. Finally, the carbohydrates and protein fractions were recovered after the ionic liquid lipid extraction process using microalgae and it was demonstrated that the resulting sugars primarily in the form of starch could be directly fermented to biobutanol, bioethanol, and acetone using a traditional ABE fermentation process.

Keywords

Microalgae, oleaginous yeast, ionic liquids, biodiesel, pyrolytic sugars, Nile Red assay, response surface methodology, direct transesterification, wet extraction, ABE fermentation

Co-Authorship Statement

Sections 2.1-2.2, and 3.2-3.7 have been submitted or accepted by peer-reviewed journals and were co-authored with the Dr. Lars Rehmann who provided technical advice and revised these manuscripts. A statement of publication status is provided at the beginning of each chapter.

Section 3.4 was coauthored with several collaborators and equal authorship was assigned to both Dr. Luis Luque and myself due to equal contribution to experimental planning, execution, and manuscript preparation. Specifically, I performed all microalgae cultivations, performed the lipid quantification, and quantified sugars during cultivation. Dr. Luque provided the upgraded pyrolysis oil, performed the oil characterization and the yeast cultivations. All other coauthors provided technical advice and reviewed the manuscript.

Section 3.5 was coauthored with two researchers from Queen's University in Belfast. Dr. Natalia Plechkova synthesized all of the ionic liquids used in this study as well as provided technical advice. Dr. Kenneth Seddon revised the manuscript and provided technical advice during my tenure at Queen's University Belfast. I designed and performed all experiments and drafted the manuscript.

Section 3.6 was coauthored with researchers from the University of Manitoba. Visiting graduate student Garret Munch performed the large scale cultivation of *R. diobovatum*. I performed all ionic liquid experiments and drafted the manuscript. All other authors provided technical advice and revised the manuscript.

Section 3.7 was coauthored with fellow PhD Candidate Kai Gao and equal authorship was again assigned to us both due to equal contribution to experimental planning, execution, and manuscript preparation. Specifically, I performed solvent and ionic liquid extractions, algae cultivations, as well as lipid and protein compositional analysis. Kai Gao performed fermentations, carbohydrate analysis and performed the hydrolysis reactions.

Acknowledgments

I would first like to acknowledge the guidance and support generously provided by my supervisor Dr. Lars Rehmann, especially during our many coffee breaks.

I would sincerely like to thank the following faculty members for providing me with mentorship in various areas during my time at Western: Drs. Franco Berruti, Amarjeet Bassi, Kibret Mequanint, Mita Ray, AJ Ray, Lauren Flynn, and Jose Herrera. Thank you to Drs. AJ Ray, Paul Charpentier, Martin Duennwald, and Pascale Champagne for agreeing to sit on my thesis examination board and reviewing this work. I also immensely appreciate the help and equipment loans provided by Souhiel Afara, Brian Dixon, and Dr. Mamdouh Abou-Zaid over the past 4 years.

I appreciate the various scholarships I have received during my tenure at Western which allowed me to focus on my career development including a Canada Graduate Scholarship from NSERC and the Ontario Graduate Scholarship. I also appreciate the extensive financial resources contributed by NSERC through Biofuel Net Canada for both research costs as well as for my own personal development including BiofuelNet Travel Subsidies and the HQP Exchange program.

My work would not have been successful or at least not nearly as fun without the support of my many labmates in the last 4 years including but especially Luis Luque, Kai Gao, Erin Johnson, Jeff Wood, Thomas Krieg, Raphael Grater, Alex Nikolay, Eric Doerr, and Garret Munch. I also thank my fellow students who were always available to bounce ideas off of, particularly Pegah Saremid. I would also like to thank my family for always supporting me in my career goals even though they really don’t understand why anyone would ever want to be a university student for 10 years. Thanks for being proud of me anyways!

Finally, I would like to thank my husband Ryan Ward for standing beside me throughout my graduate studies and always supporting my career choices. I dedicate this work to him.

Table of Contents

List of Tables

List of Figures

List of Acronyms

[C_2mim][$EtSO_4$] – 1-ethyl-3-methylimidazolium ethylsulfate
5-HMF – 5-hydroxymethylfurfural
A_{680} – Absorbance at 680 nm
ABE fermentation – Acetone, butanol, ethanol fermentation
ANOVA – Analysis of variance
B&D – Bligh & Dyer
BBD – Box-Behnken design
C/N – Carbon to nitrogen ratio
C11:0Me – Undecanoic acid methyl ester
C15:0Me – Pentadecanoic acid methyl ester
CCD – Central composite design
CFPP – Cold flow plugging point
Chl*a* – Chlorophyll a
Chl*b* – Chlorophyll b
CN – Centane
CrI – Crystallinity index
DAD – Diode array detector
DCW – Dry cell weight
DMSO – Dimethyl sulfoxide
DT – Direct transesterification
EtAc – Ethylacetate
Em. – Emission wavelength
Ex. – Excitation wavelength
FAME – Fatty acid methyl esters
FID – Flame ionization detector
FTIR – Fourier transformation infrared spectroscopy
GC – Gas chromatography
H2P – Hexane 2-proponaol
HEA – Hexane extracted algae
HPLC – High performance liquid chromatography
HTP – High throughput
IL – Ionic liquid
ILEA – Ionic liquid extracted algae
IV – Iodine value
IV/G – Inhibitor value normalized to glucose
LAP – Laboratory analytical procedure
LCSF – Long chain saturation factor
LEA – Lipid extracted algae
MeOH – Methanol

μ_{max} – maximum specific growth rate
N_{max} – maximum cell density
NMR – Nuclear magnetic resonance spectroscopy
NREL – National Renewable Energy Laboratory
Ω_{glc} – Glucose consumption rate
Ω_{xyl} – Xylose consumption rate
Q_0 – Initial adaptation parameter
RID – Refractive index detector
SCOs – Single celled oils
SFE – Supercritical fluid extraction
SV – Saponification value
TAGs – Triacylglycerides
TAP – Tris-Acetate-Phosphate media
TRS – Total reducing sugars
VOCs – volatile organic compounds
XRD – X-ray diffraction

Chapter 1 – Introduction

The desire to create sustainable and renewable processes for the production of chemicals and fuels is manifold. Briefly, our dependence on fossil fuels for an array of products from plastics to transportations fuels is creating climate change, inflicting extensive environmental damage, and economic and societal instability due to the geography of its production (Kircher 2015). Biofuels and biochemical are widely regarded as environmentally friendly and their production is not nearly as strictly geographically restricted. Their lifetime ecological impact is thought to be less than fossil fuels as they are produced using at least partially renewable processes and their consumption creates less emissions than petroleum fuels (Stoeglehner and Narodoslawsky 2009). However, biofuels production continues to be limited by the demand for low cost fuels and chemicals supplied by the petrochemical industry which is largely permitted to disregard the long term environmental impacts of their processes and products. In this context, the biofuels industry will not thrive until it can compete with the economy of the petroleum industry while offering the benefits of the positive environmental, social, and health footprints of their production (Erickson et al. 2012; Kircher 2015). Additionally, in order to make biofuels as sustainable as possible, their renewability must be continually assessed. Care should be taken to use renewable feedstocks such as waste products and to reduce the consumption of fossil fuels during the production process, commonly used for heating/cooling operations.

A brief introduction to biodiesel, lipids, lipid feedstocks, lipid extraction technologies, transesterification, and value added products is first presented in this chapter, followed by the general and specific objectives of this work.

1.1 Research Background

1.1.1 Biodiesel

Biodiesel is considered one of the leading renewable fuels for a host of reasons. It is biodegradable, relatively less toxic, a direct substitute for petroleum diesel, produces lower combustion emissions, is compatible with current distribution infrastructure, and has a lower net life cycle contribution to carbon dioxide (CO_2) emissions compared to petroleum diesel (Atabani et al. 2012). Biodiesel is conventionally synthesized from vegetable oil,

primarily composed of a class of lipids called triacylglycerides (TAGs). A general process for the production of biodiesel is illustrated in **Figure 1.1-1**.

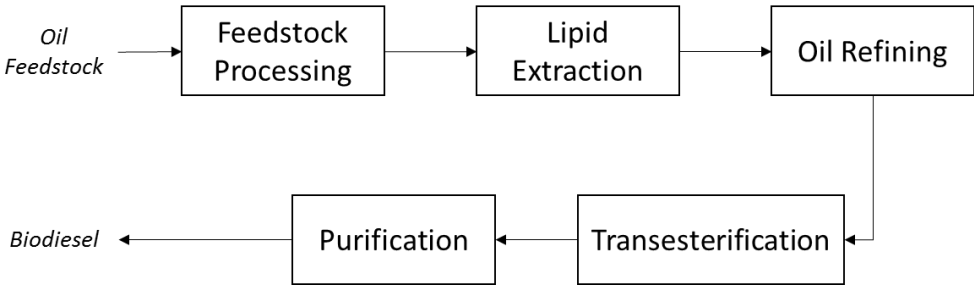


Figure 1.1-1 – A general outline of the biodiesel production process.

Generally, an oil containing feedstock like canola seeds are subjected to several steps of feedstock processing which includes harvesting, drying, and milling for example (Canola Council of Canada 2016). Then the meal is subjected to oil extraction processes like pressing and organic solvent extraction. The extracted oil may require further processing to remove impurities such as free fatty acids prior to the transesterification process. After transesterification, the resulting biodiesel must be purified to remove any residual free fatty acids, catalyst, alcohol, and glycerol. The individual operations involved in biodiesel synthesis will be highly specific to the oil feedstock being used and the oil characteristics important in the process design are discussed below.

1.1.2 Lipids and Oil Feedstocks

As previously mentioned, biodiesel is synthesized from lipids, however, many different classes of lipids can be present in an oil feedstock. Knowledge of the lipid composition of a feedstock is important for the proper selection of the extraction and transesterification methods, as well as for predicting the fuel properties of the resulting biodiesel. There are several major categories of lipids whose abundance can differ significantly between different types of feedstocks and between species.

Free fatty acids (**Figure 1.1-2A**) are typically used by plants and animals as a building block for other types of lipids, however, they can be present in refined oils in significant quantities. Fatty acids are often esterified with glycerol into triacylglycerides

(TAGs) which contain three fatty acid residues (**Figure 1.1-2B**) and are stored in TAG bodies within the cell (Sitepu et al. 2014b). This is the most abundant form of lipids found in oil seeds (Demirbas 2008). Often times TAGs are also referred to as neutral lipids due to their solubility in highly apolar solvents. Phospholipids are another major class of lipids found in cell membranes. Their basic composition is of a glycerol molecule esterified to two fatty acid residues and a phosphate molecule. Other types of lipids which are relatively less abundant than TAGs and phospholipids include shingolipids, glycolipids, and sterols (Halim et al. 2012).

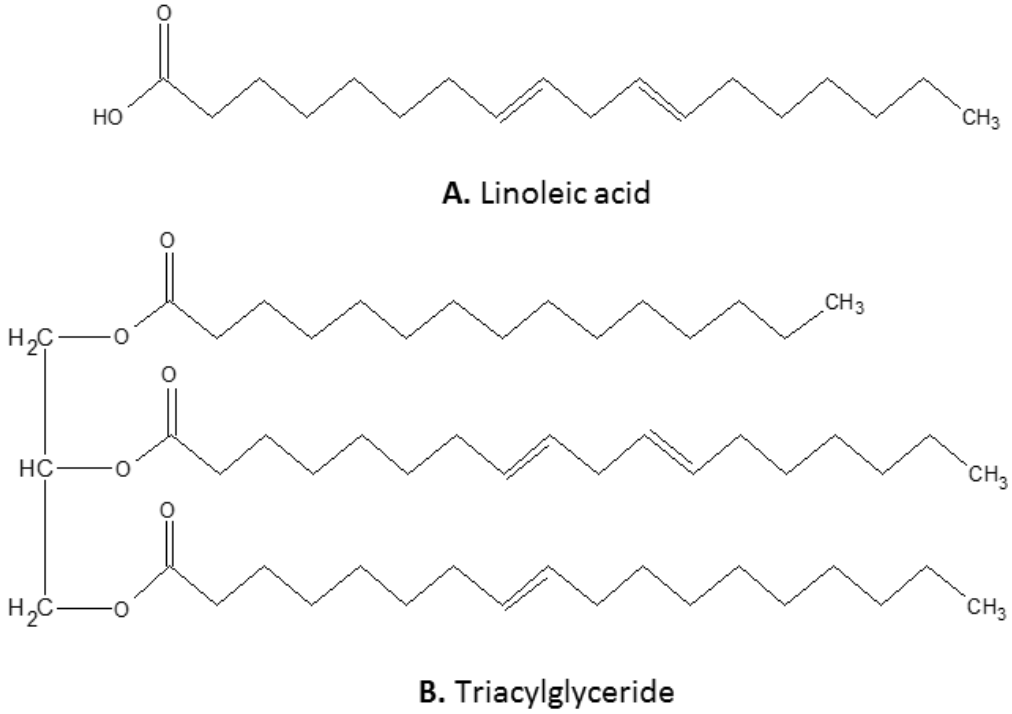


Figure 1.1-2 – Chemical structures of A. The free fatty acid linoleic acid (C18:2) and B. A TAG containing palmitic acid (C16:0), linoleic acid (C18:2), and oleic acid (C18:1).

Composition of the individual fatty acid residues will have a significant effect on the properties of the synthesized biodiesel as they can vary considerably between species. Fatty acid residues vary in chain length from generally 12 to 24 carbons, and can have varying degrees of unsaturation (Meng et al. 2009; Halim et al. 2012). These two properties are often used to quickly reference a fatty acid and written in the form of CX:Y where X represents the number of carbons in the chain length, and Y the number of double bonds

present. Thus, one of the most common fatty acid residues in plant and animal lipids; oleic acid, is designated C18:1 as it has 18 carbons and one double bond. The chain length has a significant effect on the combustibility of the resulting biodiesel and number of unsaturations affect the melting point and volatility as well as the stability of the resulting fuel (Knothe 2005a).

Feedstocks for biodiesel synthesis are typically divided into two major categories, edible oils and non-edible oils. Currently over 90% of the world's biodiesel is synthesized from edible oils like corn or canola oil (Atabani et al. 2012). Due to rising concerns over the price of edible oils and competition between food and biofuels, non-edible oil feedstocks are currently of significant interest for biodiesel synthesis. Non-edible oils can be further divided into waste lipids and purposefully grown non-edible oil crops. Common waste lipids include tallow from animal rendering processes or waste cooking oils. Processes for biodiesel production from these waste feedstocks have other complications relating their high melting point and the impurities present, respectively (Demirbas 2008). Additionally, the amount of waste oil generated is thought to be insufficient to meet the growing global demand for biodiesel (Demirbas 2008; Balat 2011; Atabani et al. 2012). Thus, development of non-edible oil feedstocks is paramount.

Several non-edible oil crops have received considerable attention such as jatropha, karanja, mahua, tabacco, cotton and castor oils (Banković-Ilić et al. 2012; Atabani et al. 2012). However, some of these crops require the use of agricultural land, have long growth cycles, and are only available seasonally (Li et al. 2008a). A promising alternative source of non-edible oil are microbial lipids. Microbial lipids present several significant advantages over traditional crops like canola or soya beans, primarily their faster growth rate, they are less affected by climate and season, and they do not require agricultural land for their cultivation (Chisti 2007; Li et al. 2008a; Sitepu et al. 2014b). Microbial oils refer to oil production by single-celled organisms like bacteria, fungi, and microalgae.

1.1.2.1 Microbial Lipids

Microalgae have long been hailed as the most promising source of lipids for biodiesel due to their ability to synthesize lipids from mainly sunlight and CO_2 (Chisti 2007; Singh and

Sharma 2012). However, low cell densities, slow phototrophic growth rates and the high cost of downstream processing of microalgae have limited their commercial applications to high end products like carotenoids, anti-oxidants, and omega fatty acids (Yen et al. 2013). While heterotrophic microalgae cultivation can produce higher lipid titers at much faster rates (Lowrey et al. 2015), some researchers argue this defeats the purpose of using a photosynthetic organism. However, the use of an organic carbon source such as glucose can be considered comparable to other bioconversion processes like the fermentation of ethanol. In such cases, while the end product differs, both processes renewability and sustainability is largely dependent on the source of the carbohydrate feedstock with the greatest advantage derived when waste feedstocks are used. A second alterative platform for bioconversion of glucose to lipids are oleaginous yeasts as some species have been shown to accumulate over 50% of their weight in lipid and they are less fastidious than microalgae (Sitepu et al. 2014a; Sitepu et al. 2014b). While both microalgae and yeasts are promising alternatives for microbial lipid production they will differ considerably in their lipid composition. Many species of microalgae are known to have a favorable fatty acid lipid profile for fatty acid methyl ester (FAME) synthesis (Meng et al. 2009; Halim et al. 2012) resulting in biodiesel with a low cold flow plugging point (important for biodiesel use in cold climates), while yeast lipid profiles are less amenable to biodiesel production due to higher saturation and longer chain length of their synthesized fatty acids (Meng et al. 2009). Neutral lipids are used as a mechanism for the storage of chemical energy and accordingly, both microalgae and yeasts only accumulate significant amounts of lipids when at least one nutrient is limited (Meng et al. 2009). Most commonly, nitrogen limitation is used as it is readily consumed and easily regulated in cultures and bioreactors. However, the ratio of carbon to nitrogen must first be optimized so that biomass production is not limited, however, nitrogen is depleted upon the onset of the stationary phase where lipid accumulation occurs.

1.1.3 Lipid extraction process

Once the cultivation and harvesting of the oil feedstock is complete, traditionally the oils are extracted and refined prior to being transesterified into biodiesel. The method of lipid extraction is chosen based on the feedstock used. Traditional oil crops such as canola or soya are first subjected to particle size reduction followed by a mechanical disruption method such as extrusion or pressing and the remaining oil is extracted using an organic solvent (Demirbas 2008; Canola Council of Canada 2016). The main goal of most pretreatments used prior to solvent extraction is generally to disrupt the cellular structure to improve the diffusion of organic solvent into the material allowing dissolution of the lipids into the organic solvent.

1.1.3.1 Conventional processing of microbial oils

Unlike oil seed crops, microalgae and yeasts are grown in aqueous media. Furthermore, with our current cultivation capabilities, cell densities of microalgae grown under phototrophic conditions are typically under 2 g/L and heterotrophic cultures of both yeasts and microalgae are under 20 g/L in batch cultures (Halim et al. 2012; Sitepu et al. 2014b). Thus, the first step in downstream processing of microbial lipids must be a harvesting and/or dewatering step. Liquid solid separations can be energy intensive operations, therefore, a lot of effort is currently focused on the development of low cost dewatering technologies such as sedimentation, flocculation, centrifugation, and filtration (Milledge and Heaven 2012). After the dewatering step, the microbial cells should be present as a thick concentrate. Following dewatering, the concentrate may be subject to one or more pretreatment steps designed to lyse the cells and release intracellular lipids or more commonly, it is dried and milled into a powder in preparation for organic solvent extraction. Drying is essential prior to organic solvent lipid extraction as water significantly impedes the solvent extraction process (Halim et al. 2012). Following lipid extraction, the residual solids are separated from the extraction solvent containing the crude microbial lipids and the lipids are recovery by distillation of the solvent or another similar process. Following recovery of the lipids, the crude extract may require some upgrading to remove

impurities in order to achieve high yields and efficiency during transesterification however, this is largely dependent on the choice of solvent and extraction process used.

1.1.3.2 Organic solvent extraction

The organic solvent extraction process is based on the diffusion of the organic solvent into the cell and the basic solubility concept of *like dissolving like* (Halim et al. 2012). Lipids containing long hydrophobic chains are soluble in most common non-polar organic solvents like hexane, chloroform, and toluene (Kumar et al. 2015). It is believed that the organic solvent first diffuses into the cell, interacts and solvates the lipids present in the cell through van der Waals interactions, then driven by a concentration gradient the lipid/solvent complex diffuses back into the bulk organic solvent (Halim et al. 2012). Accordingly, as mass transfer is limited by the rate of diffusion in the cell and through the boundary layer surrounding the cell, the extraction process can be lengthy. Further complicating the extraction process, lipids can be present in the cell in a variety of structures. TAGs are typically stored in lipid bodies and are believed to be most readily extracted when using a single organic solvent such as hexane, whereas other lipids particularly polar lipids can be complexed with other cellular components like carbohydrates or proteins through stronger forces like hydrogen bonding. If extraction of polar lipids is also desired, the use of a single non-polar solvent will not be sufficient to break these bonds and typically a mixture of solvents is used in this case (Medina and Grima 1998; Halim et al. 2012; Kumar et al. 2015). Two common mixtures used for combined neutral and polar lipid extraction are chloroform and methanol or hexane and isopropanol (Folch et al. 1956; Bligh and Dyer 1959; Hara and Radin 1978).

1.1.3.3 Pretreatment methods for cell disruption

In order to improve the mass transfer efficiency of organic solvent extraction, various methods of cell disruption have been investigated. In theory, cell disruption should increase the surface area of the interface between the lipids and the organic solvent and thus lead to faster extraction times (Halim et al. 2012). However, many cell disruption methods can be energy intensive, therefore any increase in lipid extraction efficiency must be substantial to justify their use. Cell disruption techniques used for microbial cells are

substantially different from those used on oil crops due to the much smaller size of microalgae, bacteria and yeasts. Furthermore, cell disruption processes are typically performed before drying, as many require some water in order to work. Such is the case for ultrasonication, microwave irradiation, bead-milling, high-pressure homogenization, steam explosion, acid/alkaline hydrolysis, osmotic shock, and enzymatic lysis (Lee et al. 2010; Zheng et al. 2011; Halim et al. 2012; Aguirre and Bassi 2014; Taher et al. 2014; Kumar et al. 2015). The advantages and disadvantages of these pretreatments are presented in

Table 1.1-1. After cell disruption, drying of the disrupted concentrate is still required prior to lipid extraction using an organic solvent due to the formation of strong emulsions when water is present (Halim et al. 2012).

Table 1.1-1 – Overview of pretreatment technologies for cell disruption (Chisti and Moo-Young 1986; Halim et al. 2012)

Method	Advantages	Disadvantages
Ultrasonication	· Short processing times	· Energy intensive · High equipment costs
Microwave irradiation	· Short processing times	· Energy intensive · High equipment costs · Possible product degradation
Bead-milling	· Works with difficult to lyse species	· Difficult scale up · Energy intensive · High equipment costs
High-pressure homogenization	· Short processing times	· Energy intensive · High equipment costs · Possible product degradation
Steam explosion	· Short processing times	· Energy intensive · Potential · High equipment costs · Possible product degradation
Acid/alkaline hydrolysis	· Low cost of equipment · Low energy	· Neutralization of waste needed · Possible product degradation
Osmotic shock	· Low cost of materials and equipment · Low energy · Mild conditions	· Long treatment time
Enzymatic lysis	· Low cost of equipment · Low energy · Mild conditions	· High cost of enzymes · Long treatment time

1.1.3.4 Emerging technologies

Several emerging technologies for lipid extraction from microalgae are worth mentioning. They include supercritical or subcritical fluid extraction (SFE), the OriginOil Electro Water Separation, direct transesterification, and ionic liquid aided lipid extraction. All of these emerging technologies seek to either eliminate energy intensive drying processes or reduce the total number of processing steps in converting microalgal/yeast oils into biodiesel.

SFE typically uses pressurized CO_2 in a liquid state to dissolve the lipids present in the biomass packed into a pressure vessel (Couto et al. 2010; Cheng et al. 2011; Halim et al. 2012). As a non-polar solvent, the extraction process is thought to be similar to organic solvent extraction, however, SFE presents several key advantages primarily the use of a non-toxic, non-flammable extraction solvent, and the ease of separation of the extraction solvent from the lipids by pressure decomposition (Couto et al. 2010; Cheng et al. 2011; Halim et al. 2012). Liquid CO_2 and CO_2 expanded methanol have recently been demonstrated to favour extraction of neutral lipids from *Botryococcus braunii* and requires significantly less pressure than supercritical CO_2 extraction, however the normalized extraction compared to the standard Folch extraction method only yielded up to 50% recovery of the available oil (Paudel et al. 2015). Alternatively, supercritical methanol has previously been demonstrated to allow the auto-transesterification of lipids into fatty acid methyl esters (FAME) in the absence of a catalyst (Demirbas 2008). Recently it was demonstrated to be possible using whole wet microalgae biomass, (Patil et al. 2011; Patil et al. 2012; Reddy et al. 2014; Nan et al. 2015) and although it requires much higher processing temperatures and pressures than supercritical CO_2 extraction, it is a growing area of interest.

The OriginOil single step extraction process uses a unique approach combining pulse electromagnetic fields with pH modification using CO_2 to disrupt microalgae cells in a cell concentrate which are then separated using a gravity clarifier (OriginOil 2010). However, it is unclear if this process had been implemented commercially yet and the scale of separation times required for the gravity clarifier operation is unclear. Direct

transesterification was originally demonstrated in the mid-1980s (Lepage and Roy 1984), however it is currently receiving renewed attention as pressure to develop renewable fuels increases (Griffiths et al. 2010; Hidalgo et al. 2013; Suh et al. 2015). In this process, transesterification is performed using whole biomass and biodiesel is recovered rather than lipids. The fastest reaction times and yields have been achieved using dried biomass, however, the use of wet biomass is also being investigated.

Finally, ionic liquids are a novel class of solvents which have been proposed as a low energy green alternative to solvent extraction for the separation of microalgae lipids and have seen some preliminary success (Kim et al. 2012; Teixeira 2012b; Choi et al. 2014b; Choi et al. 2014c; Olkiewicz et al. 2015). However, higher processing temperature are still required for these ionic liquids albeit for shorter processing times. Furthermore, little is known about the compatibility of ionic liquids with wet biomass or the effects of processing conditions on the ability to recovery lipids. This thesis directly addresses this lack of understanding of ionic liquid based cell disruption in order to achieve greater understanding of this process.

1.1.4 Transesterification

Transesterification is the most commonly used process for the generation of biodiesel from lipids. Historically, vegetable oil was the first biofuel used in one of the earliest combustion engines, the diesel engine. Today vegetable oils are transesterified into fatty acid esters in order to increase their volatility and decrease their viscosity. The process of transesterification for biodiesel production was first patented in the 1930s by a Belgian researcher; G. Chavanne at the University of Brussels (Xiao and Gao 2011). Fatty acid esters can be synthesized through either an acid or base catalyzed mechanism from fatty acids and/or glycerides and a short chain alcohol, although methanol is typically preferred (Demirbas 2008). These two mechanisms differ significantly and the choice of acid or base catalyst is typically dependent on the composition of the oil feedstock. The acid catalyzed mechanism can act on both free fatty acids (**Equation 1.1-1**) and glycerides (**Equation 1.1-2**) allowing a greater flexibility in the feedstock composition than alkaline catalysis which can only act on glycerides.

$$Fatty\ acid\ (R_1COOH) + Alcohol\ (ROH) \leftrightharpoons Ester\ (R_1COOR) + Water\ (H_2O)$$

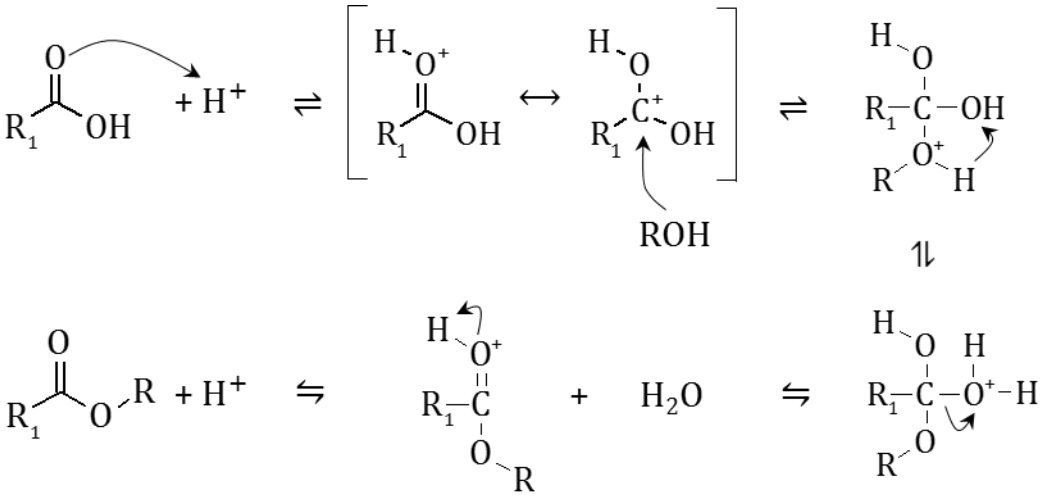
Equation 1.1-1 - Overall esterification process for a free fatty acid only possible with an acid catalyst.

$$Triglyceride\ + ROH\ \leftrightharpoons R_1COOR + Diglyceride$$
$$Diglyceride\ + ROH\ \leftrightharpoons R_2COOR + Monoglyceride$$
$$Monoglyceride\ + ROH\ \leftrightharpoons R_3COOR + Glycerol$$

Equation 1.1-2 – General transesterification process for a triglyceride for both acid and alkaline catalysis. Each fatty acid is transesterified one by one. The conversion of the monoglyceride is considered to be the rate limiting step as the monoglyceride is the most stable intermediate.

1.1.4.1 Acid catalyzed transesterification

Typically, sulfuric, hydrochloric, or sulfonic acids are used in acid catalyzed transesterification processes as they result in high yields (Demirbas 2008; Xiao and Gao 2011). Although the reaction is reversible, an excess of alcohol allows the reaction to go virtually to completion. The major disadvantages of acid catalyzed transesterification are the higher temperatures and much longer reaction times required compared to alkaline catalysis. The mechanism of acid esterification of a free fatty acid is shown in **Scheme 1-1.1**



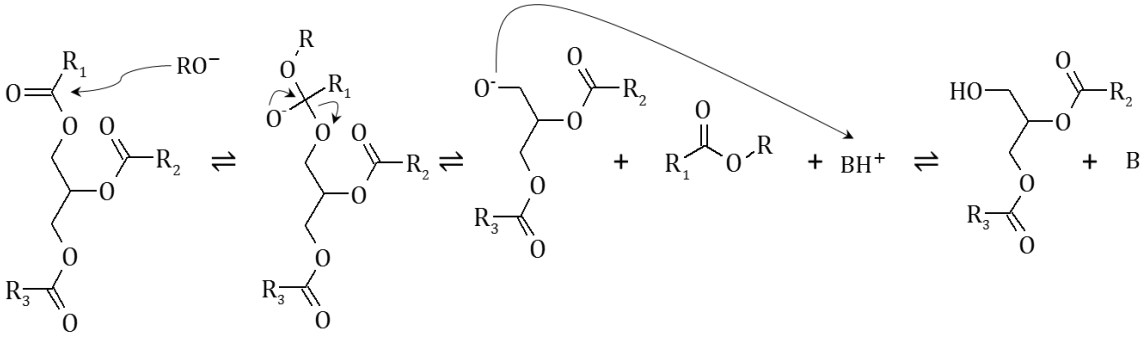
Scheme 1.1-1– Mechanism of acid catalyzed esterification of a fatty acid. Transesterification of glycerides by acid catalysis proceeds in a similar manner only differing in the production of another glyceride rather than water as a final product.

1.1.4.2 Alkaline catalyzed transesterification

Base catalyzed transesterification requires the formation of an alkoxide anion (**Equation 1-1.3**) in order to form the reaction intermediate (**Scheme 1-1.2**) which can either dissociate into the original ester or form a new ester. As a result, esterification of free fatty acids cannot occur.

$$ROH + B \rightleftharpoons RO^- + BH^+$$

Equation 1.1-3 - Formation of the alkoxide in the pre-step.



Scheme 1.1-2– Mechanism of base catalyzed transesterification of a triglyceride.

However, base transesterification is the preferred method for biodiesel synthesis in industry due to the fast reaction rates, high yields, and low molar concentrations of catalyst required (Demirbas 2008; Xiao and Gao 2011). Typically, alkaline metal hydroxides like KOH, or NaOH are preferred due to their low cost and while they are less active than alkoxides like CH_3ONa, they are less sensitive to water which can never be completely eliminated from the lipid feedstock (Demirbas 2008; Xiao and Gao 2011). The presence of water can lead to hydrolysis of the esters and consequently soap formation which reduces the reaction yields and can cause the formation of emulsions which impede downstream purification steps (Demirbas 2008; Xiao and Gao 2011).

1.1.5 Value added products

Finally, it is one of the goals of the emerging field of biorefining, to produce as many value added products as possible during the bioprocessing of any feedstock in order to generate multiple revenue streams and improve the overall economics of the process. Microalgae in particular produce several high value products that could possibly be recovered along with the major biochemicals: lipids, carbohydrates, and proteins. Some of the possible products and their applications are presented in **Figure 1.1-3** (Spolaore et al. 2006; Milledge 2011; Gong and You 2015). Lipids as previously discussed can be used for the production of biodiesel, however, further separation of the high value lipids such as omega fatty acids or sterols may have applications as nutritional supplements or in the cosmetics or pharmaceutical fields.

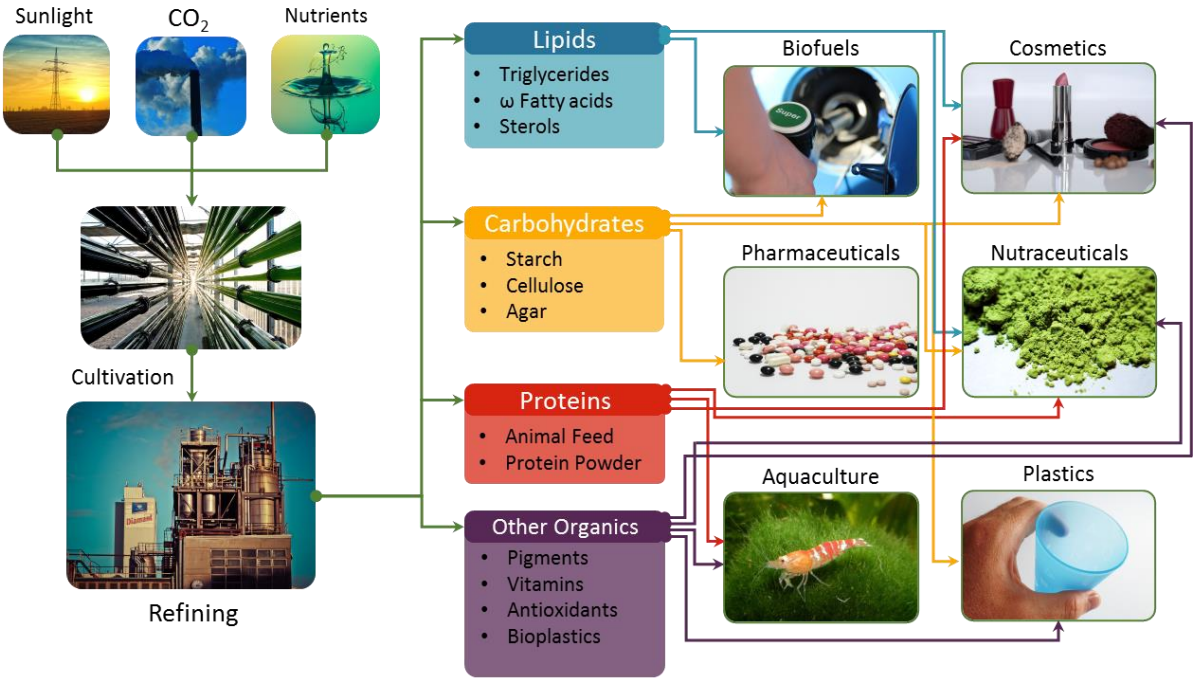


Figure 1.1-3 – Potential value added products in a microalgae based biorefinery and their applications.

Carbohydrates produced from microalgae can be used as substrate for the production of alcohol fuels, however they may also have applications in food processing or cosmetics as fillers. Proteins recovered from other types of feedstocks have been used extensively as components in animal feed, however, microalgae are currently under study as feed for fish and shrimp in the aquaculture industry. Finally, many specialty organics are present in microalgae and to some extent oleaginous yeasts such as carotenoids, vitamins, antioxidants and even monomers for bioplastics. In particular, the production of nutritional supplements from a *natural* source like microalgae is highly valued.

1.2 Research Objectives

The overall objective of this work was to produce microbial lipids and convert them into biodiesel in a low energy, water compatible process, thus eliminating the need for an energy intensive drying step. Furthermore, it was the goal of this work to explore other methods of improving the sustainability of this process when possible such as using

renewable feedstocks for oil production and the conversion of the process waste streams into value-added products.

1.2.1 Specific Objectives

1. Production of microbial lipids
    a. Development of a reliable method for high-throughput lipid quantification
    b. Optimization of mixotrophic/heterotrophic lipid production in *Chlorella vulgaris*
    c. Production of microbial lipids using renewable feedstocks
2. Development of a low energy ionic liquid based lipid extraction process
    a. Identify room temperature ionic liquids capable of cell disruption
    b. Assess the compatibility of ionic liquid based lipid extraction with water
    c. Reduce the total operational steps by direct transesterification
    d. Evaluate the reuse/recycling of the ionic liquid and catalyst
3. Recover residual solids for further applications in biofuel production

Objective 1a: Development of a reliable method for high-throughput lipid quantification.

Previously, reliable quantification of lipids was limited to materially intensive methods such as the Folch method or Soxhlet extraction which due to the low cell densities of algae cultures, required cultivation in excessive volumes impeding the rapid optimization of lipid production in microalgae cultures. A previously developed method, the Nile Red assay which allowed high-throughput processing of small samples sizes (< 1 mL) was reported but showed high variability between species and poor reproducibility. Therefore, it was necessary to improve the Nile Red assay in order to hasten future cultivation work.

Objective 1b: Optimization of mixotrophic/heterotrophic lipid production in *C. vulgaris*

In order to study lipid extraction processes, sufficient amounts of algal biomass with a reasonable lipid content was required. Due to the higher densities and faster growth rates

exhibited under mixotrophic or heterotrophic conditions, optimization of lipid production was undertaken using glucose as a carbon source.

Objective 1c: Production of microbial lipids using renewable feedstocks

Due to growing concerns over the use of glucose from edible crops, and an overall desire to improve the sustainability of the production of microbial lipids for biodiesel production, the use of glucose derived from the fast pyrolysis of waste lignocellulosic biomass was investigated. Microalgae were contrasted with oleaginous yeast as a promising alternative for heterotrophic lipid production as yeasts are generally much less fastidious and less sensitive to growth inhibition commonly experienced with lignocellulosic feedstocks.

Objective 2a: Identify room temperature ionic liquids capable of cell disruption

A very small number of publications had previously identified ionic liquids as a possible means of microalgal cell disruption when this work was first undertaken. However, due to lack of proper controls, low recoveries, and the extensive use of cosolvents, it was difficult to discern which ionic liquids were actual capable of aiding lipid extraction. Furthermore, only imidazolium based ionic liquids had been explored which require high processing temperatures. Therefore, it was necessary to screen a larger number of room temperature ionic liquids for their ability to disrupt microalgae and facilitate lipid extraction at low temperatures.

Objective 2b: Assess the compatibility of ionic liquid based extraction with water

After the identification of ionic liquids capable of aiding lipid extraction, it was the primary goal of this work to improve the compatibility of the lipid extraction process with water in order to reduce the process energy requirements by eliminating biomass drying operations. Therefore, the effects of water content and other process parameters on the ionic liquid extraction process was investigated.

Objective 2c: Reduce the total operational steps by direct transesterification

In order to reduce the total number of operational steps in the developed process, the recovery of FAME directly from whole biomass was investigated. The previously developed process was augmented by the addition of a catalyst to the lipid extraction

solvent (ionic liquid, methanol mixture) and FAME was directly recovered rather than having three separate operations, cell disruption, lipid extraction, and transesterification.

Objective 2d: Evaluate the reuse/recycling of the ionic liquid and catalyst

In order to reduce the process cost, material reduction was investigated. The ionic liquid was recovered from impurities by solvent precipitation of the proteins and carbohydrates followed by evaporation/distillation of the organic solvent. The reuse of the ionic liquid was investigated and the recovery of the catalyst was quantified.

Objective 3: Recover residual solids for further applications in biofuel production

Finally, the residual solids recovered during the ionic liquid recovery process were assessed for their gross composition. Microalgae which can also contain readily fermented carbohydrates like starch were subjected to compositional analysis before and after ionic liquid extraction and compared to traditional drying/organic solvent extraction. The recovered carbohydrates were fermented to produce butanol and ethanol as a value added compounds and to reduce the overall waste of the process.

1.3 Outline of Thesis

This thesis is divided into four chapters. The introduction which outlines the research background and the specific objectives of this work. Secondly, a literature review of ionic liquid processing of biomass is presented. In the third chapter, the research methodology is documented and the experimental results are presented and discussed. Finally, in chapter four, the research is summarized, conclusions are made, and possible avenues of future work are outlined. In several cases, the work conducted was done in collaboration with fellow researcher and **Figure 1.3-1** is an overview of the interconnections between each work presented in this thesis.

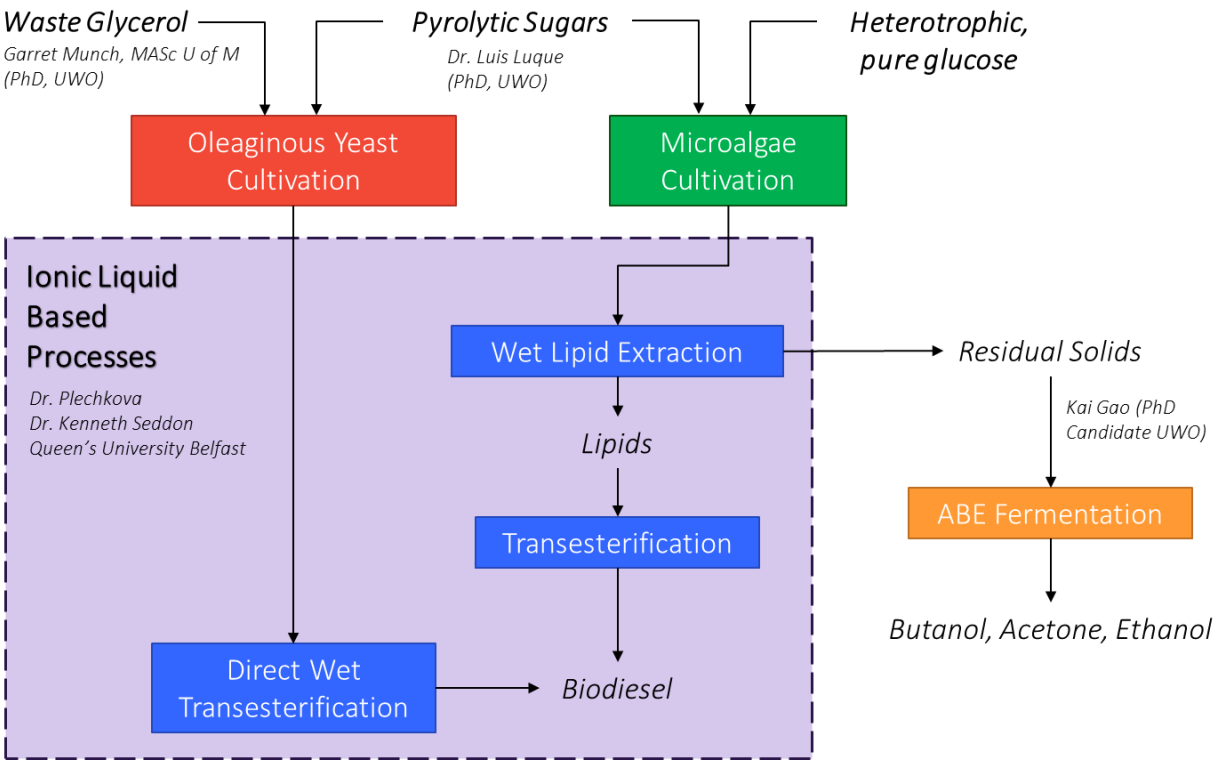


Figure 1.3-1 – An overview of the work presented in this thesis. First, yeast and algae were produced as feedstocks for biodiesel production using waste glycerol, pyrolytic sugars, and glucose. These feedstocks served as the inputs for the development of an ionic liquid aided lipid extraction process, followed by a direct transesterification process. Finally, residual solids were recovered and fermented into biobutanol.

Chapter 2 – Literature Review

2.1 Preface to Chapter 2

The nature of this thesis is highly interdisciplinary spanning topics from cultivation of microalgae to the chemistry of ionic liquids. As such, a literature review was prepared only for the central theme of this work, the use of ionic liquids for biomass processing in order for this thesis to remain as concise as possible.

Chapter 2 is divided into two subsections reviewing the use of ionic liquids for lignocellulosic biomass processing and then specifically algal biomass processing. *Section 2.2* was prepared and submitted as a book chapter prepared for the Handbook of Biorefinery Research and Technologies. Due to the style and reference restrictions imposed by the editors, information generally regarded as accepted such as the general chemistry of ionic liquids is not referenced from other works. This chapter was included in this thesis in order to provide a comprehensive background of the mechanisms of ionic liquids processing of biomass. Furthermore, this section presents a naming system used throughout this thesis which will allow the reader to quickly understand the chemical structure associated with an ionic liquid.

Section 2.3 was prepared as a mini-review of the current work in the field of ionic liquids and microalgae which was prepared in June 2016 for Current Opinions in Green and Sustainable Chemistry. As such, results of future chapters is cited in this work as already published data and a thorough comparison to most recent published works is made here. The use of ionic liquids for the disruption of oleaginous yeast is not reviewed here as currently no literature has yet reported the used of ionic liquids for yeast bioprocessing.

2.2 Review of Ionic Liquid Pretreatment of Biomass

Valerie Orr and Lars Rehmann

Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St, London, Ontario, Canada, N6A 5B9

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it was prepared for the Handbook of Biorefinery Research and Technologies (Submitted March 2016, currently under revision), Park, J.M (ed.), Springer. Permission to reproduce figures within this chapter was awarded.

2.2.1 Abstract

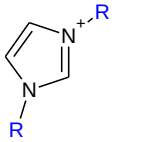
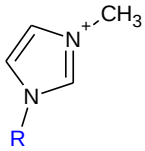
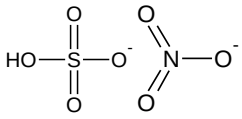
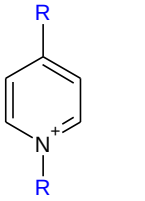
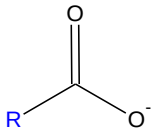
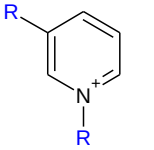
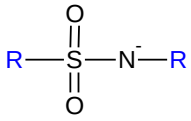
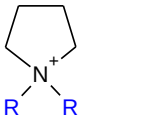
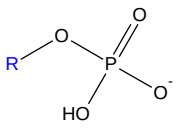
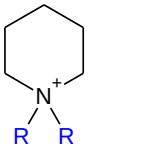
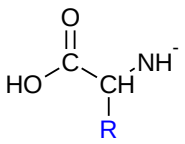
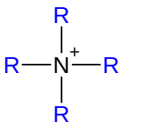
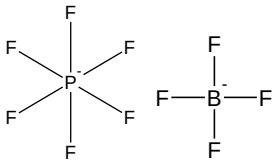
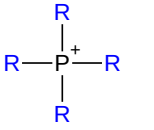
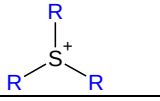
Ionic liquid pretreatment and its subsequent saccharification is a promising emerging method of biomass processing. General considerations when working with ionic liquids, the physiochemical effects of ionic liquid pretreatment as well as some of the technical considerations of ionic liquid based processes will be discussed. The effects of ionic liquid pretreatment on the subsequent acid or enzymatic saccharification as well as alternative processing schemes for saccharification are considered. Finally, applications of ionic liquid biomass processing on emerging feedstocks are discussed.

2.2.2 Introduction

Ionic liquid mediated pretreatment or saccharification of biomass is an emerging area of research and process development. Ionic liquids are liquid salts with melting temperatures below 100°C. For many ionic liquids, this means they are solids at room temperature and must be heated in order to melt them. Ionic liquids are typically viscous however, viscosity decreases with temperature and with the presence of impurities like water. Like all salts, ionic liquids are composed of two ions, however in the case of ionic liquids these ions cannot coordinate to form a regular crystal structure due to their delocalized charge. Charge delocalization is typically achieved by using large asymmetric organic groups, particularly for the cation, however, hydrogen bonding within the ionic liquid can also contribute to a low melting temperature. Several cations have proven to be excellent starting points for ionic liquid synthesis and are shown in **Table 2.2-1.** Imidazolium is the most commonly used cation for ionic liquid synthesis and unsurprisingly the ionic liquids it forms are the most highly studied for lignocellulose bioprocessing.

Researchers propose there may be millions of possible ionic liquids based on the growing list of potential cations and anion combinations. Currently ionic liquids are exhibiting such differing kinetics and thermodynamics than traditional molecular solvents that the chemistry can often be unpredictable with our current level of understanding. While at times this makes predicting applications based on structure very hit and miss, this is also a source of excitement and is spurring current advances in the fields of ionic liquid pretreatment of biomass.

Table 2.2-1 - Some common ionic liquid cation and anion structures and their abbreviations

Name of Cations	Structure	Name of Anions	Structure
$[C_nC_nim]$ 1-alkyl-3-alkyl-imidzolium		Halides	Cl^- Br^-
$[C_nmim]$ 1-alkyl-3-methyl-imidazolium		Acids	
$[C_nC_{1\beta}py]$ 1-alkyl-4-methylpyridinium		Carboxylic Acids	
$[C_nC_nC_{1\beta}py]$ 1-alkyl-3-methylpyridinium		Amides (S, P, C)	
$[C_nC_npyr]$ N-alkyl-N-alkylpyrrolidinium		Alkyl-substituted phosphate and sulfates	
$[C_nC_npip]$ 1-alkyl-1-alkylpiperidinium		Amino acids	
$[N_{nnnn}]$ Tetraalkylammonium		Fluorinated Compounds	
$[P_{nnnn}]$ Tetraalkylphosphonium			
$[S_{nnn}]$ Trialkylsulphonium			

2.2.1 General Structure and Properties of Ionic Liquids

Several structures have proven to be good starting points for ionic liquid synthesis and are shown in **Table 2.2-1**. One of the most common cation used is dialkylimidazolium cations, however six carbon heterocyclic cations like pyridinium and pyrrolidinium as well as highly substituted ammonium, phosphonium, and sulfonium ions are also commonly used. Anions vary considerably from halides like chloride to fluorinated phosphate or dicyanamides. Ionic liquids containing carboxylic acids or amino acids as anions are an emerging class of ionic liquids which are primarily being pursued as potentially biodegradable ionic liquids.

Ionic liquids have several highly desirable properties which make them unique from traditional organic or polar solvents. Solvents like water or hexane evaporate when heated close to their boiling points, this is the basic principle of distillation operations, however ionic liquids have very high boiling points sometimes greater than 400°C. The strong coulombic charges present in ionic liquids prevent them from evaporating and many ionic liquids are known for their very low vapour pressures. The low volatility of ionic liquids is why they are often considered a "*green solvents*" as they don't cause pollution in the form of volatile organic compounds (VOCs). However, recent studies of the toxicity of ionic liquids and their potentially non-renewable synthesis pathways indicates that many ionic liquids are not intrinsically green and this should be assessed on a case by case basis. Current interest in synthesis of renewable ionic liquids is largely focused on the use of cholinium as the cation combined with amino acid anions which have been shown to have excellent biodegradability (Petkovic et al. 2010; Liu et al. 2012). Regardless, one distinct advantage of replacing organic solvents in a process with ionic liquids is their low flammability. Finally, ionic liquids may also exhibit high electrical conductivity.

Melting temperature of ionic liquids will play a large role in determining the operational range of any process and thus the effect of various structural changes on melting temperature has been thoroughly studied. The anion has a greater effect on melting temperature than the cation, however, melting temperatures increase with shorter chain lengths or greater degree of side chain branching. The presence of oxygen or double bonds

in the side chains can also reduce viscosity, while the use of small anions such as chloride or bromide increase viscosity and melting temperature (Pinkert et al. 2009). Accordingly, the presence of halide impurities will also increase viscosity while solvent contaminants have the opposite effect. For this reason, significant efforts into the development of halide free synthesis routes are a growing area of research. Viscosity issues can be somewhat alleviated by increasing the processing temperature however this is at the expense of increased energy usage. Otherwise, branched side chains in dialkylimidazolium based ionic liquids demonstrate lower viscosities (Pinkert et al. 2009). Finally, intermolecular hydrogen bonding can be significant in imidazolium-halide ionic liquids which is thought to influence the melting temperature and viscosity of ionic liquids.

One of the most important aspects of ionic liquids are their relatively high thermal stabilities (300-400°C) (Pinkert et al. 2009). In general, phosphonium have shown greater thermal stability than their ammonium based counterparts, however few have been tested for dissolving biomass. Some anions like $[PF_6]$ and $[BF_4]$ may also degrade in the presence of water to hydrofluoric acid which may account for some of the catalytic activity seen in these ionic liquids and which also has its own particular safety concerns. Additionally, protic ionic liquids which possess at least one proton which can dissociate may not conform to these generalization as they often exhibit significant volatility and reactivity which will also affect their thermal stability (Greaves and Drummond 2008).

Ionic liquids can also exhibit a range of polarities. However, as solvent polarity has not been adequately quantified for traditional molecular solvents to date it is difficult to extend these imperfect theories to ionic liquids which add an additional coulombic aspect to the molecular dynamics of the system. However, many ionic liquids are soluble in water and exhibit significant hydroscopic behavior. Since water impurities can later effect the biomass processing capability of the ionic liquid, care must be taken after drying ionic liquids to prevent water accumulation. Miscibility with water is highly dependent on the hydrophobicity/hydrophilicity of the anion and cation. Generally, hydrophobic ionic liquids are made with cations with long side chains. One sub-category of ionic liquids called *switchable solvents* are ionic liquids which are capable of changing polarity or

hydrophilicity when a trigger is applied. These are typically made from amidines where carbon dioxide is used as a trigger to convert them to carbonate or bicarbonate salts (Dominguez de Maria 2014; Paudel et al. 2015). These types of solvents could be useful when a single process requires solvents with multiple polarities. However, most biomass processing studies to date use hydrophilic ionic liquids and water is used as an anti-solvent to recover the ionic liquid and separate it from the fractionated biomass. The use of more volatile solvents for this step may be much less energy intensive but has not been thoroughly investigated. Many ionic liquids which are soluble in water are also soluble in short chain alcohols or acetone.

2.2.2 Lignocellulosic Biomass Processing

While the study of the utilization of lignocellulosic biomass for biofuels and chemical production has been an area of intense study since the 1970s oil crisis, ionic liquids have only begun to receive increased attention since the 1990s due to advances in their synthesis. Before this, ionic liquids research was a more neoteric field of study and only a limited number of ionic liquids were known. Furthermore, the reactivity of these ionic liquids with air and water made their applications limited. Ionic liquids as cellulose solvents were revived in the early 2000s with the report from of Swatloski et al. (2002) who demonstrated several imidazolium based ionic liquids capable of dissolving cellulose at concentrations up to 25 wt%. Before this, cellulose dissolution was limited to a handful of commercial processes which all have severe disadvantages. First, the viscose process which solubilized cellulose through a derivitizing process which converts it to xanthate using carbon disulfide and alkali treatment. Cellulose is regenerated by neutralization with a strong acid. The lyocell process is a non-derivizating process whereby cellulose is dissolved in N-methylmorpholine N-oxide. The lack of suitable solvents for cellulose hydrolysis is a direct reflection of the highly recalcitrant structure of cellulose.

2.2.2.1 Structure of lignocellulosic biomass

Lignocellulosic biomass is a complex mixture of three main biopolymers, cellulose or glucan, hemi-cellulose or xylan, and lignin. The relative amounts of each biopolymer is species and environment specific. Cellulose is a polymer of glucose and it is by far the

polymer of greatest interest in bio-refining. Cellulose is a structural carbohydrate found the cell wall of plants and accordingly is one of the most abundant sources of glucose in the world. Cellulose is a polymer of cellobiose; a disaccharide formed by the β(1-4)-glycosidic linkage of two glucose molecules. Accordingly, cellulose degradation generally requires multiple enzymes, some to cut within the cellulose molecule and reduce the degree of polymerization (endocellulase), some to depolymerize the cellulose (exocellulase), and some to degrade cellobiose into glucose (glucosidase).

The three-dimensional structure of cellulose is complex and directly affects future processing steps. Through hydrogen bonding, cellulose β-sheets are packed into microfibers which are highly crystalline. In nature, cellulose is mainly present in the form of cellulose I (**Figure 2.2-1**- Left). This is a direct result of its mechanism of synthesis. Cellulose is synthesized adjacent to the plant cell wall by an enzyme protein complex called the rosette terminal complex (TC). The cytoplasmic portion of the complex synthesized multiple cellulose sheets positioned in a parallel manner. Crystallization occurs shortly after the parallel sheets are forced through the rosette TC aperture. The second most common form of cellulose crystal structure is cellulose II which is for all extensive purposes a synthetic creation (**Figure 2.2-1**- Right). It is essentially formed by anti-parallel β-sheets positioning which is more thermodynamically stabile as it allows three intermolecular hydrogen bonds to form.

Cellulose II crystal structure is the polymorph found in regenerated cellulose such as that produced by ionic liquid pretreatment. Cellulose II forms upon regeneration as it is the more thermodynamically stable form of cellulose due to shorter intermolecular hydrogen bond lengths. Finally, cellulose can also be found in a disorganized state called amorphous cellulose. Crystallinity index (CrI) is used to quantify the proportion of crystalline cellulose and amorphous cellulose. There are several methods for measuring crystallinity but commonly X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR) are used. Crystallinity index can be calculated in different manners and may encompass the crystalline peaks caused by both cellulose I and the formation of cellulose II making it difficult to relate the CrI to

future digestibility as cellulose I is believed to be more hydrophobic making it much less digestible. Separate quantification of the CrI of cellulose I and II may provide a better picture of the mechanisms of cellulose digestion.

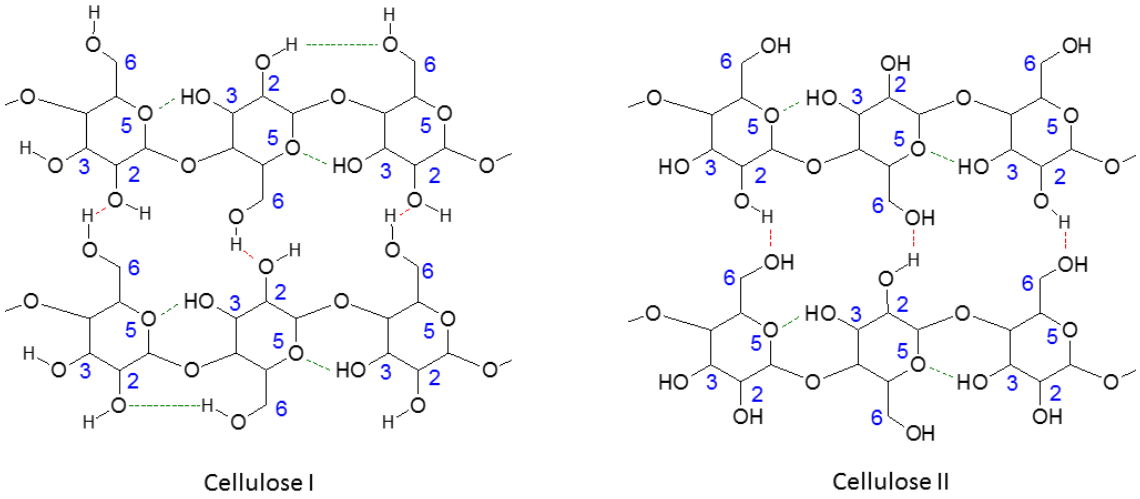


Figure 2.2-1 – *Cellulose lattice structures*. Cellulose lattice structures. Both polymorphs contain O(3)H-O(5)' intramolecular hydrogen bonds and cellulose I contains an additional O(2)H-O(6)' intramolecular hydrogen bond but they differ in the location of the intramolecular bonds. Cellulose I contains a O(3)H-O(6)" intermolecular hydrogen bond while cellulose II forms a O(2)H-O(6)" intermolecular hydrogen bond and thus does not form the O(2)H-O(6)' intramolecular hydrogen bond.

Another further challenge in lignocellulose biomass processing is the frequent associations between cellulose, hemicellulose, and lignin. Lignin in particular surrounds the cellulose fibers waterproofing them and making them highly resistant to enzymatic and chemical degradation. Lignin is a polymer composed mainly of guaiacyl, syringyl, and p-hydroxyphenyl subunits which may be useful as precursors for resins, epoxies, adhesives, or flavoring molecules (Brandt et al. 2013). However, cellulose and hemi-cellulose which are composed of sugar subunits are of greater interest for bioconversion to biofuels or other chemicals. Only a few species of lignin degradation enzymes (called laccases) have been identified which attests to the highly resistive structure of this polymer. While chemical hydrolysis is possible, phenol based inhibitors can form as by-products which can be detrimental during subsequent fermentation or cultivation steps. Hemi-cellulose which is

also interconnected with cellulose and lignin is a short amorphous branched polymer composed of several different types of sugars. The most common sugar subunits are xylose; a five carbon sugar, and mannose, but glucose, galactose, arabinose, and rhamnose are all possible subunits. The composition of hemi-cellulose varies significantly between soft and hard wood and varies with species and also structural location (stems, leaves, roots, etc.). Hemi-cellulose is much more susceptible to hydrolysis than cellulose due to its low degree of polymerization and lack of crystallinity (Brandt et al. 2013). It may be desirable to recover hemi-cellulose in robust biochemical processes using organism capable of utilizing five carbon sugars like xylose and a variety of hexoses, however in cases where these sugars cannot be utilized it may be more practical to recover them separately from cellulose.

Due to the complexity of lignocellulosic biomass, the three main goals of pretreatment are typically as follows: (i) removal of lignin, (ii) reduction of cellulose crystallinity, and (iii) increase accessible surface area of cellulose. With these goals in mind, several possible processing schemes for lignocellulosic biomass with ionic liquids are illustrated in **Figure 2.2-2**.

The most common strategy uses an antisolvent to precipitate the cellulose and wash the ionic liquid, hemi-cellulose, and lignin from the regenerated cellulose. Regenerated cellulose (as Cellulose II) can then be easily hydrolyzed to glucose using enzymatic or acid hydrolysis. The less common strategy is shown below and involves the direct catalysis of dissolved cellulose into glucose followed by separation and purification of the glucose typically using ion-exchange chromatography (discussed in *Section 2.2.3.1*). The direct fermentation of ionic liquid containing sugars has been attempted but due to toxicity problems results have been poor in highly concentrated ionic liquids.

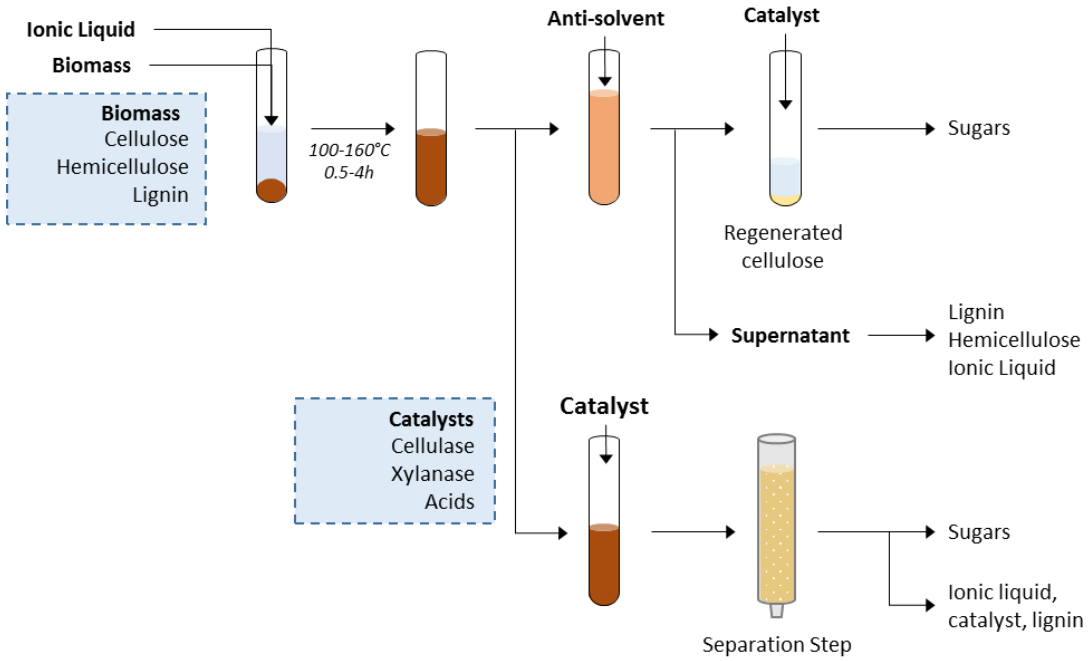


Figure 2.2-2 – The two main strategies used for lignocellulosic biomass processing using ionic liquids.

2.2.2.2 Cellulose solubility in ionic liquids

[C_4mim][Cl] was the first reported ionic liquid capable of solubilizing cellulose up to weights of 25% (Swatloski et al. 2002). Since then more than 30 ionic liquids have demonstrated some cellulose dissolution ability. Cellulose solubility in many ionic liquids has been recently tabulated (Wang et al. 2012). The anion structure is thought to play a more significant role in the solubility of cellulose than the cation. Typically, ionic liquids with coordinating anions like Cl, Br, acetate and phosphates exhibit greater cellulose solubility while shorter side chain length cations are most suitable. More extensive studies have demonstrated that the chloride ion in [C_4mim][Cl] or [C_2mim][Cl] does in fact interact with the hydroxyl groups of the cellulose in a 1:1 stoichiometric ratio (Remsing et al. 2006). This indicates that a strong hydrogen bond acceptor is necessary for cellulose solvation.

A popular empirical method for determining solvent polarity is the Kamlet-Taft solvatochromatic relationship where hydrogen bond donor/acidity (α), hydrogen bond acceptor/basicity (β), and dipolarity/polarizability (π*) are measured using optical

measurement of closely related dyes in the ionic solvent. As predicted by the reports of Remsing, (2006), high hydrogen bond basicity (otherwise known as a strong hydrogen bond acceptor) has been correlated to high cellulose solubility (Pinkert et al. 2009). Ionic liquids containing anions with good hydrogen bond acceptor ability like Cl^- or acetate which have $\beta > 0.8$ as depicted in **Figure 2.2-3** are capable of dissolving cellulose such as [C_2mim][Ac] or [C_4mim][Cl] (Xu et al. 2010; Brandt et al. 2013). The cation polarity is thought to have a lesser effect on cellulose solubility, however, increasing the side chain length of [C_2mim][Ac] decreases cellulose solubility, as does the substitution of hydroxyl groups. It has been proposed that any functionalization which decreases the difference between hydrogen bond acidity (α) and basicity (β) of the ionic liquid will decrease cellulose solubility (Pinkert et al. 2009; Brandt et al. 2013).

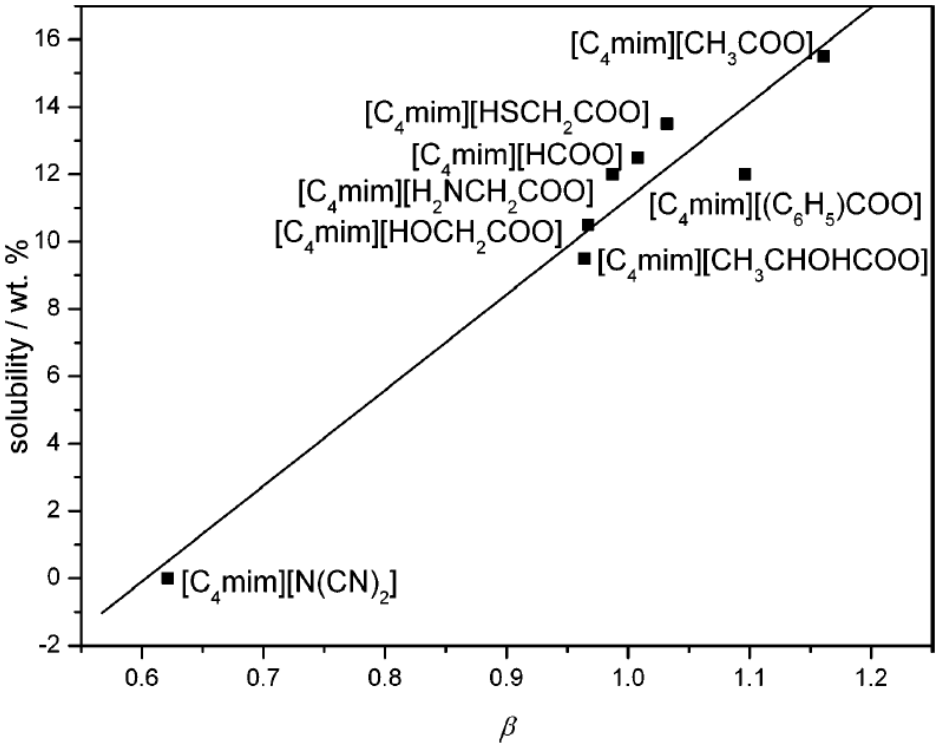


Figure 2.2-3 – Solubility of cellulose for [C_4mim] based ionic liquids in terms of the Kamlet Taft hydrogen bond basicity (β). Reproduced from (Xu et al. 2010).

High variability of Kamlet-Taft parameters reported in the literature is likely due to the presence of impurities such as water and halides which can severely affect the polarity of the solvent. Impurities such as water can also significantly reduce the ionic liquid's ability to dissolve cellulose. It is though that competitive hydrogen bonding impedes the disruption of the cellulose hydrogen bonding network and thus, non-hydrated anions and ionic liquids are more effective solvents.

Finally, one of the most significant limitations of an ionic liquid based process is the high viscosity of the ionic liquid biomass mixtures when high weight percentages of cellulose are dissolved in the ionic liquid. Cellulose dissolved in 1-allyl-3-methylimidazolium chloride or [Amim][Cl] at 4% and 8% wt. were reported to have viscosities of 110 and 14880 Pa·s, respectively (Zhang et al. 2005). The zero shear viscosity of ionic liquids containing cellulose is highly dependent on the cellulose concentration in solution and increases with higher weight percentages, effectively limiting the maximum cellulose concentrations which can be practically managed due to economic limitations. However, ionic liquid/cellulose solution viscosity does decrease with increasing processing temperature.

2.2.2.3 Cellulose regeneration and delignification

The vast majority of ionic liquid studies have focused on the regeneration of cellulose from the ionic liquids prior to hydrolysis. Regeneration is typically achieved by the addition of an anti-solvent such as water which competes with hydrogen bonds between the ionic liquid and the cellulose resulting in the precipitation of the cellulose. This also accounts for the low cellulose solubility in wet ionic liquids (water content >1% wt.). While the effects of various polar solvents on regeneration of cellulose from ionic liquids have not been studied in detail, several have been shown to be good anti-solvents including water, acetone, and ethanol (Swatloski et al. 2002). The macroscopic morphology of the regenerated cellulose was dependent on the method of mixing. Simple mixing of the cellulose containing ionic liquid with an anti-solvent results in the formation of a powdery floc while fibers can be generated when using extrusion (Swatloski et al. 2002). Regeneration of cellulose in thin films has been studied more extensively. Using water and

[C_4mim][Cl] or [C_2mim][Ac], regeneration of cellulose indicated this process is controlled by diffusion which can be described using Fick's law similar to the lyocell process (Sescousse et al. 2011). Accordingly, regeneration of cellulose occurs faster when the anti-solvent is heated or mixing is applied increasing mass transport. Regardless, the process is not entirely understood.

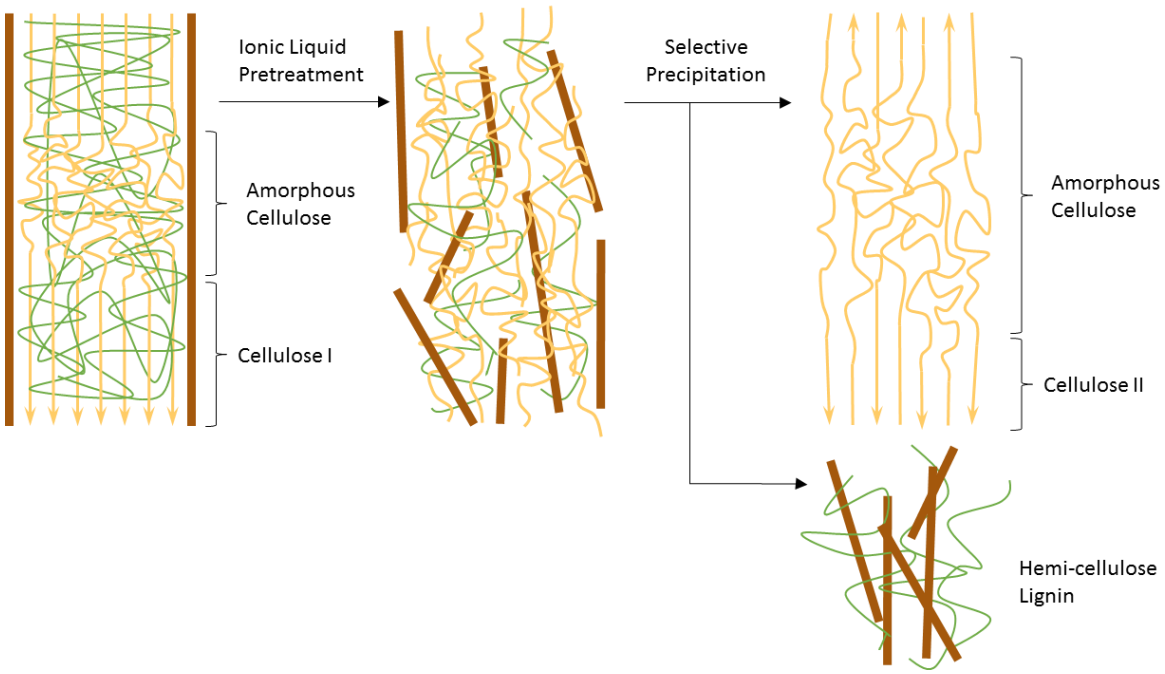


Figure 2.2-4 – *Proposed mechanism of lignin rejection and cellulose regeneration*. Cellulose, hemicellulose, and lignin are solubilized by the ionic liquid pretreatment. Cellulose is selectively precipitated from the mixture and reforms as a mixture of cellulose II and amorphous regions. Lignin and hemicellulose which are more soluble in aqueous ionic liquids can be further precipitated and through this mechanism cellulose is purified and transformed into a more digestible feedstock (Singh et al. 2009).

Dynamic modelling of this system indicates that cellulose is regenerated as a mixture of cellulose II and amorphous cellulose as depicted in **Figure 2.2-4** (Ding et al. 2012b). This is consistent with experimental results which have demonstrated a marked decrease in cellulose crystallinity after ionic liquid pretreatment and regeneration. This allows much more facile chemical or enzymatic hydrolysis of the cellulose. High sugar yields during saccharification are correlated to the overall decrease in CrI (increase in amorphous cellulose) and cellulose II is more susceptible to hydrolysis than cellulose I

(Kim et al. 2010). Conversion of cellulose I to cellulose II which may not be reflected in the CrI may be a better indicator of cellulose digestibility (Wada et al. 2010). It is hypothesized that cellulose I crystal structure is more hydrophobic than cellulose II which inhibits the penetration of water, enzymes, or catalysts needed for cellulose hydrolysis making it less susceptible to hydrolysis.

One of the most promising functions of ionic liquid pretreatment is the possibility of separating the lignin and the hemi-cellulose from the cellulose. Lignin can be recovered from the mixture of ionic liquid, antisolvent, and hemi-cellulose after cellulose precipitation by further dilution using larger quantities of dilutant than needed for cellulose precipitation. This allows a selective two step fractionation of the deconstructed biomass, however, a drawback of greater solvent use is the greater energy consumption necessary for their subsequent distillation in order to recover and reuse of the ionic liquid. Another two step process employed is the selective precipitation of cellulose using water followed by evaporation of the water from the ionic liquid and precipitation of lignin from the ionic liquid using ethanol (Lateef et al. 2009). Alternatively, liquid/liquid separation (or similar processes) using an organic solvent which is immiscible with water (the most commonly used antisolvent) but has a high affinity for lignin can be used to recover the lignin.

2.2.2.4 Effect of Ionic Liquid Pretreatment on Cellulose Crystallinity, DP, and surface area

As previously stated, ionic liquid pretreatment has been associated with increased digestibility of cellulose through a reduction of cellulose crystallinity. However, ionic liquid structure, operating conditions, and type of biomass have a profound effect on the effectiveness of the pretreatment. More specifically, solid loading (wt%), reaction time (h) and temperature (°C), as well as antisolvent choice will likely affect the subsequent digestibility of cellulose. The relative biomass composition of lignin, cellulose, and hemi-cellulose, the biomass moisture content, as well as the crystallinity of the biomass will also likely affect the effectiveness of the pretreatment, however, few of these factors have been extensively studied.

Firstly, crystallinity was general found to reduce while surface area and pore size generally increased under modest ionic liquid pretreatment conditions for several types of lignocellulosic biomass. In switchgrass, crystallinity was significantly reduced from 26.2 to 2.6% after pretreatment with [C_2mim][Ac] at 160°C for 3h with a solid loading of 3% followed by regeneration using water (Li et al. 2010a). Dilute acid pretreatment of the same biomass increased the CrI to 39%. Similarly, an average reduction of CrI of 75% was achieved with [C_2mim][Ac] pretreatment at 120°C for 30 min followed by regeneration for switchgrass, popular, and avicel (Samayam and Schall 2010). Surface area of cellulose from bagasse was significantly increased after treatment with [C_2mim][Ac] at 120°C for 120 min from 1.38 in untreated bagasse to 131.84 m^2/g (da Silva et al. 2011). This treatment ultimately resulted in almost complete enzymatic hydrolysis of the cellulose in only 48h. Similarly, switchgrass treated with [C_2mim][Ac] at 160°C for 3h at 3% solid loading had significantly higher surface area after ionic liquid pretreatment and regeneration as well as greater average pore volume (Arora et al. 2010). Larger increases in surface area and pore size were found with a higher processing temperature of 160°C and only a minor difference was noted at 120°C.

The ionic liquid choice will also affect the efficiency of the pretreatment. [C_4mpy][Cl] was found to decrease the degree of polymerization when bagasse or eucalyptus was treated at 120C for 60 min at 5% solid loading and but had little effect on the crystallinity while [C_2mim][Ac] was found decrease the crystallinity to a greater extent than the degree of polymerization (Uju et al. 2012). A study using avicel showed that the degree of polymerization was also more greatly affected by pretreatment with [C_4mim][Cl] (21% decrease) than [C_2mim][Cl] (14% decrease) or [C_2mim][Et_2PO_4] (4% decrease) (Vitz et al. 2009).

The general consensus in the literature indicates that crystallinity and degree of polymerization tends to decrease with increasing processing time and temperature, while surface area and porosity increase. The relationship between operating conditions and these factors is specific for each ionic liquid and biomass species. However, these trends are not surprising since solubility generally increases with temperature and increased residence

time should allow for greater diffusion of the solvent into the biomass. Furthermore, small improvements in yield gained by optimization for each biomass and ionic liquid must be evaluated for increases in process efficiency or energy consumption.

2.2.2.5 Solubility and extraction of lignin and xylan in ionic liquids

Many of the ionic liquids used for cellulose dissolution also exhibit moderate lignin solubility (Pu et al. 2007). While this area has not been studied as extensively as cellulose solubility in ionic liquids, delignification of biomass after ionic liquid pretreatment has been observed to increase the digestibility of the regenerated cellulose (**Figure 2.2-5**). Generally, water impurities have a lesser effect on lignin solubility which could be exploited if only the delignification of biomass is desired. Lignin solubility is often determined in ionic liquids using lignin generated from the Kraft pulp process. However, scientists should be cautious when interpreting lignin solubility in this way as it is not necessarily a good indicator of performance using whole biomass. For example, it was found that large, non-coordinating anions like $[PF_6]$ or $[BF_4]$ are not suitable for southern Kraft pine lignin dissolution, but alkyl sulfate anions or fluorinated sulfates like $[MeSO_4]$ or $[CF_3SO_4]$ were effective. Similar results were found with pinewood Kraft lignin, however, higher lignin extraction efficiency was found with chloride containing ionic liquids when extracting lignin from maple wood flour which is likely a result of the higher overall solubility of the whole biomass rather than a single component (Lee et al. 2009). This is also true of $[C_2mim][Ac]$ pretreatment of switchgrass which extracted 62% wt. of the lignin after 3h at 160°C while dilute acid pretreatment was ineffective at removing lignin (Li et al. 2010a). More recently, several renewable ionic liquids synthesized from amino acids and chloninium have exhibited good affinity for both xylan (>5% wt) and lignin (>15% wt.) but have generally low affinity for cellulose (<0.5% wt.) including those made with glycine, alanine, serine, threonine, leucine, methionine, phenylalanine, proline, and lysine (Liu et al. 2012). Higher solubility of lignin was associated with amino acids possessing higher acid dissociation constants (pK_a) except in the case of [Ch][Trp] where the resulting ionic liquid was highly viscous (Liu et al. 2012).

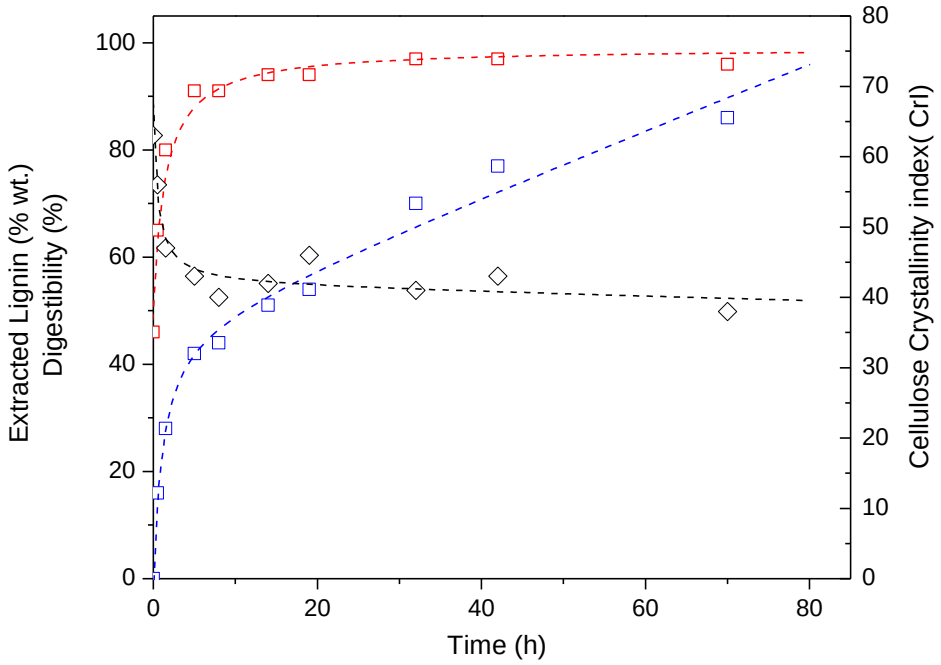


Figure 2.2-5 – Effect of increased processing time on lignin extraction (blue square), digestibility (red square), and cellulose crystallinity (black diamond) with pretreatment using [C_2mim][Ac] at 90°C. Adapted from data provided in (Lee et al. 2009).

Hemicellulose, as a carbohydrate with lower degree of polymerization and a lack of crystallinity has generally exhibited excellent solubility in ionic liquids such as up to 21% wt. in [Amim][Ac] (Zakrzewska et al. 2010). Accordingly, many studies have reported significant hemicellulose extraction during ionic liquid pretreatment of whole biomass. A mass balance analysis of this ionic liquid pretreatment for corn stover was able to remove 82.9% wt. of the lignin and 89.4% wt. of the xylan which was entirely recovered in the ionic liquid fraction (Li et al. 2011a). Less than 2% of the xylan and none of the lignin was removed by ammonia fiber expansion pretreatment however, a greater proportion of the glucan was recovered using this method (Li et al. 2011a). Higher total reducing sugars were recovered from the ionic liquid pretreated corn stover using cellulase digestion than AFEX pretreatment even through glucan recovery was higher with AFEX indicating that digestion was improved by the dual remove of lignin and hemi-cellulose during ionic liquid

pretreatment (Li et al. 2011a). Increasing processing time of maple wood flour with [C_2mim][Ac] was found to correlate to lower cellulose CrI, increased lignin extraction, and increased xylan removal which resulted in higher enzymatic digestibility (Lee et al. 2009). Higher processing temperatures also resulted in higher delignification of switchgrass which in turn correlated to greater digestibility (Arora et al. 2010).

2.2.3 Effect of ionic liquid Pretreatment on Subsequent Enzymatic Saccharification

Enzymatic saccharification is a milder process and results in more readily fermented sugars in many cases than acid catalyzed saccharification. Although this process is known to produce less fermentation inhibitors, it's applications are limited by slow kinetics and high process costs. However, pretreatment of cellulosic biomass with ionic liquids has been demonstrated to significantly increase the digestibility of the biomass by cellulase enzymes. Pretreatment of switchgrass with the ionic liquid [C_2mim][Ac] for 3h at 160°C increase the digestibility from 2.7% in untreated biomass to 96.0% after ionic liquid pretreatment (Li et al. 2010a). Ionic liquid pretreatment significantly improves the enzyme kinetics of saccharification as well as demonstrated in **Figure 2.2-6**. After ionic liquid pretreatment in switch grass the initial enzyme velocity increased from 0.3 in untreated switchgrass to 18.5 mg L^{-1} min^{-1} (and increase of over 61 fold) (Li et al. 2010a). Similar results of dramatic increases in enzyme velocity have been demonstrated for wheat straw(Fu et al. 2010), corn stover, and miscanthus (Shill et al. 2011). Complete removal of the ionic liquid is essential for hydrolysis and any ionic liquid trapped within the regenerated cellulose may cause enzyme inhibition as hydrolysis occurs releasing the ionic liquid into solution (Zhao et al. 2009b). Even at low concentrations ionic liquids are capable of inhibiting cellulase activity.

Digestibility of the ionic liquid pretreated biomass is also dependent on the ionic liquid used for pretreatment. While pretreatment of bagasse with [C_2mim][Ac] for 2h at 120°C resulted in 100% conversion of available glucan by cellulase within 48h, [C_1mim][Me_2PO_4] only yielded 60% conversion(da Silva et al. 2011). The overall order of cellulase digestibility by ionic liquid was [C_2mim][Ac] > [C_1mim][Me_2PO_4] > Amim][Cl] > [C4mim][Cl] ≥ [C_2C_{1OH}py][EtSO4] > [C4mim][$(CF_3SO_2)_2$N] > untreated bagasse which

was roughly the same for hemi-cellulase digestion (da Silva et al. 2011). Increased digestibility was linked to increased extraction of water soluble solids which improved with longer processing times (da Silva et al. 2011).

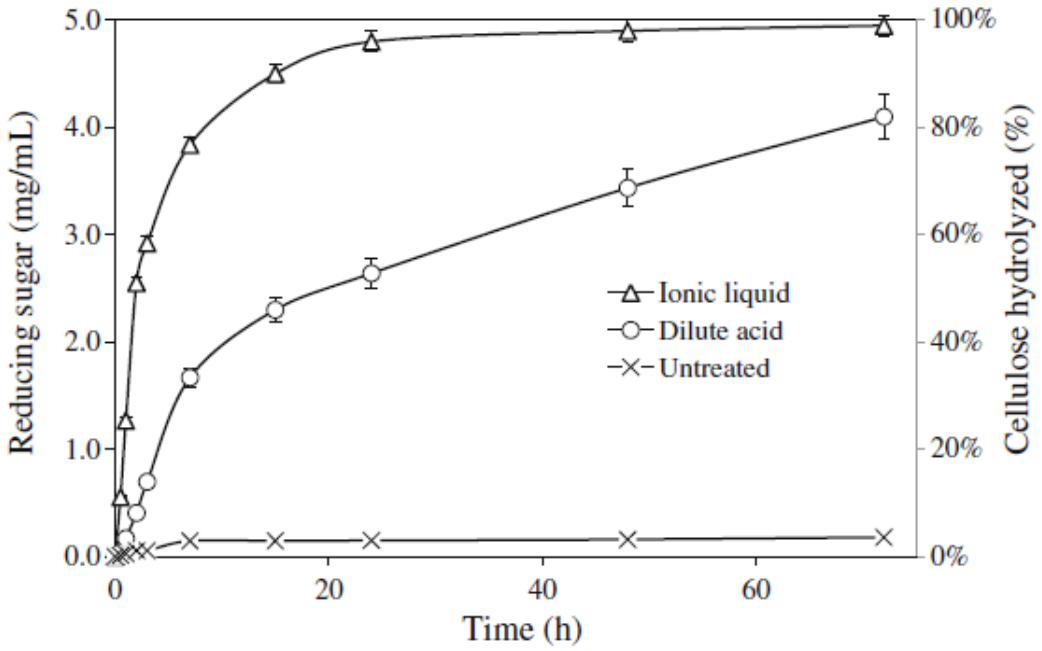


Figure 2.2-6 – *Enzymatic hydrolysis of ionic liquid pretreated switchgrass.* Comparison of dilute acid pretreated switchgrass and ionic liquid pretreated switchgrass using [C_2mim][Ac] for 3h at 160°C. Reproduced from (Li et al. 2010a).

Enzymatic hydrolysis of cellulose is affected by many factors such as the lattice structure (cellulose I, II, etc.), the surface area, the porosity, the particle size, the degree of polymerization, the extent of lignin and hemicellulose interconnections, and the crystallinity (Mansfield et al. 1999). The enzymes themselves also can exhibit product inhibition, thermal inactivation, or irreversible adsorption (Mansfield et al. 1999). Cellobiose inhibition can be prevented by the addition of β-glucosidase to form glucose and accordingly most commercial enzyme cocktails include it. Much of the variation between enzymatic hydrolysis studies using ionic liquid pretreated biomass is likely due to the differences in composition and structure of the starting materials used, however, the origin of cellulases used can also play a role. Enzymatic hydrolysis of [C_2mim][Ac] treated

popular, switchgrass, and Avicel using 5 different commercial cellulase/xylanase cocktails showed significant differences between the digestion of each type of biomass with a single enzyme cocktail as well as between a single type of biomass and different enzyme cocktails (Samayam and Schall 2010). Conversely, enzymatic hydrolysis of hemi-cellulose using xylanases after ionic liquid pretreatment has not seen similar improvements as cellulose, however, this is likely due to the lower degree of polymerization and lack of crystallinity of hemi-cellulose (Zhao et al. 2009a).

2.2.3.1 Direct Hydrolysis of Cellulose in Ionic Liquids

Most studies to date have focused on the hydrolysis of regenerated of cellulose. However, regeneration of the cellulose also allows for some cellulose II crystalline regions to reform potentially reducing rates of hydrolysis. Therefore, several researchers have attempted hydrolysis directly on cellulose dissolved in ionic liquids which theoretically should have low crystallinity and consequently high accessibility for catalysis to reaction sites required for hydrolysis to occur. However, practically, direct hydrolysis of cellulose in ionic liquids is a more complex process than regeneration of cellulose prior to hydrolysis. Direct hydrolysis is limited by the high viscosities of cellulose laden ionic liquids and requires much more complex purification processes to separate the released sugars from the ionic liquid. This is essential if the sugars are to be used for fermentation as many ionic liquids are toxic to microorganisms even in small amounts. In some cases, biocompatible ionic liquids may be used which will not adversely affect the subsequent fermentation at low enough concentrations. Regardless, separation of glucose and ionic liquid can be achieved a variety of ways such as separation on an alumina resin (Feng et al. 2011).

Finally, another further limitation to this process is the requirement for water as a reactant in the hydrolysis reaction which as discussed earlier can reduce the solubility of cellulose in the ionic liquid through competitive hydrogen bonding. Therefore, in many cases efforts to restrict the water added to the reaction mixture are undertaken, however, in most cases the addition of the catalysts which are almost always supplied as aqueous solutions (acids, enzymes) will also supply the water needed for hydrolysis.

Enzymatic hydrolysis

While enzymatic hydrolysis is generally poor in ionic liquids due to enzyme inactivation, studies of the tolerance of lignocellulosic enzymes for ionic liquids may be of use in order minimize washing steps prior to hydrolysis. Another further complication of direct enzymatic hydrolysis of cellulose in ionic liquids is the relatively high operating temperatures used for the most common ionic liquids (100-160°C) are likely to deactivate cellulase enzymes whose optimal temperature is typically around 50°C. Regardless, some ionic liquids at dilute concentration have been found to be compatible with cellulases. $[C_1mim][Me_2PO_3]$ was found to be the most biocompatible ionic liquid and allowed the *in situ* hydrolysis of corn cobs when diluted below 40% wt (Engel et al. 2010; Li et al. 2010b).

Acid hydrolysis

Acid hydrolysis of ionic liquid dissolved cellulose has seen more success than enzymatic hydrolysis. Hydrolysis of cellulose in $[C_4mim][Cl]$ using 7% wt. HCl at 100°C for 30 min generated 66% of the theoretical yield which is a substantial improvement in reaction time compared to enzymatic hydrolysis which typically takes at least 24h with ionic liquid pretreated biomass (Li and Zhao 2007). However, much like acid pretreatment steps, acid hydrolysis of cellulose in ionic liquids has also been found to generate 5-hydroxymethylfurfural (5-HMF) a known inhibitor to microbial growth (Binder and Raines 2010). Generation of 5-HMF was greatest under low water or anhydrous conditions suggesting glucose was being dehydrated during the reaction. Too much water would result in the precipitation of cellulose from the ionic liquid while intermediate amounts can create an uncooperative gel which is equally unfavorable (Binder and Raines 2010). Strategies for limiting the water content during the hydrolysis reaction has seen improved yields. Gradual water addition over time yielded ~90% conversion using HCl and minimized the generation of 5-HMF (Binder and Raines 2010). Formation of inhibitors may also be promoted by certain common ionic liquid additives or impurities like metal chlorides which promote 5-HMF formation particularly if fructose is present (Zhao et al. 2007). Interestingly, both endo and exoglycosidic activity was detected in the initial stages of acid hydrolysis within

an ionic liquid unlike traditional hydrolysis reactions which exhibit mostly endoglycosidic activity during the initial stages (Li and Zhao 2007). Finally, a strong acid is required for activation of cellulose hydrolysis with HCl, H_2SO_4, and HNO_3 exhibiting better catalytic activity than H_3PO_4 (Fan et al. 2013).

Heterogeneous catalysts are generally preferred for commercial processes due to their ease in reuse. Not surprisingly, several research groups have demonstrated heterogeneous acid catalysis for *in situ* cellulose hydrolysis (**Table 2.2-2**). To date the best results have been found using a sulfonated glucose based material which hydrolyzed 72.7% of the available sugars in 4h using [C_4mim][Cl] at 110°C (Guo et al. 2012). However, HY zeolite used in combination with [C_4mim][Cl] and microwave heating at 240W yielded 47.5% Total Reducing Sugars (TRS) in just under 8 min (Zhang and Zhao 2009). Other catalysis that have shown some promise also include Amberlyst 15 resin, Nafion NR50, and a functionalized sulfonic acid silica catalyst. In the case of Nafon NR50 it was found that this catalyst could only hydrolyze cellulose II crystalline structures and not the naturally occurring cellulose I crystalline regions (Kim et al. 2010). Use of ionic liquids composed of weakly basic anions like acetate or phosphonate were found to inhibit the hydrolysis of cellulose by capturing available H_3O^+ and preventing the activation of hydrolysis when using the ion exchange resin Amberlyt 15 as a solid acid catalyst (Rinaldi et al. 2010). Additionally, the presence of synthesis impurities such as N-methylimidazole was found to decrease the catalytic activity (Rinaldi et al. 2010).

Table 2.2-2 – Hydrolysis yields using heterogeneous catalysts in ionic liquids.

Catalyst	Ionic Liquid	Conditions	Yield (TRS[a])	Reference
Sulfonated carbon	[C_4mim][Cl]	110°C, 4h	72.7%	(Guo et al. 2012)
Superparamagnetic sulfonated carbon	[C_4mim][Cl]	130°C, 3h	68.9%	(Guo et al. 2013)
Sulfonated Silica	[C_4mim][Cl]	70°C, 6h	67%	(Amarasekara and Owereh 2010)
Amberlyst 15	[C_4mim][Cl]	100°C, 5h	28%	(Rinaldi et al. 2008)
Nafon NR50	[C_4mim][Cl], water	160°C, 4h	35%	(Kim et al. 2010)
HY Zeolite	[C_4mim][Cl]	Microwave heating 240W, 8 min	47.7%	(Zhang and Zhao 2009)
Silica encapsulated sulfonated magnetic iron nanoparticles	[C_4mim][Cl]	110°C, 8h	73.2%	(Xiong et al. 2014)
Sulfonated(styrene-co-divinylbenzene)	[C_4mim][Br]	120°C, 8h	92.9%	(Fan et al. 2013)

[a] Total reducing sugars

2.2.4 Technical Considerations for Ionic Liquid Mediated Lignocellulosic Processes

The largest obstacle for the adoption of lignocellulose derived sugars in today's bioprocesses remains their cost-effective degradation and purification. The viability of any ionic liquid based process will depend on several factors including number of operations, processing efficiency, and reuse of the ionic liquid. The cost of ionic liquid synthesis is often cited to discount ionic liquids as a solvent. While the cost of ionic liquids is considerably more than traditional organic solvents, most ionic liquids are only synthesized on demand and not at any appreciable scale. Large scale production of ionic liquids with commercial applications will generate some economy of scale through process intensification, however, it is generally agreed that much of the value of ionic liquids is generated through their reuse. Regardless of their reuse, much of the cost of ionic liquid synthesis is derived from the raw materials costs of the precursors (Chen et al. 2014). Therefore, cost can be somewhat controlled by selecting ionic liquids synthesized from low cost precursors like choline, glucose, amino acids, and ammonia. Another consideration

during the synthesis of ionic liquids is the copious amounts of solvents often needed to wash impurities from the ionic liquid which some argue mitigate the positive environmental impact ionic liquid based processes may achieve.

There are several energy implications of using ionic liquids. One significant energy requirement is during heat addition for pretreatment and the energy requirements needed for separation and recycling of the ionic liquid. While in most cases ionic liquid pretreatment is conducted at temperatures greater than 100°C, the processing times are also typically shorter than other pretreatment processes and may lead to considerable energy savings. With the increasing number of known room temperature ionic liquids, further study of these ionic liquids ability to dissolve cellulose may be of use. Recycling of ionic liquids is typically achieved by precipitation of dissolved biomass using an antisolvent and thus requires separation of the antisolvent prior to reuse of the ionic liquid. The energy requirement for separating the antisolvent will be highly dependent on the volatile nature of that solvent. Currently water is used in most studies however, separation of water will be costlier than separation of more volatile polar solvents like acetone or ethanol. On the other hand, while these solvents are not overly toxic, these solvents are flammable whereas water is not. Thus requirements for fire safety may be higher for some antisolvents while energy costs may be lower. Consideration must be given to the level of purity required during regeneration of the ionic liquid as solvent impurities can significant affect the biomass dissolution ability of the ionic liquid which may be costly. Another significant energy requirement of an ionic liquid based lignocellulose process is related to the high viscosity of ionic liquid/biomass solutions. As viscosity decreases with increasing processing temperatures or the addition of suitable cosolvents, energy demands between heating and power consumption of mixing will have to be balanced.

Separation and purification may become a greater challenge when using an *in situ* approach to saccharification of cellulose dissolved in ionic liquids without regeneration. One particular advantage of this approach is that an anti-solvent may not be necessary for sugar removal eliminating the need for subsequent distillation of the anti-solvent. However, separation of the sugars from the ionic liquid will likely require a higher cost operation like

chromatography. The desire for the separation of additional value-added products such as lignin may require further processing as well, particularly if these impurities impede subsequent rounds of ionic liquid reuse.

Finally, each ionic liquid may exhibit particular safety concerns which should be dealt with on a case by case basis such as protic ionic liquids which exhibit significant amount of volatility, or fluorinated compounds which may degrade into hydrofluoric acid under some process conditions and require specially constructed vessels.

2.3 Ionic liquids for the fractionation of algae biomass

Valerie C. A. Orr and Lars Rehmann

Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St., London, Ontario, Canada, N6A 3K7

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it was prepared for *Current Opinions in Green and Sustainable Chemistry* (Submitted June 2016, currently under revision).

2.3.1 Abstract

Microalgae have emerged as one of the most promising sources of renewable biomass. However, considerable challenges must be addressed in order to improve the commercial outlook for the production of commodity chemicals. The largest challenge remains the energy intensive and consequently costly process of microalgae harvesting and drying. Ionic liquids have found a niche application in this area by allowing the extraction of lipids from wet biomass at low temperatures in less time than traditional lipid extraction method. Multiple recent studies have advanced the study of wet extraction of microalgae using ionic liquids and elucidated some of the limitations of this process. However, the most promising avenue for ionic liquid based wet extraction lies in the fractionation and recovery of multiple biomass products such as lipids, carbohydrates, and carotenoids, in a single process.

Keywords: Ionic liquids, Microalgae, lipid extraction

2.3.2 Introduction

Algae have received considerable attention in recent years as one of the most promising source of sustainable biomass for the production of fuels and chemicals. Algae present several advantages over terrestrial crops for sustainable biomass production. Firstly, algae do not require soil and are capable of photosynthetic growth using sunlight, carbon dioxide, water, and inorganic salts as their major nutrients allowing for a potentially carbon negative growth process (Chisti 2007). Algae are also capable of producing an assortment of desirable biochemicals for a variety of industries, but of particular interest is the accumulation of lipids by microalgae for the production of biodiesel. However, although they are capable of growth rates much exceeding traditional land crops used for biodiesel production such as soya bean, their overall low cell density in their liquid growth media presents certain challenges during downstream processing. This has so far limited with applications at the commercial scale to a handful of high end products such as the carotenoid astaxanthin, or as a source of protein and antioxidants (Milledge 2011).

Since the late 1990s, greater general awareness of the significant impacts of chemical refining processes on the environment has led to a shift from post-process remediation to the development of greener processes. As the overarching goal of algal based products is to use a renewable resource for the production of chemicals and fuels, it is unsurprising that the development of algae refining processes are also heavily influences by the principles of green engineering. Ionic liquids which have been often touted as green solvents are becoming increasingly used as solvents for lipid extraction, refining of carbohydrates, or extraction of high value products from algal biomass.

Ionic liquids are salts which generally consist of a large asymmetrical organic cation and a variety of anions which have a melting point below 100°C. Cations structures most commonly use nitrogen containing ring structures such as imidazolium or pyridinium structures with various alkyl substitutions, or in contrast alkyl substituted ammonium or phosphonium based structures. Anions can vary considerably from small alkali metals such as chloride or bromide ions, to more complex structures like alkyl esters, carboxylic acids, fluorinated anions, or amides. They have often been described as *green*

solvents due to their low volatility, however, they can vary considerably in their physiochemical properties such as polarity, hydrophobicity, toxicity, and thermostability (Petkovic et al. 2011). Accordingly, a full life cycle analysis of the impacts of both the ionic liquid synthesis and the developed process must be independently assessed before being classified as truly "***green***".

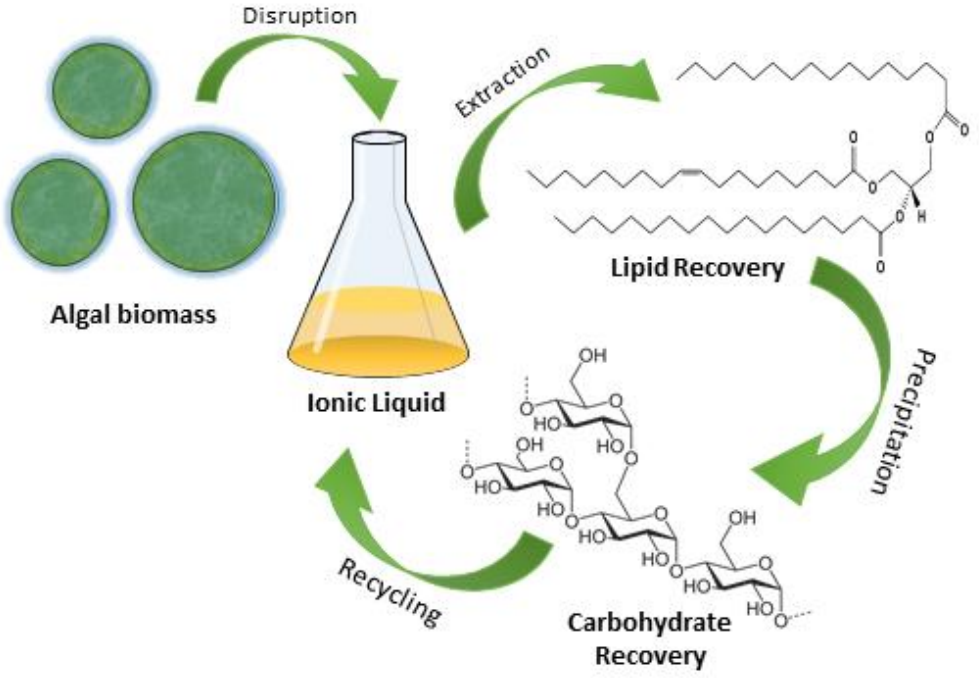


Figure 2.3-1 – Summary of an ionic liquid based biomass fractionation process. After cell disruption with the ionic liquid, the lipids are recovered and the residual biomass containing carbohydrates and proteins is precipitated allowing for recycling of the ionic liquid.

Several specific applications of ionic liquids in algal biomass processing have emerged and is the basis for the organization of this critical review. Firstly, ionic liquid based lipid extraction processes for biodiesel production are discussed in the most detail as the greatest number of publications are focused on this area. This is followed by the emerging areas of carbohydrate refining using ionic liquids, and the extraction of high value or specific end-products is discussed (**Figure 2.3-1**). All available literature was considered in this review as this is an exciting emerging field without any current review and the total publications relating to algae and ionic liquids number less than 25.

2.3.3 Lipid Extraction

Several species of microalgae exhibit the ability to accumulate large portions of their dry weight as intracellular lipids (up to 75% wt.) (Chisti 2007). Due to their small size, single celled algae must first be harvested from their liquid growth medium using solid liquid separation techniques. Traditionally, microalgae are then dried and subjected to organic solvent based extraction. Cell disruption can greatly aid in facilitating the recovery of lipids by improving the mass transfer properties of the system as solvent extraction is primarily based on diffusion (Halim et al. 2012). However, due to their small size, cell disruption processes can be either materially or energy intensive processes such as enzymatic degradation, ultrasonication, or microwave irradiation (Halim et al. 2012; Kumar et al. 2015). Most solvent based extraction processes are also incompatible with wet biomass requiring extensive drying the algae prior to extraction which can add significant costs to the overall process. Dewatering and drying is thought to be responsible for up to 70% of the biodiesel production cost from microbial lipids (Haas et al. 2006).

Ionic liquids have been recently shown to facilitate the extraction of lipids from microalgae, primarily by disrupting microalgae cell structure (Teixeira 2012a; Orr et al. 2016) allowing either auto partitioning of the lipids or presumably improving access of co-solvents to the intracellular lipids. However, case must be taken to compare ionic liquid extraction techniques to the proper controls, particularly, lipids extracted should be compared to total cellular lipid content as determined by an appropriate analytical technique such as the Bligh & Dyer (B&D) method (Bligh and Dyer 1959), the Folch method (Folch et al. 1956) or direct transesterification (van Wychen and Laurens 2013) using ground and dried biomass. One common issue in many publications is the application of the B&D method with wet cultures of microalgae. This method was originally created to extract lipids from wet tissues containing up to 80% water, however, it has yet to be shown that this method is acceptable using a wet culture of single celled algae which are much more resistant to cell lysis than animal tissues. It has also been shown to be significantly less efficiency in extracting lipids from fish tissues containing >2% lipids by dry weight and as it is expected that industrial production of microalgae would contain >20% wt. lipids

this method may not be appropriate for the study of microalgae systems (Iverson et al. 2001).

It should also be kept in mind that these methods do not necessarily represent the same fractions of lipids that can be extracted from biomass. For example, hexane extraction is highly specific for neutral lipids (triacylglycerides) while the Folch method results in an oil with relatively higher level of non-lipid impurities (Hara and Radin 1978). In most cases, only the neutral lipids will be used for biodiesel production as transesterification yields from polar lipids require more expensive catalysts, longer reaction times, and higher reaction temperatures (Hidalgo et al. 2013). Furthermore, as these methods (when used on dry biomass) are believed to extract almost all of the available lipids, reports of ionic liquids extracting significantly higher amounts of lipids than these methods should be further scrutinized, as it is possible that further impurities from the biomass or from the synthesis of the ionic liquid are present in the extracted oil (Orr et al. 2016). Whenever possible, direct transesterification methods such as the laboratory protocol developed by the National Renewable Energy Laboratory should be adopted as it uses an acid catalyst which is known to convert all lipids including free fatty acids and phospholipids to fatty acid methyl esters and reduces loses of lipids experiences during extraction by adopting appropriate recovery standards. Otherwise, transesterification of the oil to FAME using a base catalyst will allow identification of the relative purity of the extracted oil.

2.3.3.1 Ionic liquid structure

The majority of the ionic liquids studied to date have focused on imidazolium based ionic liquids likely due to their commercial availability with only two publications describing the use of pyridinium, ammonium, and phosphonium based ionic liquids (Olkiewicz et al. 2015; Orr et al. 2016). A summary of ionic liquids and their normalized lipid yields can be found in **Table 2.3-1.** *Chlorella vulgaris* has been the most commonly studied microalgae to date for ionic liquid extraction likely due to its notoriety as one of the most difficult species to lyse.

Table 2.3-1 – Summary of single ionic liquid extractions using dried algae

Ionic liquid (IL)	Solid loading (% wt.)	Pretreatment Conditions	Algal Species	Normalized Yield*	Reference
Imidazolium Cations					
[Amim]Cl	5	65°C, 18h	*C. vulgaris*	13% DT	(Choi et al. 2014c)
[Amim]Cl	5	120°C, 2h	*C. vulgaris*	13% DT	(Choi et al. 2014c)
$[C_2mim]Cl$	5	120°C, 2h	*C. vulgaris*	80% DT	(Choi et al. 2014c)
$[C_4mim]Cl$	5	120°C, 2h	*C. vulgaris*	13% DT	(Choi et al. 2014c)
$[C_6mim]Cl$	10	AT, 16h	*C. vulgaris*	53.4% H2P	(Orr et al. 2016)
$[C_2mim][NTf_2]$	5	110°C, 2h	*C. vulgaris*	50.7% DT	(Choi et al. 2014b)
$[C_2mim][NTf_2]$	5	120°C, 2h	*C. vulgaris*	59% DT	(Choi et al. 2014c)
$[C_2mim][BF_4]$	5	65°C, 18h	*C. vulgaris*	62% DT	(Choi et al. 2014c)
$[C_2mim][BF_4]$	5	120°C, 2h	*C. vulgaris*	75% DT	(Choi et al. 2014c)
$[C_2mim][HSO_4]$	5	110°C, 2h	*C. vulgaris*	10.0% DT	(Choi et al. 2014b)
$[C_2mim]][HSO_4]$	5	120°C, 2h	*C. vulgaris*	13% DT	(Choi et al. 2014c)
$[C_2mim][O_2CH]$	10	AT, 16h	*C. vulgaris*	66.7% H2P	(Orr et al. 2016)
$[C_4mim][O_2CH]$	10	AT, 16h	*C. vulgaris*	30.1% H2P	(Orr et al. 2016)
$[C_2mim][O_2CMe]$	5	65°C, 18h	*C. vulgaris*	69% DT	(Choi et al. 2014c)
$[C_2mim][O_2CMe]$	5	110°C, 2h	*C. vulgaris*	65.3% DT	(Choi et al. 2014b)
$[C_2mim][O_2CMe]$	5	120°C, 2h	*C. vulgaris*	77% DT	(Choi et al. 2014c)
$[C_2mim][O_2CMe]$	10	AT, 16h	*C. vulgaris*	39.2% H2P	(Orr et al. 2016)
$[C_4mim][O_2CMe]$	10	AT, 16h	*C. vulgaris*	34.8% H2P	(Orr et al. 2016)
$[C_2mim][O_2CEt]$	10	AT, 16h	*C. vulgaris*	12.3% H2P	(Orr et al. 2016)
$[C_4mim][O_2CCH_2OH]$	10	AT, 16h	*C. vulgaris*	6.5% H2P	(Orr et al. 2016)
$[C_2mim][CH_3SO_3]$	5	120°C, 2h	*C. vulgaris*	30% DT	(Choi et al. 2014c)
$[C_1mim][MeOPO_3]$	10	AT, 16h	*C. vulgaris*	60.1% H2P	(Orr et al. 2016)
$[C_2mim][MeSO_4]$	5.8	65°C, 18h	*Duniella sp.*	71.8%^	(Young et al. 2010)
$[C_4mim][MeSO_4]$	10% w/v	60°C, 3h	*C. vulgaris*	166.3% B&D	(Kim et al. 2013)
$[C_4mim][MeSO_4]$	10% w/v	60°C, 3h + sonication	*C. vulgaris*	259.6% B&D	(Kim et al. 2013)
$[C_2mim][EtSO_4]$	10	AT, 16h	*C. vulgaris*	81.9% H2P	(Orr et al. 2016)
$[C_2mim][EtSO_4]$	5	120 C, 2h	*C. vulgaris*	21% DT	(Choi et al. 2014c)

$[C_2mim][Et_2PO_4]$	5	120°C, 2h	*C. vulgaris*	86% DT	(Choi et al. 2014c)
$[C_2mim][Et_2PO_4]$	5	110°C, 2h	*C. vulgaris*	72.7% DT	(Choi et al. 2014b)
$[C_2mim][SCN]$	5	120°C, 2 h	*C. vulgaris*	15% DT	(Choi et al. 2014c)
$[C_2mim][SCN]$	5	110°C, 2h	*C. vulgaris*	12.1% DT	(Choi et al. 2014b)
$[C_2MIM]][AlCl_4]$	5	120°C, 2 h	*C. vulgaris*	60% DT	(Choi et al. 2014c)
Pyridinium Cations					
$[C_2py]Cl$	10	AT, 16h	*C. vulgaris*	57.5% H2P	(Orr et al. 2016)
$[C_4m_\beta py]Br$	10	AT, 16h	*C. vulgaris*	101.8% H2P	(Orr et al. 2016)
$[C_4m_\beta py][N(CN)_2]$	10	AT, 16h	*C. vulgaris*	93.6% H2P	(Orr et al. 2016)
Ammonium Cations					
$[N_{1\,4\,8\,8}]Cl$	10	AT, 16h	*C. vulgaris*	100.4% H2P	(Orr et al. 2016)
$[N_{1\,1\,2OH\,4OH}]Cl$	10	AT, 16h	*C. vulgaris*	2.8% H2P	(Orr et al. 2016)
$[N_{0\,0\,0\,3}][NO_3]$	10	AT, 16h	*C. vulgaris*	92.3% H2P	(Orr et al. 2016)
$[N_{0\,1\,1\,2}][O_2CH]$	10	AT, 16h	*C. vulgaris*	97.3% H2P	(Orr et al. 2016)
$[N_{1\,4\,\,4\,\,4}][OH]$	10	AT, 16h	*C. vulgaris*	1.9% H2P	(Orr et al. 2016)
$[N_{0\,1\,1\,2}][MeOCH_2CO_2]$	10	AT, 16h	*C. vulgaris*	76.2% H2P	(Orr et al. 2016)
$[N_{1\,2\,2\,2}][MeSO_4]$	10	AT, 16h	*C. vulgaris*	78.0% H2P	(Orr et al. 2016)
$[N_{1\,1\,4\,4}][tau]$	10	AT, 16h	*C. vulgaris*	0.0% H2P	(Orr et al. 2016)
$[N_{0\,0\,0\,1}][pro]$	10	AT, 16h	*C. vulgaris*	52.6% H2P	(Orr et al. 2016)
Phosphonium Cations					
$[P(CH_2OH)_4]Cl$	10% w/v	100°C, 24h	*C. vulgaris*	166.7% B&D	(Olkiewicz et al. 2015)
$[P(CH_2OH)_4]Cl$	10% w/v	100°C, 24h	*N. oculata*	96.4% B&D	(Olkiewicz et al. 2015)
$[P_{1\,4\,4\,4}]Cl$	10	AT, 16h	*C. vulgaris*	7.1% H2P	(Orr et al. 2016)
$[P_{4\,4\,4\,4}][NO_3]$	10	AT, 16h	*C. vulgaris*	87.4% H2P	(Orr et al. 2016)
$[P_{1\,4\,4\,4}][O_2CH]$	10	AT, 16h	*C. vulgaris*	46.3% H2P	(Orr et al. 2016)
$[P_{4\,4\,4\,4}][OH]$	10	AT, 16h	*C. vulgaris*	129.8% H2P	(Orr et al. 2016)
$[P_{1\,4\,4\,4}][OH]$	10	AT, 16h	*C. vulgaris*	25.9% H2P	(Orr et al. 2016)
$[P_{1\,4\,4\,4}][OH]$	10	AT, 16h	*C. vulgaris*	25.9% H2P	(Orr et al. 2016)
$[P_{1\,4\,4\,4}][HO_2CCH_2CH2CO_2]$	10	AT, 16h	*C. vulgaris*	22.1% H2P	(Orr et al. 2016)

* Normalized yield according to B&D: Bligh & Dyer; DT: direct transesterification; H2P: hexane 2-propanol extraction. When necessary, data was extracted from plots using Web Plot Digitizer. http://arohatgi.info/WebPlotDigitizer

^ Based on maximum yield of 11.0% wt. lipid

All of the reported ionic liquids so far have been hydrophilic, water soluble ionic liquids with the exception of $[C_2mim][NTf_2]$. Dialkyl imidazolium cations combined with alkyl ester sulfates or phosphates can be used extensively. $[C_2mim]$ paired with either $[EtSO_4]$, $[MeSO_4]$, or $[Et_2PO_4]$ have generally resulted in high yields (> 70%) of lipids when compared to traditional solvent extraction methods. $[C_2mim][Cl]$ one of the most commonly known ionic liquids has also exhibited high lipid extraction efficiency, although other dialkylimidazolium chlorides worked poorly. Interesting 1-hexyl-imidazolium exhibited moderate ability (53.4%) to extract lipids are room temperature even with 30% water content present in the IL (Orr et al. 2016). Acidic ionic liquids composed of carboxylic acid anions have also exhibited some lipid extraction ability which increased within increasing processing temperature, and decreased with increasing carboxylic acid chain length.

Few reports of pyridinium, ammonium, and phosphonium based ionic liquids for lipid extraction exist. However, ammonium and phosphonium based ionic liquids may be significantly cheaper to synthesize than their imidazolium based counterparts. Similar to dialkylimidazolium ionic liquids studied so far, $[N_{1\,2\,2\,2}][MeSO_4]$ showed good lipid recovery, as did ammonium or phosphonium cations paired with carboxylic acids. Finally, only three pyridinium based ionic liquids have been tested at room temperature, however, $[C_4m_\beta py][Br]$, showed significant disruption of the microalgae cell structure and allowed recovery of 100% of the available lipids (Orr et al. 2016).

2.3.3.2 Reaction conditions and cosolvents

Reaction conditions likely play a significant role in the success of screening studies using ionic liquids. Lower temperatures are generally desirable in order to minimize the energy required for the overall process. However, as many ionic liquids exhibit high viscosities at low temperatures, the majority of studies have focused on the processing of algal biomass at temperatures in the range of 100-120°C. Viscosity can also be reduced to some extent by using a cosolvent such as methanol. However, cosolvent addition can potentially affect lipid extraction through mechanisms other than reducing the ionic liquid viscosity such as increasing the auto-partitioning behavior of the lipids or increasing the

rate of transfer between the ionic liquid and the immiscible hexane layer often used in these studies for easy handling of lipids. The use of hexane for lipid extraction should be kept in mind when considering these studies, as in many cases the hexane extraction time far exceeded the pretreatment time and potentially even the time required to extract all of the lipids with hexane alone. Studies employing the use of cosolvents in ionic liquid based lipid extraction are summarized in **Table 2.3-2**. Generally, methanol was found to have little effect on the yield of lipids compared to the ionic liquid alone, while hexane was found to decrease lipid extraction and chloroform to increase It (Choi et al. 2014c). Acetone also appeared to be a promising cosolvent for $[C_2mim][MeSO_4]$ (Young et al. 2010). Ionic liquid mixtures have not seen much improvement over the use of a single ionic liquid, however only a single study has explored this area. One interesting exception was the case of the mixture of hydrophilic ionic liquid $[C_2mim][Ac]$ and the hydrophobic ionic liquid $[C_2mim][NTf_2]$ which increased the extraction efficiency from 76.3 and 59.5% with each ionic liquid alone respectively, to 87.5% together compared to Bligh & Dyer extraction (Choi et al. 2014c).

Chloride salts have also been explored as additives in lipid extraction with $FeCl_3 \cdot 6H_2O$ increasing the overall efficiency of $[C_2mim][Ac]$ extraction by to 70% of the available FAME (Choi et al. 2014b). This system was further applied to microalgae biomass containing significant portion of the omega-3 fatty acid docosahexanoic acid (DHA) but DHA recovery was found to be mostly dependent on reaction temperature and time (Choi et al. 2014a).

Generally, the addition of further mechanical or chemical treatments to the ionic liquid based extraction process which increase the speed of cell disruption such as ultrasonication, microwave irradiation, or addition of mineral acids have had a positive effect, however, care should be taken to limit energy intense processes (Pan et al. 2016). In a further study, pressurization via CO_2 addition to $[C_4mim][BF_4]$ was found to increase the extraction efficiency from 68% when $[C_4mim][BF_4]$ was used alone to 76% compared to soxhlet extraction (Yu et al. 2014).

Table 2.3-2 – Summary of ionic liquid extraction using cosolvents or additives using dried algae

Ionic liquid (IL)	Cosolvent or Additive	Solid loading (% wt.)	Pretreatment Conditions	Algal Species	Normalized Yield*	Reference
[Amim]Cl	$CHCl_3$	n.a	n.a	*Unknown*	138.2% B&D	(Teixeira 2012b)
[C_2mim]Cl	$CHCl_3$	n.a	n.a	*Unknown*	288.2% B&D	
[C_{2OH}mim]Cl	$CHCl_3$	n.a	n.a	*Unknown*	294.1% B&D	
[C_4mim]Cl	$CHCl_3$	n.a	n.a	*Unknown*	261.8% B&D	
[C_2mim][$MeSO_4$]	Methanol	5.8	65°C, 18h	*Duniella sp.*	78.2% ^	(Young et al. 2010)
[C_2mim][$MeSO_4$]	Acetic acid	5.8	65°C, 18h	*Duniella sp.*	50.9% ^	
[C_2mim][$MeSO_4$]	Acetone	5.8	65°C, 18h	*Duniella sp.*	83.6% ^	
[C_2mim][$MeSO_4$]	$CHCl_3$	5.8	65°C, 18h	*Duniella sp.*	76.4% ^	
[C_2mim][$MeSO_4$]	DMSO	5.8	65°C, 18h	*Duniella sp.*	54.5% ^	
[C_2mim][$MeSO_4$]	2-Propanol	5.8	65°C, 18h	*Duniella sp.*	77.3% ^	
[C_2mim][O_2CMe]	none	5	120°C, 2h	*C. vulgaris*	76.4% DT	(Choi et al. 2014c)
[C_2mim][O_2CMe]	Methanol	5	120°C, 2h	*C. vulgaris*	75.6% DT	
[C_2mim][O_2CMe]	Hexane	5	120°C, 2h	*C. vulgaris*	64.7% DT	
[C_2mim][O_2CMe]	CHCl3	5	120°C, 2h	*C. vulgaris*	81.5% DT	
[C_2mim]Cl	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	89.1% B&D	(Kim et al. 2012)
[C_2mim]Br	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	46.3% B&D	
[C_4mim]Cl	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	5.5% B&D	
[C_4mim][BF_4]	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	49.0% B&D	
[C_4mim][NTf_2]	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	29.8% B&D	
[C_4mim][PF_6]	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	36.0% B&D	
[C_4mim][CF_3SO_3]	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	111.5% B&D	
[C_4mim][CH_3SO_3]	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	58.4% B&D	
[C_2mim][$MeSO_4$]	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	118.1% B&D	
[C_2mim][O_2CMe]	Methanol	10 w/v	65°C, 18h	*C. vulgaris*	26.6% B&D	
[C_2mim][Et_2PO_4]	$FeCl_3{\cdot}6H_2O$	5	90°C, 1h	*C. vulgaris*	48.5% DT	(Choi et al. 2014b)
[C_2mim][HSO_4]	$FeCl_3{\cdot}6H_2O$	5	90°C, 1h	*C. vulgaris*	39.4% DT	
[C_2mim][NTf_2]	$FeCl_3{\cdot}6H_2O$	5	90°C, 1h	*C. vulgaris*	42.5% DT	
[C_2mim][O_2CMe]	$FeCl_3{\cdot}6H_2O$	5	90°C, 1h	*C. vulgaris*	68.0% DT	
[C_2mim][SCN]	$FeCl_3{\cdot}6H_2O$	5	90°C, 1h	*C. vulgaris*	47.3% DT	

* When necessary, data was extracted from plots using Web Plot Digitizer. http://arohatgi.info/WebPlotDigitizer

^ Based on maximum yield of 11.0% wt. lipid

2.3.3.3 Ionic liquid disruption of wet algae

As stated earlier, one of the most significant costs in microalgae lipid extraction is the dewatering and drying process. Accordingly, the extraction of lipids from wet algal biomass using ionic liquids is receiving significant attention, however, in many studies, lack of through comparison to appropriate positive and negative controls make it difficult to ascertain the actual effect of the ionic liquid on the process. Care should be taken to study the same treatments without the ionic liquid present if additional mechanical pressures are exerted.

First, the disruption of primarily *Chlorella sp.* structure using wet cultures was demonstrated by Teixeira in 2012. Several species were lysed in an hour or less at temperatures above 100°C mainly using chloride based imidazolium ionic liquids (Teixeira 2012b). Shortly afterwards, wet disruption of the cyanobacteria *Synechocystis sp.* was achieved using $[C_2mim][MeO(H)PO_2]$ and $[C_2mim][O_2CMe]$ at room temperature in 30 min (Fujita et al. 2013). It was also reported that the hydrophobic ionic liquids $[C_2mim][NTf_2]$ and fluorinated ionic liquid $[C_2mim][BF_4]$ were not effective but have since exhibited some lipid extraction ability (Kim et al. 2012; Choi et al. 2014c).

More recent reports have provided greater analytical insight into the extraction efficiencies and the role was water in ionic liquid based lipid extractions. Lipids were successfully extracted from fresh *N. oculata* biomass containing 71.7% water content using the ionic liquid $[P(CH_2OH)_4]Cl$ (Olkiewicz et al. 2015). Approximately 75% of the available lipids were extracted using the ionic liquid based method compared to the B&D method. Both methods showed a higher propensity for extracting shorter chain fatty acids when wet biomass was used. The ionic liquid $[C_2mim][EtSO_4]$ has also been shown to successfully extract lipids from wet biomass when combined with the cosolvent methanol (Orr et al. 2016). This study also demonstrated that water contents above 82% wt. drastically decreased extraction efficiencies, as did decreasing the solid loading ratio and methanol to ionic liquid ratios above and below 2:1 (Methanol:IL). In both studies, ionic liquid treatments at room temperature were possible in under 1 h and these ionic liquids were successfully reused up to 4 times.

Other reports combining ionic liquid extraction of wet biomass with other means of disruption have also been recently explored. For example, ultrasonication or microwave irradiation was combined with ionic liquid extraction using [C_4mim][HSO_4] and resulted in increased yields over the ionic liquid alone and this was not compared to the maximum theoretical yield of lipids extracted from dried biomass (Pan et al. 2016). Furthermore, previous studies using a related ionic liquid [C_2mim][HSO_4] showed very poor extraction of lipids with dried biomass (<15% of total available lipids) (Choi et al. 2014b; Choi et al. 2014c) and microwave irradiation and ultrasonication are known to promote disruption of wet algae. Since no negative controls were done with microwave irradiation or ultrasonication without the ionic liquid present using the same extraction procedure, it is difficult to discern whether the ionic liquid actually aided this process. In a further report using ultrasonication, again, there was no extraction performed without the presence of ionic liquid using the same conditions as the IL extraction (for example the same solvents) and the total lipid content of the algae biomass used was less than 3% wt. using the B&D method indicating that either the control extractions worked poorly, or there was no neutral lipids present in the biomass used (Wahidin et al. 2016). Finally, in a report concerning the use of [$N_{0\,2\,2\,2}$][HSO_4] combined with high pressure extraction (1 MPa) for 1h at 110°C resulted in extraction of all of the available lipids, however, again, no negative control was used to distinguish the role of the ionic liquid from that of the high pressure and temperature extraction conditions used (Chen et al. 2015b; Yu et al. 2016). Furthermore, the ionic liquid concentrations used in this study; 1% wt. in water, are extremely low and thus these ionic liquids would mainly be present in their dissociated form which is thought to greatly diminish their unique solvent properties.

2.3.3.4 Switchable solvents

Switchable solvents are a subclass of ionic liquids which can be divided into two categories, switchable polarity solvents (SPS) or switchable hydrophilicity solvents (SHS). SPS are a class of solvents which upon the addition of a trigger; most notably carbon dioxide, change polarity of the solvent. They present a novel method of lipid extraction as they are capable of extracting the lipids in one form and subsequently separating the lipids from the solvent in the more polar form (Samorì et al. 2010; Samori et al. 2013). N,N-

dimethylcyclohexylamine mixed with octanol was much better suited to extracting lipids from wet *Nannochloropsis gaditana*, and *Tetraselmis suecica*, *Desmodesmus communis* biomass compared to 1,8-diazabicyclo-[5.4.0]-undec-7ene (DBU) octanol extraction of *Botryococcus braunii*, however it is difficult to ascertain whether this was due to differences in these microalgae species. This process may present a unique method of lipid separation, however, the process could be lengthy taking over 24 h to dissolve *D. communis*. Additionally, SHS show poor compatibility with water due to the formation of the bicarbonate salt in the presence of water and the feedstock and solvent must be kept rigorously dry (Boyd et al. 2012). Alternatively, SPS work in a similar manner, however, upon addition of CO_2 they switch from a hydrophobic solvent to a hydrophilic one. These SPS were designed specifically for use with wet biomass however, they were only capable of extracting 42% compared to the total available oils as determined by chloroform methanol extraction (Boyd et al. 2012).

2.3.4 Applications of ionic liquids for extraction of other value added compounds

Due to the vast diversity of ionic liquids, they are also currently under investigation for the extraction and recovery of various other biomass constituents from algae such as carbohydrates and carotenoids. Carbohydrates can compromise a significant portion of the biomass composition of both microalgae and microalgae depending on their cultivation conditions. Recent reports indicate that ionic liquids can be used to facilitate the recovery of carbohydrates from both microalgae and macroalgae for the production of renewable sugars (Malihan et al. 2014; Gao et al. 2016).

2.3.4.1 Carbohydrates and sugar products

The ionic liquid $[C_2mim][EtSO_4]$ was used for the room temperature lipid extraction of *Chlorella vulgaris* followed by recovery of the carbohydrates and proteins via anti-solvent precipitation (Gao et al. 2016). It was found that the selection of the anti-solvent could alter the purity of the recovered carbohydrates mainly present in the form of starch. Water reduced the protein fraction by 50% while ethanol resulted in recovery of 100% of

the available protein. The recovered algal starch was successfully fermented to butanol, acetone, and ethanol using traditional ABE fermentation.

Agarose was successfully extracted from the macroalgae *Gracilaria dura* using the ionic liquids $[C_2mim][O_2CMe]$, [Ch][O_2CMe], and $[C_2mim][Et_2PO_4]$ with efficiencies of 39%, 12.5%, and 18.5% respectively using microwave heating at 80°C for 2h (Trivedi and Kumar 2014). In the same study, the ionic liquid $[C_2mim][O_2CMe]$ could be recycled four times with only a 27% decreased in extraction efficiency, likely due to the 55% reduction in recovered mass of ionic liquid after 4 cycles.

The macroalgae *Gelidium amansii* which is primarily composed of agar and fibrin; two polysaccharides of galactose and glucose, was subjected to acidic ionic liquid treatment for the simultaneous hydrolysis and recovery of sugars (Malihan et al. 2014). $[C_2mim][HSO_4]$ and $[C_2mim][H_2PO_4]$ were both shown to exhibit high catalytic activity towards agar resulting in high yields of galactose (> 75%) however, as reaction temperature and time were increased, increasing amounts of sugar degradation occurred. The sugar degradation products, 5-hydroxymethylfurfural (5-HMF), formic acid, and levulinic acid were observed. In one alternative study, the sugars in macroalgae derived agar was converted too the end product 2,5-dimethylfuran via 5-HMF using the ionic liquid catalyst $[DMA][CH_3SO_3]$ as an alternative fuel product (De et al. 2012).

2.3.4.2 Extraction of microalgae pigments

Astaxanthin is one of the most well-known high value products current produced from microalgae. It is widely used as an additive in aquaculture for pigmentation of farmed salmon or shrimp, and is cosmetic and nutraceutical industries as a pigment and potent antioxidant (Desai et al. 2015). Similar to lipids, carotenoids are highly hydrophobic compounds and in many cases are extracted under similar conditions as lipids. Accordingly, ionic liquid based extraction has recently been applied to astaxanthin (Desai et al. 2015). The ionic liquid $[C_2mim][Bu_2PO_4]$ was used to increase the permeability of the microalgae *Haematococcus pluvialis* allowing subsequent extraction of astaxanthin using ethyl acetate. Greater than 70% of the available astaxanthin was recovered using this process which was not found to disrupt the microalgae cell structure. The ionic liquid pretreatment was

performed at mild temperatures (45-65°C) for relatively short periods of time and was found to significant increase the recovery compared to ethyl acetate extraction of untreated algae.

2.3.5 Conclusions

Recent advances in the processing of microalgae using ionic liquids have demonstrated some of their unique prospects compared to traditional solvents. They are recyclable and capable of disrupting wet microalgae biomass under mild conditions faster than organic solvent extraction processes. Furthermore, in one case it has been demonstrated that the ionic liquid can be used to facilitate the recovery of both the lipid and carbohydrate fractions which could be further fermented into biobutanol. Other promising applications include the use of acidic ionic liquids as catalysts for the hydrolysis of sugars from macroalgae and the extraction of high cost pigments. If these process can be combined using a single ionic liquid based extraction/fractionation process in the coming years, this could greatly advance the commercial outlook of microalgae based production of renewable chemicals.

2.3.6 Acknowledgements

The authors are grateful to the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, BioFuelNet Canada and the Alexander von Humboldt Foundation for financial support.

Chapter 3 – Experimental Results

3.1 Preface to Chapter 3

Chapter 3 presents the experimental results obtained in this work. It is organized into 6 subsections each representing a separate published or submitted contribution as follows:

Section 3.2 presents the development of an improved high throughput optical assay for the determination of lipid content. It identifies the absorbance of the emitted fluorescent light by chlorophyll as the major cause of variability in this assay. Removal of the chlorophyll prior to lipid determination by simple bleaching allows accurate quantification of the lipid content of the cells.

Section 3.3 presents the use of the Nile Red assay and response surface methodology for the optimization of three media components for *C. vulgaris* cultivation under mixotrophic conditions. The optimized conditions are then confirmed and *C. vulgaris* is cultivated under heterotrophic conditions using pure glucose in a bench scale reactor for the production of large quantities of biomass.

Section 3.4 investigates the use of an alternative waste source of glucose; sugars derived from fast pyrolysis of lignocellulosic biomass, as a more sustainable source of glucose for heterotrophic microalgae cultivation. This is contrasted to a promising alternative for microbial lipid production, the oleaginous yeast *Rhodosporidium diobovatum*. The effects of lignocellulose derived inhibitors on growth, lipid production, and lipid composition are quantified.

Section 3.5 uses the biomass produced in the bioreactors presented in *Section 3.4* in order to study the disruption of *C. vulgaris* by room temperature ionic liquids. First, over 25 ionic liquids are screened and the quality of the extracted oil is quantified for the top 9 candidates. From these results the most promising ionic liquid was identified and the effects of process parameters on the extraction of the algal lipids was investigated. It was

further identified that a co-solvent, methanol was required for full extraction of lipids from fresh wet algal biomass. The reuse of the recovered ionic liquid was examined.

Section 3.6 builds upon the work in the previous section and explores the addition of an alkaline catalyst to the previous extraction protocol in order to convert cellular lipids directly to FAME. This work was performed using *R. diobovatum* biomass produced in the pilot scale in collaboration with researchers from the University of Manitoba using waste glycerol from a biodiesel plant. Response surface methodology was used to optimize the reaction conditions in terms of methanol and KOH loading, as well as reaction temperature. The effects of water content and ionic liquid loading ratio on the reaction time profile and FAME yield was further studied. Recovery of the catalyst and ionic liquid was quantified.

Section 3.7 concludes this investigation by exploring the recovery and use of the residual algal solids for the production of additional value-added products; in this case, butanol production via ABE fermentation. The gross chemical composition of the raw and lipid extracted residual solids was quantified. The ionic liquid process was compared to the traditional hexane solvent extraction process. The recovered solids composed mainly of starch were directly fermented to butanol using an amylase secreting species of *Clostridium*. The lipid extracted algae was further processed by dilute acid hydrolysis and detoxification in order to achieve higher butanol productivity.

3.2 Improvement of the Nile Red fluorescence assay for determination of total lipid content in microalgae independent of chlorophyll content

Valerie Orr and Lars Rehmann

Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St, London, Ontario, Canada, N6A 5B9

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in the *Journal of Applied Phycology* 28: 2181-2189 (2015)

3.2.1 Preface to Section 3.2

A major limitation in the optimization of microalgae lipid production was the lack of a reproducible analytical method for measuring intracellular lipids using a small sample size in a high-throughput method. Traditional lipid quantification methods use organic solvents to extract lipids from dried biomass. The solvents are removed by evaporation and the lipids are quantified gravimetrically. While these methods are generally regarded to be accurate, for reproducible results, particularly for low lipids content samples (< 10% wt.), an excess of 100 mg of biomass is required in order to ensure reasonable precision when using a standard analytical balance. As most algal cultures in shaker flasks or using other batch cultivation processes have maximum cell densities of less than 10 g/L (Yuvraj et al. 2016), a sample size of greater than 10 mL would be required in order to harvest sufficient biomass to extract the lipids a single time at the highest culture density. In order to study the lipid accumulation over time in a culture using these methods, large cultures volumes are needed to support sampling of such large volumes.

Fortunately, a high throughput method for lipid quantification was developed called the Nile Red assay (Chen et al. 2009). The original assay provided several valuable improvements over traditional solvent extraction methods such as adopting small culture samples sizes (< 1 mL) and adapting the assay to a microtiter plate allowing high-throughput (HTP) quantification. However, high variability between studies and species was attributed to variable uptake of the dye in different species of microalgae (Chen et al. 2009; Laurens et al. 2015). In this work, it is shown that the high variability of this assay was primarily due to interference of chlorophyll in the optical measurement of Nile Red fluorescence. Nile Red emission is measured in the same range in which chlorophylls absorb light, therefore it was proposed that species which had a high level of chlorophyll content (such as green algae) were able to absorb a greater proportion of the emitted light resulting in underestimation of the lipid content. This theory aligned well with the observations that green algae were the least amenable species to this assay, previously attributed to their strong cell wall structures.

Removal of chlorophyll was achieved by bleaching using hypochlorite prior to the measurement of Nile Red and shown to drastically increase the relative fluorescence of the dye in both *Chlorella vulgaris* and *Nannochloropsis oculata* compared to unbleached cells. It was further demonstrated that optical density measurements and their correlation to dry cell weight (DCW) were also highly influenced by the chlorophyll content of the cell which can be easily manipulated through media composition and cultivation conditions. Upon chlorophyll removal, the precision of cell density measurements using optical density was dramatically improved and became uniform regardless of original chlorophyll content of the cell. It was further demonstrated that Nile Red fluorescence became linearly proportional to cell density only after chlorophyll removal confirming the interference of chlorophyll in this assay. As chlorophyll content of the cell is highly dependent on the media composition, cultivation conditions, culture age, and species, interference of chlorophyll was proposed to be the most likely cause of the high variability of this assay in the literature. Thus, the improvements established in this chapter could lead to overall greater accuracy of this highly convenient assay for the greater algal research community.

3.2.2 Abstract

Rapid determination of the specific lipid content of microalgae can accelerate screening and development of algae fuel application. Until recently, specific lipid content was commonly measured using gravimetric solvent extraction. Such methodology is time consuming and requires relatively large sample volumes. The Nile Red lipid assay has recently gained acceptance as it requires minute sample sizes. However, the current methodology is highly biased through interference of chlorophyll. Chlorophyll content can significantly vary between species, cultivation conditions, and culture age, remaining a serious but accepted problem. The assay was improved in this study in two ways. The cell dry weight determination was generalized by chemical chlorophyll removal prior to optical cell density measurements. Algae samples with different chlorophyll content were used to correlate optical measurements to cell dry weight and the correlation was substantially improved compared to non-treated samples (97% decrease in sum of squared residuals). Degradation of chlorophyll was also found to increase the emission intensity of Nile Red presumably by removing a source of molecular absorption of red light, potentially increasing the detection threshold. Secondly, milk fat was used as a standard for correlating lipid concentration to Nile Red fluorescence. It was cheaper, more precise, easier to accurately prepare and linearly correlated to lipid concentration over a broader range than triolein, largely due to the fact that it forms a stable lipid/water micro-emulsion. The improved methodology for the Nile Red lipid assay was found to significantly increase the working range and accuracy of the assay.

Keywords: Nile Red; lipid content; microalgae; dry cell weight, high throughput, fluorometry

3.2.3 Introduction

The overproduction of lipids in microalgae is currently a matter of great interest amongst alternative fuels researchers (Chisti 2007; Hu et al. 2008; Singh and Gu 2010; Amaro et al. 2011; Makareviciene et al. 2013; Liu and Benning 2013). Lipids extracted from algae, mainly in the form of triacylglycerides, can be converted to biodiesel via a simple transesterification reaction (Chisti 2007). However, most algae only accumulate high amounts of TAG lipids under high stress conditions such as nitrogen starvation in the presence of excess carbon source (Hu et al. 2008); and while several candidate species have been identified such as *Chlorella vulgaris*, the search for novel species which rapidly accumulate lipid is still underway. Rapid high-throughput (HTP) screening of novel strains and the optimization of environmental conditions will be critical in the improvement of biodiesel productivity as the field advances. The Nile Red lipid assay has gained popularity as a fast and simple screening method in the microalgae field (Guckert and Cooksey 1990; Lee et al. 1998; Rosenbaum et al. 2005; Chiu et al. 2009; Huang et al. 2009; Montero et al. 2010; Mutanda et al. 2011; Chen et al. 2011b; Li et al. 2011b; Moazami et al. 2011; Doan et al. 2011; Ren et al. 2013; Roleda et al. 2013), however, the currently existing methodology is highly affected by the microalgae pigments.

Nile Red (9-diethylamino-5H-benzo [α]-phenoxazine-5-one) is a fluorescent dye which has been widely for lipid staining in histological studies of microalgae (Wang et al. 2009; De la Hoz Siegler et al. 2012) and more recently to evaluate volumetric lipid concentration (Chen et al. 2009; Huang et al. 2009; Chen et al. 2011b; Chen et al. 2011c; Bertozzini et al. 2011; De la Hoz Siegler et al. 2012). These assays are based on the divergent nature of the dye which fluoresces yellow-red in hydrophobic environments and whose fluorescence is quenched in aqueous environments. The fluorescence maximum is dependent on the polarity of the hydrophobic environment and is blue-shifted as the polarity of the solvent decreases (Greenspan and Fowler 1985; Cooksey et al. 1987). Carrier solvents such as DMSO, acetone, and ethanol, have been used to increase penetration of the dye into the microalgae, as lipids are stored intracellularly. These solvents do not interfere with the assay as the produce Nile Red emissions >590 nm, do not overlap with neutral lipid emissions (Chen et al. 2009; Chen et al. 2011c; De la Hoz Siegler

et al. 2012). By choosing the appropriate emission and excitation wavelengths, neutral lipids (emission 550-590 nm) may be distinguished from polar lipids (emission >590 nm) such as phospholipids (Cooksey et al. 1987). Thus at the wavelengths selected in this work; excitation with 530 nm and emission at 570 nm, Nile Red fluorescence will be primarily due to TAGs.

It was previously shown by Chen et al. (2009) that volumetric lipid concentration is proportional to fluorescence intensity of Nile Red. However, the current methodology suffers from a very limited range of accuracy for samples between 5×10^4 and 4×10^5 cells/mL (0.01-0.08 OD_{750}), meaning that all cultures must first be diluted into this narrow range prior to determination introducing new errors. Furthermore, fluorescence intensity of triolein, the standard currently used, is only linear in the concentration range of 2-20 μg/mL due to the separation of the lipid from the aqueous environment of the assay at higher concentrations during the incubation period. The resulting droplet formation at higher triolein concentration consequently leads to erratic measurements of fluorescence while droplets floats in or out of the range of the photo-detector. While the use of triolein as a standard does improve the volumetric determination of lipid concentration in microalgae cultures, interference of algal pigments with the fluorescence of Nile Red through both absorption and fluorescence emission can lead to skewed results. Nile Red based fluorescence assays alone can at best provide a measure of lipid contend per reaction volume. Measuring the specific lipid content of algae requires lipid and biomass determination. Biomass concentration can be correlated to optical density measurements. However, interference of chlorophyll in optical methods of dry cell weight determination prevent accurate determination of specific lipid content of a microalgae suspension (Griffiths et al. 2011).

Optical density is often used as a rapid and convenient indicator of cell density in microbiology in order to forego lengthy and tedious gravimetric measurements of dry cell weight (DCW). Typically for unicellular organisms such as *Escherichia coli* or *Saccharomyces cerevisiae,* absorbance at 600 nm is directly proportional to the mass concentration of cells. Absorbance of a colloid suspension is a function of molecular

adsorption and attenuation such as scattering. Molecular adsorption is directly proportional to concentration of the adsorbing particle while light scattering of a solution of particles is dependent on several factors such as size, shape, refractive index, and concentration of the particles (Myers et al. 2013). In non-pigmented organisms like *E. coli*, absorbance of red light is dominated by light scattering and molecular absorption is assumed to be minimal (Myers et al. 2013). However, for pigmented organisms such as microalgae, absorbance becomes a function of both molecular absorption and light scattering in most regions of the UV-Vis-IR spectrum typically used for these measurements. In microalgae this is primarily due to the presence of chlorophyll, however, other carotenoids which absorb light may also be present. It is well-known that microalgal chlorophyll content can vary widely based on external factors such as media composition, light intensity, and cultivation time (Griffiths et al. 2011). Due to the high molecular extinction coefficient of chlorophylls, miniscule changes in cellular chlorophyll content can dramatically impact any dry cell weight measurements through optical means. Therefore, normalization of volumetric lipid concentration by the quotient of Nile Red fluorescence and optical density will be skewed based on the chlorophyll content on the cells.

Herein we present two improvements upon the Nile Red assay, bleaching of cell prior to optical density and fluorescence measurements to prevent interference from pigments, and use of a cheaper and easier to handle standard for volumetric lipid determination, milk fat.

3.2.4 Results & Discussion

3.2.4.1 Interference of pigments in optical density measurements

As microalgae based processes move from more fundamental studies to applications based research, a method for accurate and rapid quantification of the concentration of microalgae under various cultivation conditions is necessary. As pigmented organisms, microalgae possess chlorophyll a and potentially other chlorophylls such as b or c, as well as accessory pigments which results in a high absorption background during optical measurements of cell density. The three main chlorophylls, a, b, and c, have in *vivo* absorption peaks between 570-700 nm which is the region typically employed for optical

density measurements (Bricaud 2004; Myers et al. 2013). In microalgae, typically optical density at 680 nm is used as it gives a highly linear response to cell number in this range, however, the correlation between OD_{680} and cell number is highly affected by chlorophyll content of the culture (Griffiths et al. 2011; Myers et al. 2013). Chlorophyll content is highly dependent on species, culture media, environmental factors, and is also known to change with age of a culture (Griffiths et al. 2011). Thus, users of this method must create a new correlation for each cultivation condition or species used for accurate measurement of cell density. *Chlorella vulgaris* was cultivated under a variety of conditions in order to get low (A-D) and high (E and F) levels of chlorophyll and various lipid compositions as summarized in **Table 3.2-1**. Cultures A-D were confirmed to have low total chlorophyll content below <1 μg/mg of algae while cultures E-H were confirmed to have high total chlorophyll content >10 μg/mg of algae (Porra et al. 1989).

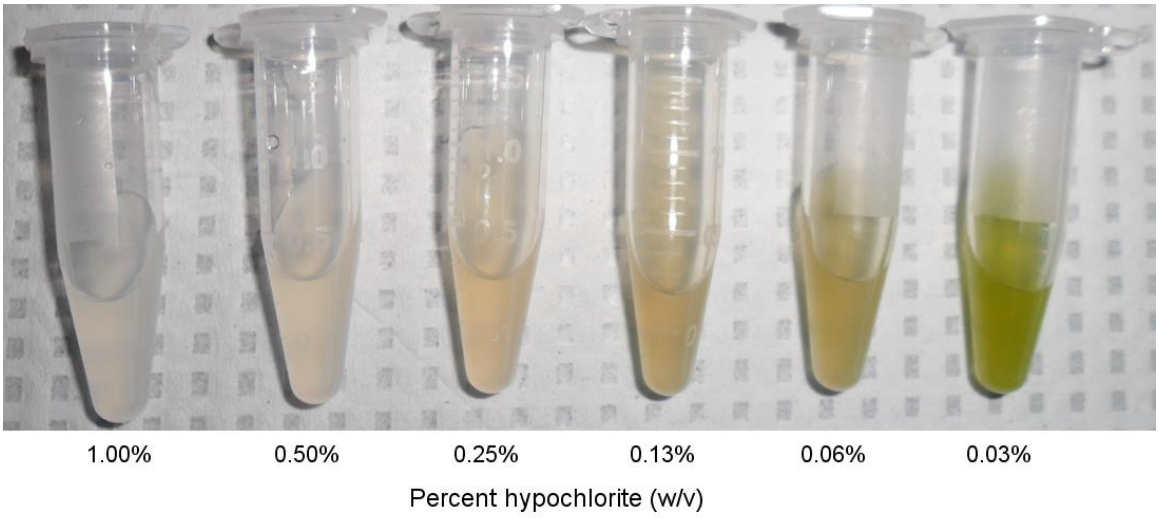


Figure 3.2-1 – Bleaching of a single algae suspension (~2 g DCW/L) with hypochlorite after 1 min incubation at room temperature. The final concentration of hypochlorite is indicated in % (w/v).

C. vulgaris cultures were harvested and resuspended to a cell density of approximately 10 g/L before constructing a 2 fold serial dilution in order to determine OD_{680} as a function of DCW for each cultivation condition. Cultures were bleached white using a final concentration of 0.3% (w/v) hypochlorite before subsequently measuring the OD_{680} without interference from pigments. Hypochlorite was diluted in a serial dilution

from 6% to 0.01% (w/v) in phosphate buffer pH 6.8 to confirm that there was no interference of hypochlorite in the determination of OD_{680}. All of the concentrations tested did not absorb at this wavelength as expected from the literature (Hussain et al. 1970). **Figure 3.2-1** shows the effect of hypochlorite addition to a 2 g/L algae suspension. Within moments, the culture with a final concentration of 1% (w/v) hypochlorite was bleached white. After a 20-minute incubation the remaining cultures with >0.06% (w/v) bleach content were rendered colourless as well. However, it should be noted that cultures with higher chlorophyll content might require larger amounts of hypochlorite in order to be fully bleached and the volume added should be recorded for later calculations of cell density. Finally, the effect of bleach on cell integrity was determined by staining with crystal violet and observing the cultures under a light microscope (data not shown).

Table 3.2-1 – Comparison of least squares non-linear regression for bleached algae and non-bleached algae at 680 nm

	Unbleached	**Bleached**
Model	$DCW\left[\frac{g}{L}\right] = \frac{A_{680}p}{q - A_{680}}$	
Parameters		
p	5.972 (4.294, 7.65)	2.155 (2.007, 2.304)
q	10.74 (5.736, 15.75)	5.363 (4.598, 6.128)
Goodness of Fit		
Adjusted R^2	0.9101	0.9837
RMSE	0.3089	0.0619
SSE	5.917	0.2375

Hypochlorite bleaching did not appear to cause cell lysis (data not shown), only degradation of the chlorophyll although this is possibly due to the presence of the cell wall which appeared intact. Cell integrity is critical for the success of this assay as it prevents the lipid bodies within the cell from aggregating and partitioning out of the solution, therefore, bleaching with hypochlorite may not be an appropriate method of removing chlorophyll from cell wall-less species. In those cases, the more laborious but less destructive method of chlorophyll removal using solid-liquid extraction with DMSO is recommended.

Figure 3.2-2 illustrates the effect of chlorophyll on OD measurements at 680 nm. Unbleached cultures with low chlorophyll content were found to have a lower slope than

the cultures with high chlorophyll content, showing the direct contribution of molecular absorbance to the measurement. However, when the cultures were bleached, the correlation between DCW and OD_{680} was much stronger over a larger range for both low and high chlorophyll content cultures, as can be seen be the substantially reduced spread of the data generated under different cultivation conditions. **Table 3.2-1** presents the rational fit used to predict DCW from OD_{680} and summarizes the non-linear least squares regression of both the unbleached and bleached cultures. It can be seen from the goodness of fit testing that bleaching the algae prior to OD_{680} measurement significantly increases the precision of predicting DCW for all cultures regardless of their original chlorophyll content (96% reduction of SSE, increase of adjusted R^2 from 0.91 to 0.98, narrowed prediction limit (**Figure 3.2-2**). Once chlorophyll is removed from the system, measured absorption should be a direct result of light scattering (at the respective wavelength) and be directly proportional to the particle concentration. However, light scattering is also dependent on particle size and shape, therefore, a new correlation curve must be constructed for each new species.

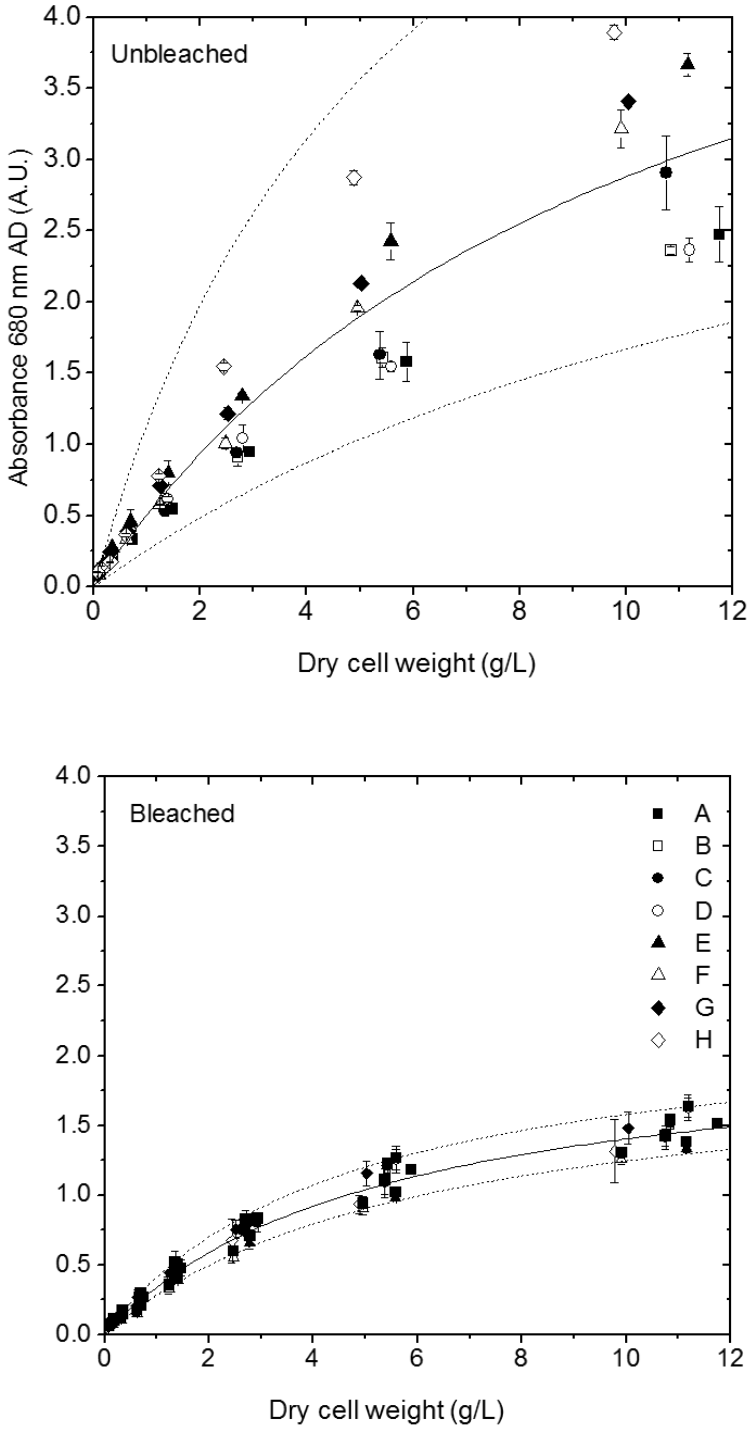


Figure 3.2-2 – Absorbance of two fold serial dilutions of algal suspensions from various cultivation conditions. Cultivation conditions and symbol legend are described in **Table 3.2-2**. Data represents mean values and standard deviations of triplicate measurements.

Table 3.2-2 – Media composition and culture performance after 5 days of cultivation. Cells grown in media A-D were low in chlorophyll content while cells grown in media E-H were high in chlorophyll content. All media used in this study was based on the TAP media (2.42 g/L Tris, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $CaCl_2 \cdot 2H_2O$, 1 mL/L glacial acetic acid, 1 mL/L Hutner's Trace Element Solution. Carbon and nitrogen sources were supplemented as indicated.

Code	Symbol	Glucose [%]	NH_4Cl [mM]	Yeast Extract [%]	DCW [g/L]	Lipid Content [%]	Lipid Conc. [g/L]
A	■	1	2.5	-	1.83 ± 0.31	24 ± 3	0.42 ± 0.08
B	□	1	5	-	3.93 ± 0.12	19 ± 2	0.75 ± 0.02
C	●	2	2.5	-	1.83 ± 0.31	24 ± 0	0.45 ± 0.07
D	○	2	5	-	3.67 ± 0.15	19 ± 1	0.69 ± 0.03
E	▲	1	2.5	1%	6.77 ± 0.25	15 ± 0	1.01 ± 0.03
F	△	1	5	1%	6.27 ± 0.32	15 ± 1	0.94 ± 0.05

3.2.4.2 Interference of Chlorophyll in Nile Red fluorescence

In order to allow rapid determination of neutral lipid content using the Nile Red assay, it was most desirable to bleach the algae, read the absorbance in a plate reader, and subsequently perform the Nile Red assay on the bleached sample. However, it was quickly determined that fluorescence of Nile Red was dependent on the concentration of bleach in the sample as illustrated in **Figure 3.2-3**. Since the remaining hypochlorite after bleaching is variable depending on the chlorophyll content and cell density of the sample, the extent of the effect of hypochlorite on fluorescence in each sample would vary considerable. Therefore, it was determined that the remaining hypochlorite must be removed from the culture by washing the cells prior to the Nile Red assay. Oxidation/halogenation of unsaturated fatty acids was also considered, however, the rate of reaction of hypochlorite with TAGs is reported to be very slow at room temperature (Wang and Tao 1998).

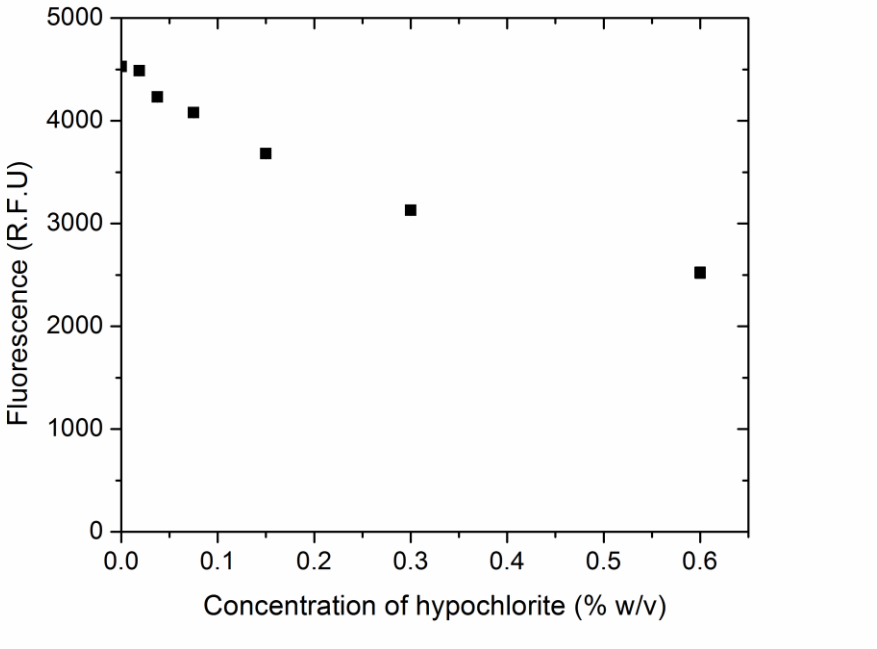


Figure 3.2-3 Effect of hypochlorite on Nile Red fluorescence of 250 µg/mL milk fat. Data represents the mean values and standard deviations of triplicate measurements.

The protocol used in the Nile Red assay is based on that outlined by Chen et al. (2009) and BioTek (Held and Kerri 2011). One hundred microliters of algal suspension was mixed with an equal amount of 5 µg/mL Nile Red in 50 mM phosphate buffer pH 6.8 with 50% DMSO, and incubated in the dark for 10 min prior to detecting the fluorescence of Nile Red in the solution. The fluorescence emission spectrum, using an excitation of 530 nm, was recorded for the unbleached and bleached version of *C. vulgaris* and *Nannochloropsis gaditana,* as depicted in **Figure 3.2-4.** The fluorescence of the bleached culture is significantly higher than the fluorescence of the unbleached algae for all wavelengths measured. As it was previously reported that species belonging to the phylum *Chlorophyta* such as *C. vulgaris* exhibit very little fluorescence contrary to their high lipid content, while species belonging to other phyla which do not possess Chl*b* did not exhibit this behavior (Alonzo and Mayzaud 1999; Chen et al. 2009), it was hypothesized that Chl*b* which is known to absorb red light might be responsible for poor absorbance. However, as both *C. vulgaris* which possesses both Chl*a* and Chl*b* and *N. gaditana* which only contains

Chl*a* both exhibited an increase in fluorescence when pigments were removed by bleaching, Chl*a* and Chl*b* must both contribute to this dampening of fluorescence through their absorbance of red light. As species of *Chlorophyta* tend to possess larger amounts of chlorophyll as well as two chlorophyll species which absorb red light, it is likely that they are more difficult to measure purely due to their higher chlorophyll content; up to 4% dry cell weight (Myers and Graham 1958), and not due to penetration of Nile Red into the cell as previously hypothesized (Chen et al. 2009).

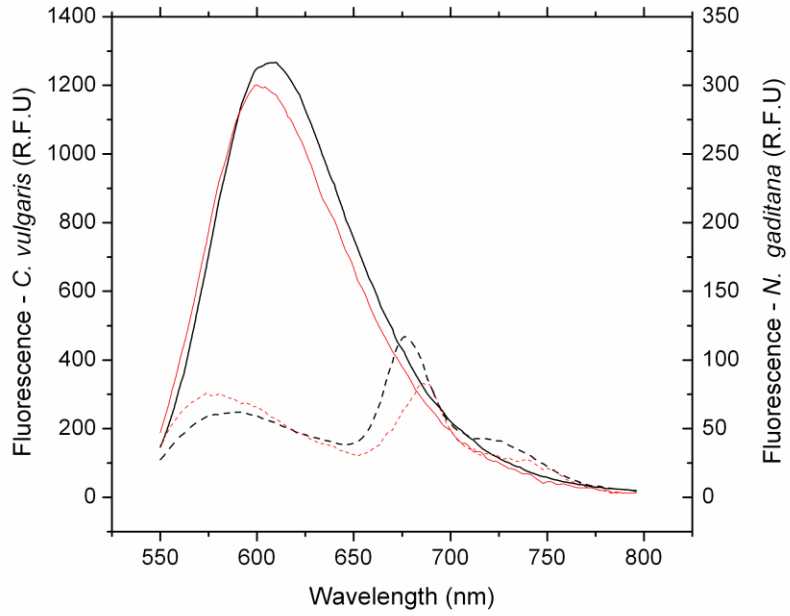


Figure 3.2-4 – Emission spectrum of Nile Red fluorescence of *C. vulgaris* (black) and *N. gaditana* (red) of unbleached (dashed) and bleached (solid) cultures excited at 530 nm.

3.2.4.3 Lipid standard for the Nile Red assay

While it is reported that triolein fluorescence is only linear between a range of 2-20 µg/mL (Chen et al. 2009), it was found that if prepared in anhydrous ethanol at 20 fold higher concentration and diluted into the aqueous assay buffer in the wells, that generally, a good linear correlation between triolein and fluorescence can be found between 4-250 µg/mL as can be seen in **Figure 3.2-5.** In the previous study a lower concentration of Nile Red was used and this may be the reason for the disparity (Chen et al. 2009). The main

challenging for the Nile Red assay is that lipids have to be present as a distinct phase. The triolein emulsion is no longer homogenous once the concentration exceeds a threshold and large droplets form. A natural stable fat in water emulsion can be found in milk. Milk fat was found to be a superior standard for lipid fluorescence as it does not require any special preparation. However, the observed slope was twice the numerical value compare to the equivalent concentration of lipids in the triolein standards, most likely due to the presence of other hydrophobic molecules in table cream such as cholesterol for example. However, milk is inexpensive compared to analytical reagents, while also highly standardized and regulated due to strict food standards in most parts of the world. This was confirmed by testing three different suppliers of milk fat (Parmalat, Nelson, and Metro Brands) to find they performed identically under the assay conditions (data not shown). It is easy to handle as it is already a stable emulsion making it a simple to use standard for this assay.

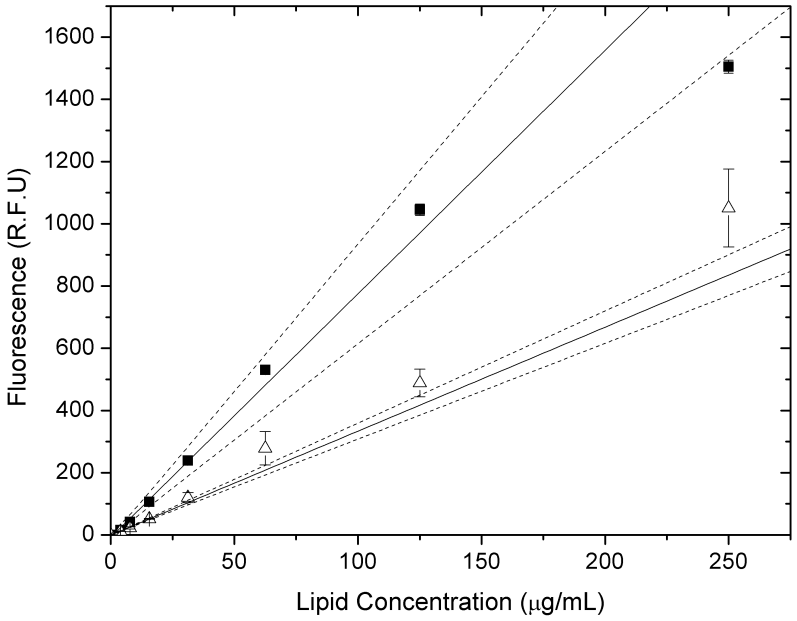


Figure 3.2-5 – Comparison of triolein (open triangle) and milk fat (closed square) as standards for the Nile red lipid assay. Data represents the mean values and standard deviations of triplicate measurements.

3.2.4.4 Effect of algal cell concentration

The effect of algal cell concentration on the fluorescence of Nile Red was measured in order to determine the working range of the Nile Red assay using bleached algae. It can

be seen from **Figure 3.2-6** that Nile Red fluorescence in unbleached cell suspensions is quickly saturated making the linear region of this assay small. This saturation effect has been reported previously (De la Hoz Siegler et al. 2012). In the bleached algae suspension, fluorescence is linearly proportional to algae cell concentration over a much larger range. Chen et al. (2009), report that the linear range of their assay is for algae cell concentrations between an OD_{750} of 0.01-0.08 A.U. or 0.5-4 × 10^5 cells/mL. This is likely due to the interference of chlorophyll at higher cell densities which previously required dilution of the algae so that Nile Red fluorescence was in excess of that which could be absorbed by the chlorophyll present in the sample. In this improved method the range for accurate determination of specific lipid concentration is between 0.1 – 2 g/L of algae making this assay much easier to perform for a broader range samples. This range is equivalent to OD_{680} between 0.04 and 0.6 A.U., and therefore increases the previously reported range by approximately 8 fold.

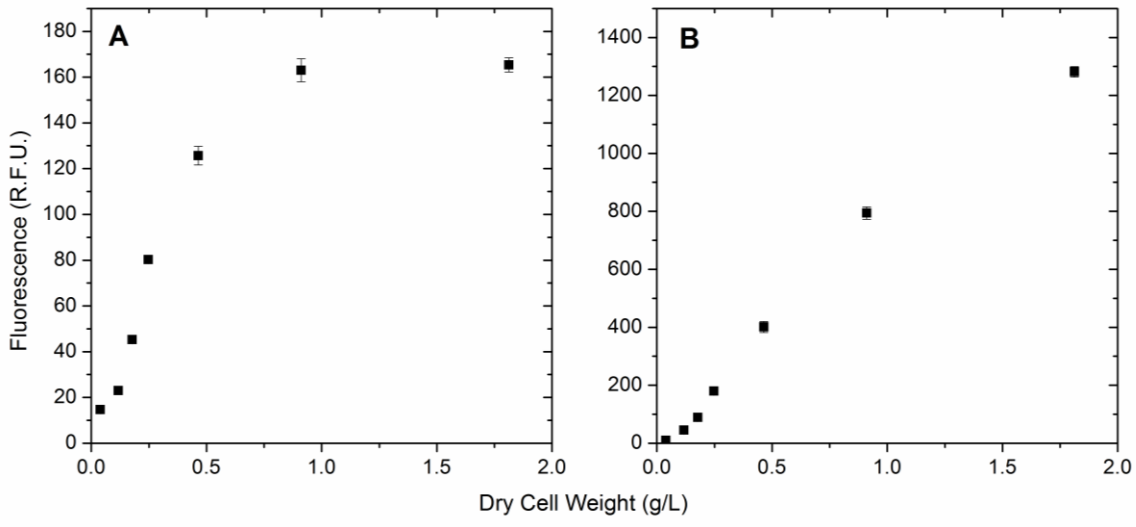


Figure 3.2-6 – Fluorescence of Nile red of unbleached (A) and bleached (B) two fold serial dilutions of a high chlorophyll content algal suspension. Data represents the mean values and standard deviations of triplicate measurements.

Specific lipid contents were calculated by converting Nile Red fluorescence intensity into volumetric lipid concentration and dividing it by the DCW as calculated from OD_{680} data. When compared to the gravimetrically determined lipid content (**Figure 3.2-7**), the low chlorophyll content cultures were not significantly different than the calculated

lipid content. The high chlorophyll content cultures, culture E was significantly lower than the hexane extraction $p < 0.05$, however, this underestimation by the calculated lipid content may be due to extraction of significant amounts of other hydrophobic molecules such as chlorophyll as the extract was nearly black in solvent extractions of cultures E and F which may cause the gravimetric determination to overestimate the neutral lipid content in some cases. In general, the optical assay was in good agreement with the gravimetric method.

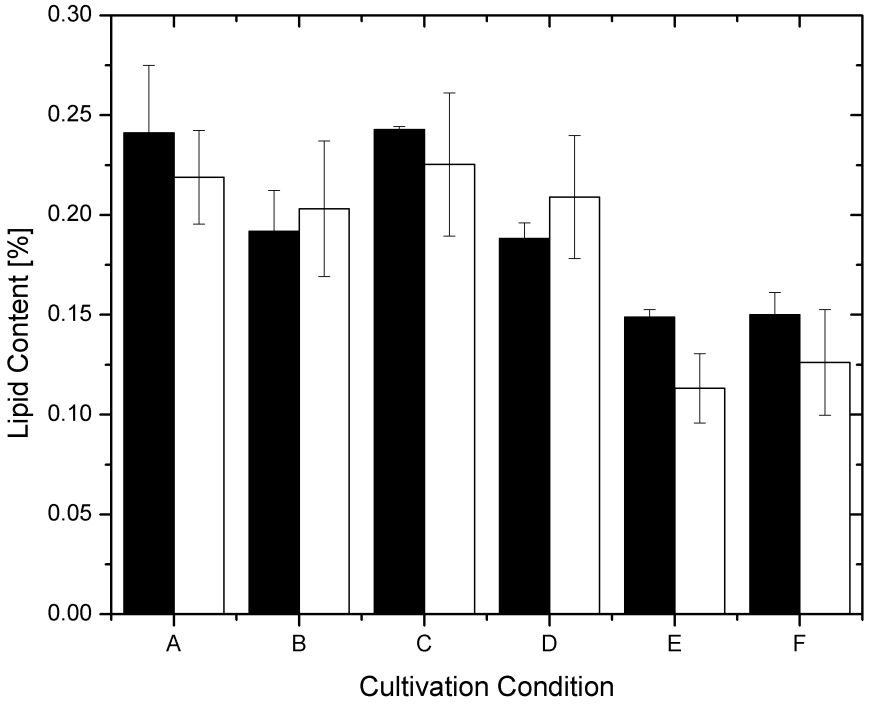


Figure 3.2-7 – Comparison between calculated lipid content using the Nile Red Assay (white) and the gravimetric lipid content using hexane/isopropanol extraction (black). Data represents mean values and standard deviations of triplicate measurements.

3.2.5 Conclusions

Gravimetric determination of lipid content in microalgae is an arduous task that requires cultivation of large volumes and is time consuming making it difficult to screen species for lipid production and to screen for factors affect lipid accumulation. The previous Nile Red assays were subject to interference by chlorophyll pigments in microalgae resulting in results dependent on the chlorophyll content of the algae. The improved Nile Red assay involves degrading chlorophyll prior to dry cell weight

determination and Nile Red fluorescence measurement which allows determination of volumetric and specific lipid concentration independent of chlorophyll content.

3.2.6 Methods

3.2.6.1 Strain and cultivation conditions

Chlorella vulgaris strain UTEX 2714 purchased from The Culture Collection of Algae at the University of Texas Austin was purified to remove bacterial contamination using a standard agar purification method (Guillard 2005). The culture was maintained in liquid culture using aseptic technique in 150 mL Tris-Acetate-Phosphate (TAP) media pH 7.0 in 500 mL shaker flasks. All cultures were grown at 25°C at 150 rpm under cyclic illumination consisting of 16h on: 8h off (100 μmol/m^2·s) (Multitron2, Infors Canada). TAP medium used consisted of 20 mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, 7.0 mM NH_4Cl, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1mL/L glacial acetic acid, and 1 mL/L of Hutner's trace elements solution (50 g/L Na_2EDTA, 22 g/L $ZnSO_4·7H_2O$, 11.4 g/L H_3BO_3, 5.06 g/L $MnCl_2·4H_2O$, 1.61 g/L $CoCl_2·6H_2O$, 1.57 g/L $CuSO_4·5H_2O$, 1.10 g/L $(NH_4)_6Mo_7O_{24}·4H_2O$, 4.99 g/L $FeSO_4·7H_2O$, and 20 g/L KOH). Inoculum cultures were prepared in TAP media and cultivated under the above conditions for 48 h in order to have an exponential phase seed culture. All cultures were inoculated at 10% (v/v) after the old media was removed by gentle sterile centrifugation at 2000 × g for 5 min and resuspended in nitrogen free TAP media. To study the accuracy of the method under high and low lipid content, high and low chlorophyll content, and high and low cell densities, cultures were grown in various media for five days. The basal media consisted of 20 mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1mL/L glacial acetic acid, and 1 mL/L of Hutner's trace elements solution and the concentration of NH_4Cl, $NaNO_3$, and glucose was varied as indicated.

3.2.6.2 Harvesting and washing of microalgae

Microalgae were harvested by centrifugation at 2500 × *g* for 10 min at room temperature in a Sorvall R40 centrifuge (ThermoScientific, USA). Microalgae were washed three times with deionized water and dilute 0.1M HCl to remove precipitated salts. Algae

were resuspended at a high density in either water for freeze-drying and dry cell weight analysis or in 50 mM phosphate buffer pH 6.8 for the Nile Red assay.

3.2.6.3 Dry cell weight analysis

Ten milliliters of the high density algal culture in water was pipetted into a weighed pan and placed in a vacuum desiccator at room temperature for 48 h or until the weight as measured using an analytical balance (Mettler-Toledo, USA) no longer fluctuated.

3.2.6.4 Freeze Drying

Algal cultures washed three times with water were frozen at -86°C for a minimum of 8 h and lyophilized using a 4.5 L freeze-drier (Labconco) for 24 h or until the weight no longer fluctuated.

3.2.6.5 Extraction and measurement of chlorophyll

Chlorophyll content was determined in duplicate by mixing 0.05 g of freeze-dried algae in 8 mL of acetone solution (80% (v/v) acetone, 2.5 mM sodium phosphate buffer pH 7.8 (Porra et al. 1989) for 16 h on a rotary shaker (Cole-Palmer, USA). Tubes were centrifuged at 2000 × g for 2 min and the acetone solution supernatant was removed into another container. The remaining pellet was washed with 2 mL of acetone solution and centrifuged three times and the fractions of acetone were pooled. The final volume was adjusted to 15 mL with acetone. The chlorophyll absorbance was measured by scanning the sample in a glass cuvette using a UV-Vis spectrophotometer (Thermo-Scientific, USA). Total chlorophyll content was determined using the following equation:

$$\text{Total Chlorophyll Content } (\%w/w) = \frac{17.76 \times [A_{645}] + 7.34 \times [A_{663}]}{mass\ of\ sample}$$

3.2.6.6 Gravimetric extraction of neutral lipids

Lipid contents were determined in triplicate by mixing 0.1 g of freeze-dried algae in 8 mL of hexane isopropanol solution (H2P) (60% (v/v) hexane, 40% 2-propanol) for 16 h on a rotary shaker. Tubes were centrifuged at 2000 × g for 2 min and the clarified H2P was removed into a weighed container. The remaining pellet was washed with H2P a minimum of three time or until the remaining pellet was colourless and the collected H2P fractions

were pooled and the solvent was evaporated. The mass of extractable lipids was measured using an analytical balance after the solvent was fully removed and the weight no longer fluctuated.

3.2.6.7 Spectrophotometric determination of cell density

Cell density determined by spectroscopic methods was compared to that found through gravimetric analysis. High density cultures in phosphate buffer were diluted in a 2-fold standard curve. One hundred microliters of well-mixed culture was added in triplicate to each well in a black 96 well microplate (Corning, USA) and 25 µL of 50 mM phosphate buffer pH 6.8 was added. Absorbance at 680 nm was determined using an Infinite M1000 dual absorbance/fluorescence plate reader (Tecan, USA). In parallel, another plate was prepared in the same manner except 25 µL of 1.5% (w/v) hypochlorite solution was added to each well, and the plate was shaken for 5 minutes in a mini-orbital shaker (VWR, USA) prior to measuring the absorbance at 680 nm.

3.2.6.8 Fluorescence measurements with Nile Red dye

Nile Red fluorescence was determined in triplicate for all samples. For bleached algae, suspensions were centrifuged at 10,000 × g for 5 min and resuspended in 50 mM sodium phosphate buffer pH 6.8. This was repeated two times in order to ensure >10,000-fold dilution of any remaining hypochlorite. Using a black 96 well microplate (Corning, USA), 100 µL of the algae samples were added to the wells. A standard curve of milk fat was constructed by diluting 5% or 10% milk fat cream (Nelson, Canada) in a twofold in series in 50 mM phosphate buffer pH 6.8 and adding 100 µL of each standard to the wells. A standard curve of triolein was constructed by dissolving analytical grade (>99%) triolein (Sigma-Aldrich, Canada) in anhydrous ethanol and creating a twofold serial dilution in anhydrous ethanol such that addition of 5 µL to 95 µL of phosphate buffer in the wells gave the desired concentrations. A working solution of 50% DMSO (Sigma-Aldrich, Canada) and 5 µg/mL Nile Red (Enzo-Scientific, USA) was prepared fresh daily and 100 µL was added to each well. The plate was incubated in the dark with shaking at 40°C and fluorescence was read (Ex. 530 nm, Em. 570 nm) after 10 minutes.

3.3 Optimization of media for mixotrophic cultivation of *Chlorella vulgaris*

Valerie C. A. Orr and Lars Rehmann

Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St., London, Ontario, Canada, N6A 3K7

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it was prepared for submission to Algal Research (Submitted June 2016). Additional results included in this chapter, not submitted for publication are described in the preface to this section.

3.3.1 Preface to Section 3.3

Microalgae have been praised as a renewable source of biomass which can be grown from the simplest elements, sunlight, carbon dioxide, and mineral salts. However, microalgae grown under photosynthetic conditions suffer from problems with light attenuation, low carbon dioxide solubility, slow growth rates, and low cell densities. However, many species of microalgae are also capable of heterotrophic cultivation using an organic carbon source. When microalgae are grown under heterotrophic conditions, growth rates increase, much higher cell densities can be achieved, and light penetration into the culture is no longer a priority concern. However, the vast majority of studies are focused on the phototrophic cultivation of microalgae, and literature elucidating the effects of heterotrophic media composition and cultivation conditions on lipid production are limited.

Therefore, it was undertaken to investigate the effects of three major media components, glucose, nitrate, and magnesium, on the mixotrophic cultivation of *Chlorella vulgaris*. *Chlorella sp.* are an industrially relevant group of microalgae reportedly capable of producing approximately 30% lipid by weight (Chisti 2007). Media composition can affect the culture performance in many ways, including the growth rate, maximum cell density, and lipid productivity. Nutrient limitation is most often used to induce the accumulation of lipids, however, nutrient limitation also limits the synthesis of proteins and consequently can limit the maximum cell density achieved (Azma et al. 2011). Maximizing volumetric lipid productivity therefore becomes a multivariate optimization problem. Thus, response surface methodology was employed to study the effect of media composition on multiple culture responses including logistic growth parameters, maximum cell density, lipid content, lipid accumulation, and glucose consumption rate.

The improved Nile Red assay presented in the previous chapter was used to observe lipid accumulation throughout the growth of the cultures, however, the end point lipid content was determined using a more accurate method of quantification; direct transesterification using only 10 mg of biomass (van Wychen and Laurens 2013), which became available only shortly after the improvements to the Nile Red assay were completed. However, the collection of 10 mg of biomass during the cultivation process in

shaker flasks can still be challenging at the beginning of the cultivation period therefore the Nile Red assay was still used to monitor lipid production during growth.

The results of this chapter show that lipid accumulation in heterotrophic cultures only begins with the onset of stationary phase if glucose is still present. In cultures which glucose was quickly depleted, lipid content remained low and glucose was first dedicated to biomass generation. The results of the Nile Red assay correlated well with the end point lipid content determined by direct transesterification. Growth parameters determined by fitting optical density data to a logistic growth model were highly influenced by all three factors, glucose concentration, magnesium concentration, and nitrate concentration. As expected, high nitrogen concentrations resulted in the highest biomass densities but the lowest lipid contents. Therefore, optimization of the volumetric lipid production (gL-1) resulted in a compromise between achieving the highest cells density possible and the highest lipid content possible. Shaker flasks experiments using the optimized conditions resulted in excellent agreement between the actual and predicted culture performance, however, lipid content was underestimated due to higher variability in in this response's polynomial.

Bioreactor cultivation is presented in this chapter however, was not included in the manuscript submitted to Algal Research as the cultivation in the bioreactor was purely heterotrophic without light which likely contributed to the differences in culture performance. The modifications in this chapter from the original manuscript include **Table 3.3-1** which includes the bioreactor performance data, and the addition of **Figure 3.3-8** which presents the reactor cultivation profile. Bioreactor cultivation using the same media mimicked the shaker flasks experiments in all performance parameters except lipid content which was drastically lower than that achieved in shaker flasks. It was revealed that bioreactor cultivation resulted in greater flux towards carbohydrate synthesis (starch) than lipid accumulation possibly linking the production of lipids to the limited light penetration, the greater oxygen availability or shear stress found in stirred tank reactors.

3.3.2 Abstract

Microalgae can accumulate large proportions of their dry cell weight as storage lipids when grown under appropriate nutrient limiting conditions. While a high ratio of carbon to nitrogen is often cited as the primary mode of triggering lipid accumulation in microalgae little information on the effect of initial substrate concentration independent of C/N ratio is available. Response surface methodologies provide a power tool for assessing complex relationships such as the interaction between the carbon source and nitrogen source. A Box-Behnken design was employed to study the effect of carbon, nitrogen, and magnesium on the growth rate, culture adaptation, maximum cell density, lipid accumulation rate, and glucose consumption rate in shaker flasks. End point dry cell weight and total lipid content were assessed for each culture and numerical optimization was used to select an optimal media for the highest cell density and lipid content under the ranges studied. Both shaker flasks and a bench scale stirred tank reactor was conducted using the optimal media and resulted in an overall conversion of glucose to lipids of 0.116 g/g and 0.068 g/g respectively. High lipid productivities were achieved in this study of 383 and 218 $mgL^{-1}d^{-1}$ for shaker flasks and bioreactor cultivations respectively.

***Keywords**:* Response surface methodology, Nile Red, *Chlorella vulgaris*, heterotrophic cultivation, mixotrophic cultivation, lipid production

3.3.3 Introduction

Lipid production in oleaginous microorganisms has been under investigation as a potential source of renewable lipids for biodiesel synthesis. Currently, biodiesel is predominately synthesized from vegetables oils or waste fats which has created a global increase in the demand and ergo the price of these oils (Atabani et al. 2012; Sitepu et al. 2014b). Accordingly, production of second generation biodiesel from non-edible oils such as those derived from oleaginous microbes is of growing interest to the biodiesel industry.

Almost all microbial species are capable of production of lipids, however, those capable of accumulating over 20% of their dry weight as lipids are designated oleaginous microorganisms (Meng et al. 2009). Those species which accumulate such significant portions generally use these lipids as an alternative energy storage molecule in the form of triacylglycerides (TAGs) (Sitepu et al. 2014b). TAGs extracted from these organisms can be upgraded into fatty acid methyl esters (FAME); the most common form of biodiesel, using traditional transesterification processes. Microalgae have received a significant amount of attention as oleaginous microbes due to their ability to accumulate lipids from inorganic carbon (CO_2) using photosynthesis. However, low growth rates and lipid productivity has increased interest in the heterotrophic or mixotrophic (combined heterotrophic and phototrophic growth) cultivation (Liang 2013). Supplementation with organic carbon like glucose or acetate can greatly augment the growth rate and lipid productivity of these strains, particularly when grown under mixotrophic conditions (Liang et al. 2009). Overall conversion of glucose to lipid has been relatively low for most single celled oils (SCOs) between 0.12-0.17 g/g compared to the theoretical maximum of 0.3 g/g for *Rhodosporidium toruloides* (Bommareddy et al. 2015). However, a much higher yield; 0.298 g/g glucose, has been obtained for the microalgae *Chlorella protothecoides* when using a mixed mode photosynthetic-heterotrophic cultivation process (Xiong et al. 2010). However, much like other microorganisms grown on organic carbon sources, use of carbon derived from waste sources like lignocellulosic biomass, waste water, pyrolytic sugars or other waste streams will ultimately be preferable (Liang 2013; Luque et al. 2016).

While reports of the effects of media composition on the heterotrophic cultivation of microalgae have been steadily increasing, many questions remain as to the complex relationship between carbon and nitrogen on the metabolism of these species. While it is known that lipid production increases with nitrogen or phosphorus limitation, excessive restriction of these substrates will also affect maximum cell density and consequently overall volumetric lipid productivity ($gL^{-1}d^{-1}$) (Li et al. 2008b). While a high ratio of carbon to nitrogen (C/N) is often cited as the main method for triggering lipid production, this downplays the essential role of nitrogen concentration plays in determining the overall growth performance of the culture (Li et al. 2008b; Chen et al. 2011a).

In order to further elucidate the effects of nitrogen and carbon as independent factors and the effects of their interaction, response surface methodology was used. Three major nutrients, glucose, sodium nitrate, and magnesium sulfate was conducted using a Box-Behnken design. Multiple responses were analyzed including: glucose consumption, lipid accumulation, growth rate, end point dry cell weight and lipid content in mixotrophic shaker flask cultivations.

3.3.4 Materials and Methods

3.3.4.1 Strain and culture conditions

Chlorella vulgaris strain UTEX 2714 was purchased from The Culture Collection of Algae at the University of Texas Austin. The culture was maintained as an actively growing cultures in liquid media using aseptic technique in 150 mL Tris-acetate-phosphate (TAP) media (20 mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, 7.0 mM NH_4Cl, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1 mL/L glacial acetic acid, and 1 mL/L of Hutner's trace elements solution) at pH 6.5 in 500 mL shaker flasks. All cultures were grown and maintained at 25 °C at 150 rpm under cyclic illumination consisting of 16 h on: 8 h off (100 $\mu mol\ m^{-2}\ s^{-1}$).

3.3.4.2 Box-Behnken Design

The effect of nitrate, glucose, and magnesium on various culture parameters were measured using a Box-Behnken design (BBD). This design was chosen due to poor predictability at the factor extremes when using a central composite design (data not

shown) and in order to reduce the number of runs required. The factors and concentrations tested in this study are summarized in **Table 3.3-2** and the design was replicated twice with 3 center point repeats.

Table 3.3-2 – Coded and uncoded concentrations of media components used in the Box-Behnken design

		Coded Levels and Concentration		
Component	**Label**	**-1**	**0**	**1**
Glucose (gL^{-1})	x_1	2	11	20
$NaNO_3$ (gL^{-1})	x_2	0.225	1.375	2.55
$MgSO_4 \cdot 7H_2O$(gL^{-1})	x_3	0.25	1.375	2.5

Media components studied at different levels were added to a basal media according to the BBD and autoclaved prior to inoculation. The basal media contained 20 mM Tris base, 1.74 gL^{-1} KH_2PO_4, 0.04 gL^{-1} $CaCl_2$ $2H_2O$, and 1 mL/L of Hutner's Trace element solution and was adjusted to a pH of 6.8 using 5 M NaOH. A seed culture was prepared in TAP media 48h prior to inoculation of the BBD media and cells were harvested aseptically by sterile centrifugation and resuspended in an equal volume of sterile water in order to minimize carryover of residual acetate and ammonium from the preculture. BBD cultures were prepared in a working volume of 75 mL in a 250 mL shaker flasks and inoculated with 1% v/v of the centrifuged seed culture.

3.3.4.3 Bioreactor cultivation

C. vulgaris was cultivated in a 5 L bench top stirred tank reactor (Labfors 4, Infors HT) equipped with two 6 blade Rushton turbines in the optimized media with an agitation speed of 350 rpm, an aeration rate of 0.2 vvm, and a temperature of 25 °C. No additional light was provided. The optimal media contained 20 mM Tris base, 18.8 gL^{-1} glucose, 2.5 gL^{-1} $MgSO_4$ $7H_2O$, 1.11 gL^{-1} $NaNO_3$, 1.74 gL^{-1} KH_2PO_4, 0.04 gL^{-1} $CaCl_2$ $2H_2O$, and 1 mL/L of Hutner's Trace element solution and was adjusted to a pH of 6.8 using 5 M H_2SO_4. A seed culture was prepared in TAP media 48h prior to inoculation of the media and cells were harvested aseptically by sterile centrifugation and resuspended in 25 mL of sterile water prior to inoculation. Bioreactors were inoculated with the equivalent of 2.5% v/v seed culture and were conducted in triplicate.

3.3.4.4 Analytical procedures

Samples of the culture were taken every 12 hours after 24 of incubation for up to 192h. Samples were processed for several measurements according to the following general procedure: first optical density was measured followed by centrifugation and measurement of glucose concentration in the supernatant. The cell pellet was then bleached and used to measure lipid accumulation using the Nile Red assay. At the culture end point (glucose consumption ceased), the endpoint dry cell weight was measured by centrifugation and washing of the cell pellet followed by lyophilization. The freeze-dried cells were used for total lipid determination using a direct transesterification protocol developed by NREL. Specific procedures documented below.

Optical density and Growth Rate

Cell suspensions were appropriately diluted with 50 mM phosphate buffer pH 6.8 and 100 μL was measured in a microtiter plate for optical density at 680nm using a dual absorbance fluorescence plate reader (M1000 Infinite, Tecan, USA).

The growth rates were fit using optical density at 680 nm using the model developed by Baranyi and Roberts (1994) where N represents the cell density, N_{max} represents the maximum cell density, μ_{max} is the maximum specific growth rate, and Q is a physiological adaptation parameter used to accommodate lag time:

$$\frac{dN}{dt} = \mu_{max}\left(\frac{Q}{1+Q}\right)\left(1-\frac{N}{N_{max}}\right)N \quad (1)$$

$$\frac{dQ}{dt} = \mu_{max}Q \quad (2)$$

Glucose Determination

The supernatant was filtered with PES filters and diluted at least 1:10 v/v using 5 mM H_2SO_4 prior to glucose analysis by HPLC using a HiPlex H column operated under previously described conditions (Wood et al. 2014).

Nile Red Assay for Lipid Accumulation

Nile Red fluorescence assay was performed using bleached algae as previously described (Orr and Rehmann 2015). In order to improve throughput, a minor modification

was made. The bleached algae were washed by transferring the cultures into a 96 well filter plate with a pore size of 0.2 μm (Millipore, USA) and centrifugation at 3500 rpm for 5 min. Residual hypochlorite was removed by mixing 1 mL of phosphate buffer (50 mM pH 6.5) to each well to wash the retained cells followed by centrifugation. This washing process was repeated 3 times. Cells were resuspended in an appropriate amount of phosphate buffer (dependent on cell concentration) and 100 μL was transferred to a clear bottom black microtiter plate. Absorbance at 680 nm was used to determine the optical dry cell weight as previously described. Following absorbance measurements, 100 μL of the Nile Red (Enzo Scientific) working solution (10 $\mu g mL^{-1}$ in 50% (v/v) DMSO, 50% (v/v) 50mM phosphate buffer pH 6.5) was added to each well. A standard lipid curve created using 5% (w/v) cream (Nelson, Canada) was included in duplicate on each plate. The plate was incubated in the dark with shaking at 40°C and fluorescence was measured at Ex = 530 nm and Em = 570 nm after 10 min. The lipid content was determined using the following equations:

$$optical\ DCW\ \left[\frac{g}{L}\right] = \frac{2.155 \times A_{680}}{5.363 - A_{680}} \quad (3)$$

$$Lipid\ (\%\ wt) = \frac{F\ (RFU)}{b(RFU \cdot Lg^{-1})\ \times\ optical\ DCW(gL^{-1})} \quad (4)$$

Where A680 is the absorbance at 600 nm using 100 μL of culture, b is the extinction coefficient for lipid concentration, optical DCW is the calculated dry cell weight of the culture prior to Nile Red addition using the optical method, and F is the relative fluorescence.

Total Lipid Content by Direct Transesterification to FAME

The fatty acid methyl ester (FAME) content by weight was determined for triplicate cultures using a slightly modified standard FAME laboratory analytical procedure (LAP) from the National Renewable Energy Laboratories (NREL) (van Wychen and Laurens 2013). Briefly, approximately 10 mg of dried cells were mixed with 20 μL of the recovery standard pentadecanoic acid methyl ester (C15:0Me at 10 mg/mL), 300 μL of 0.6 M HCl, and 200 μL of a trichloromethane methanol mixture (2:1 v/v) and subsequently incubated for 1h at 85°C in a water bath with stirring on a magnetic hot plate at 1000 rpm. After

cooling, 1 mL of hexane was added to each sample and mixed at ambient temperature at 1000 rpm. Samples were centrifuged and 450 μL of the clear top hexane phase was spiked with 50 μL of the internal standard undecanoic acid methyl ester (C11:0Me) to have a final concentration of 100 μg/mL. FAME was separated and analyzed using an FID equipped Agilent 7890 Series GC and an Agilent DB-Wax capillary column (30m, 0.25 mmm, 0.25 μm). Helium was used as the carrier gas at a constant pressure of 119 kPa, and the FID was operated at 280°C. Samples were injected in split mode with a 1:10 split ratio and eluted using the following oven ramp: 50°C, 1 min, 10°C min-1 to 200°C, 3°C min-1 220°C, 10 min. Individual FAMEs were quantified using analytical standard mixture (Supelco 37, Sigma Aldrich) and the internal standard. Unidentified FAME were quantified by applying the RF factor of the closest known peak. Total FAME content by weight was calculated according to the NREL LAP by adjusting the cumulative FAME mass using the recovery standard C15:0Me and dividing the total by the weight of cells used in the assay.

Starch determination

Total starch content was determined using the NREL LAP for enzymatic starch determination (Sluiter and Sluiter 2008). Glucose concentration was determined using the previously described method.

3.3.5 Results and Discussion

Previous work using a standard central composite design for the optimization of glucose, nitrate, phosphate, magnesium, calcium and iron concentration was found to have poor prediction ability at the extremes resulting in significant lack of fit and analysis of the factorial design indicated no significant effect of phosphate, calcium, and iron on maximum optical density and end point lipid content under the conditions studied (data not shown). Therefore, in order to improve prediction in the extremes for the remaining factors, glucose, nitrate, and magnesium, a Box-Behnken design (BBD) was chosen to gain greater insight into the interactions and effects of these factors on heterotrophic growth performance and culture productivity.

Several measurements of culture performance were determined every 12 h such as optical density, glucose consumption, as well as lipid accumulation. Lipid accumulation

was measured using the Nile Red lipid assay in shaker flasks during the cultivation period as it uses small sample sizes and the end point total lipid content was confirmed using an NREL Laboratory Analytical Procedure (van Wychen and Laurens 2013). End point lipid content determined using the NREL LAP showed reasonable correlation with the end point lipid content as determined using the Nile Red method ($R^2 = 0.7463$). Optical density which is particularly prone to interference by cellular chlorophyll content was used for continuous monitoring during the cultivation period and dry cell weight measurement was conducted only at the culture end point for a more accurate determination of cell density as it requires a substantial sample size.

3.3.5.1 Box-Behnken Design Response Surface Polynomials

The overall goals of this optimization was to maximize the growth rate and volumetric lipid productivity in order to achieve a high overall lipid productivity ($gL^{-1}d^{-1}$). Growth rate was determined in all 15 shaker flask runs by optical density and fit to a logistic growth model (Baranyi and Roberts 1994) using nonlinear least squares regression in MATLAB (MathWorks, USA). Optical density data was collected every 12 h and the fitted parameters are summarized in Table 3.3-3 along with media composition, glucose consumption rate, end point DCW, end point lipid content, and volumetric lipid productivity. A comparison of the fitted regression curves and collected data and the lipid accumulation according to the Nile Red assay are illustrated in **Figure 3.3-1** for the three center point replicates. The high variability of the lipid content in the early phase of cultivation is due to the low cell densities found in these samples.

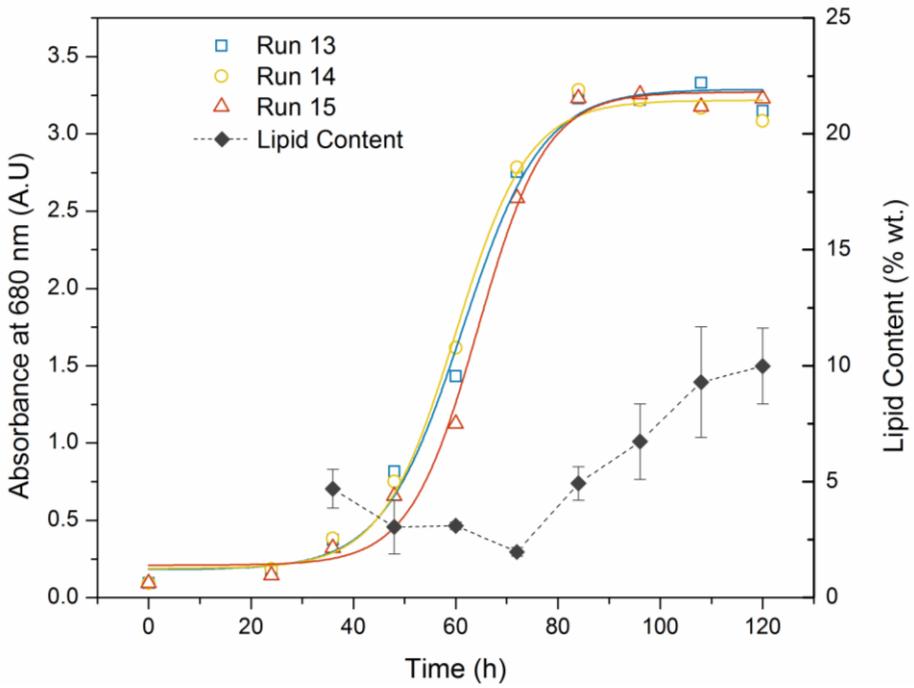


Figure 3.3-1 – Growth and lipid content (Nile Red Assay) profiles for center point replicates (runs 13-15) discrete data (open symbols) and their best fit according to the Baranyi model (Baranyi and Roberts 1994) as determined by least squares non-linear regression (corresponding coloured line).

Table 3.3-3 – Media composition of BBD and culture performance parameters measured in this study

Run	Glucose (gL^{-1})	$NaNO_3$ (gL^{-1})	$MgSO_4\ 7H_2O$ (gL^{-1})	Baranyi Fit Parameters (A_{680})		Glucose Consumption Rate; Ω_{Glc} ($gL^{-1}h^{-1}$)	End point DCW (gL^{-1})	End Point Lipid Content (% wt.)	Volumetric Lipid Productivity (gL^{-1})
				μ_{max} (h^{-1})	N_{max} (A.U)				
1	2	0.23	1.38	0.227	0.773	-0.0375	1.15 ± 0.01	26.3 ± 3.7	0.30 ± 0.04
2	20	0.23	1.38	0.179	0.749	-0.1984	1.59 ± 0.02	30.2 ± 1.4	0.48 ± 0.02
3	2	2.55	1.38	0.119	1.141	-0.0292	1.10 ± 0.02	10.8 ± 1.2	0.12 ± 0.01
4	20	2.55	1.38	0.116	5.277	-0.1987	9.54 ± 0.15	14.9 ± 1.6	1.42 ± 0.13
5	2	1.39	0.25	0.085	1.767	-0.0396	1.28 ± 0.03	10.8 ± 0.2	0.14 ± 0.00
6	20	1.39	0.25	0.117	3.757	-0.2126	8.80 ± 0.09	27.8 ± 5.7	2.44 ± 0.51
7	2	1.39	2.5	0.119	1.258	-0.0263	1.23 ± 0.06	14.1 ± 0.8	0.17 ± 0.02
8	20	1.39	2.5	0.130	3.513	-0.2008	8.08 ± 0.02	27.8 ± 3.2	2.25 ± 0.25
9	11	0.23	0.25	0.094	0.919	-0.1425	1.79 ± 0.05	30.0 ± 0.4	0.54 ± 0.02
10	11	2.55	0.25	0.082	3.848	-0.1402	4.05 ± 0.09	11.0 ± 1.2	0.45 ± 0.06
11	11	0.23	2.5	0.198	0.694	-0.0345	1.51 ± 0.00	23.7 ± 0.7	0.43 ± 0.11
12	11	2.55	2.5	0.117	4.007	-0.1541	5.07 ± 0.08	11.9 ± 1.1	0.60 ± 0.06
13	11	1.39	1.38	0.149	3.462	-0.1447	5.27± 0.06	19.3 ± 2.2	1.02 ± 0.13
14	11	1.39	1.38	0.170	3.381	-0.1555	5.28 ± 0.00	17.3 ± 0.5	0.91 ± 0.03
15	11	1.39	1.38	0.204	3.360	-0.1488	5.46 ± 0.05	18.8 ± 0.6	1.03 ± 0.03

3.3.5.2 Growth Parameters

Analysis of variance was performed on the fitted Baranyi coefficients and is summarized for μ_{max} and N_{max} in **Table S 3.3-1 and 2**. The other parameters fit to the Baranyi model were the initial cell concentration (N_0) and the initial culture adaptation parameter (Q_0) however, as these parameters are affected primarily by the initial seeding density and the cultivation conditions and media composition of the seed culture respectively, they were not investigated any further. A comparison of the actual and predicted values for the response surface polynomial fit values and μ_{max} and N_{max} are shown in **Figure S 3.3-1**. Perturbation plots were constructed with the resulting models in order to illustrate the relationship between each factor and the growth parameters (**Figure 3.3-2**). To generate the plots, one factor level was changed while all others were held at the center point.

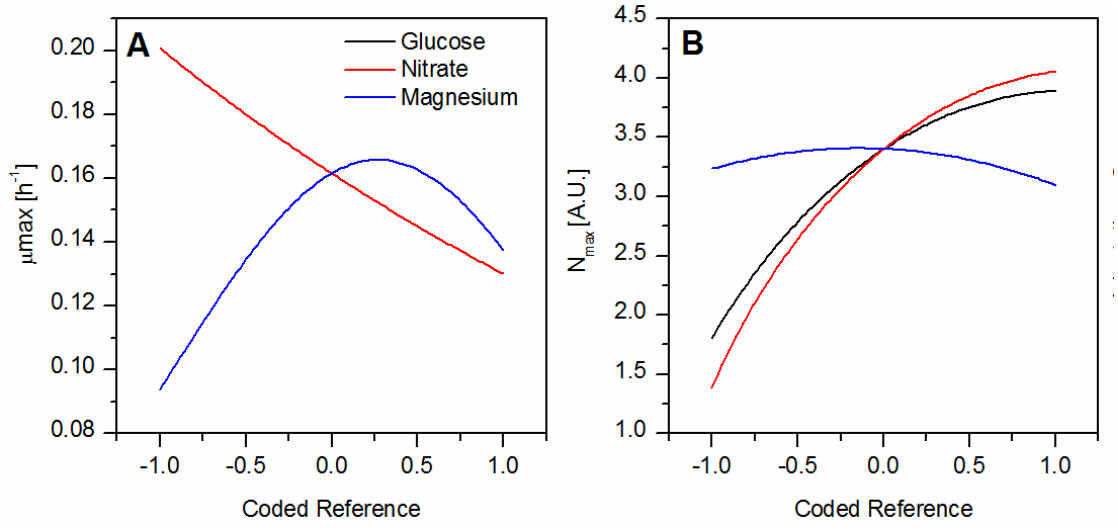


Figure 3.3-2 – Perturbation plots of each growth parameter with respect to changes in each factor. Other factors were held at the center point (0,0) as a single factor was incremented. **A**. Growth rate (μ_{max}) **B.** Maximum optical density (N_{max}).

Growth rates ranged from 0.085 to 0.227 h^{-1} depending on the media composition. The lowest growth rates were experienced in cultures containing the lowest level of magnesium sulfate as is illustrated in the perturbation plot in **Figure 3.3-2A**. However, this may be due to artificially inflated absorbance in cultures which had high chlorophyll

content as magnesium is an important cofactor in chlorophyll synthesis (Haque and Bangrak 2012). The major differences in chlorophyll content between cultures can seen in **Figure 3.3-3**. Accordingly, growth parameters fitted using optical density measurements can vary significantly from the actual dry cell weight measurements which is likely why high variance is often found in microalgae studies. With this potential interference in mind, the highest growth rates are predicted for moderate levels of magnesium (1.5-1.7 gL^{-1} $MgSO_4 \cdot 7H_2O$) and moderate levels of nitrate (1.1-1.2 gL^{-1} $NaNO_3$).

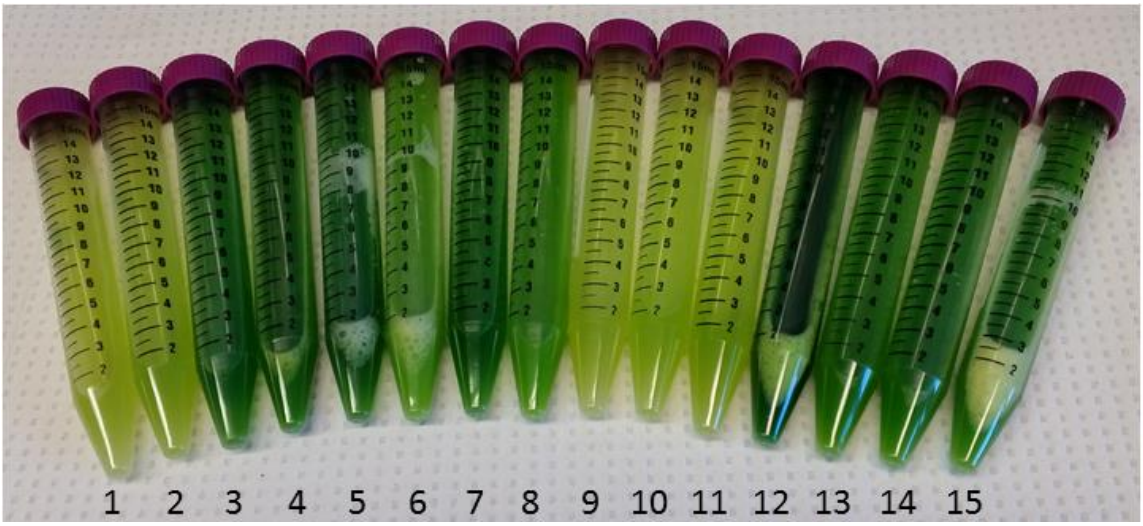


Figure 3.3-3 –Comparison of *C. vulgaris* grown under study conditions at the culture end point. The large variation in intensity and colour is indicative of a large variation in chlorophyll content of cells grown under different conditions.

The maximum optical density was found to be affected by all three factors, with glucose and nitrate concentrations exhibiting the strongest effects. Maximum optical densities occurred at the highest concentrations of glucose and nitrate (**Figure 3.3-2B)**. Overall, the highest maximum optical density was predicted at the highest level of nitrate (2.5 gL^{-1} $NaNO_3$), the highest level of glucose (20 gL^{-1}), and moderate levels of magnesium (0.7-1.7 gL^{-1} $MgSO_4 \cdot 7H_2O$). Interference of chlorophyll in the determination of maximum optical density can be seen by comparing the N_{max} to the actual DCW measurements of the culture. Run 12 which had the highest chlorophyll content as can be seen visually in **Figure 3.3-3**, had a higher fitted N_{max} than run 6 which had a much higher DCW and appeared much more yellow.

3.3.5.3 Glucose Consumption and Lipid Accumulation Rate

Glucose consumption was monitored throughout fermentation (**Figure 3.3-4** and Table 3.3-3) for two purposes, firstly to determine the fermentation end point, and secondly, in order to determine which conditions if any resulted in incomplete depletion of the added glucose.

Unsurprisingly, glucose consumption rates were highest in the cultures containing the highest initial glucose concentration. However, ANOVA analysis using a reduced linear interactions model indicated that nitrate concentration and magnesium concentration also played a role in the consumption of glucose (**Table S 3.3-3**). Two cultures did not consume all of the available glucose even after 168h; culture 8 which had 1.64 gL^{-1} of glucose remaining (8.8% of starting concentration) and culture 11 which left over 55% of the original glucose unconsumed (5.53 gL^{-1} remaining). All other cultures consumed all glucose within 144 h of inoculation, however, in cultures with an initial concentration of 2 gL^{-1}, glucose was depleted within 48 h, in cultures with initial glucose concentration of 11 gL^{-1}, glucose was depleted within 72-84 h (except culture 11), and glucose depletion at the highest concentration (20 gL^{-1}) took between 96-144 h (except culture 8).

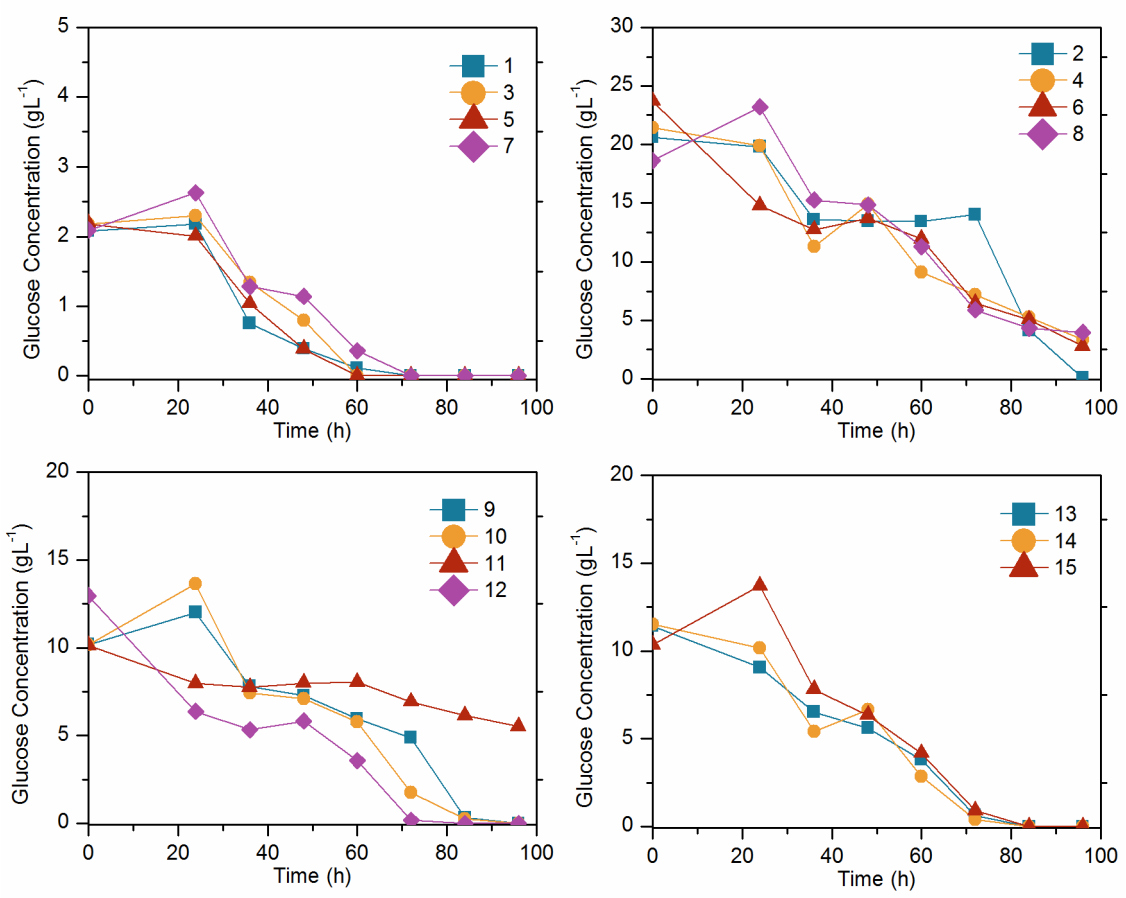


Figure 3.3-4 – Glucose consumption profiles for all 15 run conditions. Runs are organized based on the starting glucose concentration and replicate center points are runs 13-15.

Lipid accumulation during the cultivation was monitored using the Nile Red assay (data not shown). Very little change in lipid content was found in most cultures, particularly those which depleted their glucose rapidly. The five cultures which did not deplete their glucose within 144 h were found to be the cultures with the highest lipid levels and lipid content increased significantly in the period after 96 h in these cultures as seen in

Figure 3.3-5. High lipid contents in the beginning of cultures 2 and 11 is likely due to the very low cell densities found in these two cultures. These results suggest that the ratio of carbon to nitrogen in these cultures was adequate for lipid accumulation to begin and that significant lipid accumulation only occurs during stationary phase. A

similar phenomenon was recorded for *C. protothecoides* which only began accumulating lipids during the stationary phase (Xiong et al. 2010).

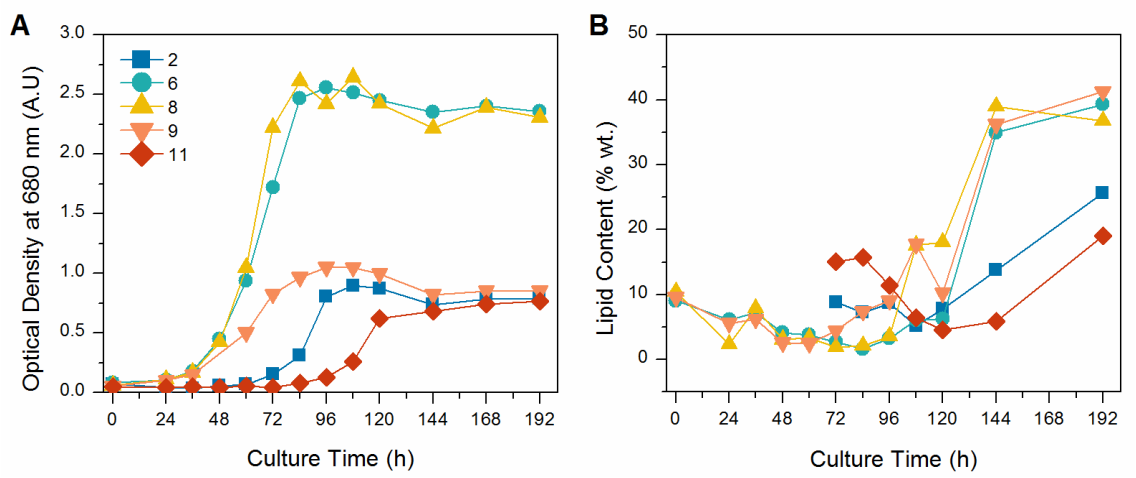


Figure 3.3-5 – Growth (**A**) and lipid accumulation (**B**) during cultivation of selected runs as determined by the Nile Red assay.

3.3.5.4 Endpoint Dry Cell Weight, Lipid Content, and Lipid Productivity

As previously stated, the end point DCW is a much more accurate representation of the culture performance than optical density. Similarly, the Nile Red (NR) assay is also subject to significant error (Orr and Rehmann 2015), therefore end point lipid content was also determined using a direct transesterification method. End point DCW was measured in triplicate for each culture and averaged (Table 3.3-3). The DCW was fit to a reduced cubic polynomial following ANOVA analysis (**Table 3.3-4**) which resulted in the following uncoded equation:

$$DCW^{0.2} = 0.9634 + 0.0185 \times [Glc] + 0.0177 \times [NaNO_3] + 0.0362 \times [MgSO_4 \cdot 7H_2O] + 0.0315 \times [Glc] \times [NaNO_3] + 0.0188 \times [NaNO_3] \times [MgSO_4 \cdot 7H_2O] - (9.7769 \times 10^{-4}) \times [Glc]^2 - 0.0242 \times [NaNO_3]^2 - 0.0231 \times [MgSO_4 \cdot 7H_2O]^2 - (7.2155 \times 10^{-3}) \times [Glc] \times [NaNO_3]^2$$

where $[Glc]$ is the concentration of glucose (gL^{-1}), $[NaNO_3]$ is the concentration of sodium nitrate (gL^{-1}), and $[MgSO_4 \cdot 7H_2O]$ is the concentration of $MgSO_4$ $7H_2O$ (gL^{-1}).

Table 3.3-4 – ANOVA for end point dry cell weight after a box-cox transformation of $\lambda = 0.2$ and elimination of insignificant terms using backward elimination, $\alpha = 0.1$

Source	Sum of Squares	DF	Mean Square	F-Value	*p*-value (Prob > F)
Model	**0.60**	**9**	**0.066**	**471.70**	**< 0.0001**
Glucose (x_1)	*0.23*	*1*	*0.23*	*1670.16*	***< 0.0001***
Nitrate (x_2)	*0.12*	*1*	*0.12*	*821.21*	***< 0.0001***
Magnesium (x_3)	1.764×10^{-5}	*1*	1.764×10^{-5}	*0.13*	*0.7377*
$x_1 x_2$	*0.058*	*1*	*0.058*	*414.36*	***< 0.0001***
$x_2 x_3$	2.425×10^{-3}	*1*	2.425×10^{-3}	*17.23*	***0.0089***
x_1^2	*0.023*	*1*	*0.023*	*164.59*	***< 0.0001***
x_2^2	*0.072*	*1*	*0.072*	*514.03*	***< 0.0001***
x_3^2	3.168×10^{-3}	*1*	3.168×10^{-3}	*22.51*	***0.0051***
$x_1 x_2^2$	*0.015*	*1*	*0.015*	*109.48*	***0.0001***
Residual	$\mathbf{7.035\times10^{-4}}$	**5**	$\mathbf{1.407\times10^{-4}}$		
Lack of Fit	6.392×10^{-4}	*3*	2.131×10^{-4}	*6.63*	*0.1339*
Pure Error	6.429×10^{-5}	*2*	3.214×10^{-5}		
Total	**0.60**	**14**			

The DCW varied significantly from 1.1 gL^{-1} to 9.5 gL^{-1} depending on the media composition. There was excellent fit between a reduced cubic model and the data and no significant lack of fit was detected (R^2 of 99.88% and R^2 adjusted of 99.67%). Correlation between the predicted values and the actual values was excellent (R^2 of prediction of 98.10%). The higher order polynomial needed in this case is a direct reflection of the complex relationship between nitrate and glucose concentrations ($x_1 x_2$, $p < 0.0001$ *and* $x_1 x_2^2$, $p = 0.0001$). The effect of glucose and nitrate on end point DCW is illustrated in **Figure 3.3-6A** which shows that the highest cell densities were achieved when nitrate was at a moderately high level (1.15-2.5 gL^{-1}) and glucose was at the maximum concentration (17-20 gL^{-1}).

End point lipid content was also determined in triplicate for each culture using direct transesterification to FAME followed by quantification using GC (Table 3.3-3). End point lipid content fit reasonably well to a simple linear polynomial:

$$\ln(lipid\ content) = 3.07856 + 0.029036 \times [Glc] - 0.35311 \times [NaNO_3]$$

where $[Glc]$ is the concentration of glucose (gL^{-1}), $[NaNO_3]$ is the concentration of sodium nitrate (gL^{-1}), and lipid content measured as percent dry weight.

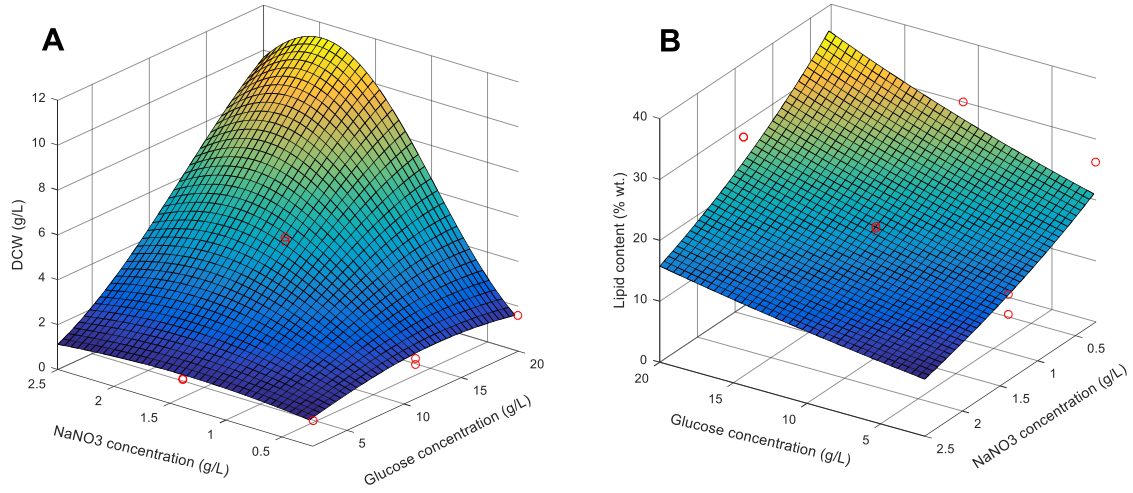


Figure 3.3-6 – Effects of glucose and nitrate concentration on DCW and lipid content according to generated response surface polynomials. Actual data points are shown as red circles.

There was reasonable fit between the data and the linear model (R^2 of 87.66% and R^2 adjusted of 85.60%), prediction was adequate (R^2 of prediction of 78.45%), and there was no significant lack of fit (**Table 3.3-5**). End point lipid content varied significantly from 10.8 – 30.2% wt. depending on media composition and was found to be most highly affected by nitrate concentration with high nitrate concentrations resulting in the lowest lipid contents as expected. The highest levels of lipid accumulation can be seen in **Figure 3.3-6B** occurred at the highest concentration of glucose (20 gL^{-1}) and the lowest concentrations of nitrate (0.255 gL^{-1}).

Table 3.3-5 – ANOVA for end point lipid content (% wt.) after a box-cox transformation of $\lambda = 0$ and elimination of insignificant terms using backward elimination, $\alpha = 0.1$

Source	Sum of Squares	DF	Mean Square	F-Value	*p*-value (Prob > F)
Model	**1.89**	**2**	**0.95**	**42.62**	**< 0.0001**
Glucose (x_1)	*0.55*	*1*	*0.55*	*24.58*	***0.0003***
Nitrate (x_2)	*1.35*	*1*	*1.35*	*60.65*	***< 0.0001***
Residual	**0.27**	**12**	**0.022**		
Lack of Fit	*0.26*	*10*	*0.026*	*7.50*	*0.1233*
Pure Error	*6.928×10^{-3}*	*2*	*3.464×10^{-3}*		
Total	**2.16**	**14**			

Lastly, the end point volumetric lipid production (g/L) was also considered as the product of DCW and lipid content. As demonstrated by the response surface polynomials generated for DCW and lipid content, maximizing these two outcomes requires some compromise as the lowest concentrations of nitrate resulted in the highest lipid contents but the lowest cell densities, and vice versa. Thus, the maximization of DCW and lipid content should mirror the optimization of volumetric lipid production. Indeed, ANOVA analysis of volumetric lipid production yields a polynomial almost identical to DCW (except the addition of the interaction term $x_2 x_3$) and the highest volumetric productivities coincide with the highest DCW (data not shown).

3.3.5.5 Optimization of Media Composition and Bench Scale Reactor

The media composition with respect to glucose, nitrate, and magnesium was optimized using the following restrictions: the culture must achieve a cell density above 4 gL^{-1} and lipid content was maximized with a minimum content of 20% wt. With these constraints only a single general solution existed (several individual solutions within a small range), with a median of 18.8 gL^{-1} glucose, 1.11 gL^{-1} $NaNO_3$, and 2.5 gL^{-1} $MgSO_4 \cdot 7H_2O$. The predicted values based on the previous polynomials was compared to the experimental values obtained with this optimized media recipe in **Table 3.3-6**. In order to assess the quality of the prediction, these cultures were performed in triplicate in shaker flasks (**Figure 3.3-7**).

The lipid content achieved in the shaker flasks was much higher than the predicted range according to the response surface polynomial. However, it should be noted that the polynomial generated for lipid content was also found to have the poorest prediction ability of all of the responses studied in this investigation and may be improved by additional data points as more conditions are assessed. It was further confirmed that lipid accumulation only began with the onset of stationary phase after 48h. Furthermore, over 10 g/L of glucose was consumed prior to the onset of stationary phase therefore an initial glucose concentration above this threshold are required in order to accumulate lipid contents above ~ 10 % wt.

Table 3.3-6 – Comparison of predicted and experimental values for the developed polynomials for [Glc] = 18.8 gL^{-1}, [$NaNO_3$] = 1.11 gL^{-1}, and [$MgSO_4 7H_2O$] = 2.5 gL^{-1} in both shaker flask cultivations and 5 L stirred tank reactors.

Parameter	P.I. (α_2 = 0.05)	Shaker Flasks	Bioreactor
Baranyi Growth Parameters			
μ_{max} [h^{-1}]	0.123-0175	0.136	0.095
N_{max} [A.U.]	2.81-3.31	2.41	2.26
Continuous Measurements			
Glucose Consumption Rate; Ω_{Glc} ($gL^{-1}h^{-1}$)	n.a*	-0.182 ± 0.02	-0.185 ± 0.02
Endpoint Measurements			
DCW [gL^{-1}]	5.85-6.81	6.12 ± 0.12	6.13 ± 0.28
Lipid Content [% wt.] (NREL)	22.4-29.3	37.6 ± 2.1%	21.5 ± 1.3%
Starch Content [% wt.]	n.a	20.5 ± 2.2%	48.5 ± 0.4%
Volumetric Productivity [g/L]	1.02-2.59	2.30 ± 0.11	1.31 ± 0.08

* Inverse transformations do not allow prediction interval calculation

^ lipid accumulation rate as determined by Nile Red (NR) in shaker flasks and NREL LAP in bioreactor samples

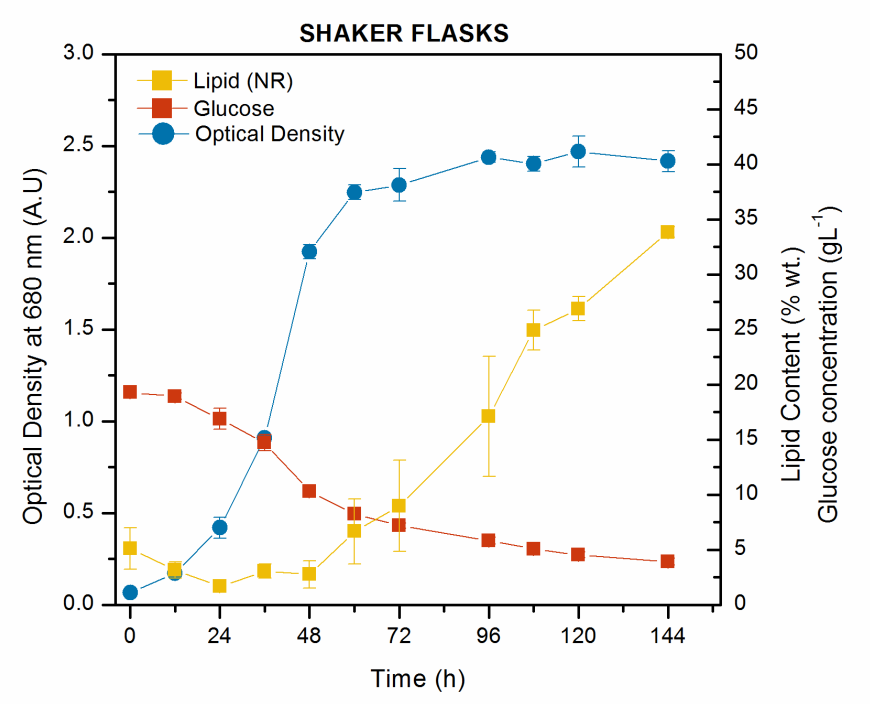


Figure 3.3-7 – Culture performance in shaker flasks under optimal growth conditions: 18.8 g/L glucose, 1.11 g/L $NaNO_3$, and 2.5 g/L $MgSO_4$ $7H_2O$ with light.

3.3.5.6 Bioreactor cultivation

Lipid content, glucose consumption, DCW, and lipid content according to the direct transesterification procedure were monitored in the stirred tank reactor (*n=3*, **Figure 3.3-8**). Growth was slower in the bioreactor, taking over 120h to reach stationary phase compared to 60 h in the shaker flasks. While glucose consumption and end point DCW were found to be identical in the shaker flasks and bioreactors, the lipid content differed significantly (**Table 3.3-6**). While it is expected that shaker flask cultivation and bioreactor cultivation would differ to some degree due to the large difference in aeration rates, there is also some evidence that increased aeration rates can result in lower lipid accumulation in heterotrophic cultures of *Chlorella sorokiniana* (Chen and Johns 1991). In order to determine where the additional carbon flux was directed as the glucose consumption and biomass densities were identical in both the shaker flasks and bioreactor, the end point biomass was subjected to further analysis, specifically starch determination. Unsurprisingly, the bioreactors produced microalgae biomass which was composed of primarily starch, while the shaker flasks produced biomass with significantly more lipids. Three main differences between the shaker flask cultivations and bioreactor optimization were (i) the lack of additional light provided in the bioreactor (purely heterotrophic culture), (ii) greater aeration in the bioreactor, and (iii) greater shear stress in the bioreactor. If starch accumulation can be triggered by increased aeration, then it may be possible to control the carbon flux of heterotrophic cultures between starch and lipids depending on the desired product using highly mutable parameters like aeration rate and agitation speed, however, further study is required to confirm this hypothesis.

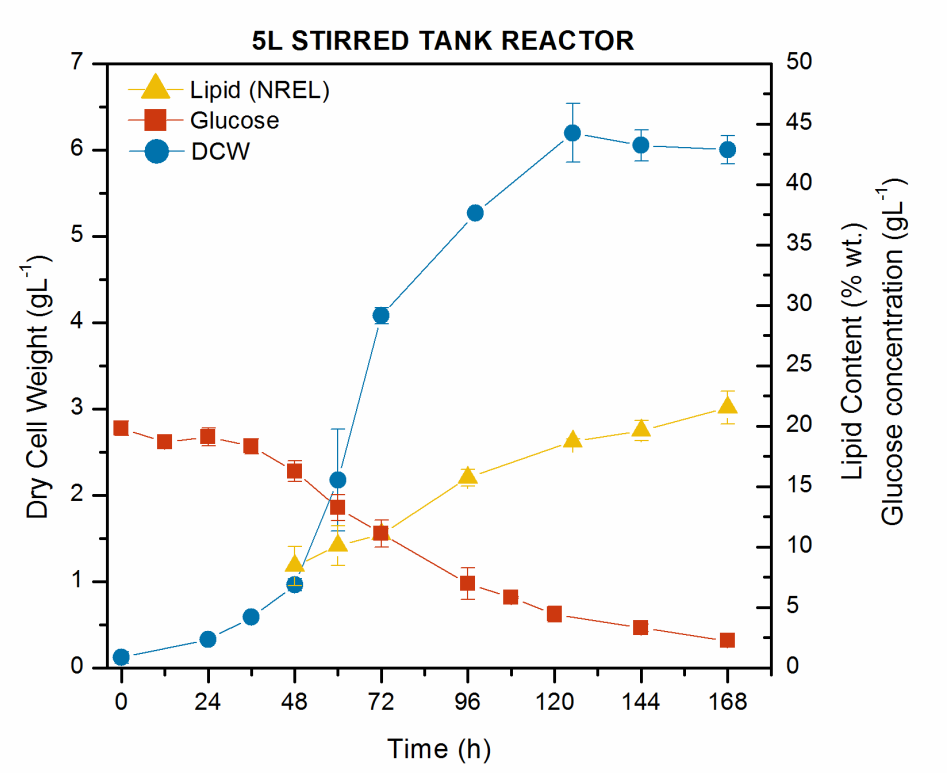


Figure 3.3-8 – Culture performance in 5L stirred tank reactor cultivation under optimal growth conditions: 18.8 g/L glucose, 1.11 g/L $NaNO_3$, and 2.5 g/L $MgSO_4$ $7H_2O$ without light.

3.3.6 Conclusions

In summary, the strategy proposed in this study was able to optimize the volumetric lipid production of mixotrophic *C. vulgaris* culture using an easily manageable number of runs and simple measurement tools. Concentration of nitrate and glucose were the most significant factors in both the production of lipids and biomass which were found to be competing interests. The optimal conditions in shaker flasks resulted in a high biomass density (6.1 gL^{-1}) and lipid content (37.6% wt.) in 6 days.

3.3.7 Supplementary Information

Table S 3.3-1 – ANOVA for Baranyi growth rate using optical density (μ_{max}) after a box-cox transformation of $\lambda = 0$ and elimination of insignificant terms using backward elimination, $\alpha = 0.1$

Source	Sum of Squares	DF	Mean Square	F-Value	p-value (Prob > F)
Model	1.13	3	0.38	15.04	**0.0003**
Nitrate (x_2)	0.38	1	0.38	15.03	**0.0026**
Magnesium (x_3)	0.29	1	0.29	11.57	**0.0059**
x_3^2	0.46	1	0.46	18.52	**0.0012**
Residual	0.28	11	0.025		
Lack of Fit	0.23	9	0.025	1.00	0.5948
Pure Error	0.050	2	0.025		
Total	1.41	14			

Resulting uncoded equation for factors in [gL^{-1}]

$$\ln(\mu_{max}) = -2.3234 - 0.1867 \times [NaNO_3] + 0.9357 \times [MgSO_4 \cdot 7H_2O] - 0.2787 \times [MgSO_4 \cdot 7H_2O]^2$$

Table S 3.3-2 – ANOVA for maximum cell density using optical density (N_{max}) after a box-cox transformation of $\lambda = 1.5$ and elimination of insignificant terms using backward elimination, $\alpha = 0.1$

Source	Sum of Squares	DF	Mean Square	F-Value	*p*-value (Prob > F)
Model	182.95	7	26.14	147.47	**< 0.0001**
Glucose (x_1)	55.03	1	55.03	310.49	**< 0.0001**
Nitrate (x_2)	85.31	1	85.31	481.38	**< 0.0001**
Magnesium (x_3)	0.27	1	0.27	1.52	0.2579
$x_1 x_2$	29.90	1	29.90	168.73	**< 0.0001**
x_1^2	5.57	1	5.57	31.41	**0.0008**
x_2^2	7.00	1	7.00	39.52	**0.0004**
x_3^2	1.50	1	1.50	8.46	0.0227
Residual	1.24	7	0.18		
Lack of Fit	1.20	5	0.24	10.79	0.0870
Pure Error	0.044	2	0.022		
Total	184.19	14			

Resulting uncoded equation for factors in [gL^{-1}]:

$$(N_{max})^{1.5} = -1.367 + 0.262 \times [Glc] + 2.763 \times [NaNO_3] + 1.222 \times [MgSO_4 \cdot 7H_2O] + 0.261 \times [Glc][NaNO_3] - 0.015 \times [Glc]^2 - 1.019\,[NaNO_3]^2 - 0.504 \times [MgSO_4 \cdot 7H_2O]^2$$

Table S 3.3-3 – ANOVA for glucose consumption (Ω_{Glc}) after a box-cox transformation of $(y+1)^{-1}$ and elimination of insignificant terms using backward elimination, $\alpha = 0.1$

Source	Sum of Squares	DF	Mean Square	F-Value	*p*-value (Prob > F)
Model	0.11	4	0.027	38.70	< 0.0001
Glucose (x_1)	0.097	1	0.097	139.68	< 0.0001
Nitrate (x_2)	2.275×10^{-3}	1	2.275×10^{-3}	3.29	0.0999
Magnesium (x_3)	2.603×10^{-3}	1	2.603×10^{-3}	3.76	0.0812
x_2 x_3	5.592×10^{-3}	1	5.592×10^{-3}	8.08	0.0175
Residual	6.921×10^{-3}	10	6.921×10^{-4}		
Lack of Fit	6.807×10^{-3}	8	8.509×10^{-4}	14.92	0.0643
Pure Error	1.141×10^{-4}	2	5.703×10^{-5}		
Total	0.11	14			

Resulting uncoded equation for factors in $[gL^{-1}]$:

$$\frac{1}{(\Omega_{Glc}+1)} = 1.071 + 0.012 \times [Glc] - 0.025 \times [NaNO_3] - 0.056 \times [MgSO_4 \cdot 7H_2O] + 0.029 \times [NaNO_3][MgSO_4 \cdot 7H_2O]$$

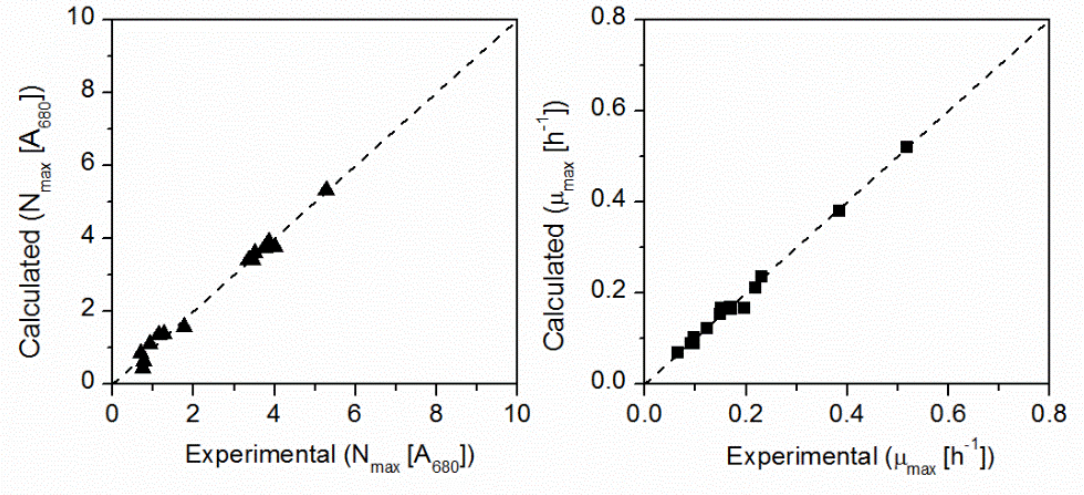


Figure S 3.3-1 – Parity plots for calculated and experimental Baranyi growth parameters.

3.4 Lipid accumulation from pinewood pyrolysates by *Rhodosporidium diobovatum* and *Chlorella vulgaris* for biodiesel production

Luis Luque,[a§] Valerie C. A. Orr,[a§] Sean Chen,[a] Roel Westerhof,[b] Stijn Oudenhoven,[b] Guus van Rossum,[c] Sascha Kersten,[b] Franco Berruti,[a] and Lars Rehmann[a]

[a] Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St., London, Ontario, Canada, N6A 3K7

[b] Sustainable Process Technology, Faculty of Science and Technology, University of Twente, P.O. Box 217, 7500 AE Enschede, Netherlands

[c] Shell Global Solutions International

§ These authors contributed equally to this work

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in *Bioresource Technology* 214: 660-669 (2016)

3.4.1 Preface to 3.4

The work presented in the previous chapter presents the optimization of media composition for heterotrophic cultivation of *Chlorella vulgaris*. Although lipid production and biomass productivity were excellent under optimized conditions, the use of pure glucose for the production of biofuels is also a contentious issue. Biofuels have begun to transition from readily fermentable glucose feedstocks produced from starchy crops to more challenging but sustainable biofuels production using waste feedstocks such as lignocellulosic biomass or other waste products.

Lignocellulose is one of the most abundant waste sources of glucose in the world. While cellulose is a polymer composed solely of glucose, it is almost always found intertwined with hemicellulose and lignin, two much less desirable polymers. Lignin in particular is the main source of aromatic compounds that can be inhibitory to microbial growth. Fast pyrolysis is a process by which lignocellulosic biomass can be decomposed into gases, char, and a carbohydrate rich liquid phase called biooil. Prior to their use as a carbon source for cultivation, sugars in the biooil must be upgraded to convert anhydrous sugars to glucose and remove inhibitory compounds.

The microalgae *C. vulgaris* and the oleaginous yeast *R. diobovatum* were cultured using pyrolysis derived glucose blended with pure glucose media in order to study the effects of inhibitors on biomass productivity, growth rate, glucose consumption, lipid accumulation, and lipid composition. *R. diobovatum* is a newly characterized yeast species capable of accumulating high levels of intracellular lipids (up to 50%) (Munch et al. 2015). Oleaginous yeasts reportedly yield higher biomass densities and lipid productivities than microalgae (Sitepu et al. 2014b). Therefore, the conversion of glucose to lipids using pyrolytic sugars was compared using these two species as competing bioconversion platforms.

C. vulgaris was much more affected by the inhibitors than the yeast and could be cultivated on blends with pyrolytic sugars up to 30% (v/v) but these cultures experienced increasing growth inhibition. *R. diobovatum* in contrast could be cultivated on pure pyrolytic sugars when using complex media, but experienced significant growth and glucose consumption inhibition in nitrogen limited media. However, in complex media,

lipid production (4.08 gL^{-1}) and cell density (19.21 gL^{-1}) were the highest yet reported for this species and much higher than the control cultivation. This was due to the simultaneous consumption of xylose derived from the hemicellulose which *R. diobovatum* readily consumed. Increasing amounts of pyrolytic sugar substitution also affected the lipid composition of the biomass and consequently affected the predicted biodiesel performance. In this respect, microalgae derived lipids were superior to yeast lipids for the production of biodiesel as then tended to be shorter and more unsaturated which favours a much lower cold flow plugging point.

The results of this chapter indicate that oleaginous yeast are better suited to production of lipids from pyrolytic sugars than microalgae, however, that with greater investigation of the effects of inhibitors on microalgae growth, microalgae cultivation on pyrolytic sugars may yet be possible.

3.4.2 Abstract

This study evaluated the suitability of pinewood pyrolysates as a carbon source for lipid production and cultivation of the oleaginous yeast *Rhodosporidium diobovatum* and the microalgae *Chlorella vulgaris*. Thermal decomposition of pinewood and fractional condensation were used to obtain an oil rich in levoglucosan which was upgraded to glucose by acid hydrolysis. Blending of pyrolytic sugars with pure glucose in both nitrogen rich and nitrogen limited conditions was studied for *R. diobovatum*, and under nitrogen limited conditions for *C. vulgaris*. Glucose consumption rate decreased with increasing proportions of pyrolytic sugars increasing cultivation time. While *R. diobovatum* was capable of growth in 100% (v/v) pyrolytic sugars, *C. vulgaris* growth declined rapidly in blends greater than 20% (v/v) until no growth was detected in blends > 40%. Finally, the effects of pyrolysis sugars on lipid composition was evaluated and biodiesel fuel properties were estimated based on the lipid profiles.

***Keywords**:* Biodiesel, pyrolytic hydrolysates, oleaginous microbes, *Rhodosporidium, Chlorella vulgaris*

3.4.3 Introduction

Biodiesel is an established alternative to petroleum-derived diesel. It is renewable and matches the fuel properties of diesel (Atabani et al. 2012). Furthermore, it is easily adopted by consumers as it can be used directly in an unmodified diesel engine or blended with petroleum diesel (Atabani et al. 2012). Currently, biodiesel is largely derived from vegetable oils, wastes fats, and animal fats (Sitepu et al. 2014b). Increased demand for edible vegetable oils as a feedstock for the growing biodiesel industry world-wide has resulted in a dramatic increase in the cost of these oils (Atabani et al. 2012). The agricultural production of some oil feedstocks, particularly palm oil are highly controversial (Balat 2011). Not only does the increase in demand affect the price of available foodstuffs, it also affects the economics of biodiesel production as the cost of feedstock can contribute as much as 75% of the overall cost of a biodiesel process (Atabani et al. 2012). Consequently, the focus of many researchers has shifted towards the development of second generation biodiesel processes which use waste or non-edible oils as their feedstock (Meng et al. 2009; Atabani et al. 2012; Sitepu et al. 2014b).

An alternative to vegetable oil for biodiesel production is the use of oleaginous microorganisms (microorganisms which can amass more than 20% lipid by dry weight) (Meng et al. 2009). Lipids extracted from single celled organisms can be trans-esterified into fatty acid methyl esters (FAMEs) using the same processes developed for vegetable oils (Sitepu et al. 2014b). While microalgae have received the most attention as oleaginous organisms, many others species of yeast, bacteria, and fungi are also capable of high lipid productivity. Several oleaginous yeast species have emerged as promising strains for lipid production as they are capable of growing on a variety of different carbon sources and can be grown to higher biomass densities than microalgae in a similar amount of time (Sitepu et al. 2014a; Munch et al. 2015). While lipid production has been discovered in many yeast species, the amount of lipid produced is highly dependent on the media composition; requiring either carbon or nitrogen limitation much like microalgae, making it difficult for direct comparison (Chen et al. 2011a). Many microalgae species are also capable of heterotrophic growth using an organic carbon source, however these cultivation processes receive less attention than phototrophic cultivation processes (Heredia-Arroyo et al. 2011). Higher lipid contents are generally

found using phototrophic growth conditions, however, heterotrophic growth rates are much higher yielding higher lipid productivities (Heredia-Arroyo et al. 2011). Finally, mixotrophic conditions combining both CO_2 and an organic carbon source with light yield the highest lipid productivities and biomass densities (Heredia-Arroyo et al. 2011). *Chlorella vulgaris* has been reported to accumulate between 5-58% lipid depending on the cultivation conditions with biomass productivities of up to 0.20 g/L/d under phototrophic conditions and 1.6 g/L/d under heterotrophic conditions (Chen et al. 2011a).

Overall lipid yield per glucose molecule has been relatively low (0.12-0.17 g/g compared to the theoretical yield of 0.30 g triacylglyceride/g glucose for *R. toruloides* (Lian et al. 2010a; Bommareddy et al. 2015) from heterotrophically grown single celled-oils (SCOs), therefore it is necessary to offset this low productivity with inexpensive feedstocks (Huang et al. 2013). A variety of low cost carbon sources have already been explored as alternatives for SCO lipid production including lignocellulosic biomass, molasses, whey, waste glycerol, and even waste oils (Papanikolaou and Aggelis 2011; Huang et al. 2013; Munch et al. 2015). Lignocellulosic feedstocks often need energy intensive pretreatment(s) to produce a fermentable substrate (Mosier et al. 2005). One unconventional pretreatment for the production of sugar from lignocellulose is fast pyrolysis (Garcia-Perez et al. 2008; Oudenhoven et al. 2013). Pyrolysis is the thermal decomposition of biomass at temperatures typically around 500 °C in the absence of oxygen. Under these conditions, a carbohydrate-rich liquid stream composed primarily of levoglucosan (1,6-anhydro-β-glucopyranose) can be recovered through factional condensation of the condensable gases or 'bio-oil', which can be used after hydrolysis and upgrading as a feedstock for several bioconversion processes generating additional value for the pyrolysis process (Luque et al. 2014).

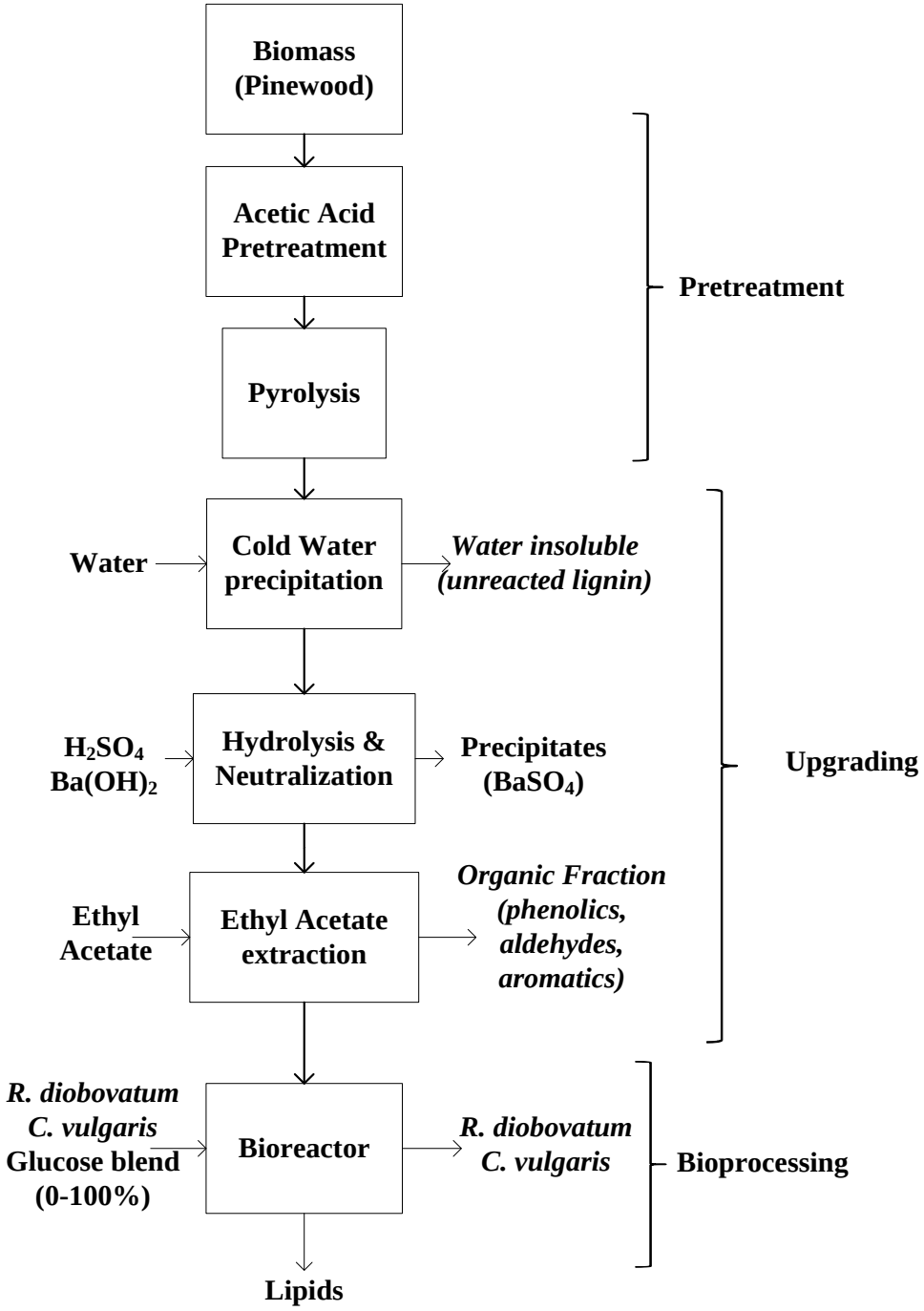


Figure 3.4-1 – Biorefinery approach for lipid production with SCOs.

The most significant challenge in the bioconversion of pyrolytic sugars is the greater multitude of possible inhibitory compounds formed during pyrolysis including the degradation products which are also formed during the more conventional acid hydrolysis of lignocellulose; such as furans, aldehydes and organic acids, but can also include greater amounts of monolignols (e.g. vanillin and cresols) (Saha et al. 2005). These inhibitory compounds can be carried over from pyrolysis through the upgrading steps and can later affect growth and production formation at sufficient concentrations (Garcia-perez et al. 2008; Luque et al. 2014; Wood et al. 2014). It was previously shown that the upgrading process shown in **Figure 3.4-1** can decrease the abundance of inhibitory compounds below their inhibitory concentration allowing unimpeded cultivation of *Saccharomyces cerevisiae* for ethanol fermentation (Luque et al. 2014).

In this study, a sugar rich fraction low in inhibitory compounds was obtained through a two-step upgrading process. Glucose obtained from this process was used as the main carbon source for lipid accumulation in *R. diobovatum* and *C. vulgaris*. The effect of increasing amounts of pyrolysis inhibitors on growth, lipid accumulation, and lipid composition in these species was evaluated by substituting increasing proportions of pure glucose for pyrolytic sugars. Furthermore, the effects of pyrolytic sugars on the estimated fuel properties (Cetane number and cold flow plugging point) were calculated from the lipid composition using a previously described model (Ramos et al. 2009).

3.4.4 Materials and Methods

3.4.4.1 Bio-oil Production

Biomass demineralization, pyrolysis oil production and upgrading was accomplished using a preexisting procedure (Luque et al. 2014).

Biomass demineralization

Leaching of pinewood biomass was achieved by mixing the biomass with an acetic acid solution 10% V/V in a jacketed stirred batch reactor, to a final biomass to leaching agent ratio of 1:10 for 2 h at 90°C. Once the leaching was completed, leachate was removed through a perforated plate in the bottom of the reactor. Biomass was then rinsed with Milli Q (Milli-Q Integral 5, EMD Millipore, USA) water in batches of 1L for 5 minutes at room temperature. The final rinse batch was determined by monitoring the

conductivity (Pinnacle Series, Nova Analytics, USA) of the output leachate stream until the value approached zero and remained constant. Excess water was removed via evaporation at 105°C for 24 h in a convection oven (Thermo Scientific, USA). Final moisture was determined using a moisture analyzer (ADAM, USA).

Pyrolysis oil production

Demineralized pinewood was pyrolyzed in a fluidized bed reactor at 480°C with a vapor residence time < 2s. Two condenser in series were used to fractionate the pyrolytic vapors according to their boiling point. In the first condenser operated at 80°C, an oil rich in anhydrous sugars and lignin-derived aromatics was obtained. The second condenser operated at 20°C, procured an oil rich fraction rich in acetic acid and water. The pressure in both condensers was held constant at 1.1±0.01 bar. The oil collected in the first condenser was used as the source of sugars for the lipid production experiments.

Upgrading of pyrolysis sugars

Pyrolysis oil rich in anhydrosugars was subjected to cold water precipitation as reported elsewhere (Luque et al. 2014). Water temperature was kept constant, 4°C, in an ice bath while oil was added dropwise, under heavy stirring (900 rpm) to 1:5 oil to cold water ratio. Insoluble lignin was recovered via vacuum filtration with a previously weighed and dried 0.45 µm cellulose nitrate membrane (Whatman®, UK) and measure gravimetrically. Resulting filtrate was centrifuged at 4°C and 3500 rpm for 20 mins (Sorval ST40R, Thermo Scientific, USA). The sugar containing supernatant was recovered from the pellet, collected in falcon tubes and store at -20°C until further use.

After the precipitation, levoglucosan present in the filtrate was acid hydrolyzed to glucose. Briefly, aliquots of filtrate were added to pressure vials. H_2SO_4 was then added to a final concentration of 0.5M. Hydrolysis was performed at 120°C for 20 mins in an autoclave. The hydrolysate pH was adjusted to 6.5 solid $Ba(OH)_2$ (Alfa Aesar, USA). Formed salts and solids were precipitated by centrifugation at a temperature of 4°C, 3500 rpm for 20 mins (Sorval ST40R, Thermo Scientific). Supernatant was recovered and transferred to a sterile 50 mL falcon tube by filtering it with a 0.2 µm cellulose syringe filter (VWR, Canada).

To remove possible growth inhibitors (e.g phenolics, furans, and aldehydes), hydrolyzate was further extracted with ethyl acetate (EtAc). A solution containing filtrate and EtAc in a 1:2 wt% ratio was prepared in a 250 mL Erlenmeyer and sealed with a rubber stopper to prevent loss of EtAc to the environment due to evaporation. Solution was homogenized for 12 h at 150 rpm and 25°C. After mixing, the sample was transferred to a 125 mL separation funnel and left standing for 24 h to secure proper phase separation. The organic layer (Top) was collected and any remaining EtAc in the rich sugar aqueous layer (bottom) was removed by evaporation at 150 rpm and 40°C. Evaporated ethyl acetate was measured gravimetrically and confirmed by samples taken every hour and measured by high pressure liquid chromatography until EA reached a constant value. Sugar concentration was kept constant by adding water.

Sugar content of the pyrolysis oil, water extract, and ethyl acetate residue were quantified by a previously described protocol using high pressure liquid chromatography using an Agilent LC 1200 infinite system equipped with a Hi-Plex H 300 × 7mm column and a RI detector (Agilent, USA) (Luque et al. 2014).

Inhibitory Value Quantification

Before hydrolysis and after EtAc extraction, spectra between 190 and 340 nm were measured for 80 minutes by high pressure liquid chromatography fitted with a Hiplex H column at 60°C, and equipped with a diode array detector (Agilent 1260 series, USA). Raw data was exported and processed in MATLAB (MathWorks Inc, USA). Removal performance was measure as changes in the volume under the surface after the detoxification process was performed. The inhibitor value normalized for glucose (or levoglucosan) concentration (IV/G) was previously defined according to equation (1):

$$IV/G = \int_{t=10min}^{t=80min} \int_{\lambda=190nm}^{\lambda=340nm} S_{DAD}\, dt\, d\lambda / C_G \qquad (1)$$

3.4.4.2 Strain and culture conditions

Rhodosporidium diobovatum cultivation conditions

*R. diobovatum (*08-225) obtained from Munch et al. (2015) were maintained using a slight modification of their reported method. Briefly, *R. diobovatum* was streaked onto YPD agar plates (10 g/L yeast extract, 20 g/L peptone, 30 g/L glucose, 15 g/L agar) and

grown at 30 °C for 2 days and stored at 4°C until further use. A seed culture grown overnight at 30 °C from a single colony was used to inoculate either YPD media (N^+; 30 g/L glucose or pyrolysis derived glucose, 20 g/L peptone, and 10 g/L yeast extract) or nitrogen limited media (N^-; 30 g/L glucose or pyrolysis derived glucose, 3 g/L yeast extract, 8 g/L KH_2PO_4, 0.5 g/L $MgSO_4$-$7H_2O$) at 10% (v/v). Media was adjusted to a pH of 5.5 and sterilized by filtration.

Chlorella vulgaris cultivation conditions

Chlorella vulgaris strain UTEX 2714 was purchased from The Culture Collection of Algae at the University of Texas Austin. The culture was maintained as an actively growing cultures in liquid media using aseptic technique in 150 mL Tris-acetate-phosphate (TAP) media (20 mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, 7.0 mM NH_4Cl, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1 mL/L glacial acetic acid, and 1 mL/L of Hutner's trace elements solution) at pH 7.0 in 500 mL shaker flasks. Cultures were grown and maintained at 25 °C at 150 rpm under cyclic illumination consisting of 16 h on: 8 h off (100 μmol m^{-2} s^{-1}). After 48 h, an exponentially growing seed culture was inoculated into Tris-nitrate-glucose (TNG) media (20 g/L glucose or pyrolysis derived glucose, 20 mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, mM, 2.4 mM $NaNO_3$, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1 mL/L Hutner's trace elements solution) at 10% (v/v) and grown under the same conditions as above. The TNG media was adjusted to a pH of 6.8 and filter sterilized.

Lipid production using pyrolytic sugars

Media prepared with pure glucose was blended with the same media prepared with pyrolysis sugars to the indicated amounts (% v/v). Final glucose concentration was kept at 30 g/L for the yeast and 20 g/L for the microalgae. Yeast cultures were grown in a 24 well plate in triplicate in a final volume of 2 mL. Plates were sealed with a sterile PCR film (VWR, Canada) and a hole was puncture to allow aeriation using a sterile 18-gauge needle (BD, USA). Plates were incubated at 30 °C and 74 rpm using a Tecan 200m Microtiter plate reader (Tecan, Austria) until glucose levels were depleted as detected by HPLC. Growth was monitored by optical density, OD_{600nm}, at 15 mins intervals. Algae cultures were grown in triplicate in a final volume of 5.5 mL in a shaker incubator at 25

°C and150 rpm with cyclic illumination of 16 h on: 8 h off (100 μmol m^{-2} s^{-1}). Small samples (20 μL) were taken every 24 h to monitor growth by optical density at 680 nm and glucose concentration was detected by HPLC.

3.4.4.3 Lipid Analysis

Harvesting and Freeze-drying

Once glucose was depleted or growth had ceased, approximately 1.5 mL of each culture was transferred to preweighed 2.0 mL centrifuge tubes. Cultures were harvested by centrifugation at 10000 × g in a Spectrafuge 24D microcentrifuge (Labnet International, USA) for 5 min. Cell pellets were resuspended with deionised water and washed three times *via* centrifugation and resuspension to remove residual salts and sugars. The washed cells were frozen at -20 °C for a minimum of 8 h and lyophilized using a 4.5 L freeze-drier (Labconco) for 24 h or until the weight no longer fluctuated. Dry cell weight was calculated based on the volume harvested and final dry weight.

Analytical Determination of Total FAME Content

The FAME content by weight was determined for triplicate cultures using a slightly modified standard FAME laboratory analytical procedure (NREL/TP-5100-60958). Briefly, approximately 10 mg of dried cells were mixed with 20 μL of the recovery standard pentadecanoic acid methyl ester (C15:0Me at 10 mg/mL), 300 μL of 0.6M HCl, and 200 μL of a trichloromethane methanol mixture (2:1 v/v) and subsequently incubated for 1h at 85°C in a water bath with stirring on a magnetic hot plate at 1000 rpm. After cooling, 1 mL of hexane was added to each sample and mixed at ambient temperature at 1000 rpm. Samples were centrifuged and 450 μL of the clear top hexane phase was spiked with 50 μL of the internal standard undecanoic acid methyl ester (C11:0Me) to have a final concentration of 100 μg/mL. FAME was separated and analyzed using an FID equipped Agilent 7890 Series GC and an Agilent DB-Wax capillary column (30m, 0.25 mmm, 0.25 μm). Helium was used as the carrier gas at a constant pressure of 119 kPa, and the FID was operated at 280°C. Samples were injected in split mode with a 1:10 split ratio and eluted using the following oven ramp: 50°C, 1 min, 10°C min-1 to 200°C, 3°C min-1 220°C, 10 min. Individual FAMEs were quantified

using analytical standard mixture (Supelco 37, Sigma Aldrich) and the internal standard. Unidentified FAME were quantified by applying the RF factor of the closest known peak. Total FAME content by weight was calculated according to the NREL LAP by adjusting the cumulative FAME mass using the recovery standard C15:0Me and dividing the total by the weight of cells used in the assay.

3.4.4.4 Estimation of Biodiesel properties based on FAME content

Two critical biodiesel properties were estimated from FAME profiles generated during lipid quantification. Cetane number (CN) and the cold filter plugging point (CFPP) were calculated using the equations reported elsewhere (Ramos et al. 2009; Nascimento et al. 2013). Briefly, the Saponification value (SV) the Iodine Value (IV) were calculated using eqs (2) and (3), where *Mw* is the molecular mass of the fatty acid, *P* is the percentage (0—100) of each individual FAME component by weight and *D* is the number of double bonds present in the fatty acid chain:

$$SV = \sum (560 \times P)/Mw \tag{2}$$

$$IV = \sum 254 \times D \times P)/Mw \tag{3}$$

Once the values were determined, the cetane number was estimated using:

$$CN = 46.3 + \left(\frac{5458}{SV}\right) - (0.255 \times IO) \tag{4}$$

The CFPP was calculated by first estimating the long-chain saturation factor (LCSF) using:

$$LCSF = (0.1 \times C16{:}0) + (0.5 \times C18{:}0) + (1 \times C20{:}0) + (1.5 \times C22{:}0) + (2 \times C24{:}0) \tag{5}$$

Cold flow plugging point was calculated by substituting the LCSF value in:

$$CFPP = (3.1417 \times LCSF) - 16.477 \tag{6}$$

3.4.5 Results and discussion

Pyrolytic sugars were produced through fast pyrolysis of demineralized pine wood followed by fractional condensation as reported elsewhere (Luque et al. 2014). The

fraction rich in levoglucosan was then upgraded to glucose through two extraction steps hydrolysis to glucose.

3.4.5.1 Upgrading of pyrolytic sugars

The upgrading process is shown in **Figure 3.4-1**. The first upgrading step consisted of removing insoluble lignin and hydrophobic compounds from the pyrolytic oil via cold water extraction (W). The sugar was further upgraded by acid hydrolysis of levoglucosan to glucose followed by neutralization. Ethyl acetate extraction was chosen as the final step in order to remove any possible inhibitory compounds carried over from the cold water extraction or the strong acid hydrolysis step (W-H-EtAc). A final aqueous solution of 35 g/L glucose was obtained which corresponded to a molar yield of 0.89 (mol glucose / mol levoglucosan) and a yield based of 0.48 based on total initial cellulose available in the biomass. The values are in agreement with previously reported data (Luque et al. 2014).

The reduction of the inhibitory compounds after upgrading was quantified using an HPLC equipped with a diode array detector (DAD). Many of the potential inhibitory compounds which may be present in pyrolytic sugars contain chromophores which can be detected using UV spectroscopy. The relative abundance of these inhibitors was quantified by numerical integration and normalized to the sugar concentration as the inhibitor value (IV/G) as described in *Section 3.4.4.1*. A reduction in the number and height of peaks shown in **Figure 3.4-1** demonstrate the removal of absorbing compounds. In **Figure 3.4-2**, the IV/G values for pinewood hydrolysates used in this study were compared to values obtained for corn cobs and switchgrass hydrolysates upgraded using a similar process. The increased abundance of inhibitors in pinewood hydrolysates may be attributable to the increased proportion of lignin in pine wood; 35 % wt., compared to between 20 to 30 % wt. for switchgrass and corn cobs (Mosier et al. 2005). The IV/G values show that the detoxification approach applied in this study substantially reduces the presence of inhibitors in pinewood pyrolysates in agreement with results obtained with other feedstocks.

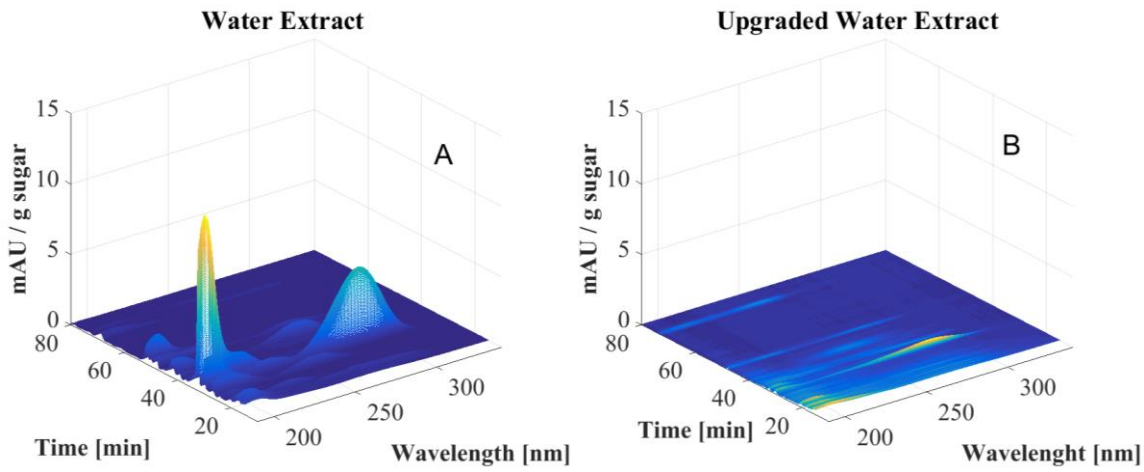


Figure 3.4-2 – Surface change as a function of upgrading train. The figure shows how the spectra of the sample changes as the detoxification process is performed.

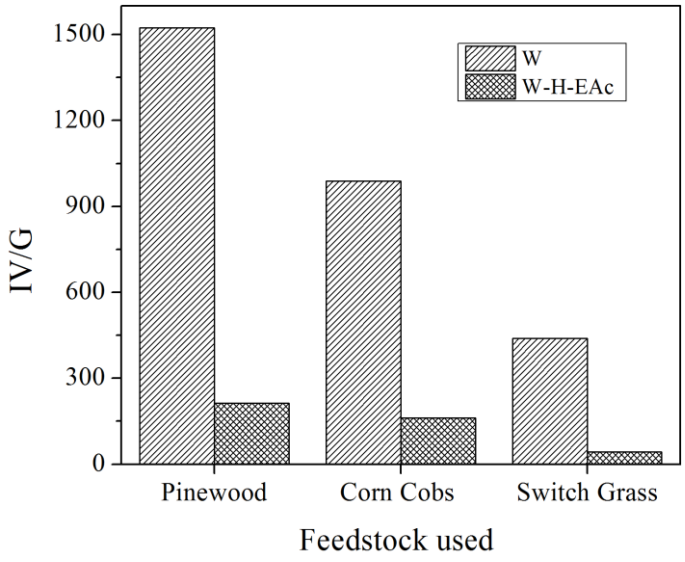


Figure 3.4-3 – IV/G values for different pyrolytic extracts from pinewood, corn cobs, and switch grass. W indicates the water extracts prior to upgrading as indicated in **Figure 3.4-1**. W-H-EtAC indicates the upgraded pyrolytic glucose solution after hydrolysis and ethyl acetate extraction.

3.4.5.2 Bioconversion of pyrolytic sugars

R. diobovatum was cultivated in either YPD media (**Figure 3.4-4A**) or nitrogen limited media (**Figure 3.4-4B**) with pyrolytic sugars as the sole carbon source. Accumulation of lipids in oleaginous yeasts is triggered by changes in the intracellular concentrations of metabolites which occur most readily when nitrogen becomes depleted in the medium (Papanikolaou and Aggelis 2011). Upon nitrogen depletion, carbon assimilation continues but is directed towards lipid synthesis rather than cell growth or other cellular processes (Ratledge 2004). Nitrogen limited media was evaluated in order to stimulate lipid production in this species. Glucose and xylose consumption is shown on **Table 3.4-1**. Media containing pure glucose was blended with media containing pyrolytic sugars in order to evaluate whether the inhibitors present in the pyrolysate affected growth, lipid accumulation, and lipid composition. Growth was marginally affected by increasing blends of pyrolytic sugars in YPD media, however final cell titers as measured by volumetric end point dry cell weight (**Table 3.4-2**) indicates that pyrolytic sugars supported significantly higher biomass densities (p <0.05) in all blends compared to the control. This is likely due to the increasing proportion of xylose present in the pyrolysis sugar media which was not added to the control media. Although *R. diobovatum* has not been extensively studied it has been shown that it can grow on a variety of carbon sources (Sitepu et al. 2014a). Indeed, complete xylose consumption as reported in **Table 3.4-1** occurred in blends up to 60% which also corresponds to the highest observed biomass density.

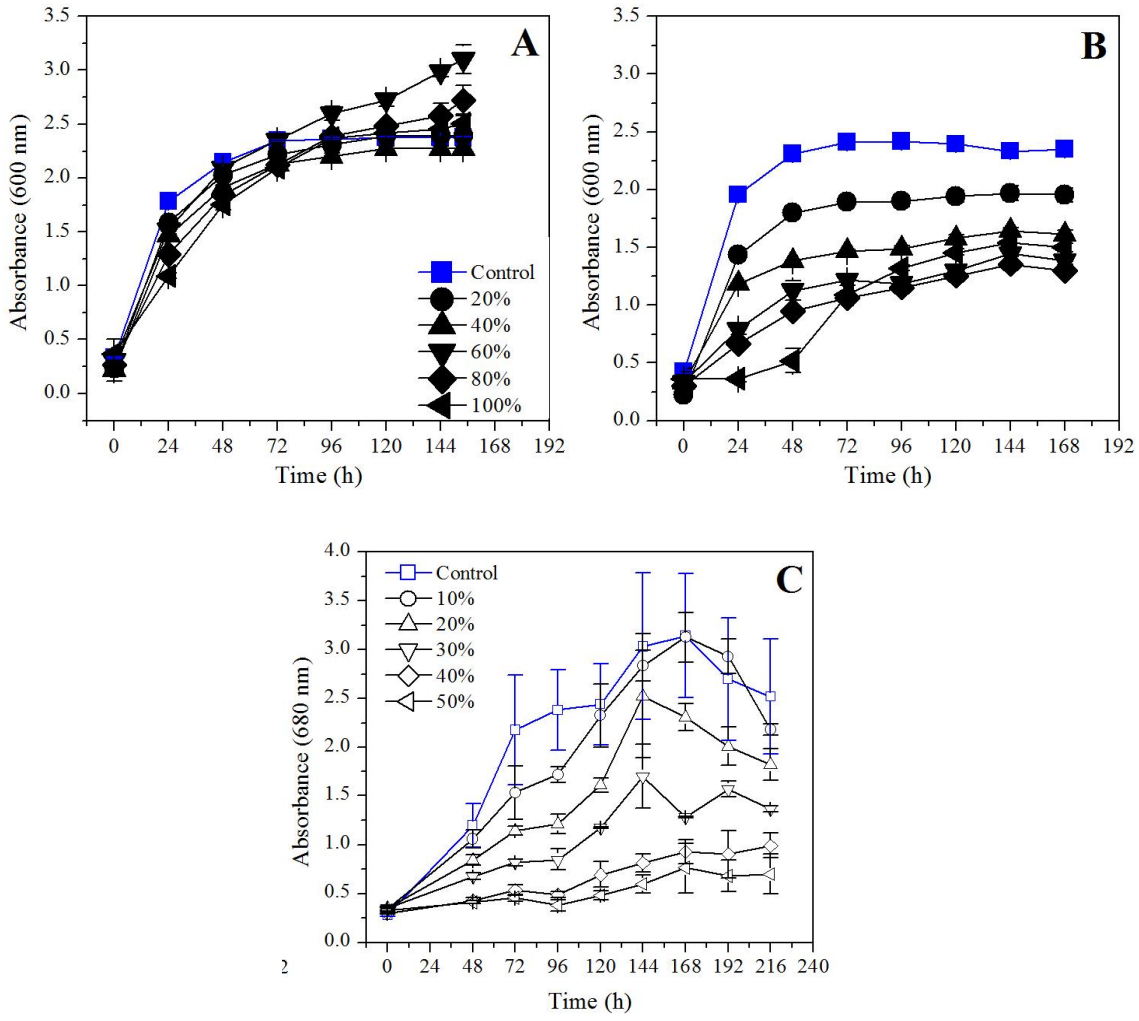


Figure 3.4-4 – Growth profiles of *R. diobovatum* (A & B) and *C. vulgaris* (C) using and increasing proportion of pyrolytic sugars (0-100%). A. Nitrogen rich YPD media B. Nitrogen limited media C. TNG media

Conversely, growth was significantly impaired with increasing blends of pyrolytic sugars under nitrogen limited conditions, **Figure 3.4-4B**, (p <0.05). However, final biomass density was identical in YPD and nitrogen limited controls (11.66 ± 0.05 g/L and 11.59 ± 0.20 g/L, respectively) indicating that on its own nitrogen limitation was not sufficient to affect cell titers. Previous results have shown *R. glutinis, R. toruloides,* and *C. curvatus* are capable of growth on 100% pyrolytic sugars (Lian et al. 2010a; Lian et al. 2013). Higher cell density has previously been used as a strategy to improve the tolerance of *S. cerevisiae* cultures to lignocellulosic hydrolysates by increasing the volumetric detoxification rates of the inhibitors present in the hydrolysate (Almeida et al. 2007). The

higher cell densities achieved in the nitrogen rich media in this study may be responsible for the increased tolerance of *R. diobovatum* to the inhibitors present in the pyrolysate in this media than in the nitrogen limited media.

Table 3.4-1 – Glucose and xylose consumption (Ω) by *R. diobovatum* (YPD, Nitrogen Limited) and *C. vulgaris* (TNG). Xylose was not present in the control media.

Media Blend	Glucose (g/L)		Ω_{Glc} (g/L/h)	Xylose (g/L)		Ω_{Xyl} (g/L/h)
	Initial	Final		Initial	Final	
***R. diobovatum* – YPD**						
Control	29.22 ± 0.05	n.d	0.37	--	--	--
20%	28.38 ± 0.38	n.d	0.32	4.08 ± 3.30	n.d	0.036
40%	30.58 ± 1.82	n.d	0.31	4.60 ± 0.57	n.d	0.043
60%	31.1 ± 0.76	n.d	0.30	7.01 ± 1.01	n.d	0.052
80%	31.7 ± 1.60	n.d	0.23	11.56 ± 1.24	1.34 ± 0.03	0.062
100%	31.9 ± 0.43	n.d	0.21	13.59 ± 0.86	3.18 ± 0.45	0.058
***R. diobovatum* – Nitrogen Limited**						
Control	33.25 ± 4.47	n.d	0.40	--	--	--
20%	31.34 ± 2.74	n.d	0.28	4.07 ± 0.56	n.d	0.03
40%	30.72 ± 2.99	5.94 ± 1.56	0.16	6.31 ± 0.42	3.18 ± 0.54	0.02
60%	31.91 ± 4.68	19.42 ± 2.74	0.08	7.01 ± 1.01	7.13 ± 0.80	0.00
80%	33.15 ± 4.06	23.76 ± 3.04	0.05	11.56 ± 1.25	8.90 ± 1.04	0.02
100%	33.12 ± 2.75	25.55 ± 0.86	0.05	13.60 ± 0.84	11.40 ± 0.5	0.02
C. vulgaris – TNG						
Control	20.49 ± 0.18[a]	13.76 ± 0.49	0.12	--	--	--
10%	20.49 ± 0.18 [a]	16.59 ± 0.59	0.11	0.76 ± 0.09	1.12 ± 0.04	--
20%	20.49 ± 0.18 [a]	15.99 ± 0.59	0.06	1.16 ± 0.07	1.46 ± 0.13	--
30%	20.2 ± 2.75	17.89 ± 0.65	0.05	1.54 ± 0.11	2.05 ± 0.12	--
40%	21.51 ± 0.60	20.25 ± 0.21	n.a	2.07 ± 0.0	2.95 ± 0.24	--
50%	20.01 ± 0.88	19.62 ± 1.66	n.a	2.44 ± 0.17	3.13 ± 0.18	--

[a] Glucose concentration was calculated based on blend ratio of glucose detected in sterile media
n.a not applicable, n.d. not detected

Table 3.4-2 – Culture performance in terms of biomass generation and lipid production by *R. diobovatum* (YPD, Nitrogen Limited) and *C. vulgaris* (TNG).

Media Blend	Dry Cell Weight (g/L)	Volumetric Lipid Production (g/L)	Lipid Productivity (mg/L/h)	Lipid Conversion (g lipid/g glucose)
***R. diobovatum* – YPD**				
Control	11.66 ± 0.05	1.43 ± 0.22	19.9 ± 3.1	0.05 ± 0.01
20%	12.15 ± 0.10	2.52 ± 0.21	35.0 ± 2.9	0.09 ± 0.01
40%	12.52 ± 0.21	3.24 ± 0.18	27.0 ± 1.5	0.11 ± 0.01
60%	19.21 ± 1.90	3.98 ± 0.27	27.7 ± 1.9	0.13 ± 0.01
80%	17.32 ± 0.39	3.98 ±0.47	26.2 ± 3.1	0.13 ± 0.01
100%	17.53 ± 0.42	4.08 ± 0.36	26.9 ± 2.4	0.13 ± 0.02
***R. diobovatum* – Nitrogen Limited**				
Control	11.59 ± 0.19	6.49 ± 0.77	67.7 ± 8.1	0.20 ± 0.02
20%	10.50 ± 0.08	6.05 ± 0.28	50.4 ± 2.3	0.19 ± 0.01
40%	9.43 ± 0.31	3.99 ± 1.23	22.1 ± 5.3	0.15 ± 0.04
60%	6.48 ± 0.20	2.45 ± 0.59	14.6 ± 3.5	0.20 ± 0.05
80%	4.83 ± 0.20	1.08 ± 0.22	6.4 ± 1.3	0.11 ± 0.02
100%	4.93 ± 0.36	1.23 ± 0.36	7.3 ± 2.2	0.16 ± 0.05
C. vulgaris – TNG				
Control	5.99 ± 0.51	1.71 ± 0.13	14.3 ± 1.4	0.18 ± 0.02
10%	4.94 ± 0.08	1.59 ± 0.05	13.3 ± 0.6	0.25 ± 0.01
20%	4.23 ± 0.31	1.22 ± 0.06	10.2 ± 0.6	0.17 ± 0.01
30%	3.00 ± 0.20	1.77 ± 0.09	6.5 ± 0.9	0.17 ± 0.02
40%	1.63 ± 0.20	0.36 ± 0.03	3.0 ± 0.3	n.d
50%	0.69 ± 0.36	0.15 ± 0.08	n.d	n.d

n.d due to lack of glucose consumption or growth

C. vulgaris was cultivated in TNG media with glucose or pyrolytic sugars under mixotrophic conditions **(Figure 3.4-4C**). Growth was only sustained with up to 30% (v/v) pyrolytic sugar blend after which growth was severely affected. It should be noted that absorbance at 680 nm is highly dependent on the chlorophyll content of the cells which may change in response to the coloration of the media and evaporation of the media over the lengthy trials was greater than 20% (v/v) (Orr and Rehmann 2015). However, dry cell weights collected at the end point indicate the same trend **(Table 3.4-2**). In comparison, growth of the microalgae *Chlamydomonas reinhardtii* on acetic acid rich pyrolytic bio-oil was only possible in blends up to 5.5% (w/w) (Liang et al. 2013; Zhao et al. 2015). The algae could be adapted to grow on up to 50% (w/w) blend of pyrolysis derived acetate, however adaptation took over 170 days and growth on pyrolytic acetate was still delayed compared to the control (Liang et al. 2013). Overall, growth rate and maximum cell density of the microalgae *C. reinhardtii* was found to be

more highly correlated with the concentration of inhibitors; particularly phenolic inhibitors, when grown on acetic acid rich bio-oils (Zhao et al. 2015).

3.4.5.3 Sugar assimilation

Glucose consumption was monitored to determine fermentation end points in order to avoid any lipid loss due to cell death or re-assimilation, **Figure 3.4-5**. Evidently, the presence of inhibitors delays glucose consumption for both species. Sugar depletion was reached in all experiments when using rich nitrogen media and *R. diobovatum* after 152 hours. This was not the case for nitrogen limited media, where only the control and 20% blend was depleted at 120 hours. *C. vulgaris* did not deplete the glucose even in the control cultivation possibly indicating the nitrogen limitation imposed was too severe. The effect of pyrolysis sugars on initial glucose consumption rate (Ω) was calculated in the linear region using linear regression (Matlab, MathWork Inc) and summarized in **Table 3.4-1**. As expected, glucose consumption rates for *R. diobovatum* in nitrogen rich media (YPD) were higher than nitrogen limited (N^-). The glucose consumption rate calculated for the 40% blend in nitrogen limited media is approximately half the value of the nitrogen rich YPD media. Glucose consumption in *C. vulgaris* showed a similar trend. Experiments were terminated before glucose was depleted if the glucose consumption rate was excessively small or in the case of *C. vulgaris*, glucose consumption ceased. Xylose consumption was only observed in the nitrogen rich media with *R. diobovatum*. Increases in xylose concentration indicated in **Table 3.4-1** are likely due to evaporation of liquid during the extensive cultivation times required for these organisms.

Using an orthogonal design, varying degrees of glucose consumption inhibition were observed during the cultivation of *R. toruloides* in the presence of multiple inhibitors and synergistic effects were detected between acetic acid, furfural, and vanillin (Zhao et al. 2012). Furthermore, cultures of *R. diobovatum* grown in the presence 5-HMF, acetic acid, and furfural under nitrogen limited conditions experienced growth delay or complete inhibition (Sitepu et al. 2014a). However, the concentrations of 5-HMF (0.04 g/L) and furfural (0.4g/L) in the 100% blend of pyrolysis sugars used in this study were below the values previously tested. However, synergies among different inhibitors

derived from biomass decomposition have been previously reported in *S. cerevisiae* and *R. toruloides* (Zhao et al. 2012; Wood et al. 2014). Furthermore, HMF and furfural which are known to directly inhibit alcohol, pyruvate and aldehyde dehydrogenases; enzymes involved in the catabolism of glucose by glycolysis (Banerjee et al. 1981) making them likely inhibitors of glucose consumption.

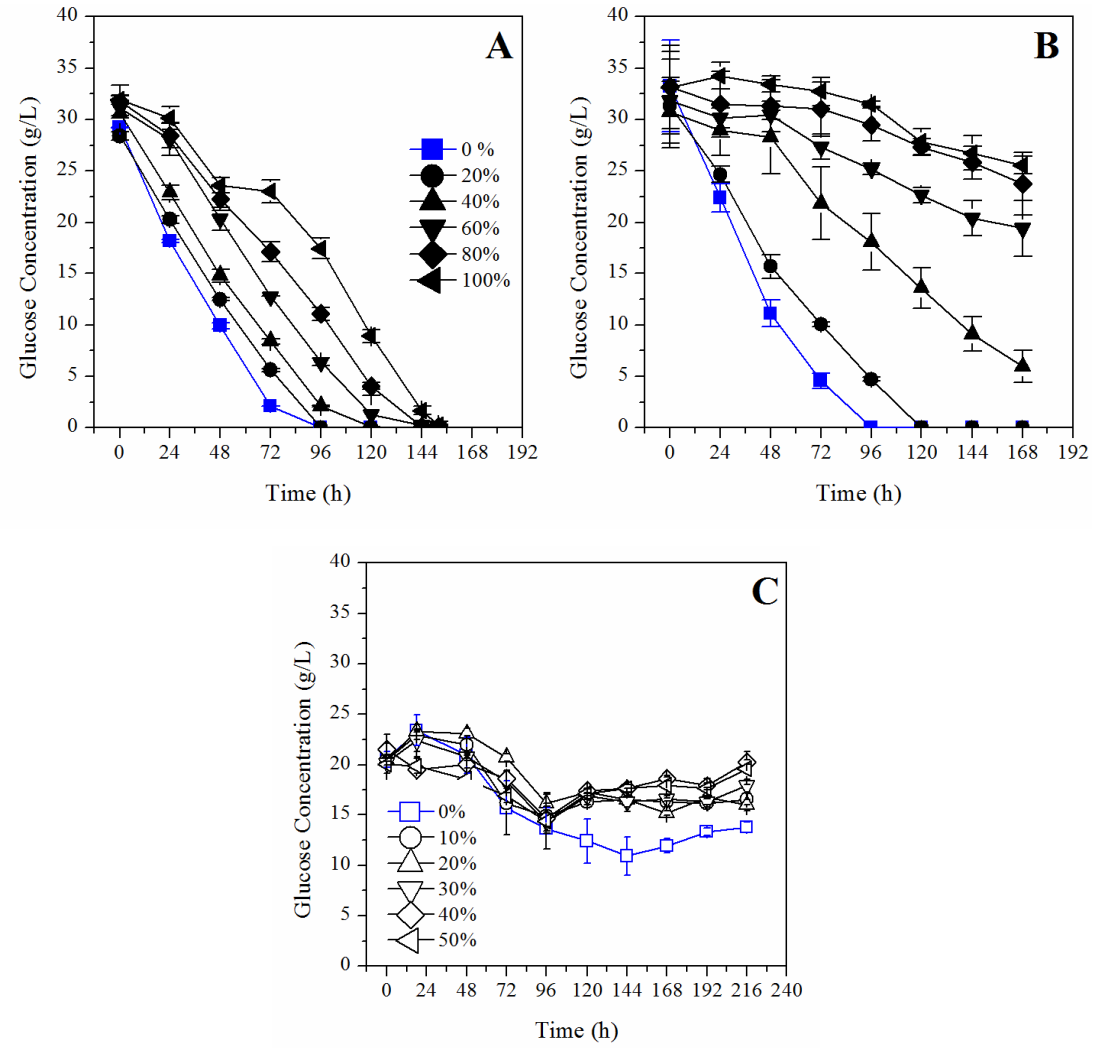


Figure 3.4-5 – Glucose consumption profiles of *R. diobovatum* (A & B) and *C. vulgaris* (C) using and increasing proportion of pyrolytic sugars (0-100%). A. Nitrogen rich YPD media B. Nitrogen limited media C. TNG media.

3.4.5.4 Effects of pyrolysis sugars on lipid accumulation

Lipid accumulation in yeasts and microalgae is significantly affected by cultivation conditions including pH, temperature, nutrient limitation, and trace metals (Beopoulos et al. 2011). Generally, lipids are accumulated when cell growth becomes limited while the carbon source is still in excess. Nitrogen limitation (Chen et al. 2011a) is most commonly used as it is simple to control and is one of the most effective means of limiting biomass growth, this is often referred to as having a high carbon to nitrogen ratio (Beopoulos et al. 2011; Chen et al. 2011a). As expected, lipid accumulation by *R. diobovatum* (**Figure 3.4-6A**) in the nitrogen limited media was much greater than the nitrogen rich media in the control cultivations (56.1% (w/w) and 12.3% (w/w) respectively). Additional stress placed on the cells by increasing the amount of blended pyrolysis sugars increased the lipid production in the nitrogen rich media, however, in the nitrogen limited media, the addition of pyrolytic sugars had a negative effect on lipid accumulation. This corresponded to the low levels of glucose consumption in these cultures and lack of glucose depletion in blends > 20% (v/v). Lipid accumulation was not affected by increasing blends of pyrolytic sugars in *C. vulgaris* cultures (**Figure 3.4-6B**).

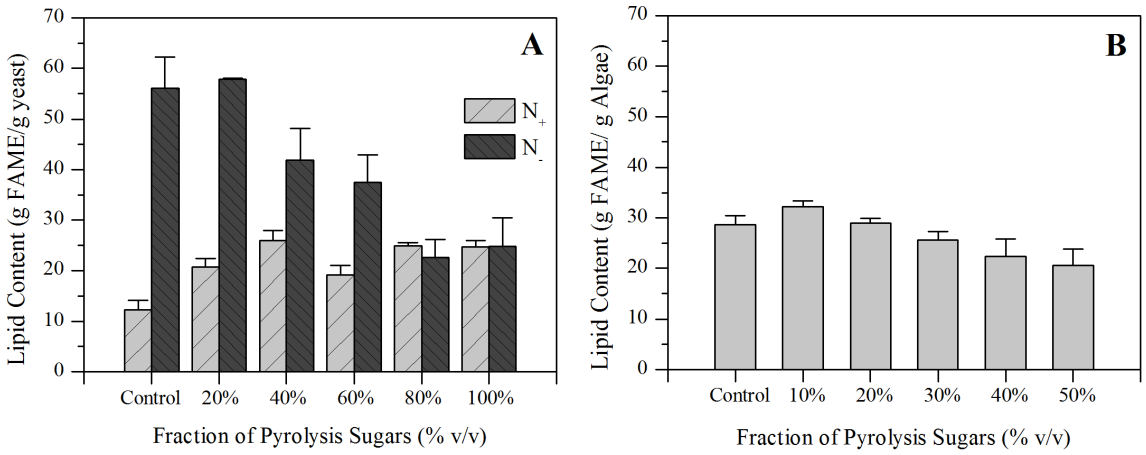


Figure 3.4-6 – Lipid accumulation of A. *R. diobovatum* and B. *C. vulgaris* using and increasing proportion of pyrolytic sugars (0-100%).

Several important indicators of culture performance (**Table 3.4-2**) were calculated for each condition including lipid productivity (g lipid/L/h) and lipid conversion (g lipid/g glucose) (Sitepu et al. 2014b). *R. diobovatum* cultures in nitrogen rich media have

a higher lipid productivity at high blend ratio with pyrolytic sugars compared to nitrogen limited media. However, as these cultures consumed more glucose and produced less lipids they had a lower conversion ratio. These differences demonstrate the need for further optimization of growth of oleaginous yeasts on pyrolytic sugars as severe nitrogen limitation may be detrimental to lipid productivity when inhibitors are present. *C. vulgaris* had the highest lipid conversion of 0.25 g/g glucose in the 20% (v/v) blend however, this may be due to simultaneous carbon fixation as they were grown under mixotrophic conditions.

In previous reports, pyrolytic sugars upgraded through an extensive process (ethyl acetate extraction, acid hydrolysis, activated carbon detoxification and rotary evaporation) were converted to lipids using *R. toruloides, R. glutinis* and *C. curvatus* (Lian et al. 2010a; Lian et al. 2013). The most promising species, *C. curvartus* accumulated up to 68% wt in lipids and produced over 16 g/L of biomass (~0.16 g lipid/g glucose) while *R. glutinis* produced 12 g/L of biomass and accumulated only 46% in lipids (~0.08 g lipid/g glucose) when cultivated on approximately 70 g/L glucose. *R. toruloides* and *R. glutinis* have also been shown to grow directly on levoglucosan, however lipid yields were significantly lower; 3.3 g/L biomass and 23.6%, than when using glucose (Lian et al. 2013).

The effect of increasing pyrolytic sugar substitution on lipid accumulation has not previously been studied. However, independently, furfural has been show to decrease lipid accumulation by up to 60% in *C. curvatus* at concentrations above 0.5 g/L while HMF concentrations up to 3 g/L had no effect (Yu et al. 2011). Further study of the upgrading process indicated that growth and lipid accumulation was severely affected by the removal of activated carbon detoxification, rotary evaporation, or ethyl acetate extraction during pyrolytic sugar upgrading (Lian et al. 2013). However, a larger study of the effects of inhibitor interactions and inhibitor concentration for oleaginous yeast is needed to provide more insight into the inhibition process.

3.4.5.5 Effect on biodiesel composition and properties

Compositional analysis of lipids based on the FAME profile obtained for each culture condition showed differences in the distributions depending on both the nitrogen content in the media and the fraction of pyrolytic glucose. Cetane number (CN) and cold flow plugging point (CFPP) were calculated from the lipid profiles of each culture in triplicate using the model proposed by Ramos et al. (2009) and are summarized in **Table 3.4-3**.

Table 3.4-3 – Estimated Cetane number (CN) and Cold Flow Plugging Point (CFPP) obtained from oils accumulated by R. diobovatum in nitrogen rich (YPD) and limited media (NL) and *C. vulgaris* in TNG media.

Media Blend	CN	CFPP (°C)
***R. diobovatum* – YPD**		
Control	49.5 ± 0.1	2.5 ± 0.3
20%	52.1 ± 0.1	8.4 ± 0.5
40%	52.9 ± 0.1	12.0 ± 0.3
60%	52.2 ± 0.1	14.9 ± 1.7
80%	53.7 ± 0.1	17.4 ± 0.4
100%	53.7 ± 0.7	18.7 ± 0.5
***R. diobovatum* – Nitrogen Limited**		
Control	53.0 ± 0.2	9.1 ± 0.6
20%	51.2 ± 0.1	7.1 ± 2.5
40%	48.1 ± 0.3	12.3 ± 0.9
60%	46.0 ± 1.5	14.6 ± 1.6
80%	42.5 ± 0.7	19.6 ± 0.7
100%	41.8 ± 0.9	16.0 ± 2.2
C. vulgaris – TNG		
Control	51.3 ± 1.0	-5.6 ± 0.64
10%	51.1 ± 0.6	-5.3 ± 1.3
20%	50.2 ± 0.2	-6.0 ± 0.1
30%	50.2 ± 0.8	-4.8 ± 0.3
40%	51.2 ± 0.5	-2.3 ± 2.1
50%	53.9 ± 0.8	7.9 ± 4.4

Fatty acid profiles are available in Tables 4-6. While the relative composition of lipids isolated from *R. diobovatum* significantly differed between the nitrogen rich and limited media similar changes due to the presence of pyrolytic sugars were detected. The differences between 0% and 100% blends were significant (two tailed heteroscedastic student T test) for palmitic acid (C16:0), stearic acid (18:0), oleic acid (C18:1), linoleic acids (C18:2), and lignoceric acid (C24:0) with $p \leq 0.01$, however many individual step sizes did not significantly alter the fatty acid profile. In both nitrogen rich (**Table 3.4-4**)

and limited media (**Table 3.4-5**), addition of pyrolytic sugars increased the proportion of C18:0, C18:1, and C24:0, but decreased the content of C18:2 with increasing amounts of pyrolytic sugars. Nevertheless, C16:0 content decreased in nitrogen limited media and increased in nitrogen rich media. Pyrolytic sugar content had no significant effect on the lipid composition of *C. vulgaris* (**Table 3.4-6**) yet, *C. vulgaris* produces shorter and more highly unsaturated fatty acids than *R. diobovatum*.

A modest decrease of palmitic acid content from 26.5 to 24.4% was previously reported when *C. curvatus* was cultured in the presence of 1 g/L of the inhibitor 5-HMF (Yu et al. 2011). Furfural had a greater effect at the same concentration and decreased C16:0 content to 21.4%. Both 5-HMF and furfural are present in the pyrolytic sugars used in this at concentrations of 0.04 g/L and 0.4 g/L respectively. While this is significantly lower than those used by Yu et al. (2011), the presences of other phenolics or synergistic effects may account for the significant effects seen in this study.

Fatty acid composition is known to significantly affect the fuel properties of the synthesized biodiesel (Knothe 2005b; Meher et al. 2006). Cetane number is one of several performance indicators regulated for biodiesel (Knothe 2005b) and is an indicator of ignition quality. Higher cetane numbers are correlated with lower emissions (Meher et al. 2006). Cetane number increased with increasing pyrolytic sugar substitution in the nitrogen rich media while it had the opposite effect in nitrogen limited media. In pyrolytic sugars blends > 60 % (v/v) in nitrogen limited media, the estimated cetane value decreased below both the American Society for Testing and Materials (ASTM) and European Committee for Standardization (EN) values of 47 and 51 respectively (Knothe 2005b).

The cold filter plugging point (CFPP) is commonly used as an indicator of biodiesel performance at low temperatures and indicates the need for additives for winterization to prevent the precipitation of FAME in cold climates (Knothe 2005b). CFPP is primarily dependent on the proportion of unsaturated fatty acids and longer chain length fatty acids of which *R. diobovatum* produced a larger proportion. Thus, the yeast produced significantly higher CFPP values than the microalgae lipid profiles indicating that microalgae derived biodiesel is more versatile. Many species of

microalgae produce much higher proportions of shorter chain length and unsaturated fatty acids resulting in biodiesel with CFPP often below 0°C (Nascimento et al. 2013). CFPP increased with increasing proportion of pyrolytic sugars for *R. diobovatum* to almost 20°C, indicating the need to further study the effects of pyrolytic inhibitors on fatty acid composition. Cetane number and CFPP were calculated for fatty acid profiles reported in the literature for oleaginous yeast and algae grown on pyrolytic sugars or acetate. *C. curvatus* produced estimate fuel properties within the same range as *R. diobovatum* (CN 62.8 and CFPP 20.8°C) as did *R. glutinis* (CN 58.4 and CFPP 9.1°C) (Lian et al. 2010a). *C. reinhardtii* grown on acetate rich pyrolytic oils had much poorer estimate biodiesel properties (CN 45.3 and CFPP 7.8°) however, this species is more typically used to study photosynthesis mechanisms than lipid production.

Table 3.4-4 – Average relative lipid composition (%) of major fatty acids in triplicate cultures of R. diobovatum cultured in YPD media. Fatty acids representing less than 1% of the total are omitted.

	Pyrolytic sugar fraction % (v/v)					
Fatty Acid	0%	20%	40%	60%	80%	100%
C16:0	9.5 ± 0.0	11.4 ± 0.1	11.9 ± 0.1	13.5 ± 0.8	14.0 ± 0.7	14.3 ± 1.3
C16:1	3.1 ±0.1	3.1±0.1	2.3 ± 0.0	1.8 ± 0.1	1.7 ± 0.0	1.6 ± 0.1
C17:1	0.8 ± 0.0	1.1 ± 0.0	0.9 ± 0.0	1.4 ± 0.1	1.2 ± 0.1	0.1
C18:0	0.6 ± 0.0	1.6 ± 0.1	2.8 ± 0.0	3.7 ± 0.2	4.1± 0.3	4.6 ± 0.6
C18:1	61.3 ± 0.4	71.6 ± 0.4	70.5 ± 0.1	68.8 ± 1.0	68.1 ± 1.5	66.6 ± 2.0
C18:2	15.3 ± 0.3	2.5 ± 0.1	3.3 ± 0.1	1.7 ± 0.1	2.0 ± 0.7	2.7 ± 0.6
C24:0	2.4 ± 0.1	3.0 ± 0.1	3.2 ± 0.1	3.6 ± 0.1	3.4 ± 0.1	3.5 ± 0.2
NI	4.4 ± 0.0	3.4 ± 0.1	2.3 ± 0.0	2.1 ± 0.2	2.1 ± 0.1	1.9 ± 0.2
Total	92.9%	94.3%	94.7%	94.5%	94.6%	94.4%

NI, non-identified

Table 3.4-5 – Average relative lipid composition (%) of major fatty acids in triplicate cultures of R. diobovatum cultured in Nitrogen Limited media. Fatty acids representing less than 1% of the total are omitted.

	Pyrolytic sugar fraction % (v/v)					
Fatty Acid	0%	20%	40%	60%	80%	100%
C14:0	1.1 ± 0.1	1.2 ± 0.0	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.0	0.8 ± 0.0
C16:0	24.0 ± 0.9	23.6 ± 0.4	19.8 ± 0.8	17.0 ± 0.7	15.5 ± 0.5	15.7 ± 0.4
C16:1	1.9 ± 0.1	1.9 ± 0.0	1.2 ± 0.0	1.0 ± 0.1	0.9 ± 0.0	0.9 ± 0.0
C17:0	0.2 ± 0.0	0.5 ± 0.0	1.0 ± 0.1	1.6 ± 0.2	2.2 ± 0.1	3.2 ± 0.1
C17:1	0.3 ± 0.0	0.9 ± 0.0	1.6 ± 0.1	2.3 ± 0.3	3.1 ± 0.2	3.7 ± 0.3
C18:0	2.9 ± 0.2	3.3 ± 0.3	4.1 ± 0.3	5.2 ± 0.6	6.3 ± 1.0	5.9 ± 0.6
C18:1	43.7 ± 0.8	46.4 ± 0.4	47.0 ± 0.7	44.7 ± 1.4	45.3 ± 1.2	49.2 ± 1.5
C18:2	19.2 ± 0.7	16.3 ± 0.2	16.7 ± 0.6	16.2 ± 1.1	17.3 ± 0.4	12.4 ± 0.9
C18:3	1.9 ± 0.1	1.4 ± 0.1	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	0.8 ± 0.1
C24:0	2.1 ± 0.1	1.7 ± 0.5	2.4 ± 0.1	2.8 ± 0.1	3.4 ± 0.2	2.9 ± 0.4
NI	1.3 ± 0.3	1.1 ± 0.2	1.8 ± 0.3	4.8 ± 4.0	2.1 ± 0.2	2.4 ± 0.1
Total	97.3%	97.3%	96.1%	93.0%	96.2%	95.6%

NI non-identified

Table 3.4-6 – Average relative lipid composition (%) of major fatty acids in triplicate cultures of *C. vulgaris* cultured in TNG media. Fatty acids representing less than 1% of the total are omitted.

	Pyrolytic sugar fraction % (v/v)					
Fatty Acid	0%	10%	20%	30%	40%	50%
C12:0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.4 ± 0.1	0.7 ± 0.2	1.6 ± 0.6
C14:0	0.4 ± 0.1	0.4 ± 01	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.4	1.1 ± 0.3
C16:0	19.2 ± 0.8	19.4 ± 0.6	18.9 ± 0.3	20.0 ± 0.4	22.8 ± 4.9	26.0 ± 1.4
C16:1	1.8 ± 0.3	1.4 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	2.7 ± 2.5	0.4 ± 0.8
C17:0	0.3 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.8 ± 0.1	1.1 ± 1.0	2.5 ± 0.5
C17:1	4.6 ± 0.5	4.3 ± 0.2	4.4 ± 0.1	4.8 ± 0.5	4.6 ± 0.3	4.7 ± 0.8
C18:0	2.8 ± 0.3	3.0 ± 0.9	2.5 ± 0.1	3.0 ± 0.3	3.9 ± 2.3	10.3 ± 32
C18:1	33.3 ± 4.0	38.4 ± 0.6	37.0 ± 0.2	31.5 ± 3.4	27.1 ± 2.3	17.9 ± 3.7
C18:2	20.8 ± 2.2	19.8 ± 0.7	22.3 ± 0.3	22.7 ± 1.6	20.7 ± 1.3	15.4 ± 1.4
C18:3	8.0 ± 0.7	7.8 ± 0.3	7.9 ± 0.2	9.2 ± 1.0	9.8 ± 2.0	12.5 ± 23
NI	7.7 ± 3.4	4.1 ± 0.3	3.7 ± 0.2	4.9 ± 0.6	5.6 ± 3.0	7.5 ± 2.2
Total	99.2%	99.4%	99.3%	99.2%	99.4%	100.0%

NI non-identified

3.4.6 Conclusion

R. diobovatum was found to tolerate upgraded pyrolytic sugar under nitrogen rich conditions, however, significant inhibition of growth and lipid accumulation was observed under nitrogen limitation. *C. vulgaris* was grown on pyrolytic glucose and demonstrated a high lipid conversion ratio however it also demonstrated strong sensitivity to pyrolysates. Inhibitors carried over from pyrolysis were found to affect glucose consumption rates, lipid accumulation and composition. Blending of pyrolysis sugars demonstrated that these effects were likely due to increasing concentrations of inhibitors and indicates a need for further studies of the effects of inhibitory compounds on both oleaginous yeasts and microalgae.

3.5 Disruption and wet extraction of the microalgae *Chlorella vulgaris* using room-temperature ionic liquids

Valerie Orr,[a] Natalia V. Plechkova,[b] Kenneth R. Seddon,[b] and Lars Rehmann[a]

[a] Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St., London, Ontario, Canada, N6A 5B9

[b] QUILL Research Centre, School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast, Northern Ireland, UK, BT9 5AG

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it will appear in the *ACS Sustainable Chemistry and Engineering:* 4(2) 591-600 (2016)

3.5.1 Preface to Section 3.5

The previous three sections investigated cultivation of oleaginous biomass for the production of microbial lipids. However, one of the most significant challenges in the production of biodiesel from microbial lipids is the high cost of the subsequent bioprocessing operations. This is mainly due to the challenge caused by their cultivation in water. Lipid extraction processes are generally incompatible with wet biomass and require a low moisture content to achieve the greatest yields. Unlike traditional oil crops, microbial species are grown in a primarily liquid medium and cannot be easily dried to low water contents by simply air drying in the fields. Their small size requires specialized equipment for their dewatering and drying which contributes significantly to their high production costs. Due to the low cost nature of commodity chemicals like biodiesel, meeting low production cost targets can be challenging.

Several strategies to improve the production process for microbial lipids can be identified. Firstly, cell titres can be improved in order to decreasing the water content of the starting feedstock. However, this will require much more extensive studies of the growth limitations of these species and will require many more years of investigation. Another possibility is to improve the dewatering processes using inexpensive strategies like flocculation or sedimentation. However, these strategies would still require extensive drying steps to reduce the water content to the necessary level for oil extraction. Finally, the oil extraction process could be improved to developing methods for lipid extraction of wet biomass. This is the strategy explored in this chapter.

Ionic liquids are a group of diverse compounds with unique properties often described as green or ionic solvents. They are most well known for their ability to dissolve cellulose using a non-derivitizing process. Many species of microalgae are known to have cellulose based cell walls which are highly recalcitrant and make microalgae resistant to cell lysis. Therefore, ionic liquids were explored as a possible means to disrupt microalgae cell structure and allow the extraction of microalgae lipids using hexane.

While several ionic liquids had been previously reported to disrupt microalgae cell structure (Young et al. 2010; Kim et al. 2012; Teixeira 2012a) and allow lipid

extraction, these processes used high temperatures (>100°C) which could also contribute significant processing costs in the form of energy required for heating which is typically supplied by fossil-fuels, further reducing the renewability of microbial based biodiesel.

Therefore, a screening study of room temperature ionic liquids was undertaken to identify ionic liquids with lower melting points which were capable of disrupting microalgae and allowing improved oil extraction at ambient temperatures. Furthermore, this study was the first study to explore the use of non-imidazolium based ionic liquids for this process which can be considerably cheaper to synthesize. Several candidates were identified and the ionic liquid [C_2mim][$EtSO_4$] was selected for further study of the water compatibility of the ionic liquid extraction process. It was found that water contents up to 82% wt. were compatible with the extraction process and the oils extracted contained a high proportion of lipids which could be converted to FAME. The developed process was able to extract lipids from the wet biomass in under 75 min.

3.5.2 Abstract

Recently, ionic liquids have been demonstrated to increase the efficiency of solvent extraction of lipids from microalgae. However, to date, mostly imidazolium-based ionic liquids have been investigated. This report extends the range of cations studied to over thirty, including imidazolium, ammonium, phosphonium, and pyridinium derivatives, which were screened for their ability to increase hexane extraction efficiency of lipids from freeze-dried microalgae *Chlorella vulgaris* at ambient temperature. Promising ionic liquids were first identified using gravimetric analysis of total extractable oils. Oils extracted after ionic liquid pre-treatment were further characterised with respect to fatty acid methyl ester (FAME) = biodiesel yield, FAME composition, and chlorophyll content. With few exceptions, all of the tested ionic liquids had lower chlorophyll content than standard solvent extraction techniques. The effect of process parameters such as mass ratio of algae to ionic liquid, incubation time, water content, and cosolvents were investigated for 1-ethyl-3-methylimidazolium ethylsulfate [C_2mim][$EtSO_4$]. The results indicate that this ionic liquid can disrupt *C. vulgaris* in conjunction with methanol and allow facile recovery of lipids over a large degree of dewatered microalgae (0-82 wt% water), in a small amount of time (75 min) at room temperature, resulting in the development of a low energy, water compatible, biodiesel production scheme.

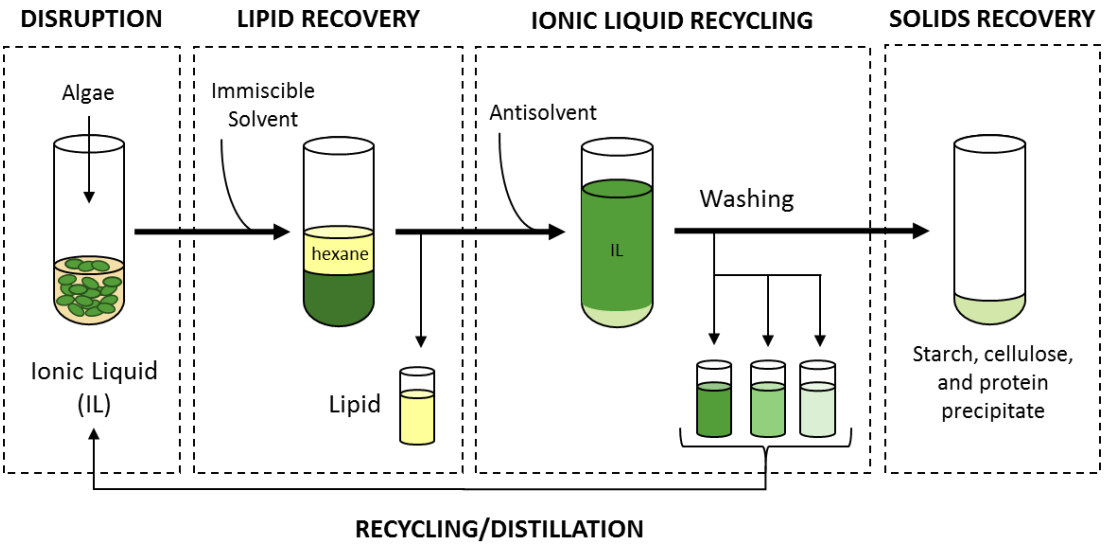


Figure 3.5-1 – Graphical Abstract – Summary of ionic liquid based lipid extraction process

Keywords: Microalgae, ionic liquids, biodiesel, *Chlorella vulgaris*, wet extraction

3.5.3 Introduction

Increasing global energy demands coupled with the environmental effects of petroleum-derived transportation fuels and chemicals have driven the development and commercialisation of alternative energy strategies, such as bioconversion (Brennan and Owende 2010; Erickson et al. 2012). Microalgae have been praised for their ability to convert solar energy and carbon dioxide into triacylglycerides (TAGs) - precursors for biodiesel - making the production of biodiesel potentially carbon-negative (Chisti 2007; Posten and Schaub 2009; Singh and Gu 2010). Microalgae offer greater photosynthetic conversion efficiency, do not require agricultural land for their cultivation, and are not limited by seasonality, unlike oil crops currently used for biodiesel production (Singh and Gu 2010). However, currently economic barriers prevent the commercial development of algal biodiesel due to the cost-prohibitive harvesting, drying, and extraction steps.(Halim et al. 2012) Unlike land plants, which are composed of large section of differentiated tissue, the single-celled microalgae are cultivated in aqueous media, requiring them to undergo more energy-intensive dewatering and drying steps (Posten and Schaub 2009; Singh and Gu 2010; Milledge and Heaven 2012). However, like most land plants, many microalgae species possess a cellulosic cell wall which is resistant to mechanical disruption and impedes diffusion during solvent extraction processes for lipid recovery (Halim et al. 2012; Grima et al. 2013). It is for this reason that extraction contributes 30-50% of the overall process cost of biodiesel from microalgae (Teixeira 2012b). Solvent extraction is one of the most commonly employed methods for TAG recovery for biodiesel production (Singh and Gu 2010; Halim et al. 2012; Grima et al. 2013). However, solvent extractions are material and time-consuming, also presenting some health and safety concerns. Due to its low polarity, hexane is the preferred solvent when the goal is to minimise the co-extraction of non-lipid contaminates. However, in order to increase the extraction efficiency, the solvent must be heated under reflux at elevated temperatures, making it even more energy-intensive (Medina and Grima 1998). Hypothetically, solvent extraction is primarily limited by diffusion, however, often times, neutral lipids are also associated with polar lipid protein complexes which require greater forces to break than interactions with a non-polar environment can provide (Medina and Grima 1998; Halim et al. 2012). It is for this reason that low dielectric constant solvents

(such as trichloromethane and hexane) are typically paired with polar cosolvents (such as methanol or 2-propanol), as they can disrupt these higher energy complexes through hydrogen bonding with the polar lipids (Bligh and Dyer 1959; Medina and Grima 1998; Halim et al. 2012). Unfortunately, oils recovered in this manner will contain other more polar biomolecules such as chlorophyll, free fatty acids, phospholipids, sterols, and gangliosides which are undesirable in the final biodiesel product or may interfere in the transesterification process (Grima et al. 2013). Cell disruption can increase extraction efficiency by increasing the interfacial area of solvent to cellular matrix for mass transfer. This can be achieved through slow or energy intense mechanisms, such as ultrasonication, electric pulse, or microwave-assisted solvent extraction, or by time-consuming enzymatic hydrolysis (Zheng et al. 2011; Grimi et al. 2014; Keris-Sen et al. 2014; Wang et al. 2014).

Recently, it was demonstrated that a number of ionic liquids can lyse both dry and wet microalgae under relatively mild conditions (80-140 °C), with and without a cosolvent (**Table 3.5-1**). Ionic liquids, also known as 'green' designer solvents due to their low volatility, excellent chemical and thermal stability (Seddon 1997), and their unique solvent properties, are best known for the ability of some ionic liquids to dissolve highly recalcitrant biopolymers such as cellulose or lignin (Plechkova and Seddon 2008; Pinkert et al. 2009; Garcia et al. 2010; Tadesse and Luque 2011). This challenging task is accomplished by the disruption of hydrogen bonding networks responsible for cellulose's structural properties (Swatloski et al. 2002; Remsing et al. 2006; Pinkert et al. 2009). To date, studies on cell disruption of microalgae biomass have so far focused mainly on imidazolium ionic liquids , likely due to their greater commercial availability as well as their highly investigated ability to dissolve cellulose, the principle component of algae cell walls (Young et al. 2010; Kim et al. 2012; Teixeira 2012b; Kim et al. 2013; Choi et al. 2014c). Long pre-treatment times and/or lengthy hexane extractions employed in these studies do not demonstrate any value over the current solvent extraction processes except where wet biomass is used (Young et al. 2010; Teixeira 2012b; Olkiewicz et al. 2015). Furthermore, the majority of studies employed the Bligh & Dyer method (Bligh and Dyer 1959) of total lipid extraction to determine the theoretical maximum yield, although it is only appropriate for biomass with low lipid content(< 2 wt%) resulting in significant

underestimation of the total lipid content (Iverson et al. 2001). With these points in mind, a vast number of room temperature ionic liquids were investigated for their ability to improve lipid extraction from microalgae by limiting the hexane extraction step to 15 minutes in order to collect only easily accessible lipids. Oils extracted with high gravimetric yields were further characterised for chemical composition and for the leading candidate, the effects of common process parameters were investigated.

Table 3.5-1 – Summary of ionic liquid based microalgae cell disruption studies to date

Algal Species	Operating Conditions	Ionic liquid (IL)	Oil Yield	Ref
Chlorella sp.	1g dry algae + 7.4g IL + 8.9g methanol, 65°C, 18h	$[C_2mim][MeSO_4]$	8.60 wt%	(Young et al. 2010)
Duniella sp.		$[C_2mim][MeSO_4]$	7.90 wt%	
Duniella sp.	1g dry algae + 7.4g IL + 8.9g acetic acid,65°C, 18h	$[C_2mim][MeSO_4]$	5.60 wt%	
Duniella sp.	1g wet algae + 7.4g IL + 8.9g acetone, 65°C, 18h	$[C_2mim][MeSO_4]$	9.20 wt%	
Duniella sp.	1g wet algae + 7.4g IL + 8.9g $CHCl_3$, 65°C, 18h	$[C_2mim][MeSO_4]$	8.40 wt%	
Duniella sp.	1g wet algae + 7.4g IL + 8.9g 2-propanol, 65°C, 18h	$[C_2mim][MeSO_4]$	8.50 wt%	
Duniella sp.	1g wet algae + 7.4g IL + 8.9g dmso, 65°C, 18h	$[C_2mim][MeSO_4]$	6.0 wt%	
Chlorella vulgaris		**FAME Yield [mg/g algae]**		(Kim et al. 2013)
		Bligh & Dyer	*28.5*	
	1g algae + 10mL IL, 60°C, 3h	$[C_4mim][MeSO_4]$	47.4	
	1g algae + 10mL IL, 60°C, 3h, sonication	$[C_4mim][MeSO_4]$	74.0	
Chlorella pyrenoidosa *Chlorella vulgaris* *Neochlorochloris pseudostigmata* *Selenastrum capricornutum* *Chlorococcum hypnosporum* *Chlamydomonas reinhardtii* *Chlamydomonas moewusii* *Scenedesmus quadricauda*	Average of various conditions between 40-120°C, 2 – 60 min, $CHCl_3$[a] was used as the oil extracting solvent	**Normalised Yield** [b]		(Teixeira 2012b)
		Bligh & Dyer	*34 ± 14%*	
		$[C_2mim]Cl$	98 ±18%	
		$[C_4mim]Cl$	89 ± 12%	
		$[C_{2OH}mim]Cl$	100 ± 10%	
		[Amim]Cl	47 ± 18%	
Chlorella vulgaris	1g dry algae + 4mL IL + 4mL methanol 65C, 18h	**FAME Yield [mg/g algae]**		(Kim et al. 2012)
		Bligh & Dyer	*106.2*	

		$[C_4mim][MeSO_4]$	125.4	
		$[C_4mim][CF_3SO_3]$	118.4	
		$[C_4mim][CH_3SO_3]$	62.0	
		$[C_4mim][BF_4]$	52.0	
		$[C_4mim][PF_6]$	38.2	
		$[C_4mim][NTf_2]$	31.6	
		$[C_4mim]Cl$	5.8	
		$[C_2mim][MeSO_4]$	118.8	
		$[C_2mim]]Cl$	94.6	
		$[C_2mim]Br$	49.2	
		$[C_2mim][O_2CMe]$	28.2	
Chlorella vulgaris	1g dry algae +19g IL 120C, 2h	**FAME Yield [mg/g algae]** [b]		(Choi et al. 2014c)
		Direct Transesterification†	*292.2*	
		Modified Folch	*206 ± 9*	
		$[C_2mim][O_2CMe]$	223 ± 5	
		$[C_2mim]][HSO_4]$	35 ± 1	
		$[C_2MIM]][AlCl_4]$	176 ± 13	
		$[C_2mim][Et_2PO_4]$	246 ± 7	
		$[C_2mim][EtSO_4]$	60 ± 2	
		$[C_2mim][SCN]$	43 ±2	
		$[C_2mim][CH_3SO_3]$	89 ± 3	
		$[C_2mim][NTf_2]$	174 ± 4	
		$[C_2mim][BF_4]$	218 ± 2	
		$[C_2mim]Cl$	235 ± 8	
		[amim]Cl	39 ± 2	
		$[C_4mim]Cl$	37 ± 0	
	1g dry algae +9.5g IL +9.5 g hexane 120C, 2h	$[C_2mim][O_2CMe]$	189 ± 9	
	1g dry algae +9.5g IL +9.5 g methanol 120C, 2h	$[C_2mim][O_2CMe]$	221 ± 13	

	1g dry algae +9.5g IL +9.5 g $CHCl_3$ 120C, 2h		$[C_2mim][O_2CMe]$	238 ± 4
	1 g dry algae + 9.5 g *IL 1* + 9.5g *IL 2*; 120C, 2h	*IL 1*	*IL 2*	**Oil Yield [mg/g algae]**
		$[C_2mim][O_2CMe]$	$[C_2mim][NTf_2]$	255.7 ± 8.4
			$[C_2mim][BF_4]$	62.5 ± 2.9
			$[C_2mim]Cl$	17.0 ± 1.4
			$[C_2mim][Et_2PO_4]$	187.8 ± 4.5
		$[C_2mim]Cl$	$[C_2mim][NTf_2]$	109.1 ± 12.7
			$[C_2mim][BF_4]$	62.3 ± 6.9
			$[C_2mim][Et_2PO_4]$	143.9 ± 5.0
		$[C_2mim][NTf_2]$	$[C_2mim][BF_4]$	163.4 ± 12.8
		$[C_2mim][EtSO_4]$	$[C_2mim][SCN]$	158.2 ± 13.8
			$[C_2mim][CH_3SO_3]$	59.2 ± 1.1
		$[C_2mim][HSO4]$	$[C_2mim][Et_2PO_4]$	221.7 ± 16.6
			$[C_2mim][EtSO_4]$	129.2 ± 2.2
			$[C_2mim][SCN]$	200.6 ± 3.9
			$[C_2mim][CH_3SO_3]$	148.4 ± 1.9
		$[C_2mim][Et_2PO_4]$	$[C_2mim][EtSO_4]$	171.4 ± 6.2
			$[C_2mim][SCN]$	158.1 ± 14.1
			$[C_2mim][CH_3SO_3]$	168.4 ± 8.6
		$[C_2mim][SCN]$	$[C_2mim][CH_3SO_3]$	78.1 ± 1.4
Chlorella vulgaris			**FAME Yield [mg FAME/g algae]**	
			Direct Transesterfication	*334.7*
	1g dry algae/20g IL, 110°C, 2h		$[C_2mim][O_2CMe]$	218.7
	Above + 5:1 (w/w) $FeCl_3$-$6H_2O$		$[C_2mim][O_2CMe]$	227.6
	1g dry algae/20g IL, 110°C, 2h		$[C_2mim][HSO_4]$	33.5
	Above + 5:1 (w/w) $FeCl_3.6H_2O$, 90°C, 1h		$[C_2mim][HSO_4]$	131.9
	1g dry algae/20g IL, 110°C, 2h		$[C_2mim][Et_2PO_4]$	243.3
	Above + 5:1 (w/w) $FeCl_3.6H_2O$, 90°C, 1h		$[C_2mim][Et_2PO_4]$	162.2
	1g dry algae/20g IL, 110°C, 2h		$[C_2mim][SCN]$	40.4

(Choi et al. 2014b)

	Above + 5:1 (w/w) $FeCl_3.6H_2O$, 90°C, 1h	$[C_2mim][SCN]$	158.3	
	1g dry algae/20g IL, 110°C, 2h	$[C_2mim][NTf_2]$	169.6	
	Above + 5:1 (w/w) $FeCl_3.6H_2O$, 90°C, 1h	$[C_2mim][NTf_2]$	142.3	
Chlorella vulgaris	1 g dry algae + 10 mL, 100°C, 24h	**FAME Yield [mg FAME/g algae]**		(Olkiewicz et al. 2015)
		Bligh & Dyer	*27 ± 1*	
		$[P(CH_2OH)_4]Cl$	45 ± 1	
Nannochloropsis oculata	1 g dry algae + 10 mL, 100°C, 24h	*Bligh & Dyer*	*84 ± 1*	
		$[P(CH_2OH)_4]Cl$	81 ± 1	
	1 g wet algae + 10 mL, 100°C, 24h		80	

[a] All other studies employed hexane to collect lipids

[b] Data obtained by digitalised plots using "WebPlotDigitizer" (Ankit Rohatgi 2015)

*$[C_{2OH}mim]$, 1-(2-hydroxyethyl)-3-methylimidazolium; [amim], 1-(prop-2-ene)-3-methylimidazolium; $[Et_2PO_4]$, diethylphosphate; $[NTf_2]$, bis{(trifluoromethyl)sulfonyl}amide

†Direct transesterification method of lipid analysis is conducted by placing a small amount of dried algae (10 mg) in a mixture of chloroform, methanol, and >1 N sulphuric or hydrochloric acid at >85 °C for 10 -60 min followed by hexane extraction. Using this method, all lipids including polar lipids are catalysed to FAME. (Lepage and Roy 1984)

3.5.4 Materials & Methods

3.5.4.1 Materials

The following ionic liquids were purchased from Sigma-Aldrich: $[P_{4\,4\,4\,4}][OH]$ (60% aqueous solution), $[P_{1\,4\,4\,4}][OH]$ (80% aqueous solution), $[N_{1\,4\,\,4\,\,4}][OH]$ (40% aqueous solution), $[C_2mim][O_2CMe]$, and $[C_2mim][EtSO_4]$. $[C_4m_\beta py)][N(CN)_2]$ was purchased from Merck. All other ionic liquids used in this study (**Table 3.5-2**) are standard materials prepared in the QUILL laboratories. Details of their synthesis are described elsewhere (Adamová et al. 2011; Ferguson et al. 2011; Adamová et al. 2012).

3.5.4.2 Strain and cultivation conditions

Chlorella vulgaris strain UTEX 2714 was purchased from The Culture Collection of Algae at the University of Texas Austin. The culture was maintained in liquid culture using aseptic technique in 150 cm^3 tris-acetate-phosphate (TAP) media pH 7.0 in 500 cm^3 shaker flasks. Seed cultures were grown at 25 °C at 150 rpm under cyclic illumination consisting of 16 h on: 8 h off (100 μmol m^{-2} s^{-1}). The TAP medium used consisted of 20 mM tris base, 1.58 mM $K_2[HPO_4]$, 2.4 mM $K[H_2PO_4]$, 7.0 mM $[NH_4]Cl$, 0.83 mM MgSO4, 0.34 mM $CaCl_2$, 1 cm^3 l^{-1} glacial ethanoic acid, and 1 cm^3 l^{-1} of Hutner's trace elements solution (Hutner et al. 1950). After 48 h, an exponentially growing seed culture was inoculated into a either 200 mL of media or a 5 l Labfors bioreactor (Infors HT) at 10% (v/v) and cultured for 5 d at 25°C and 400 rpm in TAP media with reduced $[NH_4]Cl$ (5 mM) and supplemented with glucose (20 g l^{-1}) in order to induce lipid production.

3.5.4.3 Harvesting and freeze drying

Algal cultures were harvested by centrifugation at 3500 rpm in a Sorvall RT centrifuge (Fisher Scientific) for 20 min. Cell pellets were resuspended in deionised water and washed three times *via* centrifugation and resuspension to remove residual salts. The washed cells were frozen at -86 °C for a minimum of 8 h and lyophilised using a 4.5 l freeze-drier (Labconco) for 24 h or until the weight no longer fluctuated then stored in a desiccator until further use. Fresh algae for wet extractions were harvested by centrifugation as above and resuspended in various amounts of distilled water. Dry weight was determined by overnight drying in an oven at 80 ºC.

3.5.4.4 Analytical Determination of Total Lipid Content

The standard lipid content was determined in triplicate by mixing 0.250 g of freeze-dried algae in 5 cm^3 of hexane:2-propanol solution (H2P; 3:2 v/v) (Hara and Radin 1978) or trichloromethane:methanol solution (Folch; 3:2 v/v) (Folch et al. 1956) for 16 h on a rotary shaker . The solution was filtered through a Buchner funnel fitted with a fine porosity fritted disc into a separating funnel. The remaining solids were washed with solvent until they were colourless. The H2P or Folch extracts were washed with 0.1 M NaCl and the appropriate phase was transferred to a pre-weighed vessel. The solvent was evaporated and the mass of extractable lipids was measured using an analytical balance after the solvent was fully removed and the weight no longer fluctuated.

3.5.4.5 Ionic Liquid Pre-treatment

Ionic liquids were tested in triplicate, where indicated, for their ability to aid solvent extraction by mixing 0.250 g of freeze-dried algae with the indicated mass of ionic liquid for the specified incubation time with a magnetic stirrer at ambient temperature. Solvent extraction was performed on the treated algae by adding 5 cm^3 of hexane to the ionic liquid-algae mixture and shaking for 30 s followed by 5 min of stirring before removing the top hexane layer to a new container. This was repeated three times. After three extractions, one-part water was added to the mixture in order to improve separations, as in many cases the ionic liquid and hexane formed an emulsion. This mixture was centrifuged at 3500 rpm for 10 min and the top layer was added to the pooled hexane. As a negative control, it was determined that performing the extraction in the exact same manner without ionic liquid pre-treatment, hexane by itself with such a small amount of contact time was only capable of extracting 4±1% oils. The extracted oils were measured gravimetrically as described above.

3.5.4.6 Investigation of process parameters

$[C_2mim][EtSO_4]$ was added to 0.250 g of wet algae slurry and stirred for 1h prior to extraction 3 times with 5mL of hexane. Recovery was determined gravimetrically.

3.5.4.7 Ionic Liquid Recycling

$[C_2mim][EtSO_4]$ was tested in triplicate for performance after recycling using freshly grown wet algae collected by centrifugation as follows: 0.250 g of algae paste

(56.3 ± % wt. water) was mixed with 1:10 mass ratio of dry equivalent algae to $[C_2mim][EtSO_4]$ and incubated with agitation for 1h at ambient temperature. Solvent extraction was performed on the treated algae by adding 5 cm^3 of hexane to the ionic liquid-algae mixture and shaking for 30 s and waiting 5 min for the layers to separate before removing the top hexane layer to a new container. This was repeated three times. After three extractions, 10 cm^3 of methanol was added to the mixture in order precipitate dissolved biomass and any residual hexane was pooled with the previous extraction. The methanol/ionic liquid mixture was vacuum filtered with a fine porosity Buchner funnel and the residue was washed with methanol to collect any remaining ionic liquid. The ionic liquid was then recovered by evaporation using a rotary evaporator at 200 mbar, 150 rpm, and 60°C to remove the methanol until the weight no longer fluctuated. The hexane was evaporated from the lipids and the gravimetric yield was recorded. The experiment was repeated five times to determine if performance decreased with repeated use.

3.5.4.8 Extraction and measurement of chlorophyll

Chlorophyll content of the extracted oil was determined in triplicate by resuspending oil at a concentration of 1-2 mg cm^{-3} in 100% propanone. The chlorophyll absorbance was measured by scanning the sample in a glass cuvette using a UV-Vis spectrophotometer (Thermo-Scientific, USA). Total chlorophyll content was determined using Equation (1).

$$\text{Total Chlorophyll Content } \left(\frac{\mu g}{mg}\right) = \frac{17.76A_{645}+7.34A_{662}}{mass\ of\ sample\ (mg)} \quad (1)$$

3.5.4.9 Transesterification and FAME composition

FAME was prepared by dissolving 100 mg of extracted oil in 10 cm^3 of hexane followed by the addition of 100 µl of 2M methanolic KOH. Samples were vortexed for 30 s, followed by centrifugation and 500 µl of the clear supernatant was spiked with the internal standard methyl undecanoate (Sigma) and separated on a FID equipped Agilent 7890 Series GC. The FAME mixture was separated using an Agilent DB-WAX capillary column (30 m, 0.25 mm, 0.25 µm) with helium as the carrier gas at a linear velocity of 30 cm s^{-1}. Samples were injected in split mode (50:1). The FID detector was operated at 280 °C, and FAMEs were eluted using the following program: 50 °C, 1 min, 10 °C min^{-1}

to 200 °C, 3 °C min^{-1} 220 °C, 10 min. Individual FAMEs were quantified and identified using analytical standards (Sigma) and the internal standard. Unidentified FAMEs were estimated using an averaged RF factor. Percent saponifiable lipids was calculated by dividing the sum of the mass of all of the individual detected FAMEs by the mass of extracted oil:

$$\text{Saponifiable Lipids } \left(\%\frac{w}{w}\right) = \frac{FAME\ (mg)}{Extracted\ Oil\ (mg)} \times 100 \quad (2)$$

Table 3.5-2 – Room-temperature ionic liquids investigated in this study, and their water content

Ionic Liquid	Full Name	Water Content [wt%]
Imidazolium		
$[C_1mim][MeOPO_3]$	1,3-dimethylimidazolium methyl phosphate	9.1%
$[C_2mim][O_2CMe]$	1-ethyl-3-methylimidazolium ethanoate	15.5%
$[C_2mim][EtSO_4]$	1-ethyl-3-methylimidazolium ethyl sulfate	0.3%
$[C_2mim][O_2CH]$	1-ethyl-3-methylimidazolium methanoate	8.5%
$[C_2mim][O_2CEt]$	1-ethyl-3-methylimidazolium propanoate	0.7%
$[C_4mim][O_2CMe]$	1-butyl-3-methylimidazolium ethanoate	8.8%
$[C_4mim][O_2CH]$	1-butyl-3-methylimidazolium methanoate	1.6%
$[C_4mim][O_2CCH_2OH]$	1-butyl-3-methylimidazolium glycolate	0.6%
$[C_6mim]Cl$	1-hexyl-3-methylimidazolium chloride	30.6%
Pyridinium		
$[C_2py]Cl$	1-ethylpyridinium chloride	1.3%
$[C_4m_\beta py][N(CN)_2]$	1-butyl-3-methylpyridinium dicyanamide	0.8%
$[C_4m_\beta py]Br$	1-butyl-3-methylpyridinium bromide	10.9%
Ammonium		
$[N_{0\,1\,1\,2}][O_2CH]$	dimethylethylammonium methanoate	0.7%
$[N_{0\,1\,1\,2}][MeOCH_2CO_2]$	dimethylethylammonium 2-methoxyethanoate	0.6%
$[N_{1\,2\,2\,2}][MeSO_4]$	triethylmethylammonium methyl sulfate	1.1%
$[N_{1\,4\,\,4\,\,4}][OH]$	tributylmethylammonium hydroxide	40%*
$[N_{1\,1\,2OH\,4OH}]Cl$	dimethyl-2-hydroxyethyl-4-hydroxybutylammonium chloride	0.9%
$[N_{1\,1\,4\,4}][tau]$	dibutyldimethylammonium taurinate	0.5%
$[N_{1\,4\,8\,8}]Cl$	dioctylbutylmethylammonium chloride	8.8%
$[N_{0\,0\,0\,1}][pro]$	methylammonium prolinate	26.6%
$[N_{0\,0\,0\,3}][NO_3]$	propylammonium nitrate	2.0%
Phosphonium		
$[P_{1\,4\,4\,4}]Cl$	tributylmethylphosphonium chloride	0.5%
$[P_{1\,4\,4\,4}][O_2CH]$	tributylmethylphosphonium methanote	0.2%
$[P_{1\,4\,4\,4}][O_2CEt]$	tributylmethylphosphonium propanoate	1.0%
$[P_{1\,4\,4\,4}][HO_2CCH_2CH2CO_2]$	tributylmethylphosphonium butanedioate/succinate	1.9%
$[P_{4\,4\,4\,4}][OH]$	tetrabutylphosphonium hydroxide	60%*
$[P_{1\,4\,4\,4}][OH]$	tributylmethylphosphonium hydroxide	80%*
$[P_{4\,4\,4\,4}][NO_3]$	tetrabutylphosphonium nitrate	0.8%

* - not determined by Karl Fisher titration

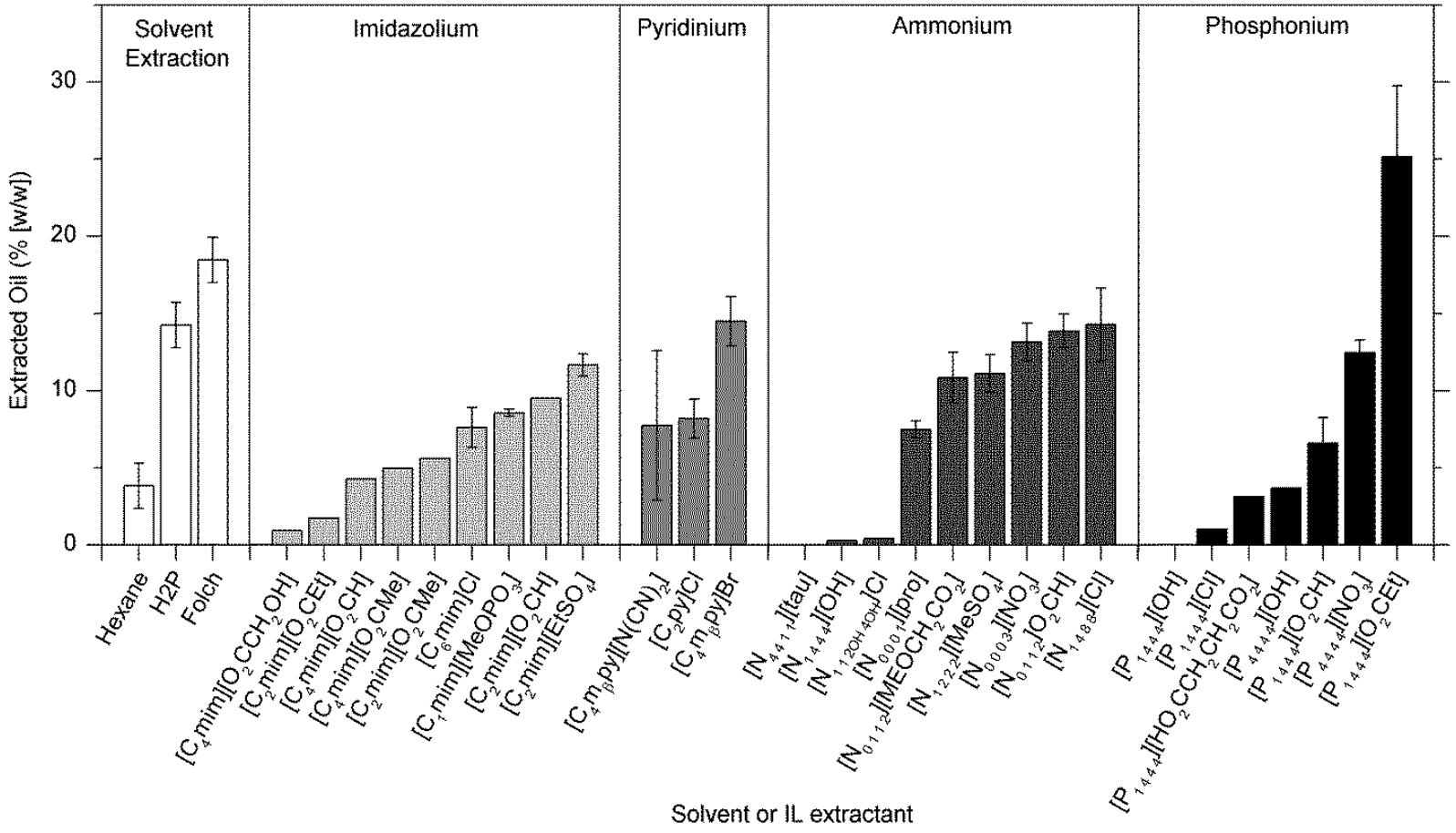


Figure 3.5-2 – Freeze-dried algae were combined with ionic liquid in a 1:10 weight ratio and magnetically stirred at ambient temperature for 16 h. Error bars (1σ) are presented for extractions performed in triplicate.

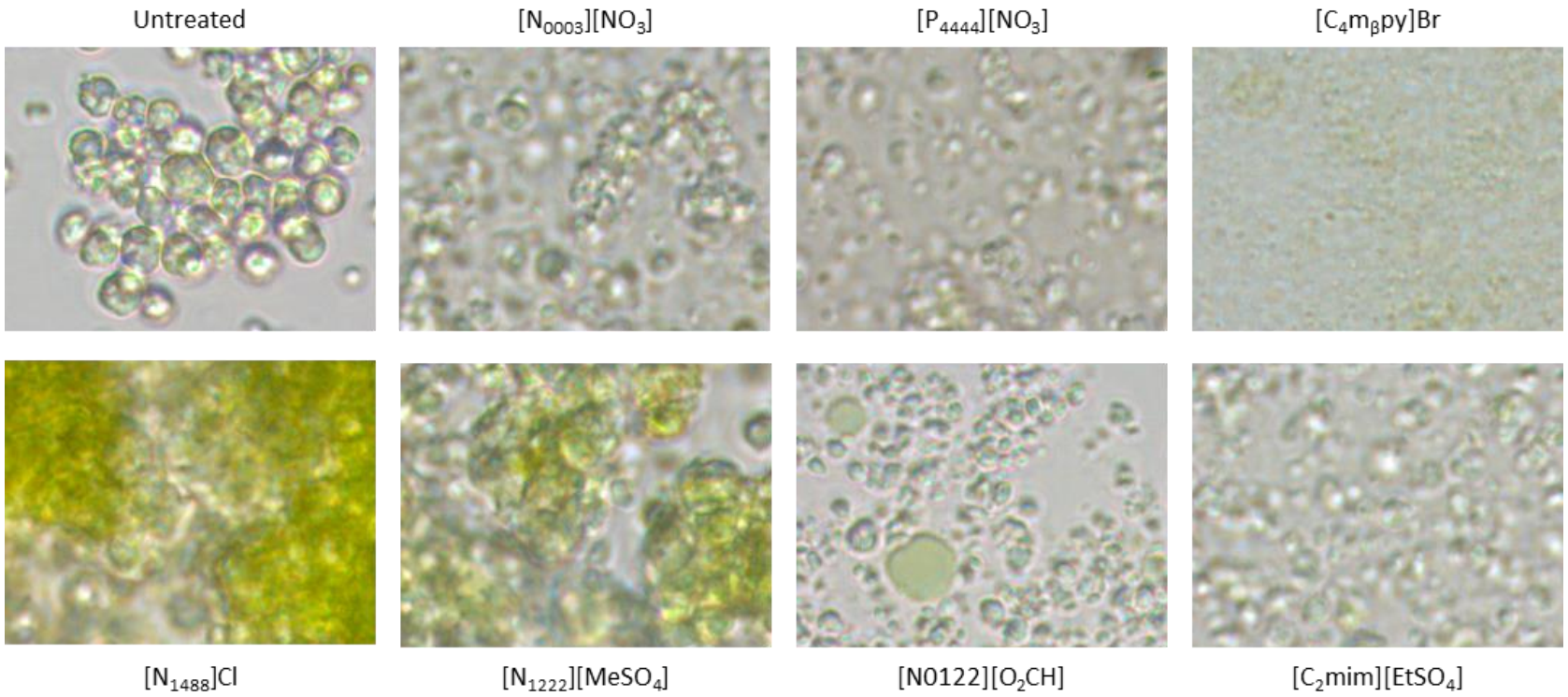


Figure 3.5-3 – Lysis of freeze-dried algae after ionic liquid pre-treatment as visualized under a standard bright field microscope.

3.5.5 Results and Discussion

3.5.5.1 Screening of room-temperature ionic liquids.

Freeze-dried microalgae were subjected to a pre-treatment with an excess of an ionic liquid (mass ratio 1:10), followed by gravimetric extraction of readily accessible lipids with hexane by only extracting oil for 15 min total (3 x 5 min). The ionic liquids screened in this study are listed in **Table 3.5-2**. A negative control was performed on untreated freeze-dried algae using solely hexane for three 5 min extractions, which yielded 4±1% (w/w) oil content. In order to compare ionic liquid extracted oil to the total lipid content of the algae, two analytical solvent extraction techniques were also employed, the Hara method {which uses a mixture of hexane and 2-propanol (H2P; 3:2 v/v)}(Hara and Radin 1978) and the Folch technique {which uses a mixture of trichloromethane and methanol (2:1 v/v)}(Folch et al. 1956). These analytical methods were found to extract 14±1% (w/w) and 18±1% (w/w) oil respectively, from the microalgal biomass. The results of ionic liquid pre-treatment on oil extraction are presented in **Figure 3.5-2.** Several leading candidate ionic liquids were also observed under an optical microscope to determine the degree of cellular dissolution, and presented in **Figure 3.5-3**. The ionic liquids have been classified by their cation class. In all cases, at least one suitable ionic liquid was found in each class with extraction efficiencies similar to the analytical methods. In most cases, oils extracted in the presence of ionic liquids were light green or yellow, indicating low levels of polar pigment impurities. Ammonium based ionic liquids performed better than the other cations, however, many of the anions in this group were small anions which were generally found surpassed longer chain anions at disrupting microalgae cell structure. [C_2mim]][O_2CMe] previously used in three separate studies performed poorly in this study and for Choi et al. (2012), however, in two studies which used higher temperatures and larger amounts of ionic liquid per gram of algae (Choi et al. 2014b; Choi et al. 2014c) found this ionic liquid to be superior to other imidazolium based ionic liquids. Unfortunately, in many studies, ionic liquids were also mixed with cosolvents making it difficult to assess the efficacy of the ionic liquid itself.

By visual inspection, [C_4m$_\beta$py]Br, **Figure 3.5-3,** yielded the most homogenous lysate after incubation compared to the other top candidate ionic liquids. However, by

gravimetric analysis, $[P_{1\,4\,4\,4}][O_2CEt]$ exhibited the highest gravimetric recovery. As mentioned earlier, gravimetric recoveries greater than the well-established protocols of Folch or Hara are likely to be caused by some interference. This was this case for $[P_{1\,4\,4\,4}][O_2CEt]$, as less than a quarter of the recovered oil were saponifiable lipids (**Figure 3.5-5**) and it is believed that this ionic liquid may be sparingly soluble in hexane accounting for the high gravimetric yield. In many cases, it was observed that the ionic liquid and hexane formed a stable emulsion which was broken by the addition of 10 mL of water. Formation of emulsions is generally not desirable in most industrial scale processes and the following ionic liquids were therefore found to be less suitable for this reason: $[N_{0\,1\,1\,2}][MeOCH_2CO_2]$, $[C_6mim]Cl$, $[N_{0\,0\,0\,1}][pro]$, $[N_{0\,1\,1\,2}][O_2CH]$, $[C_4m_\beta py)]Br$, $[P_{4\,4\,4\,4}][NO_3]$, $[C_2mim][O_2CMe]$, $[N_{1\,2\,2\,2}][MeSO_4]$, $[C_1mim][MeOPO_3]$, $[P_{1\,4\,4\,4}][O_2CEt]$, $[P_{1\,4\,4\,4}][HO_2CCH_2CH_2CO_2]$, $[P_{1\,4\,4\,4}][O_2CH]$, and $[C_2py]Cl$.

Several other unique observations were made during this screening study. In the case of $[N_{0\,0\,0\,3}][NO_3]$, the mixture of ionic liquid and algae turned brown within a minute of mixing, indicating a degradation of chlorophyll (possibly due to oxidation by nitrate). With $[N_{1\,4\,8\,8}]Cl$, it was found that it was only able to extract oils after the addition of water to the mixture. It was also observed that some ionic liquids may be capable of stabilising chlorophyll molecules; when placed on the bench for two months unshielded from natural or artificial light, they remained bright green for over 2 months. These included $[P_{1\,4\,4\,4}][O_2CH]$ and $[P_{1\,4\,4\,4}][O_2CEt]$. Finally, algae incubated in $[N_{0\,1\,1\,2}][O_2CH]$ exhibited cellular disruption, however, green spherical bodies also formed which were not observed with any other ionic liquids tested, **Figure 3.5-3.** This may be due to aggregation of chlorophyll in the presence of $[N_{0\,1\,1\,2}][O_2CH]$, indicating that this ionic liquid may have applications in chlorophyll extraction or stabilisation. These secondary traits observed in this study, could play a role if extraction and separation of coproducts is also desired.

It has been proposed that ionic liquids improve solvent extraction by disrupting the interface at which lipids contact the solvent through dissolution of the cellulosic cell wall (Young et al. 2010). A close analogue of one of the most effective ionic liquid found in this study; $[C_4m_\beta py)]Cl$, is known for one of the highest cellulose solubility up to 39 wt% at 105°C (Heinze et al. 2005; Seoud et al. 2007), while another top

candidate;[C_2mim][$EtSO_4$], is not known to dissolve cellulose (Vitz et al. 2009) indicating that dissolution of cellulose may not be the sole mechanism of action for the disruption of microalgae. This is likely as microalgae cell wall composition varies greatly between species and even between strains and can include cellulose, hemicellulose, glucosamines, lipids and proteins (Abo-Shady et al. 1993). Proteins for can represent 27% of the cell wall by dry weight in *C. pyrenoidosa* [30] and solubilisation of proteins by ionic liquids (Fujita et al. 2005; Yang 2009) could disturb the cell integrity leading to cell disruption. However, due to the differences in cell wall structure between algae strains and species, at this point it is difficult to propose a mechanism for ionic liquid disruption without first characterizing the cell wall composition of each. This highlights the need to further study the effect of a single ionic liquid on multiple algae species. Finally, heterotrophically grown microalgae were used in this study in order to generate sufficient biomass, however, while possible effects of using organic carbon sources on algal cell wall composition is unknown, the effect is expected to be minimal as the cell wall is a structural cell component unlike lipids which are used for energy storage and are effected by the media composition. However, further study on the connection between ionic liquid disruption and cell wall structure could elucidate the role of cultivation conditions on cell wall composition and the mechanism of ionic liquid disruption.

3.5.5.2 Characterization of oils extracted using ionic liquids

Oils extracted using standard solvent extraction techniques and oils recovered using the ionic liquids (which resulted in the highest yields) were characterised in terms of quality by relative FAME composition and chlorophyll content (**Figure 3.5-4**). In most cases, the composition of oils extracted using ionic liquids did not differ significantly from oil extracted using the analytical methods; H2P or Folch. When [$P_{4\,4\,4\,4}$][O_2CEt] was used, heptadecenoic acid (C17:1) and octadecanoic acids (C18:0) were preferentially extracted, but the number and amount of unidentified compounds significantly increased. It has not been previously observed that ionic liquids can affect the composition of the oil extracted, however it is to be expected that the relative extraction of individual fatty acids depends on the solvent system. The chlorophyll content of the oils was characterised as a simple indicator of the co-extraction of polar

impurities. Interestingly, [C_2mim][$EtSO_4$] was found to extract significantly more chlorophyll than that extracted by organic solvents. However, for most ionic liquids tested, the presence of the ionic liquid was found to decrease the chlorophyll content of the oil.

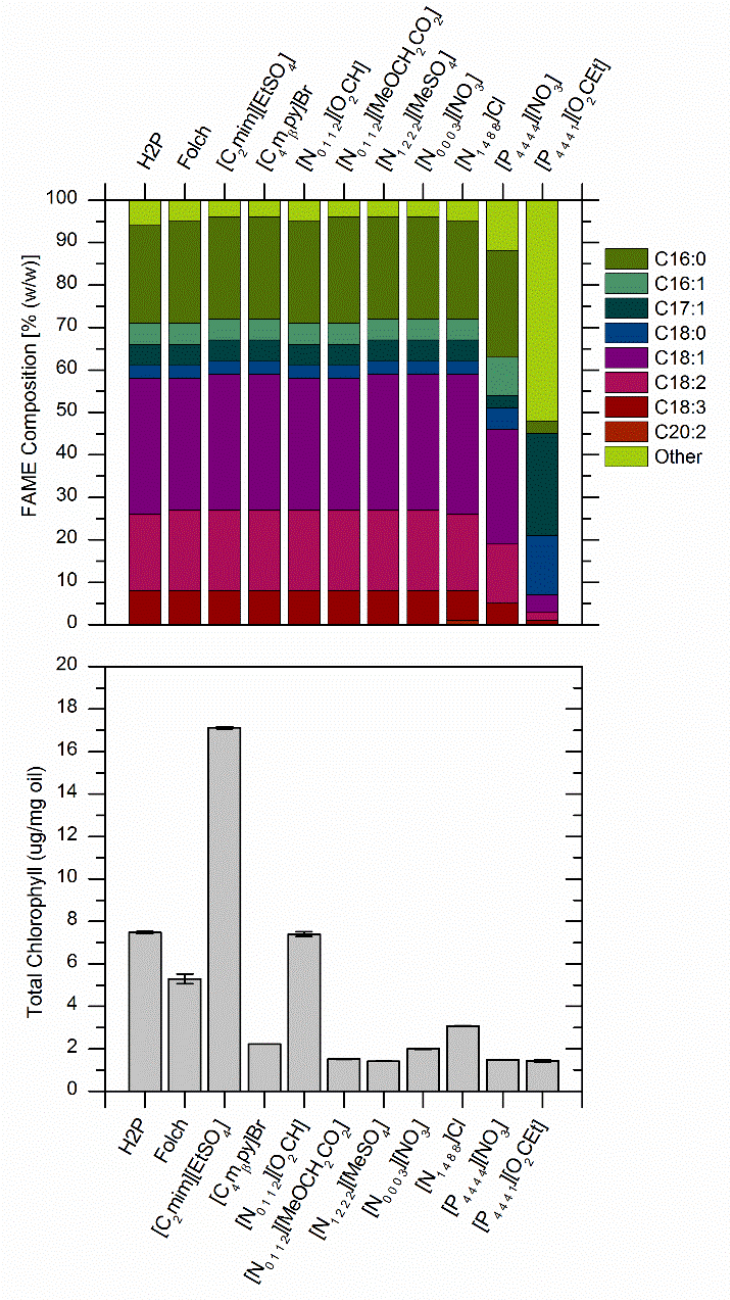


Figure 3.5-4 – Characterisation of oils extracted using ionic liquids. (Top) Relative composition of FAME synthesised from ionic liquid extracted oils. (Bot-tom) Total chlorophyll content of the ionic liquid extracted oils and the analytical solvent extracted oil (H2P and Folch).

The GC characterised oils were further analysed to determine the yield of saponifiable lipids *via* base catalysed transesterification (**Figure 3.5-5**) as it is the primary method of biodiesel synthesis due to the fast reaction times and low cost of the catalysts (Lotero et al. 2005). Calculation of FAME recovery on an algae weight basis revealed that many of the oils had lower TAG content than the oils extracted using the analytical methods (Folch/H2P). However, this is not surprising as the analytical lipid determination methods employ long extraction times (>6h), and/or vigorous mechanical disruption such as bead milling, in combination with co-solvents in order to extract all available lipids, while in this study, lipids were only extracted for a short period of time (15min) using pure hexane. This has allowed the identification of ionic liquids which may actively improve the extraction efficiency of lipids from microalgae by decreasing the processing time of oil extraction.

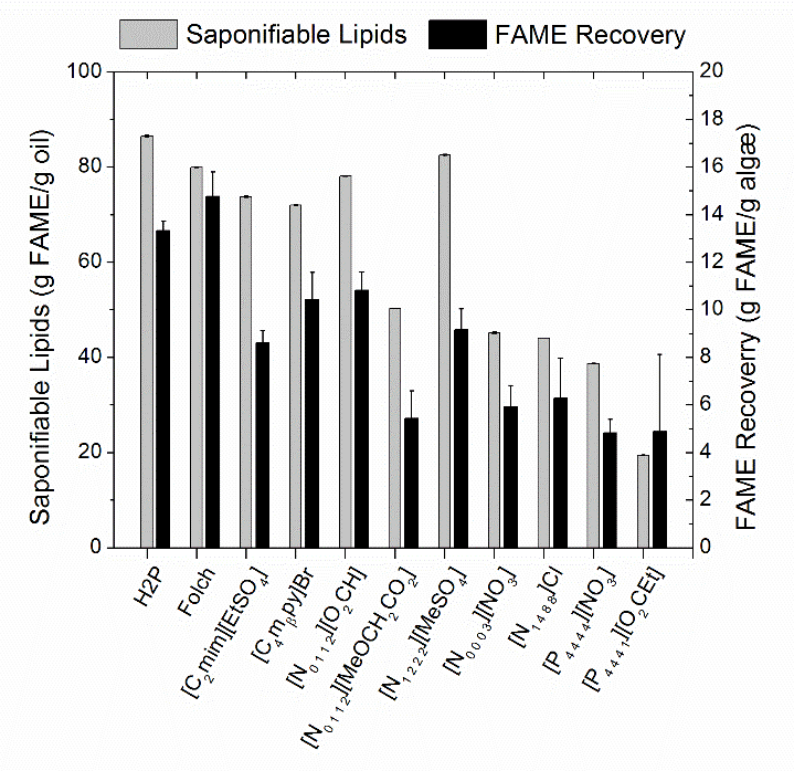


Figure 3.5-5 – Composition of oils extracted with ionic liquids was characterized with respect to percentage saponifiable lipids and the percentage total FAME recovered from microalgae biomass compared to the analytical determination of lipid content using the Hara (H2P) and Folch methods.

3.5.5.3 Investigation of Process Parameters

Typical operating ranges for a wet algae based extraction process were studied using a central composite design of experiments (CCD) and the results are presented in **Table S 3.5-1** and **Table S 3.5-2**. The model for this design was found to be significant ($p < 0.01$), however, in the case of wet algae, only a single factor was significant, the ratio of ionic liquid to algae dry equivalent ($p < 0.01$). It was found that as the amount of ionic liquid added increased up to approximately 6.5 g ionic liquid/g algae, the lipid recovery also increased. Otherwise, ionic liquid incubation times between 2-17h had no effect, nor did increasing water content up to 2 g H_2O/g of dried algae. However, in the case of no ionic liquid added to wet algae, only 0.8 wt% oil (**Table S 3.5-2**) was extracted indicating that the presence of ionic liquid is critical to extraction of oils in wet biomass. As the dewatering and drying steps are the greatest expense in microalgae based biodiesel processes, the effect of water content was further studied. Methanol is used in several studies(Young et al. 2010; Kim et al. 2012; Choi et al. 2014c) and was found to aid in the extraction of wet microalgae, thus the effect of methanol on the extraction process was also elucidated.

As observed in **Figure 3.5-6A**, extraction efficiency increased with a small addition of methanol (MeOH), however greater ratios result in a decrease in extraction yields. This may be due to the increased formation of emulsions with higher methanol concentrations. Emulsions were not formed in 1:1, 1:2, and 1:5 IL:MeOH ratios. The mixture of 2:1 IL:MeOH was further studied to minimise the amount needed per gram of wet algae (**Figure 3.5-6B**). It was found that ratios greater than 1:10 were required to extract all of the available lipids. Finally, **Figure 3.5-6C** shows the effect of increasing water content on the extraction efficiency of $[C_2mim][EtSO_4]$ on its own, with (2:1) methanol, and with only methanol. It was found that methanol extracted very little which likely consisted of purely pigments (which can represent 1-4% wt in *C. vulgaris*) (Chen et al. 2009) as the algae were depigmented by the extraction process. However, in the presence of ionic liquid, methanol acted synergistically with ionic liquid to allow extraction of lipids over broader range of dewatered algae. The range of water contents studied in this work covers both the typically solids content of microalgae cultures (0.1-1% solids) as

well as the solids loading contents found in algae dewatered by filtration (up to 27% solids) (Molina Grima et al. 2003). The results indicate the studied process will be appropriate for microalgae which have previously been dewatered.

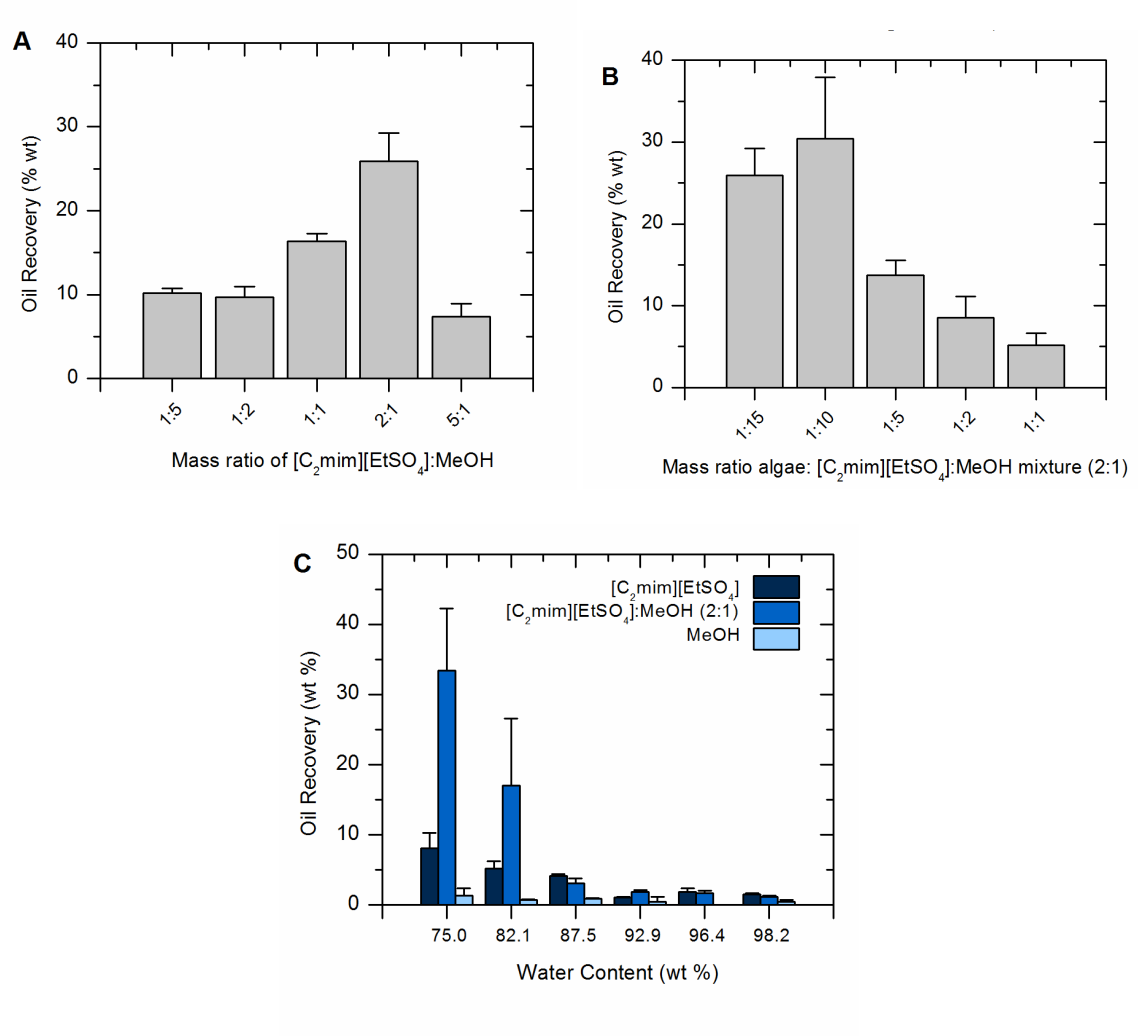


Figure 3.5-6 – Extraction of wet microalgae using [C_2mim][$EtSO_4$] A. The effect of methanol to mass ratio on the extraction of oils from wet microalgae. B. The effect of increasing mass ratios of IL:MeOH (2:1) mixture to microalgae. C. The effect of increasing water content on extraction recovery with ionic liquid, IL/MeOH mixture (2:1), and methanol (mass ratio with algae was 1:10 for all mixtures). Maximum analytical lipid yield was 26.0 ± 1.0% for this culture. (*n = 3*)

3.5.5.4 Ionic Liquid Recycling

Due to the costs of ionic liquid synthesis, recycling and reuse of the ionic liquid must be address in order to estimate the economic viability of the developed process. Using wet algae recovered by centrifugation, extraction was performed and the ionic

liquid was recovered from the biomass by anti-solvent precipitation using methanol followed by evaporation of the methanol. The recovered ionic liquid after each cycle of extraction was reused in order to judge the performance of the reused ionic liquid. The averaged recovered [C_2mim][$EtSO_4$] was 98.0 ± 5.2 wt%, however, it should be noted that chlorophyll was also recovered with the ionic liquid as methanol is used as the anti-solvent. The values reported here will therefore likely overestimate the true recovery and further study of other impurities will be needed in order to determine the true recovery. The amount of lipid recovered by the recycled ionic liquid did not change significantly over five extraction cycles, as shown in **Figure 3.5-7**, indicating that possible impurities are not negatively affecting the lipid recovery, nor are large fractions of the ionic liquid lost in each cycle.

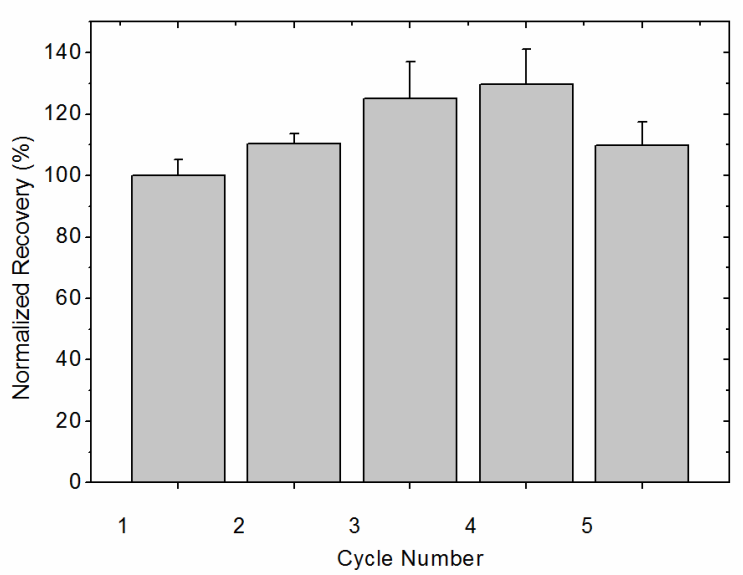


Figure 3.5-7 – Lipid recover with recycled [C_2mim][$EtSO_4$] for wet extraction of *C. vulgaris* (56.6 ± 2.5 wt %). The lipid recovery was normalized based on the values achieved with neat ionic liquid. Components were mixed in a mass ratio of dry equivalent of algae:IL:MeOH of 1:10:5 and incubated for 2 h at ambient temperature.

3.5.6 Conclusions

This work reports the results of a large screening study of ionic liquids for the disruption of microalgae, identifying several promising candidate ionic liquids with extraction efficiencies similar to analytical reference methods: [C_2mim][$EtSO_4$], [C_2m_βpy]Br, [$N_{0\,1\,1\,2}$][O_2CH], and [$N_{1\,2\,2\,2}$][$MeSO_4$]. Generally, small anions such as Br^-,

Cl^-, $[NO_3]^-$ and methanoate combined with short chain alkyl cations efficiently disrupted cell structure at room temperature and allowed the straightforward extraction of lipids from the disrupted microalgae. Key characteristics of the recovered oil indicates that the ionic liquid used for cell disruption can affect the quality and yield of the recovered biodiesel. The presence of water on the extraction efficiency of $[C_2mim][EtSO_4]$ was quantified as drying is one of the largest cost impediments to the commercialisation of microalgae derived biodiesel. It was found that this ionic liquid was effective over a wide range of water containing dewatered algae. This opens the process to a wider range of hydrophilic ionic liquids which are solid at room temperature which may become liquid when dissolved in water containing biomass. Coupled with the low energy requirements of using room-temperature ionic liquids, the ionic liquid aided extraction processes presented in this study offer several promising advantages over previously studied ionic liquid processes and the currently employed solvent extractions.

3.5.7 Acknowledgements

The authors are grateful to the Natural Sciences and Engineering Research Council of Canada, the Canada Foundation for Innovation, BioFuelNet Canada and the Alexander von Humboldt Foundation for financial support.

3.5.8 Supplementary Information

Table S 3.5-1 – Central composite design (CCD) parameters and factors effecting lipid extraction using $[C_2mim][EtSO^4]$

		Factor Levels				
Uncoded	**Coded**	*-1.68*	*-1*	*0*	*1*	*1.68*
Incubation time in IL [h]	X1	2	5	9.5	14	17
IL to algae mass ratio [X g IL/g dry algae]	X2	0	2	5	8	10
Water to algae mass ratio [X g H_2O/g dry algae]	X3	0	0.4	1	1.6	2

Table S 3.5-2 – Coded run and results for CCD

Run	Coded Factors			Lipid Content [wt%]
	X1	X2	X3	
1	1	1	1	6.80%
2	1	1	-1	6.80%
3	1	-1	1	4.74%
4	1	-1	-1	4.15%
5	-1	1	1	6.18%
6	-1	1	-1	4.22%
7	-1	-1	1	4.46%
8	-1	-1	-1	6.71%
9	-1.68	0	0	6.06%
10	1.68	0	0	7.05%
11	0	-1.68	0	0.81%
12	0	1.68	0	7.87%
13	0	0	-1.68	9.14%
14	0	0	1.68	5.33%
15	0	0	0	7.84%
16	0	0	0	7.79%
17	0	0	0	9.65%
18	0	0	0	7.37%
19	0	0	0	8.52%
20	0	0	0	6.08%

3.6 Direct conversion of the oleaginous yeast *Rhodospordium diobovatum* to biodiesel using the ionic liquid [C_2mim][$EtSO_4$]

Valerie C. A. Orr,[a] Garret Munch[a,b], Nazim Cicek[b], and Lars Rehmann[a*]

[a] Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St., London, Ontario, Canada, N6A 3K7

[b] Department of Biosystems Engineering, University of Manitoba, 66 Chancellors Cir, Winnipeg, Manitoba, Canada, R3T 2N2

* Corresponding author at: 1151 Richmond St., London, ON, Canada, N6A 3K7

Email address: lrehmann@uwo.ca

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it was submitted to *Applied Energy* (Submitted June 2016)

3.6.1 Preface to Section 3.6

The previous chapter focused on the lipid extraction of wet microalgae using ionic liquids. It was found that in order for the lipid extraction process to proceed when using wet biomass, the ionic liquid must be mixed with a cosolvent, in this case, methanol. As methanol is also a reactant in the transesterification process, direct conversion of the lipids to FAME was explored in this chapter by the addition of an alkaline catalyst to the methanol/ionic liquid mixture as demonstrated in **Figure 3.6-1**.

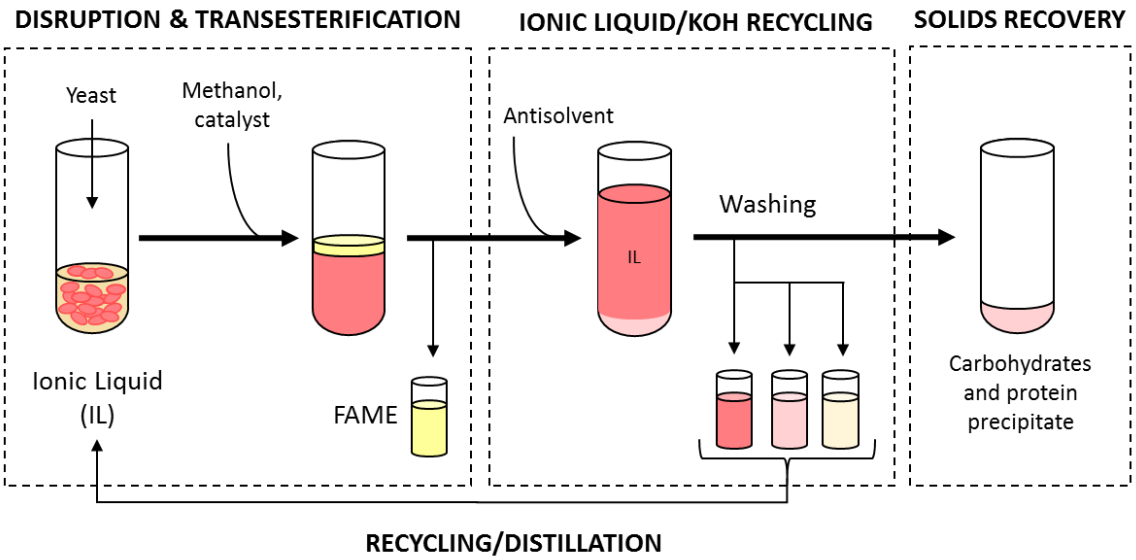


Figure 3.6-1 – A summary of the transesterification process used in this study.

First, a large amount of oleaginous yeast biomass was generated for this work at the pilot scale using waste glycerol from a first generation biodiesel plant as the carbon source. Then factors affecting the reaction were identified using a factorial design. This was followed by a thorough investigation of the effects of the reaction temperature, KOH loading ratio, and methanol loading ratio on the transesterification yield from whole yeast. This was performed using dried yeast biomass as water content is difficult to control and modify in fresh cultures. It was found that essentially all of the available lipids were transesterified to FAME using this process in under 2.5 h at moderate temperatures and moderate loading ratios of KOH. When extending this work to fresh wet yeast biomass, it was found that lower temperatures and higher KOH loading ratios were required to achieve 100% conversion within 4 h compared to the previously optimized conditions for dried biomass. The ionic liquid was found to be necessary to

achieve complete conversion with wet cultures. The reusability of the ionic liquid was investigated in order to assess whether the ionic liquid could be recycled in order to reduce the process costs. It was found that 40-60% of the KOH could be recovered (wet vs. dry biomass) along with essentially all of the ionic liquid by weight demonstrating that the ionic liquid could also facilitate the recovery of the homogenous catalyst in this work.

3.6.2 Abstract

In this study, the oleaginous yeast *Rhodosporidium diobovatum* was cultivated at the pilot scale (90L) in a stirred tank reactor on waste glycerol producing 1.49 kg of yeast biomass and 0.45 kg of neutral lipids for biodiesel synthesis. The ionic liquid, 1-ethyl-3-methylimidazolium ethylsulfate, was used to facilitate the direct conversion of oleaginous yeast biomass to fatty acid methyl esters (FAME). Factorial screening was used to identify critical factors affecting the transesterification yield and response surface methodology was employed to study the interactions of methanol, KOH, and temperature in the ionic liquid based system using dried yeast. The optimized conditions were found to be 16.9 g methanol/g yeast, 0.056 g KOH/g yeast, and 65°C which yielded 97.0% conversion of lipids to FAME in only 2.5h. The optimized system was further studied to observe the reaction profiles over time in both dry yeast and measure total FAME yield from fresh wet yeast biomass containing varying degrees of water from 65-80% wt. The ionic liquid was found to improve total overall yield of FAME compared to the negative control without ionic liquid when wet yeast was used. While 100% of the ionic liquid was recovered from the reaction, it contained only 59.3% of the catalyst suggesting a heterogeneous catalyst may be more appropriate in future work.

***Keywords**:* Single Cell Oil, pilot scale, ionic liquid, biodiesel, experimental design, lipid production, *Rhodosporidium diobovatum*

3.6.3 Introduction

Recent fluctuations in the price of crude oil and increasing environmental concerns have reinvigorated the need for greater investigation of alternative clean fuels. Biodiesel is an attractive alternative fuel for several reasons; it is highly biodegradable, has low toxicity, it can be blended with petroleum diesel or be directly substituted in diesel engines, and life cycle analysis indicates a low net contribution to various emissions (primarily SO_2 and CO_2) (Atabani et al. 2012). Currently, vegetables oils or waste fats are the primary feedstocks for biodiesel production however, additional concerns such as the environmental sustainability of farming palm oil, or the use of edible oils for fuel production has motivated further search for alternative sources of oils (Meng et al. 2009; Atabani et al. 2012; Sitepu et al. 2014b).

Microbial lipid feedstocks is a growing area of research which uses microorganisms capable of accumulating over 20% of their dry weight as lipids (Meng et al. 2009). The most studied divisions include microalgae and oleaginous yeasts. Oleaginous yeasts are particularly promising source of lipids for biodiesel production as some species are capable of higher growth rates, cell densities, and lipid accumulation rates than microalgae, and have been shown to grow on several waste feedstocks such as glycerol (Munch et al. 2015), and pyrolytic sugars (Lian et al. 2010b; Lian et al. 2013; Luque et al. 2016). Traditionally, lipids accumulated in the form of triacylglycerides (TAGs) must be extracted in a lengthy process which requires fully dried biomass prior to the transesterification of the resulting oil using a base catalyst to fatty acid methyl esters (FAME); one form of biodiesel (Ageitos et al. 2011). Due to their small size, chemical extraction methods are typically employed for lipid extraction, however, these methods are time consuming as they are limited by diffusion of the solvent into the cell and the presence of even small amounts of water can greatly impede this process (Halim et al. 2012). Cell disruption processes can aid in extraction of oils however, as yeasts have thick cell walls, they are often use energy intensive processes like bead milling or expensive additives like enzymes (Ageitos et al. 2011; Nigam and Singh 2014). The lipid refining process which includes the dewatering, drying, cell disruption, and lipid extraction processes can account for up to 70% of the overall cost of biodiesel production

from microalgae and accordingly will play a significant role in the economic viability of similar processes (Haas et al. 2006; Hidalgo et al. 2013).

Direct conversion of wet microbial biomass to biodiesel has been proposed as a method for reducing the costs of lipid refining. Direct conversion methods have recently been adopted as the preferred method for analytical determination of total cellular lipids in microbial biomass, however, these methods are not practical for large scale biodiesel production as they use unrecoverable or expensive catalysts and use high temperatures and/or long reaction times (Griffiths et al. 2010; van Wychen and Laurens 2013).

Catalysts used in transesterification can be divided by their mechanisms of action; either acid or alkaline catalysts, and further divided into homogenous or heterogeneous catalysts. Homogenous alkaline catalysts are preferred in industry due to their much faster reaction times, lower reaction temperatures, and high yields compared to acid catalyzed transesterification processes (Van Gerpen 2005). However, if oils containing high free fatty acid content (>1%) are used, an acid catalyst is preferred as the combinations of an alkaline catalyst with even a low level of moisture (>0.06%) will result in the formation of solid soaps which impede the subsequent purification processes (Van Gerpen 2005; Hidalgo et al. 2013). While few reports of direct transesterification of yeast biomass exist and focus only on the conversion of dry biomass (Liu and Zhao 2007; Thliveros et al. 2014), the direct transesterification of similar processes using wet microalgae is currently under intense investigation (Hidalgo et al. 2013; Abedini Najafabadi et al. 2015; Chen et al. 2015a; Park et al. 2015; Suh et al. 2015). These methods typically require expensive pretreatments such as microwave disruption (Chen et al. 2015a) or high operating pressures/temperatures such as in supercritical methanol extraction/transesterification (Abedini Najafabadi et al. 2015).

Recently, the ionic liquid (IL) 1-ethyl-3-methylimidazolium ethyl sulfate known as $[C_2mim][EtSO_4]$ has been shown to allow the wet disruption and extraction of lipids from the microalgae *Chlorella vulgaris* at ambient temperatures in under 1.5 h (Orr et al. 2016). The final conditions presented by this study using fresh wet microalgae found lipids were extracted at high yields (~100%) within 75 min at ambient temperature using a mass ratio of ten parts $[C_2mim][EtSO_4]$ mixed with five parts methanol and one part

microalgae. This process was compatible with water contents up to 82% wt. The ionic liquid was tested for reuse in order to aid in offsetting the high cost of ionic liquids and no decrease in performance was detected after five cycles.

In this work, growth of *Rhodosporidium diobovatum* was scaled up to 90 L and growth and lipid production using waste glycerol was characterized. The ionic liquid based lipid extraction process was advanced to allow for the direct conversion of cellular lipids within wet yeast biomass to FAME in order to remove any requirements for lipid extraction from the process. This novel *in situ* transesterification in the presence of an ionic liquid was optimized using response surface methodology and the optimal conditions were assessed for a wide range of water contents and the reuse of both the ionic liquid and the solid base catalyst was investigated.

3.6.4 Materials and Methods

3.6.4.1 Materials

All materials were purchased from Simga-Aldrich or Thermo Fisher unless otherwise stated. Crude glycerol used as the carbon source for the pilot scale cultivation was donated by the Renewable Energy Group in Danville Illinois. As a byproduct of vegetable oil based biodiesel production, it contained 78-85% glycerol, 0.3% methanol, 6-13% water, 7% ash, and 1% fatty acids. *Rhodosporidium diobovatum* 08-225 was generously donated by the Phaff Yeast Culture Collection, at the University of California at Davis (UCDFST).

3.6.4.2 Strain and culture conditions

R. diobovatum was maintained on YPG media (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, and 40 g L^{-1} glycerol) with 1.5% agar for solid media. The seed culture was grown from a single colony picked from a YPG agar plate and grown at 30°C in a baffled flask (20% v/v maximum working volume) for 24 hours at 150 rpm in a shaking incubator (Multitron, Infors HT).

3.6.4.3 Pilot scale production of *R. diobovatum*

Pilot scale cultivation of *R. diobovatum* was conducted in a refurbished 150 L, steam-sterilized stirred tank reactor equipped with four Rushton turbines and online monitoring of dissolved oxygen, temperature, and pH. The sterilized GMY media

(containing 3 g L^{-1} yeast extract, 8 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$ $7H_2O$, 50 g L^{-1} of crude glycerol, and 0.05% w/v antifoam 204) adjusted to pH 5.5 was inoculated with 0.2% (v/v) of the seed culture and grown for 6 days at 30°C. The reactor was agitated at 250 rpm and aerated with 1 vvm using sterile compressed air. Samples were taken every 12 h using a sterilizable sampling port.

3.6.4.4 Harvesting and Cell Density

Cells were harvested using a Thermo Scientific Sorvall ST 40R centrifuge for 10 min at 3500 rpm. The supernatant was used to quantify glycerol consumption using HPLC as described below. The pelleted cells were resuspended in distilled water and centrifuged as above a total of 3 times in order to remove residual salts or glycerol from the cells. The cells were dried using a Labconco Freezone 4.5 L freeze-drier for a minimum of 16 h or until the weight no longer fluctuated, this weight divided by the original volume of the sample was taken as the dry cell weight.

3.6.4.5 Quantification of Extracted Lipids

Lipid content was quantified in two manners for neutral lipid content (TAGs only) as well as total cellular lipid content (includes membrane lipids and other lipid species). Neutral lipid content was determined using the hexane:2-propanol extraction (H2P) method (Hara and Radin 1978). Briefly, 0.1 g of dried algae was mixed with 1 mL of 3:2 (v/v) hexane:2-propanol overnight (16 h). The mixture was transferred to a fine porosity Buchner funnel and the filtered solvent was collected in a separatory funnel. The residual yeast was washed with several volumes of H2P solution until a colourless precipitate was left. The filtered solvent was mixed with 0.5 M NaCl in order to remove any polar molecules from the filtrate. The top layer containing H2P and lipids was collected and the solvent was evaporated until the weight no longer fluctuated and the recovered TAGs were measured gravimetrically using an analytic balance (Sartorius, Inc.).

The total cellular lipids were determined by a direct transesterification method developed by the National Renewable Energy Laboratory (van Wychen and Laurens 2013) as previously described (Orr et al. 2016).

3.6.4.6 Gas Chromatography

FAME samples were separated and analysed using an FID equipped Agilent 7890 Series GC and an Agilent DB-Wax capillary column (30 m, 0.25 mm, 0.25 μm). Helium was used as the carrier gas at a constant pressure of 119 kPa, and the FID was operated at 280°C. Samples were injected in split mode with a 1:10 split ratio and eluted using the following oven ramp: 50°C, 1 min, 10°C min^{-1} to 200°C, 3°C min^{-1} 220°C, 10 min. Individual FAMEs were quantified using analytical standard mixture (Supelco 37, Sigma Aldrich) and the internal standard. Unidentified FAME were quantified by applying the RF factor of the closest known peak.

3.6.4.7 High Performance Liquid Chromatography

Glycerol was monitored during cultivation using a previously described method of liquid chromatography (Luque et al. 2016). Briefly, an Agilent 1260 HPLC system equipped with a Hi-Plex H column and a refractive index detector (RID) was operated at 60°C with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.6 mL min^{-1}.

3.6.4.8 H^1 NMR

The chemical structure of $[C_2mim][EtSO_4]$ recovered after use was confirmed by proton NMR using a Mercury VX 400 (400 MHz) at ambient temperature using methanol-d4 as the solvent. Peaks were assigned as follows: δ 8.99 [s, 1H, C2H], 7.71 [s, 1H, C4H], 7.63 [s, 1H, C5H], 4.31 [q, 2H, NCH_2], 4.03 [q, 2H, OCH_2], 3.98 [s, 3H, NCH_3], 1.55 [t, 3H, $OCCH_3$], 1.26 [t , 3H, $NCCH_3$].

3.6.4.9 *In situ* Transesterification Reactions

The reactions were performed as follows: 50 mg of yeast, water, and ionic liquid were mixed together at the indicated ratios in microwave vials with an extraction standard; methylated pentadecanoic acid (C15:0Me) which was used to quantify extraction efficiency. The vials were allowed to mix briefly with stirring at 800 rpm using magnetic micro-stirrers to allow the ionic liquid to disrupt the yeast cells. Once well mixed, the appropriate amount of methanol and catalyst was added to the vial which was then sealed and allowed to react at the appropriate temperature for the transesterification reaction time indicated. Thirty minutes prior to the end of the reaction, 4 mL of hexane was added to facilitate the extraction of any FAME. A sample of the hexane phase was

used to quantify the reaction conversion using GC as previously described. In the case where a reaction time profile was constructed, the hexane was added immediately at the start of the reaction and samples were taken at the indicated time (sample size of 80 μL). FAME yield (% wt.) was calculated as the FAME recovered during the transesterification reaction divided by the total available FAME derived from neutral lipids in the cells:

$$Yield\ (\%) = \frac{FAME\ (mg)}{mass\ of\ yeast\ (mg) \times maximum\ FAME\ (\%\ wt.)} \times 100\%$$

Further studies into the direct conversion of wet yeast to FAME were performed using fresh yeast cultures which were collected as a paste using centrifugation. The dry cell weight was measured as described above using a sample of this paste. Different water contents were simulated by re-addition a known volume of spent media to the cell paste and water content was calculated accordingly.

3.6.4.10 Factorial Design

The effects of ionic liquid, methanol, and catalyst ratio (KOH, H_2SO_4, and acetic acid), as well as the reaction temperature and water content were first assessed for significance using a screening factorial (2^k) with center point repeats for curvature check. These experiments were performed using freeze dried yeast and a reaction time of 3h.

The factors studied in this experiment and their uncoded levels are summarize in **Table 3.6-1.** Water content was simulated by mixing water with the yeast prior to disruption in the ionic liquid. The full factorial was performed with 14 center point repeats using KOH as the catalyst. The factorial was fit to the general interactions linear model up to 5^{th} order interactions and tested for significance and curvature using ANOVA. Insignificant terms were removed from the model using backwards elimination with a criterion of p-value < 0.05 but a hierarchical model was maintained.

Table 3.6-1 – Coded and uncoded concentrations of reaction conditions used in factorial screening experiments with using a transesterification time of 3h.

		Coded levels and concentration		
Factor	**Label**	-1	0	1
Ionic liquid (g/g yeast)	x_1	4.85	7.50	10.15
MeOH (g/g yeast)	x_2	9.0	11.88	14.75
KOH (g/g lipid)	x_3	0.16	0.25	0.34
Reaction Temperature (°C)	x_4	40	50	60
Water content (g/g yeast)	x_5	16.1	25.0	33.8

3.6.4.11 Response Surface Design and Polynomials

Following the identification of the significant factors, the ranges tested were readjusted and a quadratic response surface polynomial design was performed using a central composite design with k=3, $\alpha = (2^k)^{1/4}$, and 7 center point repeats. The design was fully replicated twice. Following this, due to lack of fit using a quadratic model, the design was augmented to include the cubic factorial points not previously included in order to model a reduced quartic response surface polynomial. The full design and experimental data is available in the supplementary information (**Table S 3.6-1**). The model used for this study was of the general form:

$$\sqrt{\mathrm{y}} = a_0 + \sum_{i=1}^{3} a_i x_i + \sum_{i=1}^{3}\sum_{j=1}^{3} a_{ij} x_i x_j + \sum_{i=1}^{3} a_{ii} x_i^2 + a_{123} x_1 x_2 x_3 + \sum_{i=1}^{3}\sum_{j=1}^{3} a_{iij} x_i^2 x_j + \sum_{i=1}^{3}\sum_{j=1}^{3} a_{iijj} x_i^2 x_j^2 + \sum_{i=1}^{3} a_{ii} x_i^3 + \sum_{i=1}^{3}\sum_{j=1}^{3}\sum_{k=1}^{3} a_{iijk} x_i^2 x_j x_k + \sum_{i=1}^{3} a_{iii} x_i^4$$

The factors studied using response surface methodology are summarized in **Table 3.6-2**. The model was fit using standard linear regression techniques in Design Expert 10 (Stat-Ease, USA), and the fit was tested for significant using ANOVA and a lack of fit test. Insignificant terms were removed from the model using backwards elimination of terms with the criterion *p*-value < 0.1. Reactions conditions were numerically optimized for the fitted model using Design Expert 10 and plots were generated of the final model using MATLAB R2016a (MathWorks, USA).

Table 3.6-2 – Coded and uncoded concentrations of reaction conditions used during the response surface study. The following parameters were kept constant; IL ratio 8 g/g yeast, using freeze-dried yeast for a reaction time of 2.5h.

		Coded levels and concentration				
Factor	**Label**	-1.682	-1	0	1	1.682
MeOH (g/g yeast)	x_1	10.8	12.5	15.0	17.5	19.2
KOH (g/g yeast)	x_2	0.027	0.043	0.068	0.093	0.110
Reaction Temperature (°C)	x_3	25	35	50	65	75

3.6.4.12 Recovery of $[C_2mim][EtSO_4]$ and KOH

The reuse of the ionic liquid was assessed after the transesterification reaction was performed in triplicate under the optimized conditions; 0.118 g KOH/g lipid, 16.9 g MeOH/g yeast, at 65°C for 2.5h using wet yeast (76% water content). Following the reaction, residual solids were recovered by the additional of methanol followed by filtration by a fine porosity Buchner funnel. The residual solids were dried and weighed to calculated recovery of residual solids. The collected ionic liquid/methanol/KOH phase was transferred to a round bottom flask and any volatile solvents (methanol & water) were evaporated using a rotary vacuum evaporator (BUCHI, Switzerland) at <70 mbar and 80°C for 1 h. The residual ionic liquid and KOH was weighed after cooling to calculate ionic liquid recovery. The ionic liquid and KOH were resuspended in approximately 25 mL of methanol and titrated using 20 mM methanolic standardized H_2SO_4 using bromocresol purple as an indicator (pH range 5.2-6.8) and the recovery of KOH was calculated accordingly. This method was standardized using the unreacted KOH/MeOH/ionic liquid mixture as a positive control and a recovery of 100.4 ± 0.2% was calculated.

3.6.5 Results and discussion

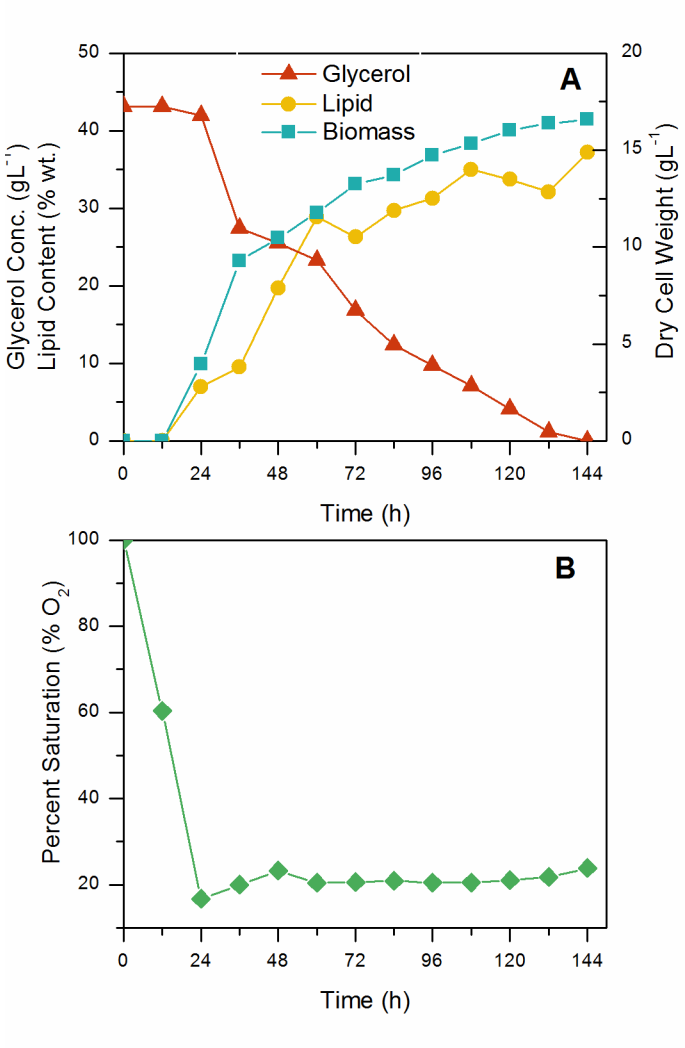


Figure 3.6-2 – Cultivation of *R. diobovatum* at the pilot scale (90L). A. Glycerol consumption (gL^{-1}), biomass dry cell weight (gL^{-1}) and neutral lipid content (% wt.). B. Oxygen saturation during cultivation.

3.6.5.1 Pilot scale cultivation of *R. diobovatum*

The large scale cultivation of *R. diobovatum* was characterized in a pilot stirred tank reactor at 90 L working volume. **Figure 3.6-2A** illustrates the growth, glycerol consumption, and lipid content of the cells over the 6 days' cultivation period. Glycerol consumption was minimal during the first 24 h likely due to the low inoculation volume used in this study while induced a lag phase of approximately 24h. Post-analysis indicates an increase in seed culture volume from 0.1% v/v to in between 1-5% v/v would drastically reduce the batch time by at least 1 day.

Oxygen limitation is often the most significant factor during scale up of aerobic cultures. Online monitoring (**Figure 3.6-2B**) during the cultivation of *R. diobovatum* reveals the dissolved oxygen content fell quickly to 20% saturation but was not completely during the course of the run, indicating that 1 vvm of aeration may not be sufficient to support the fastest growth rates possible. Oxygen became limited midway through the exponential phase which in turn can limit the maximum cell density achieved. After 36h, the culture density increased slowly throughout stationary phase likely a reflection of the greater lipid accumulation during this phase. Nitrogen was purposely limited in this media composition in order to stimulate lipid production as previously observed (Munch et al. 2015). While this encourages lipid production, it can also limit the maximum cell density. Regardless, a final cell density of 16.6 g L^{-1}, a total cellular lipid content of 40.0 ± 1.5% wt., and a neutral lipid content of 31.0 ± 1.3% wt. was achieved. This represented a 3 g/L increase (18%) in the cell density, but a 3.7% wt. decrease (7%) in total lipid content (compared to previously published results using a 7 L stirred tank reactor) (Munch et al. 2015). Overall this translated into a volumetric total cellular lipid production of 7.8 g L^{-1} and a total cellular lipid productivity of 1.30 g L^{-1} d^{-1}; an increase over the 0.87 g L^{-1} d^{-1} seen at the bench scale (Munch et al. 2015). However, it should be noted that due to the limitation in materials, the pilot scale cultivation was only conducted once.

3.6.5.2 Screening of reaction factors

Direct transesterification of the yeast biomass using KOH, H_2SO_4, or acetic acid as the catalyst was performed after a 1 h incubation of the yeast in [C_2mim][EtSO_4]. Preliminary screening indicated that the ionic liquid impeded transesterification using H_2SO_4 or acetic acid and therefore their study was discontinued. A factorial study was used to further investigate the use of KOH as a catalyst. Freeze dried yeast were used for this factorial screening and wet yeast biomass was simulated by adding water to the freeze-dried yeast prior to incubation in the ionic liquid. Following this incubation, methanol and KOH were added to the reaction vial which was sealed and allowed to react for 3 h total. At 2.5 h, hexane was added to each vial to extract any FAME produced during the reaction. The extraction standard (C15:0Me) added prior to incubation in the ionic liquid showed that the extraction efficiency using this methodology was ≥ 95% in

all cases. The end point conversion was calculated after quantification of the produced FAME using GC and total neutral lipids as determined by hexane isopropanol extraction followed by GC. The maximum theoretical FAME yield was determined to be 31.6 ± 2.6% FAME by weight (*n=3*). Linear regression and analysis of variance (ANOVA) was used to determine the significance of each factor and any interactions. The results for significant terms for base catalyzed transesterification are presented in **Table 3.6-3**.

Table 3.6-3 – Analysis of Variance for factorial screening study using base catalyst. Transesterification reaction time of 3 h.

Source	SS	DF	MS	F-value	*p*-value
Model	5	15871.88	3174.38	71.59	**< 0.0001**
x_2 (MeOH)	1	3002.59	3002.59	67.72	**< 0.0001**
x_3 (KOH)	1	5747.97	5747.97	129.64	**< 0.0001**
x_4 (Temp)	1	1117.95	1117.95	25.21	**< 0.0001**
x_2x_3 (MeOH × KOH)	1	4721.61	4721.61	106.49	**< 0.0001**
x_3x_4 (KOH × Temp)	1	1281.76	1281.76	28.91	**< 0.0001**
Curvature	1	6283.33	6283.33	141.71	**< 0.0001**
Residuals	39	1729.19	44.34		
Lack of Fit	26	1334.39	51.32	1.69	0.1614
Pure Error	13	394.79	30.37		
Total	45	23884.40			

Considering the residuals and pure error, the model fit was significant (R^2 value of 92.76% and adjusted R^2 of 94.68%) and there was no significant lack of fit (prob < F of 0.1614). However, the curvature was significant indicating that a response surface approach was required to further elucidate the relationship between these factors.

3.6.5.3 Optimization of *in situ* transesterification using base catalyst

Optimization of the reaction conditions used for base catalyzed transesterification was pursued using response surface methodology. The first design consisted of a simple central composite design, however upon analysis, lack of fit was significant (*p*-value < 0.0001) and the prediction using this model was extremely poor as observed using visual inspection of the residual plots and the correlation plot of calculated conversion versus experimental data. Therefore, the design was augmented to allow regression using the reduced quartic polynomial previously described. The results of ANOVA are presented in **Table 3.6-4**.

Table 3.6-4 – Analysis of Variance for response surface study using base catalyst and corresponding response surface coefficients. Transesterification reaction time of 2.5 h.

Source	S.S.	D.F.	M.S.	F-value	*p*-value	Coefficients	
						Label	Coded Value ± S.E.
Model	187.23	24	7.80	73.87	**< 0.0001**	a_0	9.08 ± 0.09
x_1 (MeOH)	1.53	1	1.53	14.48	**0.0004**	a_1	0.93 ± 0.25
x_2 (KOH)	0.15	1	0.15	1.41	0.2409	a_2	0.23 ± 0.20
x_3 (Temp)	2.32	1	2.32	21.97	**< 0.0001**	a_3	0.91 ± 0.19
x_1x_2	0.00	1	0.00	0.00	0.9714	a_{12}	0.00 ± 0.12
x_1x_3	0.08	1	0.08	0.79	0.3775	a_{13}	-0.06 ± 0.07
x_2x_3	0.08	1	0.08	0.72	0.4015	a_{23}	-0.06 ± 0.07
x_1^2	2.65	1	2.65	25.05	**< 0.0001**	a_{11}	1.69 ± 0.34
x_2^2	0.13	1	0.13	1.25	0.2695	a_{22}	-0.22 ± 0.20
x_3^2	0.04	1	0.04	0.39	0.5331	a_{33}	-0.04 ± 0.06
$x_1x_2x_3$	0.28	1	0.28	2.69	0.1071	a_{123}	0.13 ± 0.08
$x_1^2x_2$	2.52	1	2.52	23.82	**< 0.0001**	a_{112}	-0.58 ± 0.12
$x_1^2x_3$	0.08	1	0.08	0.77	0.3848	a_{113}	0.10 ± 0.11
$x_1x_2^2$	1.63	1	1.63	15.43	**0.0003**	a_{122}	-0.51 ± 0.13
$x_1x_3^2$	0.08	1	0.08	0.77	0.3843	a_{133}	-0.11 ± 0.13
$x_2^2x_3$	0.34	1	0.34	3.18	0.0804	a_{223}	-0.20 ± 0.11
$x_2x_3^2$	1.41	1	1.41	13.34	**0.0006**	a_{233}	-0.42 ± 0.12
x_1^3	1.16	1	1.16	11.01	**0.0017**	a_{111}	-0.33 ± 0.10
x_2^3	9.53	1	9.53	90.28	**< 0.0001**	a_{222}	0.81 ± 0.09
x_3^3	0.66	1	0.66	6.24	0.0157	a_{333}	-0.21 ± 0.08
$x_1^2x_2^2$	0.76	1	0.76	7.23	**0.0097**	a_{1122}	-0.58 ± 0.22
$x_1^2x_3^2$	3.86	1	3.86	36.59	**< 0.0001**	a_{1133}	-1.00 ± 0.17
$x_1x_2x_3^2$	9.87	1	9.87	93.47	**< 0.0001**	a_{1233}	-1.37 ± 0.14
x_1^4	2.33	1	2.33	22.09	**< 0.0001**	a_{1111}	-0.57 ± 0.12
x_2^4	2.66	1	2.66	25.15	**< 0.0001**	a_{2222}	-0.38 ± 0.08
Residuals	5.39	51	0.11				
Lack of Fit	1.19	13	0.09	0.83	0.6263		
Pure Error	4.19	38	0.11				
Total	200.89	76					

The final model employed a box cox transformation of 0.5 and the resulting model showed good correlation between the fitted values and the experimental data (**Figure 3.6-3**). Consideration of the error showed excellent model fit (R^2 value 97.20% and R^2 adjusted 95.89%) and good predication ability (R^2 prediction value 93.42%). The coefficient values and their standard error are also summarized in **Table 3.6-4**. While many insignificant terms where included in the model in order to maintain a hierarchical model, each factor's main effects could be described with either one or two terms. The

main effect of temperature was purely linear (x_3, p-value <0.0001) and conversion generally increased with increasing temperatures ($a_3 = 0.91$) . The methanol main effect was combination of quadratic and quartic ($x_1^2, x_1^3,$ and x_1^4, p-value ≤0.0017) and the relationship between conversion and methanol was more complex with both positive and negative coefficients ($a_{11} = 1.69$, $a_{111} = -0.33,$ and $a_{11111} = -0.57$) in the coded model. Finally, the main effects of KOH included a cubic and quartic term (x_2^3 and x_2^4, p-value <0.0001) again with a combination of both positive and negative coefficients ($a_{222} = 0.81$ and $a_{2222} = -0.38$) in the coded model. There were six highly significant higher order interaction terms, three between methanol and temperature, and three between all three factors. The complex relationship between temperature, methanol ratio, and KOH ratio is illustrated using contour plots for selected temperatures (**Figure 3.6-4**).

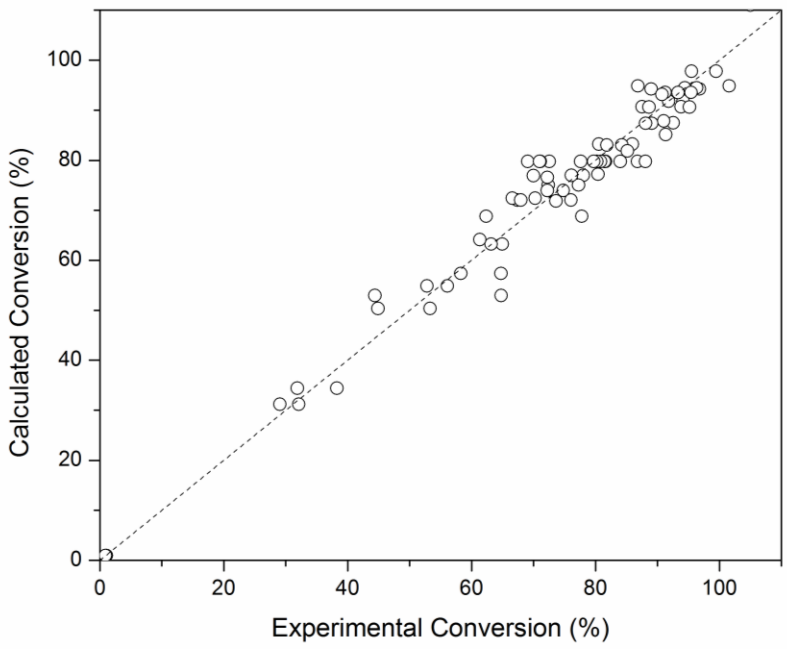


Figure 3.6-3 – Correlation plot of experimental conversion compared to predicted conversion.

Numerical optimization of conversion was performed using Design Expert 10 with the following conditions from highest importance to lowest: conversion was maximized >75%, KOH to lipid ratio was minimized, methanol ratio was minimized with the least weighting (due to the possibility of recycling unused methanol), and the

temperature was maintained in the range of 35-65 °C. This resulted in very few possible solutions. The optimal conversion under the mentioned restrictions was predicted to occur at a methanol ratio of 16.8 g/g yeast, a KOH ratio of 56 mg/g yeast at 65°C. The experimental conversion under the optimized reaction conditions was 97.1± 0.1% (*n=3*), which fell outside the 95% confidence prediction interval (100.3-131.7 % conversion) under these conditions however it did fall within the tolerance interval for 99% of the population (89.71-144.4% conversion) indicating that although the model is overestimating the conversion in this saturated region however, it is satisfactory for the purpose of optimizing reaction yield and minimizing the catalyst consumption.

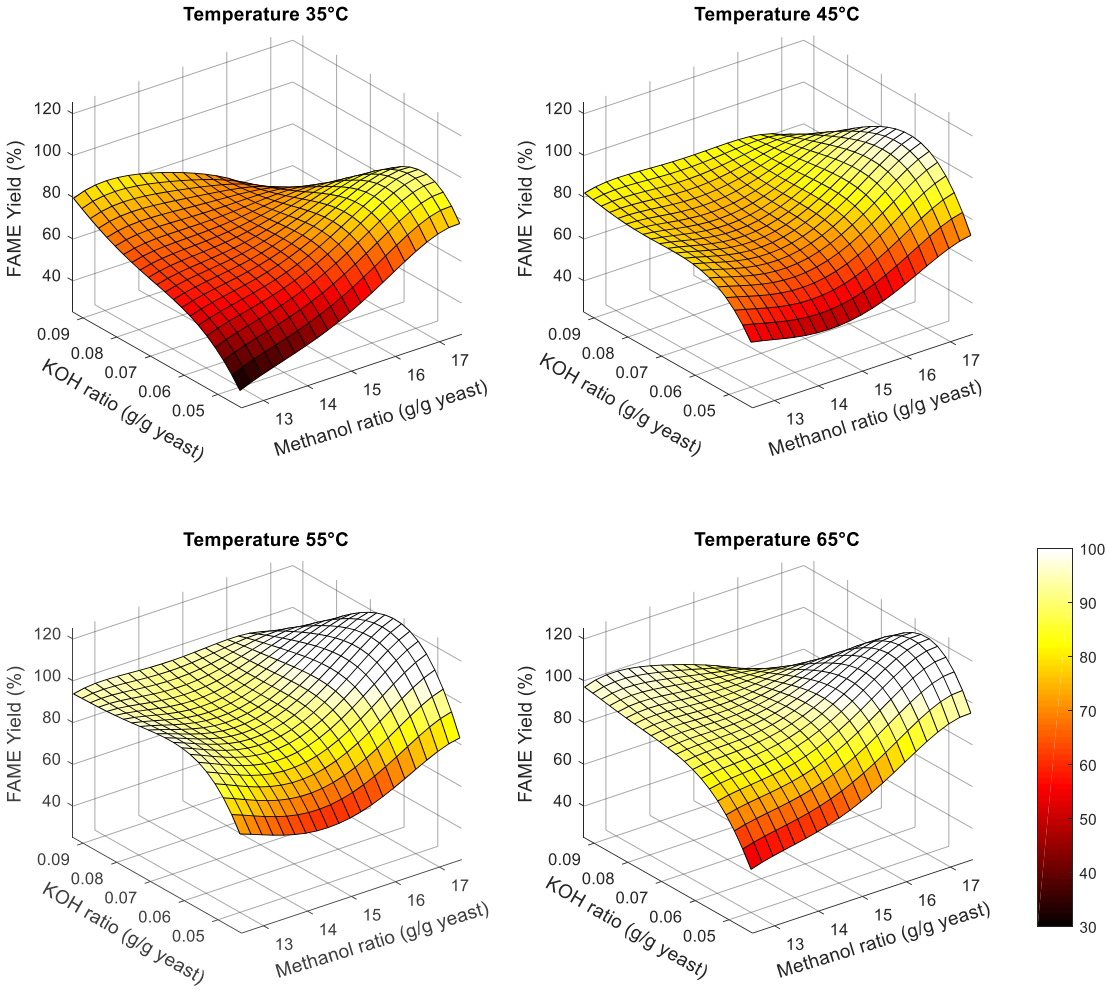


Figure 3.6-4 – Surface plots of the interaction between methanol, KOH, and temperature. Colourbar represents FAME yield from 30-100%.

Typical catalyst usage for KOH for refined oil transesterification is in the range of 0.1-5% wt. which is between 2-10 times less than the optimum found in this study (11.8% wt. based on lipid content) however, direct transesterification of biomass has been known to require much higher amounts of catalyst in the range of 20-100% wt. (Hidalgo et al. 2013). A catalyst ratio of 80 mg NaOH/g yeast (13.5% wt. for lipid) was used in the esterification of dry *Rhodosporidium toruloides* however it took 10 h to reach a yield of 97.7% (Thliveros et al. 2014). Significantly higher ratios are typically needed for acid catalysts along with longer reaction times and in one study, dry yeast and fungi were transesterified to biodiesel using acid catalysts over 20 h which used a concentration of 0.2 M H_2SO_4 or 0.4 M HCl which is approximately 14.6-78.4% wt. catalyst (Liu and Zhao 2007).

3.6.5.4 Effect of water content and ionic liquid on transesterification conversion

One of the primary objectives of this work was to increase the water compatibility of direct transesterification of wet biomass. The presence of water has a negative impact on the conversion of TAGs to biodiesel and it is considered essential to reduce water content in oil feedstocks prior to transesterification (Atadashi et al. 2012). This is primarily due to the increased hydrolysis of TAGs which produces free fatty acids which in the presence of an alkali metal can form soaps which consume the catalyst and interfere in the subsequent purification processes (Atadashi et al. 2012).

When water content was simulated during the factorial screening study by adding either 0.25 or 0.5 grams of water per gram of yeast using the center point ratios or temperatures for all other factors, it did not have a significant effect on FAME yield, (97.6%; *n=1*, with 0.5 g/g water added compared to 94.4 ± 5.1%; *n=17*, with 0.25 g/g). However, in order to determine if any difference between the simulated wet yeast and fresh cultures existed, fresh yeast cultures were harvested and directly converted to FAME using yeast containing various water contents. The results are presented in **Figure 3.6-5**. The fresh yeast was not washed or dried prior to transesterification and water content was contrived for the 80% sample by readdition of the spent media to the centrifuged cell pellet (75.9 ± 0.2% water) followed by determination of yeast dry weight by oven drying. The 76% water content sample was further centrifuged at 10,000 x *g* in

order to generate the lower water content sample (65% water). The transesterification reaction was monitored during the reaction by layering hexane above the ionic liquid/methanol phase and taking samples at the designated times using a syringe. The freeze-dried yeast was included as a positive control and reached a conversion of 87.4 ± 5.6% by 2 h which was lower than the expected 97.1% conversion at 2.5h but can be accounted for by the lower temperature used (40°C versus 65°C).

The changes in reaction conditions were found necessary for direct transesterification of wet yeasts containing >65% water content. Under the initial optimized conditions, very little FAME was detected after 2.5h with wet cultures. It is suspected that when greater amounts of water are present, a greater proportion of the catalyst will be in the form of [OH^-] instead of the methoxide anion [CH_3O^-] required for transesterification to proceed (Demirbas 2007). Therefore, the KOH loading ratio was reevaluated using wet yeast. However, at 65°C there was little to no FAME formation in the lower loading concentrations and the reaction did not proceed at all in reactions containing >20% wt. KOH (data not shown). Interestingly, when the temperature was reduced to 40°C, the transesterification reactions proceeded to competition when ≥ 20% wt. KOH was used (**Figure 3.6-5A**).

Therefore, the proceeding reactions were conducted at 40°C with 20% wt. catalyst (relative to yeast dry weight equivalent). The effects of water on the reaction profile were further elucidated in **Figure 3.6-5B.** The sample containing 65% wt. water was indistinguishable from the positive control using freeze-dried yeast. However, as water content increased to 80% there was a severe effect on the overall yield. Interestingly, under the conditions studied in this work, the reaction could proceed to ~70% yield with wet yeasts containing 76% water when no ionic liquid was present. However, the presence of IL even at the lower ratio of 2 parts ionic liquid to 1 part dry equivalent of yeast (g/g), the yield was significantly improved after a reaction time of 4h confirming the positive effect of the ionic liquid. Similar to the factorial study which did not find a difference in FAME yield at ionic liquid ratios between 5:1 and 10:1, there was no significant difference in FAME yield between 2:1 and 8:1 ratio after 4h. Due to the high cost of ionic liquids it is desirable to minimize the amount of ionic used in each reaction,

therefore the subsequent study of catalyst and ionic liquid recovery performed below used a mass ratio of IL to yeast of 2:1.

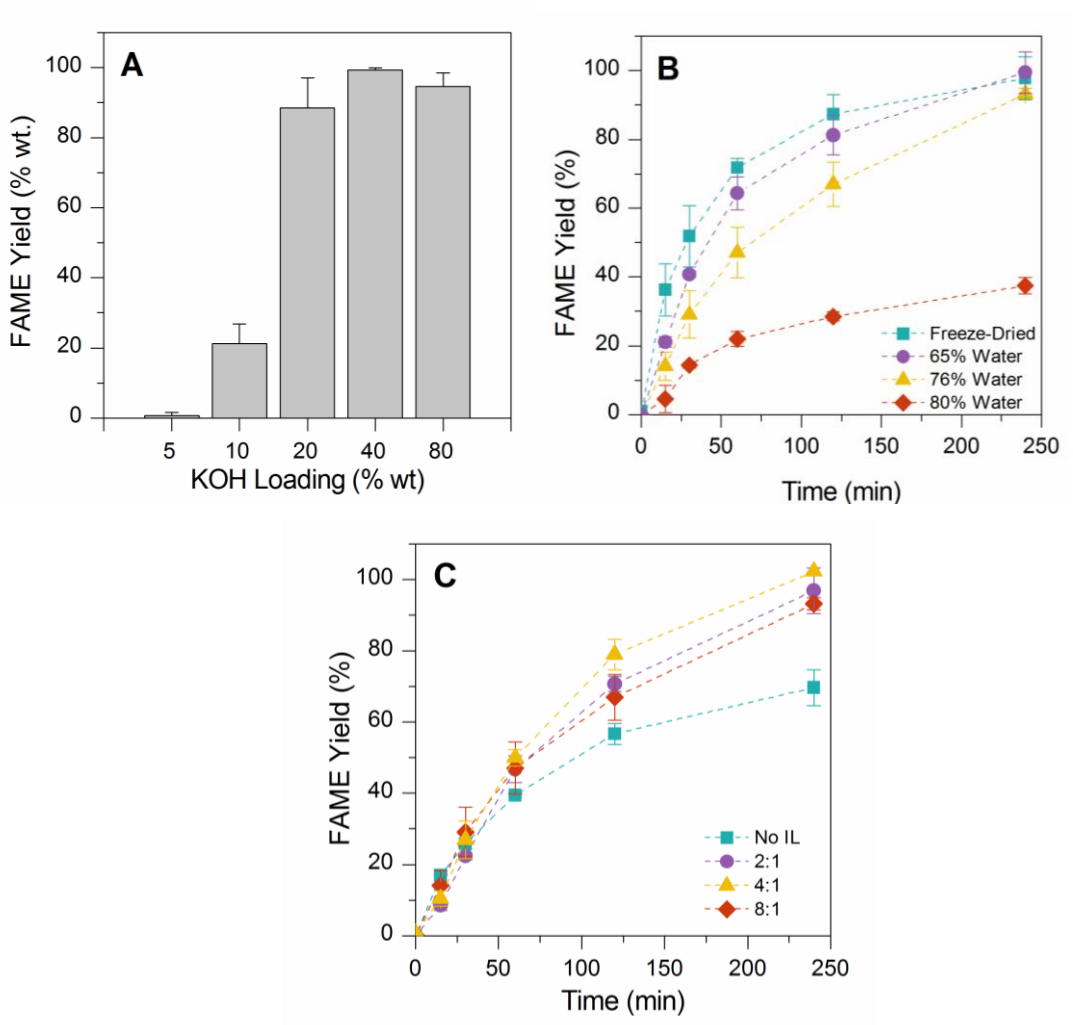


Figure 3.6-5 – The effect of KOH loading, water content, and ionic liquid ratio on the transesterification of wet yeast. A. The effect of KOH loading on wet yeast (% wt. per gram of yeast, 76% water) at 40°C B. The effect of wet yeast water content on reaction time and yield for 20% KOH loading g/g yeast at 40 °C. C. The effect of ionic liquid loading ratio on reaction time and yield (20% KOH and 40°C).

3.6.5.5 Reusability of $[C_2mim][EtSO_4]$ and KOH

Finally, due to the large amounts of catalysts used in this study, and the relatively high cost of ionic liquids, it was desirable to investigate the possibility of recycling both the catalyst and the ionic liquid used in this process. Recycling of ionic liquids has been previously studied (Olkiewicz et al. 2015; Orr et al. 2016) and therefore rather than simply repeat the transesterification process multiple times, the gravimetric recovery of

the ionic liquid and the recovery of KOH was used to determine the reusability of the ionic liquid. The transesterification was performed in triplicate with yeast containing 76% water content using a total reaction time of 4h at 40°C with 20% wt. KOH. The residual solids were recovered from the IL by adding an additional 10 mL of methanol which precipitated any dissolved biomass and the resulting mixture was vacuum filtered using a fine porosity Buchner funnel and washed with methanol to remove any residual ionic liquid and KOH. After oven drying at 40°C overnight, the recovered solids accounted for 50.4 ± 2.2% of the original yeast mass. FAME yield was 101.0 ± 1.1% of the neutral lipids present (accounting for ~30% of the original yeast mass). The ionic liquid and KOH were recovered from the methanol by vacuum evaporation was 103.4 ± 3.1% of the original mass of ionic liquid and KOH was recovered by gravimetric analysis. The chemical structure of the ionic liquid was confirmed to be unchanged by H^1 NMR (ratio of anion ($OCCH_3$) to cation ($NCCH_3$) was 1:1). Methanol recovery was not determined due to significant loses through the laboratory grade vacuum system used, however, methanol recovery by condensation is a well-established process in industry (Van Gerpen 2005). The ionic liquid and KOH recovered was re-dissolved in methanol and quantified by end point titration in triplicate with 20 mM $H_2SO_{4\,(MeOH)}$ using Bromocresol purple as an indicator. The recovery of the KOH was 44.8 ± 0.6% indicating that approximately 55% of the catalyst was lost during the reaction and/or recovery process but this is likely due to side reactions with other constituents in the biomass and possibly soap formation with free fatty acids which are not considered part of the neutral lipid fraction and thus did not decrease the over FAME yield. This is supported by the fact that 59.3 ± 0.4% of the KOH was recovered using the same reaction conditions with dry yeast biomass indicating that much of the catalyst consumption was not related to the presence of water. One potential solution for the excessive catalyst loss found in this study may be the use of heterogeneous catalysts instead of the homogenous catalyst used in this study.

3.6.5.6 Overall productivity

As this trial was conducted using a pilot scale cultivation of oleaginous yeast biomass from waste glycerol, the overall process performance from waste glycerol to biodiesel was calculated. The total process from glycerol to biodiesel took less than 150 h. From 3.88 kg of glycerol, 0.45 kg of FAME was produced giving an overall

conversion of 0.116 g TAGs/g glycerol. This is below the predicted theoretical maximum of 0.32 g TAGs/g for glycerol (Bommareddy et al. 2015). However, while the production of FAME was optimized in this study, it was evident that the cultivation of *R. diobovatum* would benefit from a more intensive investigation of operational parameters like oxygen saturation, however, these initial results are promising.

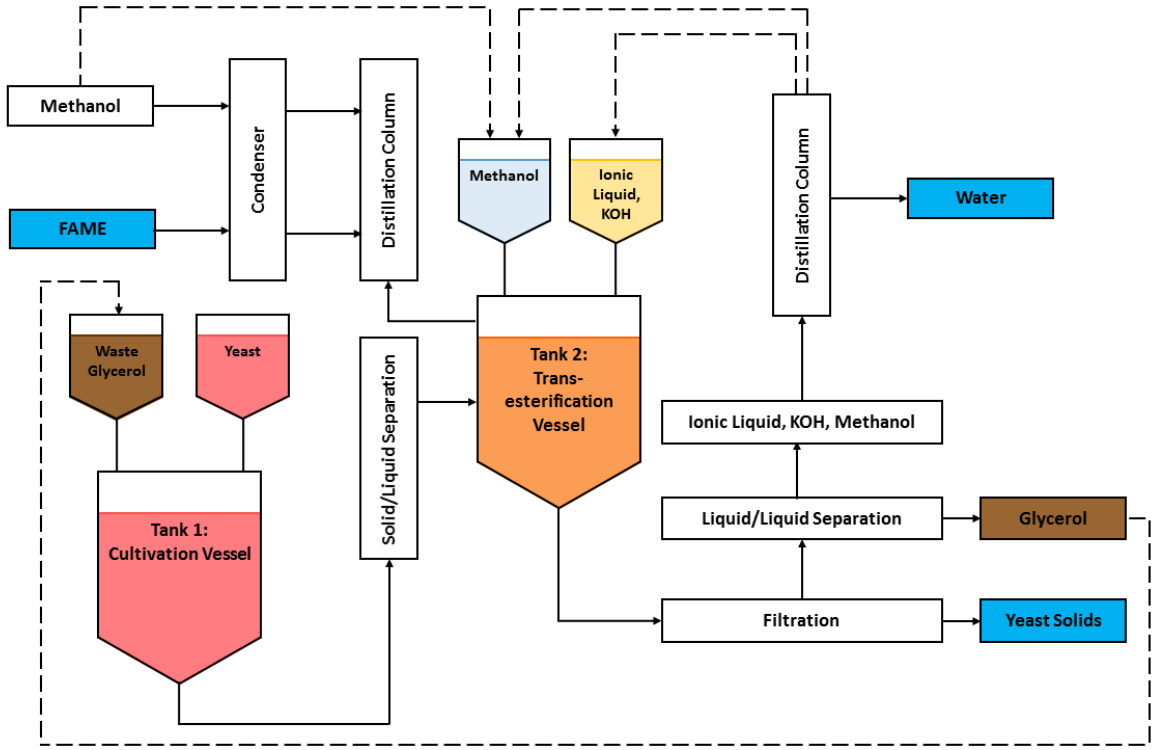


Figure 3.6-6 – Overview of proposed process for future work.

An overview of a potential process resulting from this work is illustrated in **Figure 3.6-6**. Yeast cultivated on waste glycerol would be separated from the spent media using a solid/liquid separation technique like filtration or centrifugation, then wet biomass would be transferred to a vessel where it would be mixed with methanol, KOH, and ionic liquid for transesterification. FAME produced in this reaction would be collected by distillation and methanol would be recycled. Additional methanol would be added to the vessel to precipitate dissolved proteins and other biomass components. The residual solids would be filtered from the mixture of ionic liquid, methanol, KOH, and glycerol. Although a significant portion of the KOH was consumed during the reaction in this study, the higher catalyst consumption may be offset by the lack of drying used in

this study. Glycerol could be recovered from this mixture potentially by ion exchange chromatography, however, this will be the subject of further studies. The glycerol recovered could also be reused for yeast cultivation as a carbon source to reduce waste. A full mass balance of this system would be required to properly assess the feasibility of an ionic liquid based direct conversion process for yeast lipids and will be the subject of future studies.

3.6.6 Conclusions

The present work describes the successful cultivation of *R. diobovatum* at the pilot scale and the subsequent conversion of accumulated lipids to FAME. The conversion of lipids to FAME using a novel direct transesterification process conducted in the ionic liquid $[C_2mim][EtSO_4]$ was found to increase the yield and compatibility of the process with water. The ionic liquid facilitated the recovery of the residual biomass and unconsumed catalyst potentially allowing reuse of the ionic liquid and catalysts and multiple product formation in a single process. The developed transesterification must be scaled up and the downstream purification of the biodiesel must be characterized in greater detail to determine the large scale feasibility of this promising process.

3.6.7 Acknowledgements

The authors would like to thank BioFuelNet Canada, the Natural Science and Engineering Research Council of Canada (NSERC), and the Canada Foundation for Innovation (CFI) for their financial support.

3.6.8 Supplementary Information

Table S 3.6-1 – Complete data set for all reactions conditions

MeOH	KOH	Temp	FAME Yield (%)	MeOH	KOH	Temp	FAME Yield (%)
-1	-1	-1	29.1	0	0	0	71.1
-1	-1	-1	32.1	0	0	0	81.7
1	-1	-1	72.4	0	-1	1	66.6
1	-1	-1	77.3	1	1	0	96.3
-1	1	-1	76.2	0	0	-1	76.1
-1	1	-1	78.0	0	1	1	92.6
1	1	-1	31.9	-1	0	0	86.9
1	1	-1	38.2	1	0	1	112.2
-1	-1	1	44.4	0	-1	0	64.7
-1	-1	1	64.8	0	1	0	91.0
1	-1	1	94.1	1	0	0	94.6
1	-1	1	91.8	0	-1	-1	52.8
-1	1	1	89.0	0	1	-1	73.7
-1	1	1	96.8	1	0	-1	72.3
1	1	1	53.3	-1	1	0	88.7
1	1	1	44.9	0	0	1	99.5
-1.682	0	0	80.6	-1	0	-1	65.0
-1.682	0	0	86.0	1	-1	0	67.4
1.682	0	0	84.2	-1	0	1	91.3
1.682	0	0	81.8	0	0	0	91.4
0	-1.682	0	0.9	-1	-1	0	72.2
0	-1.682	0	1.1	1	1	0	94.5
0	1.682	0	87.5	1	0	1	105.1
0	1.682	0	93.9	1	0	0	56.1
0	0	-1.682	62.4	1	0	-1	85.2
0	0	-1.682	77.8	1	-1	0	80.4
0	0	1.682	89.1	0	1	1	90.7
0	0	1.682	88.1	0	1	0	93.4
0	0	0	80.2	0	1	-1	70.0
0	0	0	84.1	0	0	1	95.5
0	0	0	80.9	0	0	0	0.0
0	0	0	86.8	0	0	-1	68.0
0	0	0	79.7	0	-1	1	70.3
0	0	0	88.1	0	-1	0	58.3
0	0	0	72.6	0	-1	-1	56.1
0	0	0	81.5	-1	1	0	95.2
0	0	0	69.1	-1	0	1	95.5
0	0	0	71.0	-1	0	0	101.6
0	0	0	84.0	-1	0	-1	63.2
0	0	0	77.6	-1	-1	0	74.8

3.7 Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction

Valerie Orr*, Kai Gao*; and Lars Rehmann

* These authors contributed equally to this work

Department of Chemical and Biochemical Engineering, University of Western Ontario, 1151 Richmond St., London, Ontario, Canada, N6A 3K7

With minor editorial changes to fulfill formatting requirements, this chapter is substantially as it appears in the *Bioresource Technology*: 206, 77-85 (2016)

3.7.1 Preface to Section 3.7

Finally, in the previous two sections, the development of the ionic liquid based process was mainly focused on the recovery of the lipid fraction for biodiesel synthesis. However, the lipid fraction only accounts for 15-50% of the total biomass composition depending on the cultivation conditions. The residual solids remaining after lipid extraction should be enriched in carbohydrate and protein, two potentially value-added products. A further advantage of the ionic liquid based extraction process is that it can be used to facilitate the recovery of the remaining solids using simple precipitation.

In this section, the gross biochemical composition of the microalgae biomass was assessed before and after ionic liquid lipid extraction and compared to the traditional hexane extraction process. It was found that the choice of antisolvent used for the precipitation and recovery of the residual solids mainly affected the protein fraction with water resulting in lower protein recovery than ethanol presumably due to higher solubility of proteins in water. The resulting lipid extracted algae (LEA) from the ionic liquid process (ILEA) and the hexane extraction process (HEA) was used directly for fermentation to produce the additional biofuels: butanol and ethanol, using ABE fermentation. As algal carbohydrates derived from *C. vulgaris* were identified to be primarily starch, these carbohydrates were readily fermented by *Clostridium saccharobutylicum* directly from starch or yields could be further improved by subjecting the LEA to dilute acid hydrolysis followed by detoxification. Finally, this study achieved the highest butanol productivity (0.32 $gL^{-1}h^{-1}$) and conversion (0.25 g/g glucose) to date using algal carbohydrates as a feedstock.

3.7.2 Abstract

Lipid extracted algae (LEA) is an attractive feedstock for alcohol fuel production as it is a non-food crop which is largely composed of readily fermented carbohydrates like starch rather than the more recalcitrant lignocellulosic materials currently under intense development. This study compares the suitability of ionic liquid extracted algae (ILEA) and hexane extracted algae (HEA) for acetone, butanol, and ethanol (ABE) fermentation. The highest butanol titers (8.05 g L^{-1}) were achieved with the fermentation of the acid hydrolysates of HEA, however, they required detoxification to support product formation after acid hydrolysis while ILEA did not. Direct ABE fermentation of ILEA and HEA (without detoxification) starches resulted in a butanol titer of 4.99 and 6.63 g L^{-1}, respectively, which significantly simplified the LEA to butanol process. The study demonstrated the compatibility of producing biodiesel and butanol from a single feedstock which may help reduce the feedstock costs of each individual process.

Keywords: Lipid-extracted algae; Ionic liquid; Hexane; ABE fermentation; Acid hydrolysis; Resin L-493

3.7.3 Introduction

Concerns as to the sustainability of first generation biofuel production from edible crops has led to the investigation of alternative feedstocks for ethanol and butanol production. Lignocellulosic residues primarily derived from agricultural or forestry wastes have received a great deal of attention in recent years, however, economical lignocellulose conversion to free sugars remains a challenge due to the recalcitrant nature of cellulose, hemi-cellulose, and lignin (John et al. 2011). Microalgae have been primarily investigated for their ability to accumulate a significant portion of their dry weight as lipids suitable for biodiesel production. However, in order to advance the sustainability of microalgae biofuel production, the carbohydrate and protein rich residues remaining after lipid extraction should also be utilized. While a lot of attention has been focused on the use of lipid extracted algae (LEA) for high protein animal and fish feeds (Brennan and Owende 2010), only a few studies have focused on the fermentation of algal biomass to other biofuels such as ethanol (Brennan and Owende 2010; John et al. 2011; Daroch et al. 2013), butanol (Jernigan et al. 2009; Potts et al. 2012; Ellis et al. 2012; van der Wal et al. 2013; Cheng et al. 2015; Wang et al. 2016), as well as methane and hydrogen (Nguyen et al. 2010; Lakaniemi et al. 2013).

Microalgae residues are an attractive feedstock for fermentation for several reasons. While the composition of algal carbohydrates varies considerably with species, many industrially relevant species of green microalgae such as *Chlorella vulgaris* can accumulate a significant portion of their dry weight as readily digestible glucans such as starch (John et al. 2011). Unlike lignocellulosic biomass, other less readily fermented sugars such as xylose, mannose, and galactose are typically present in only small amounts (Foley et al. 2011). In contrast, macroalgae species such as *Ulva lactuca* use alternative storage molecules like alginate and agarose which contain significant proportions of xylose and rhamnose (van der Wal et al. 2013). Microalgae carbohydrates present some further advantages as they are not associated with lignin (Foley et al. 2011). Lignin plays a major part in preventing catalysts and enzymes from accessing cellulose and hemicellulose and in the formation of inhibitors during hydrolysis (Ding et al. 2012a). Furthermore, lignin is a major source of phenolic inhibitory compounds which can affect the growth and production formation of microorganisms (Ezeji et al. 2007).

Finally, microalgae have significant potential as a sustainable feedstock as they can be cultivated in a potentially carbon negative process, they do not require agricultural lands, they are not eaten in any substantial quantity, and they can be cultivated year-round with continuous harvesting (climate dependent) (Foley et al. 2011; John et al. 2011).

Butanol has been proposed as a superior alternative to ethanol as it has a heating value closer to gasoline, is less volatile, and is less corrosive to distribution and storage infrastructure (Dürre 2007). Historically, high substrate cost combined with the development of petroleum based synthesis pathways resulted in the permanent decommissioning of commercial scale acetone, butanol, ethanol (ABE) fermentation plants (Jones and Woods 1986). However, increasing concerns over carbon emissions and the environmental impacts of petroleum based fuel production has renewed interest in butanol production from inexpensive sustainable feedstocks (Gao and Rehmann 2014; Gao et al. 2014). Some progress has been made in the characterization of microalgae derived substrates for ABE fermentation using whole waste water algae (Jernigan et al. 2009; Ellis et al. 2012), macroalgae (Potts et al. 2012; van der Wal et al. 2013), and lipid extracted algae (Cheng et al. 2015). With the exception of Wang et al. (2016); who used whole algae, these studies have observed poor butanol productivity or conversions.

Finally, one of the greatest challenges in the advancement of the economic feasibility of a microalgae based biofuel process is reducing the cost of harvesting, drying, and lipid extraction. Traditional methods of lipid extraction such as hexane refluxing or extraction with a mixture of a polar solvent and an apolar solvent like hexane/2-propanol are time and energy consuming, use copious amounts of flammable or toxic solvents whose volatile organic compounds contribute to growing air pollution problems, and require well dried algae (<5% moisture). In contrast, in our recent work, the ionic liquid; 1-ethyl-3-methylimidazolium ethylsulfate (C_2mim $EtSO_4$), was used to develop a novel extraction process which is fast and water compatible. The final conditions investigated found fresh wet microalgae treated with C_2mim $EtSO_4$ at a mass ratio of 1:2 with methanol for 1h at ambient temperature, disrupted the microalgae cellular structure and the process was compatible with water contents up to 82 wt% (Orr et al. 2016). This allowed the facile extraction of the lipid using a small amount of hexane for 15 min. Hexane was used to facilitate the extraction of the lipids for analytical

purposes which will auto-partition from the polar hydrophilic ionic liquid microalgae mixture. The ionic liquid could be recycled up to 5 times without any decrease in performance which will aid in offsetting the high cost of ionic liquids (Orr et al. 2016). However, it should be noted that the cost of ionic liquid synthesis has been known to decrease by over 10 fold when scaled up to commercial scale production indicating that the high cost of ionic liquids during the research and development stage is due to their custom synthesis (Wagner and Hilgers 2008).

In this study, algal biomass derived from *Chlorella vulgaris* was subjected to two lipid extraction processes and tested for its subsequent digestibility by *Clostridium saccharobutylicum*. Traditional solvent extraction which uses a mechanism of diffusion and is therefore limited by long residence times or high temperatures was contrasted with a previously developed low energy ionic liquid extraction process (Orr et al. 2016). Gross chemical composition of the raw and LEA was quantified. LEAs were subsequently hydrolysed to glucose using acid hydrolysis and detoxified using the resin: L-493 as shown in **Figure 3.7-1.** HEA and LEA, acid hydrolysates, and detoxified hydrolysates were tested for substrate performance during ABE fermentation.

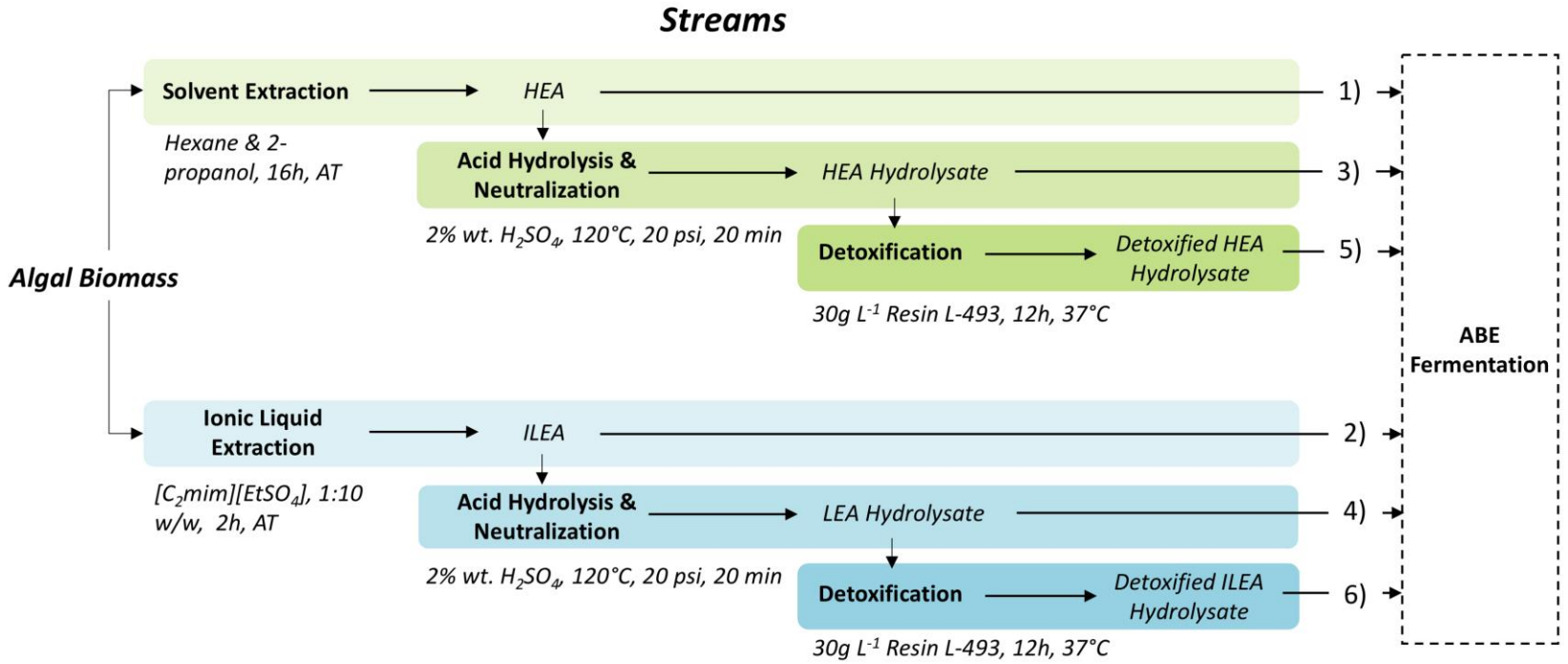


Figure 3.7-1 – Schematic diagram of the experimental design used in this study. Substrates used for subsequent fermentation are numbered 1-6.

3.7.4 Materials and Methods

3.7.4.1 Microalgae cultivation and Fractionation

Microalgae strain and cultivation conditions

Chlorella vulgaris strain UTEX 2714 was purchased from The Culture Collection of Algae at the University of Texas Austin. The culture was maintained as an actively growing cultures in liquid media using aseptic technique in 150 mL Tris-acetate-phosphate (TAP) media pH; 7.0, in 500 mL shaker flasks. Cultures were grown and maintained at 25°C at 150 rpm under cyclic illumination consisting of 16 h on: 8 h off (100 μmol m^{-2} s^{-1}). The TAP medium used consisted of 20 mM Tris base, 1.58 mM K_2HPO_4, 2.4 mM KH_2PO_4, 7.0 mM NH_4Cl, 0.83 mM $MgSO_4$, 0.34 mM $CaCl_2$, 1 mL L^{-1} glacial acetic acid, and 1 mL L^{-1} of Hutner's trace elements solution. After 48 h, an exponentially growing seed culture was inoculated into either 200 mL of media or a 5 L Labfors bioreactor (Infors HT) at 10% (v/v) and cultured for 5 d at 25°C and 400 rpm in TAP media with reduced NH_4Cl (5 mM) and supplemented with 1% (w/v) glucose in order to induce lipid production.

Harvesting and freeze drying of algal cultures

C. vulgaris cultures were harvested by centrifugation at 3,500 rpm in a Sorvall RT centrifuge (Fisher Scientific) for 20 min. Cell pellets were resuspended in deionised water and washed three times via centrifugation and resuspension to remove residual salts. The washed cells were frozen at -86°C for a minimum of 8 h and lyophilised using a 4.5 L freeze-drier (Labconco) for 24 h or until the weight no longer fluctuated then stored in a desiccator for further use. Dry weight was determined by overnight drying in an oven at 80 ºC.

3.7.4.2 Analytical Determination of Total Lipid Content

The total lipid content was determined as fatty acid methyl ester content in triplicate using a slightly modified protocol from the National Renewable Energy Laboratory (NREL/TP-5100-60958). Briefly, approximately 10 mg of dried algae was mixed with 20 μL of the recovery standard pentadecanoic acid methyl ester (C15:0Me at 10 mg mL^{-1}), 300 μL of 0.6 M HCl, and 200 μL of a trichloromethane methanol mixture (2:1 v/v) and subsequently incubated for 1h at 85°C in a water bath with stirring on a

magnetic hot plate at 1,000 rpm. After cooling, 1 mL of hexane was added to each sample and mixed at ambient temperature at 1,000 rpm. Samples were centrifuged and 450 μL of the clear top hexane phase was spiked with 50 μL of the internal standard undecanoic acid methyl ester (C11:0Me) to have a final concentration of 100 μg mL^{-1}. FAME was separated and analysed using an FID equipped Agilent 7890 Series GC and an Agilent DB-Wax capillary column (30 m, 0.25 mm, 0.25 μm). Helium was used as the carrier gas at a constant pressure of 119 kPa, and the FID was operated at 280°C. Samples were injected in split mode with a 1:10 split ratio and eluted using the following oven ramp: 50°C, 1 min, 10°C min^{-1} to 200°C, 3°C min^{-1} 220°C, 10 min. Individual FAMEs were quantified using analytical standard mixture (Supelco 37, Sigma Aldrich) and the internal standard. Unidentified FAME were quantified by applying the RF factor of the closest known peak. Total FAME content by weight was calculated according to the NREL LAP by adjusting the cumulative FAME mass using the recovery standard C15:0Me and dividing the total by the weight of cells used in the assay.

3.7.4.3 Hexane/2-propanol and Ionic Liquid Extraction

Hexane extractions were performed in triplicate by mixing 0.250 g of freeze-dried algae in 5 mL of hexane:2-propanol solution (H2P; 3:2 v/v) (Hara and Radin 1978) for 16 h at ambient temperature with agitation by a magnetic stirrer. Ionic liquid extractions were performed by mixing 0.250 g of algae with 2.5 g of $[C_2mim][EtSO_4]$ (Sigma-Aldrich) and incubating at ambient temperature for 2 h with agitation. Hexane extractions were filtered through a Buchner funnel fitted with a fine porosity fritted disc into a separating funnel. The remaining solids were washed with solvent until they were colourless (typically 3 times with 5 mL of H2P). The lipids were recovered from the ionic liquid extractions by the addition of 5 mL of hexane followed by agitation by hand. The extraction was repeated three times and the pooled hexane phase was transferred into a separating funnel. The organic extracts were washed with 0.1 M NaCl and the organic phase was transferred to a pre-weighed vessel. The solvent was evaporated and the mass of extractable lipids was measured using an analytical balance after the solvent was fully removed and the weight no longer fluctuated. Residual HEA was recovered from the funnel by resuspension in hexane and evaporating the solvent at ambient temperature using an evaporation dish. Residual ILEA was recovered by adding 10 mL of water or

ethanol to the ionic liquid mixture to precipitate the residual solids. This solution was filtered through a fine porosity Buchner funnel and washed with additional solvent to remove the ionic liquid. The solids were dried using the same method as the HEA. Dried solids were suspended in water at a concentration of 1 mg mL^{-1} and mounted on a slide for observation under a light microscope (Zeiss, Canada).

3.7.4.4 Gross Compositional Analysis

The glucan, starch, and protein compositions of the freeze-dried algae, ILEA, and HEA were determined in triplicate by following the procedures outlined by the National Renewable Energy Laboratory (Sluiter et al. 2004; Sluiter and Sluiter 2008; Laurens 2013). Protein content was measured by elemental analysis and a conversion factor of 6.35 was used as previously determined for *Chlorella vulgaris* (Safi et al. 2013).

3.7.4.5 Acid Hydrolysis and Detoxification

The LEA was subjected to acid hydrolysis to obtain monomeric sugars. Eight grams of LEA was mixed with 100 mL of 2% (v/v) H_2SO_4 and autoclaved at 121°C for 20 min. After cooling to ambient temperature, the slurry was neutralized to a pH of 6.0 with 4 M NaOH, followed by centrifugation at 3,500 rpm to remove the sediments. The supernatant was either used directly for fermentation or detoxified by mixing with 30 g L^{-1} oven-dried resin L-493 (Sigma-Aldrich) for 12 h.

3.7.4.6 Fermentation of LEA

Clostridium strain and cultivation conditions

Clostridium saccharobutylicum DSM 13864 was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). The strain was stored as a spore suspension at 4°C. The spore suspension was inoculated at 10% (v/v) into seed media (3 g L^{-1} yeast extract, 5 g L^{-1} peptone, 5 g L^{-1} soluble starch, 5 g L^{-1} glucose, 2 g L^{-1} ammonium acetate, 2 g L^{-1} NaCl, 3 g L^{-1} $MgSO_4$ $7H_2O$, 1 g L^{-1} of K_2HPO_4, 1 g L^{-1} of K_2HPO_4, and 0.1 g L^{-1} $FeSO_4$ $7H_2O$) adjusted to a pH of 6.0, autoclaved, and transferred into an anaerobic chamber (Plas-Labs, Inc., Lansing, MI) for anaerobiosis. The seed culture was incubated for 12 h at 37°C in order to generate an actively growing seed culture. Exponentially growing cells were transferred again to fresh media at 10% (v/v) and this second seed culture was used to inoculate shaker flask experiments in this study.

Batch fermentation

Direct fermentation of LEAs were carried out in shaker flasks in triplicate with LEA at 80 g L^{-1} substituted for glucose. LEA media was boiled on a hot plate to remove dissolved oxygen and transferred into an anaerobic chamber for anaerobiosis. Acid hydrolysates were prepared by dissolving solid media components into the liquid hydrolysate. For the investigation of the role of algal peptides and amino acids in supporting fermentation, yeast extract and peptone were not added to the media, however, NH_4CH_3COO remained as an inorganic nitrogen source. Samples were taken periodically for ABE and sugar analysis. Biomass density was not monitored due to the turbidity of the media.

Sugar and ABE quantification

Concentrations of glucose, acetic acid, butyric acid, acetone, ethanol, and butanol was measured by HPLC as described previously (Gao and Rehmann 2014). Briefly, an Agilent 1260 HPLC system equipped with a Hi-Plex H column and a refractive index detector (RID) was operated at 15°C with 5 mM H_2SO_4 as mobile phase and a flow rate of 0.5 mL min^{-1}.

3.7.5 Results and Discussion

3.7.5.1 Lipid extraction and compositional analysis

The results of the gross compositional analysis of untreated *C. vulgaris*, hexane extracted algae (HEA), and ionic liquid extracted algae (ILEA) are presented in **Figure 3.7-1**. All compositional methodology was based on the validated standard procedures for biomass compositional analysis available from the National Renewable Energy Laboratory (NREL). Total available lipids were quantified by direct transesterification as FAME using a protocol recently developed by NREL (Wychen and Laurens, 2013). Gravimetric lipid recovery is reported for both solvent (13.6 ± 0.1% wt, *n=3*) and ionic liquid extraction (12.4 ± 0.6% wt, *n=3*) processes and were not found to be significantly different than the theoretical maximum recovery (13.8 ± 1.7% wt, *n=3*). Ionic liquid extraction offers several advantages over the traditional solvent extraction processes; namely, greater compatibility with wet biomass (up to 82% wt water content), shorter processing times, and lower process temperatures (Teixeira 2012b; Orr et al. 2016). The

residual biomass was recovered from the hexane extraction by filtration, however, during ionic liquid extraction the ILEA was recovered by a two-step process. Firstly, an anti-solvent (water or alcohol) is added to the mixture in order to dissolve the ionic liquid but precipitate the residual carbohydrates and proteins. Secondly, this mixture is separated by filtration and the ionic liquid is recovered for reuse from the anti-solvent. Recovered ionic liquid can be reused at least 5 times without any decrease in performance (Orr et al. 2016). The composition of the residual algae in terms of total glucan, starch, and protein content is summarized in **Table 3.7-1.**

Table 3.7-1 – Gross chemical composition of untreated and LEA; *n*= 3.

	Lipid [% wt]	Glucan (Starch) [% wt]	Protein [% wt]
Unprocessed Algae	13.8 ± 1.7[a]	51.4 ± 1.0 (41.3 ± 1.4)	13.6 ± 0.8
HEA	n.a	59.9 ± 0.0 (36.1 ± 1.6)	27.4 ± 1.6
ILEA	n.a	57.5 ± 0.4 (45.1 ± 2.3)	13.2 ± 0.8

[a], determined as total FAME using NREL LAP (van Wychen and Laurens 2013)
n.a, not applicable

Interestingly, protein recovered after the hexane extraction process was higher than the ionic liquid process. Ionic liquids hypothetically aid lipid extraction by disrupting the cellular structure of the algae, which may allow water soluble proteins to be removed during the washing step when water is used as the anti-solvent. Indeed, the use of ethanol as an alternative anti-solvent resulted in the exact same amount of protein recovered as the hexane extraction (27.4 ± 1.6% wt). Thus, water was subsequently used for a scaled up ionic liquid extraction to produce adequate amounts of ILEA for fermentation. More than 80% of the carbohydrates present in the LEAs were in the form of starch for both HEA and ILEA. The lipid extraction method did not significantly alter the total recovery of sugars with 102.1% recovered after hexane extraction and 96.7% recovered after ionic liquid extraction, however, the ionic liquid extraction recovered a higher proportion of starch than the hexane extraction and resulted in higher total sugar content in the subsequent fermentation of the ILEA hydrolysate (A schematic of the process is shown in **Figure 3.7-1**). The compositional analysis is consistent with previously reported values for *C. vulgaris* (Illman et al. 2000). As the extraction of lipids did not significantly differ between hexane and ionic liquid extractions and the

processing time is much shorter for the ionic liquid process, compositional analysis indicates this to be the preferable lipid extraction process.

3.7.5.2 Direct fermentation of HEA and ILEA

Some species of *Clostridia* are capable of direct fermentation of starch through the excretion of amylolytic enzymes which degrade the extracellular starch into its glucose monomers (Paquet et al. 1991). Direct fermentation of starch has several advantages over the fermentation of hydrolysates; starch substrates are inexpensive as they require less processing steps and they potentially can be supplemented at higher concentrations than monosaccharides without causing substrate inhibition (Thang et al. 2010). Thang et al. (2011) observed that increasing the starch concentration above 50 g L^{-1} had neither a negative nor positive effect on solvent production. The ability of *C. saccharobutylicum* to consume starch was first confirmed through a control fermentation using 50 g L^{-1} soluble starch **(Figure 3.7-2A)** and compared to a fermentation using 50 g L^{-1} of glucose **(Figure 3.7-2B)**.

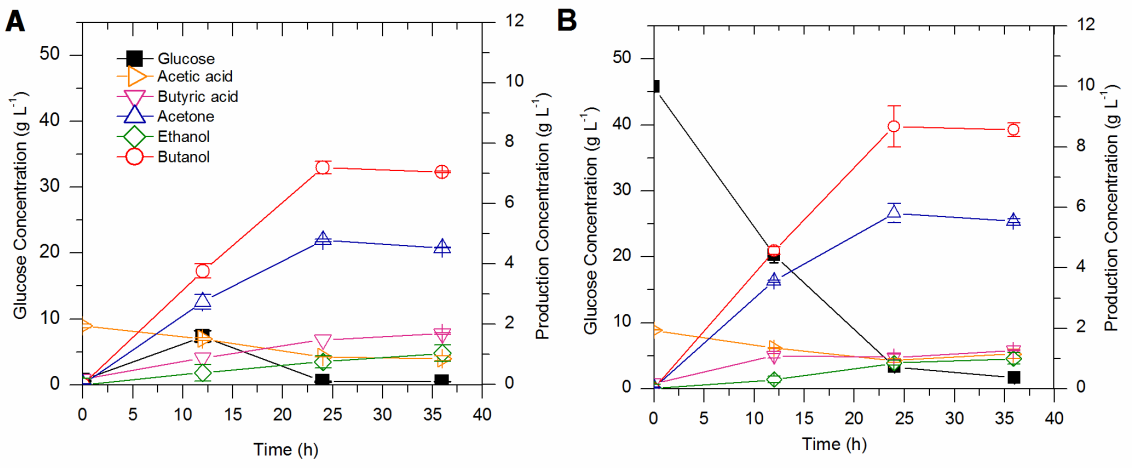


Figure 3.7-2 – Control fermentations with (A) 50 g L^{-1} soluble starch or (B) 50 g L^{-1} glucose by *Clostridium saccharobutylicum* 13864 at 37°C, 150 rpm

Glucose was detected within the first 12 h of the starch fermentation indicating *C. saccharobutylicum* is capable of extracellular starch degradation. Fermentations of both starch and glucose were complete within 24 h of inoculation, however, the final ABE production was higher in the glucose fermentations than the starch fermentations (15.33 g L^{-1} and 12.72 g L^{-1}). Similar fermentation profiles were observed with both sago and

cassava starch using *C. saccharoperbutylacetonicum* (Thang et al. 2010). HEA (Stream 1 – **Figure 3.7-1**) and ILEA (Stream 2 – **Figure 3.7-1**) were assessed as substrates for direct fermentation. The starch present in HEA (**Figure 3.7-3A**) and ILEA (**Figure 3.7-3B**) containing 28.88 g L^{-1} and 36.08 g L^{-1} of starch respectively was readily consumed by *C. saccharobutylicum* in both cultures. In both cases the available carbohydrates could directly be fermented reaching final butanol concentrations of 6.63 and 4.99 g L^{-1} respectively. Butanol formation started earlier in case of ILEA. Notably, HEA was also found to contain a higher starting concentration of acetate, presumably derived from the HEA solids. Acetate was likely removed during the washing step of the ionic liquid extraction and thus accounts for the higher starting concentration of acetate found in the HEA fermentations.

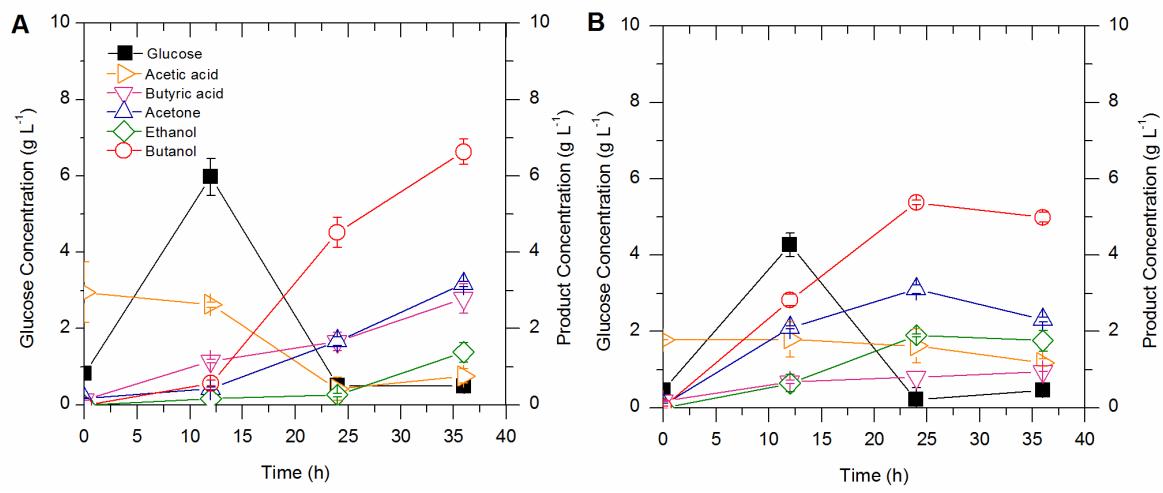


Figure 3.7-3 – Direct fermentation of lipid extracted algae with 8% solid loading (w/v) at 37°C, 200 rpm. (a), hexane extracted algae (HEA) and (b) ionic liquid extracted algae (ILEA).

3.7.5.3 Acid hydrolysis and detoxification of LEA

Dilute acid hydrolysis is known as an efficient method for starch hydrolysis and sugar yields greater than 90% are readily obtained using low solid loading concentration (Ho et al. 2013). In this study, LEA was hydrolysed using a final concentration of 2% (v/v) H_2SO_4 and a solid loading of 8% (w/v). The dilute acid hydrolysis used in this study is too mild for the complete hydrolysis of cellulose and therefore it is expected that glucose was predominately derived from the starch present in the LEA (Tsoutsos and Bethanis 2011). Harsher hydrolysis conditions were avoided in order to limit the

formation of fermentation inhibiting by-products (Tsoutsos and Bethanis 2011). Consequently, ILEA generated a higher glucose concentration from the same solid loading than HEA (46 g L^{-1} vs. 37 g L^{-1} from 80 g L^{-1} solid loading) which corresponded to the higher starch recovery of the ionic liquid based process as determined by enzymatic hydrolysis (45% and 36% by weight respectively, **Table 3.7-1**). Both samples were readily hydrolysed and the glucose recovery was between 115-119% based on the available starch, indicating that a small portion of the glucose was derived from non-starch carbohydrates present in the algae.

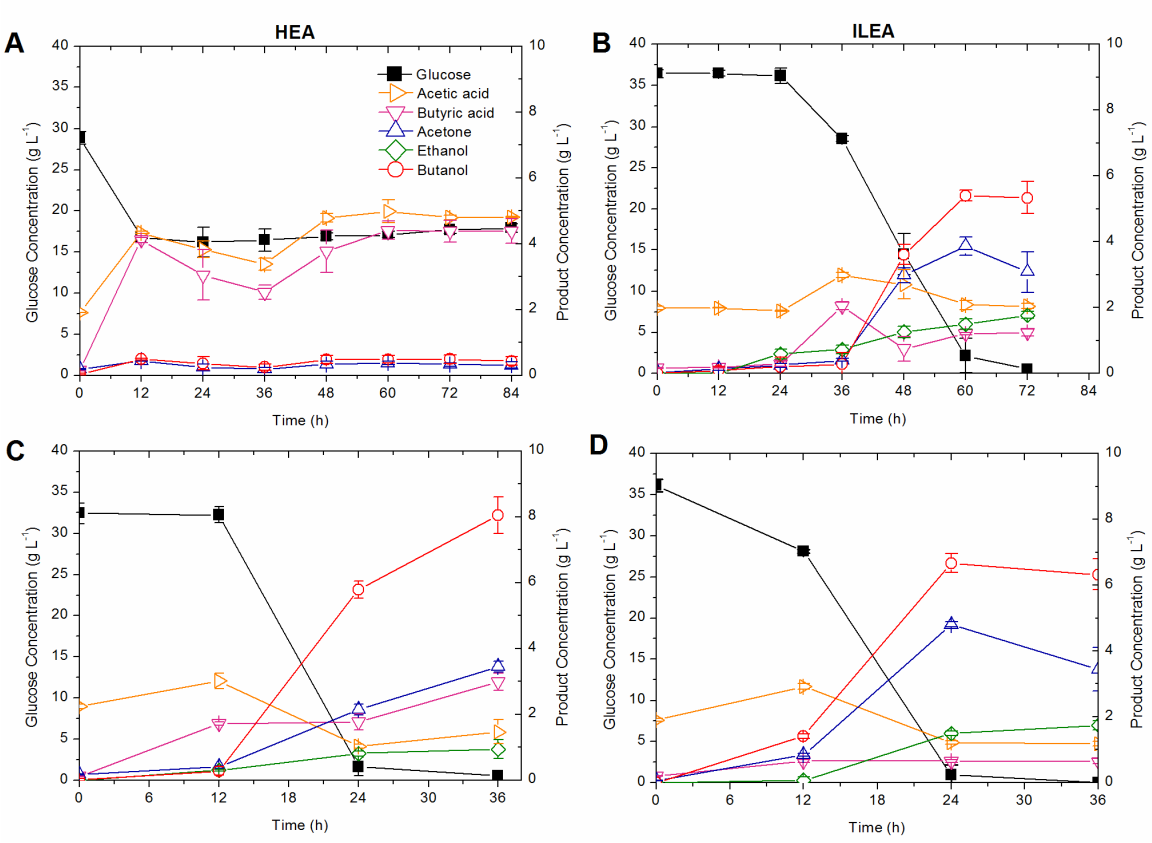


Figure 3.7-4 – Fermentation of acid hydrolysates (A & B) and detoxified acid hydrolysates (C & D) of lipid extracted algae at 37°C, 200 rpm. (A & C) HEA; (B & D) ILEA.

Acid hydrolysates were fermented after neutralization and centrifugation (**Figure 3.7-4 A and C)** or subjected to an additional detoxification step for 12 h using the resin L-493 (**Figure 3.7-4B and D**). Partial glucose consumption (42.0%) was detected during the fermentation of the acid hydrolysate of the HEA (Stream 3 – **Figure 3.7-1**, **Figure**

3.7-4A) but fermentation was arrested in acidogenesis with unexpectedly high organic acid production (4.34 g L^{-1} acetic acid and 4.11 g L^{-1} butyric acid at 12 h). This phenomenon, often called "acid crash" or "acidogenesis fermentation", can be caused by an accumulation of undissociated acids or the presence of formic acid (Wang et al. 2011). However, the reason for this occurring with HEA hydrolysates is unclear as ILEA acid hydrolysate (Stream 4 **Figure 3.7-1**, **Figure 3.7-4B**) was readily consumed after a short delay of 24 h. In order to reduce the lag phase, hydrolysates were detoxified using the resin L-493 prior to fermentation. Previous results indicate greater than 95% of total phenolic compounds are removed by the resin L-493 from the hydrolysates of lignocellulosic materials (data not shown). The delay was eliminated for both HEA (Stream 5 – **Figure 3.7-1**, **Figure 3.7-4C**) and ILEA (Stream 6 – **Figure 3.7-1**, **Figure 3.7-4D**). Detoxification allowed fermentation of HEA hydrolysates indicating that acid hydrolysis of HEA may create inhibitor compounds which acid hydrolysis of ILEA does not. As HEA was readily consumed in the direct fermentation scheme, it is unlikely that residual solvents (hexane/2-propanol) trapped within the biomass could effect this phenomenon. Detoxified hydrolysates further differed in their organic acid production with ILEA accumulating a lower concentration of butyric acid and resulted in lower final butanol concentrations (6.32 g L^{-1} compared to 8.05 g L^{-1} for HEA at 36 h). Butyric acid supplementation is known to effect higher butanol yields during ABE fermentation (Cheng et al. 2015; Regestein et al. 2015). These results combined with the results from undetoxified hydrolysates indicate that the lipid extraction process can significantly affect their subsequent fermentation.

3.7.5.4 Effects of algal derived amino acids on ABE fermentation

Compositional analysis of HEA indicated a considerable portion of protein recovered after lipid extraction. While *Clostridia* are unlikely to consume whole proteins in HEA, acid hydrolysis is also commonly employed for the degradation of proteins. Therefore, the effect of peptide and amino acids present in the acid hydrolysate of HEA were studied in order to minimize the cost of media components. Acid hydrolysate of HEA containing media with yeast extract and peptone and without yeast extract and peptone were compared to controls using pure glucose (**Table 3.7-2**). The acid hydrolysate of ILEA was not tested due to the low protein recovery. As seen in **Figure**

3.7-5A and B, yeast extract and peptone do not contribute to higher alcohol production when glucose is used as a control, however, it does reduce total culture time and consequently increased ABE productivity (**Table 3.7-2**). When the detoxified hydrolysate is used culture time increases. However, in this case, supplementation with yeast extract and peptone does increase the butanol yield from 5.42 to 8.05 g L^{-1} (**Figure 3.7-4C)** and does not alter the culture time. It should also be noted that for detoxified hydrolysates of HEA, 23% of the glucose was not consumed in the absence of yeast extract and peptone whereas glucose was completely depleted when yeast extract and peptone were used. A higher butanol titer was achieved during fermentation when using detoxified acid hydrolysate supplement with yeast extract and peptone compared to the glucose controls (**Table 3.7-2**). This was also associated with higher butyric acid production during the fermentation of the detoxified hydrolysate than the glucose control. Interestingly, Wang et al (2016) found that protein concentrations greater than 500 mg L^{-1} decreased butanol production and yield when fermenting lipid extracted *C. vulgaris* using *C. acetobutylicum* and included a NaOH wash step prior to acid hydrolysis in order to remove protein. This effect was not seen in this work.

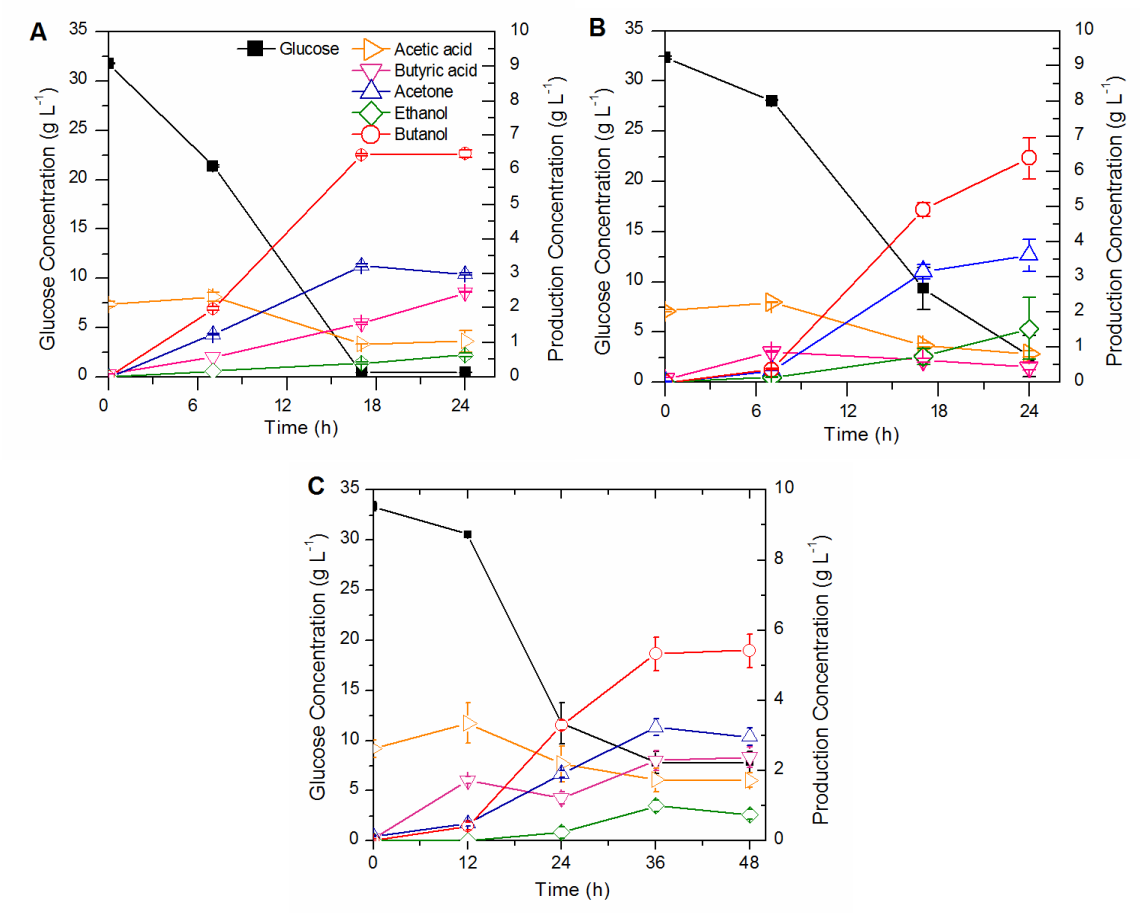


Figure 3.7-5 – The effect of supplementation of yeast extract and peptone on fermentation at 37°C, 200 rpm. A. Glucose control with yeast extract and peptone, B. Glucose control without yeast extract and peptone, C. Detoxified acid hydrolysate of HEA without yeast extract or peptone.

Table 3.7-2 – Summary of fermentation end points from triplicate cultures growth with (+YEP) or without (-YEP) yeast extract and peptone supplementation in control cultures with glucose or with the acid hydrolysate of HEA.

Media Composition	Glucose (Controls)		Detoxified Hydrolysate of HEA	
	+YEP	-YEP	+YEP	-YEP
Substrate Concentration				
Initial glucose Conc. [g L^{-1}]	31.78 ± 0.08	32.33 ± 0.15	32.44 ± 1.25	33.35 ± 0.46
Final glucose Conc. [g L^{-1}]	0.44 ± 0.01	2.63 ± 2.05	0.5 ± 0.00	7.83 ± 1.07
Organic Acids				
Acetic [g L^{-1}]	0.96 ± 0.01	0.81 ± 0.08	1.47 ± 0.37	1.72 ± 0.21
Butyric [g L^{-1}]	1.55 ± 0.03	0.44 ± 0.10	2.99 ± 0.26	2.38 ± 0.3
Organic Solvent				
Acetone [g L^{-1}]	3.23 ± 0.05	3.61 ± 0.47	3.45 ± 0.16	2.96 ± 0.25
Ethanol [g L^{-1}]	0.41 ± 0.04	1.52 ± 0.88	0.94 ± 0.29	0.75 ± 0.13
Butanol [g L^{-1}]	6.45 ± 0.04	6.38 ± 0.59	8.05 ± 0.56	5.42 ± 0.48
Culture Performance				
Total Time [h]	17	24	36	36
Butanol [g g^{-1}]	0.21	0.21	0.25	0.21
ABE [g L^{-1} h^{-1}]	0.59	0.48	0.35	0.25

3.7.6 Batch Fermentation of Algal Substrates

Recently, several attempts at the batch ABE fermentation of both micro- and macroalgae have been reported and are summarized and compared to the yields obtained in this study in **Table 3.7-3**. Only a small number of studies have begun to explore the use of algae biomass as a substrate for ABE fermentation. Only one recent study explored the use of lipid extracted algae for ABE fermentation. In this work, lipids were extracted from *C. sorokiniana* using microwave assisted solvent extraction. Several methods of hydrolysis were tested including enzymatic hydrolysis, however, no detoxification schemes were employed. Interestingly, the enzymatic hydrolysis using cellulase which should contain the lowest proportion of inhibitory compounds was amongst the worst hydrolysis strategies for subsequent butanol production (Cheng et al. 2015). *C. acetobutylicum* ATCC 824 used in this study is known to be capable of producing amylolytic enzymes (Paquet et al. 1991) which should enable them to digest starch, the major carbohydrate present in *C. sorokiniana* (Choix et al. 2012). This suggests that perhaps residual solvents were not removed from the biomass prior to

hydrolysis. In fact, increasing the proportion of hydrolysate resulted in poorer butanol yields (Cheng et al. 2015) which would be expected if toxic solvents were not completely removed.

The butanol concentrations and ABE productivities achieved in this study are amongst the highest reported thus far. This is partly due to the use of *C. saccharobutylicum* which can achieve complete fermentation in less time as well as the inclusion of a detoxification step. Wang et al. (2016) reported the fermentation of the whole biomass of *C. vulgaris* to butanol using *C. acetobutylicum* resulting in a higher butanol production 13.1 g L^{-1} but a similar conversion of 0.24 g g^{-1} (Wang et al. 2016) which is in line with the results presented in this study. The differences in butanol production is due to the lower sugar concentration used in this study (33-36 g L^{-1} vs. 55.6 g L^{-1}) (Wang et al. 2016).

Finally, feedstock cost is currently one of the biggest challenges in butanol production and by producing multiple biofuel productions from a single feedstock the feedstock cost can be split between each process. Previous work found low butanol conversion from microalgal sugars or very low productivity. The current study work demonstrates that production of biodiesel and butanol from a single feedstock is definitely feasible.

Table 3.7-3 – Summary of batch ABE fermentation from algal based substrates

Algal Species	Species (Strain)	Pretreatment	Substrate Concentration (Composition)*	Butanol Conc. (ABE) [g L^{-1}]	ABE Productivity [g L^{-1} h^{-1}]	Butanol yield or (Total ABE yield) [g g^{-1} glucose[1], starch[2], total sugars [3]]	Ref.
C. vulgaris (Ionic liquid extracted algae)	*C. saccharobutylicum* (DSM 13864)	Untreated	8% (w/v)	4.99 (9.06)	0.25	0.15[2] (0.29[2])	This study
		2% H_2SO_4	36.45 g L^{-1} Glc	5.34 (10.19)	0.14	0.15[1] (0.28[1])	
		2% H_2SO_4 & L-493	36.14 g L^{-1} Glc	6.32 (11.50)	0.32	0.17 [1] (0.32[1])	
C. vulgaris (Hexane/2-propanol extracted algae)	*C. saccharobutylicum* (DSM 13864)	Untreated	8% (w/v)	6.63 (11.18)	0.31	0.23[2] (0.31[2])	This study
		2% H_2SO_4	28.88 g L^{-1} Glc	0.44 (1.52)	0.02	0.04[1] (0.14[1])	
		2% H_2SO_4 & L-493	32.44 g L^{-1} Glc	8.05 (12.44)	0.35	0.25[1] (0.39[1])	
C. vulgaris	*C. acetobutylicum* (ATCC 824)	Enzymes	38.6 g L^{-1} TS	n.d	n.a	0.00 (n.a)	(Wang et al. 2016)
		3% H_2SO_4	32.7 g L^{-1} TS	1.5	n.a	0.05 (n.a)	
		3% H_2SO_4	69.4 g L^{-1} TS	0.5	n.a	0.01[1] (n.a)	
		1% NaOH + 3% H_2SO_4	55.6 g L^{-1}	13.1 (19.9)	0.27	0.24[1] (n.a)	
C. sorokiniana (Hexane/Methanol extracted algae)	*C. acetobutylicum* (ATCC 824)	2% H_2SO_4 & 2% NaOH	37.93 g L^{-1} TS	3.35 (4.97)	0.07	0.13[1] (0.19[1])	(Cheng et al. 2015)
		2% H_2SO_4 & 2% NaOH	52.97 g L^{-1} TS	1.45 (1.89)	0.02	0.06[1] (0.08[1])	
		2% H_2SO_4 & 2% NaOH	60.81 g L^{-1} TS	2.75 (4.09)	0.01	0.10[1] (0.15[1])	
		2% H_2SO_4 & 2% NaOH	71.21 g L^{-1} TS	2.42 (3.16)	0.02	0.12[1] (0.13[1])	
		2% H_2SO_4 & 2% NaOH	89.08 g L^{-1} TS	3.86 (6.32)	0.02	0.09[1] (0.14[1])	
Mixed culture		Untreated	10% (w/v)	0.52 (0.73)	0.01	0.17[1] (0.26[1])	

waste water	*C. saccharoperbutylacetonicum* (ATCC 27021)	1 M H_2SO_4 & 5 M NaOH	10% (w/v)	2.26 (2.74)	0.03	0.20[1] (0.24[1])	(Ellis et al. 2012)
		1 M H_2SO_4 & 5 M NaOH	10% (w/v) + 1% (w/v) Glc	5.61 (7.27)	0.08	0.21[1] (0.27[1])	
		1 M H_2SO_4 & 5 M NaOH + cellulase, xylanase	10% (w/v)	7.79 (9.74)	0.10	0.25[1] (0.31[1])	
Mixed culture waste water	*C. saccharoperbutylacetonicum* (ATCC 27021)	4% H_2SO_4, 3 h, 120°C	n.a	n.a	n.a	0.13[3] (n.a)	(Jerniga n et al. 2009)
Ulva lactuca (Macroalgae)	*C. beijerinckii* (NCIMB 8052)	10 min 150°C + cellulase	15.8 g L^{-1} TS (50% Glc, 35% Rha, 15% Xly)	3.3 (5.5)	0.02	0.21[3] (0.35[3])	(van der Wal et al. 2013)
	C. acetobutylicum (ATCC 824)	10 min 150°C + cellulase	15.6 g L^{-1} TS (50% Glc, 35% Rha, 15% Xly)	0.3 (0.3)	0.00	0.03[3] (0.03[3])	
Ulva lactuca (Macroalgae)	*C. saccharoperbutylacetonicum* (ATCC 27021)	1% H_2SO_4	15.2 g L^{-1} TS (27% Glc, 57% Ara, 16% Xyl)	4.51 (n.a)	n.a.	0.29[3] (n.a)	(Potts et al. 2012)

-, no pretreatment or hydrolysis for the substrate; na, not available; *TS, Total sugar concentration; Glc, Glucose; Ara, arabinose; Xly, xylose; Rha, rhamnose

3.7.7 Conclusions

Algal residues were assessed as ABE fermentation substrates. Fermentation results differed significantly between hexane extracted algae and ionic liquid extracted algae indicating the extraction method affects the subsequent fermentation. The highest conversion and ABE productivity was obtained using detoxified hydrolysates of HEA demonstrating the need for hydrolysate detoxification prior to fermentation. Amino acids and peptides released by acid hydrolysis were found to have little effect on fermentation. Direct fermentation of starch was possible using the LEAs but only generated modest levels of butanol, however direct fermentation reduced the number of preparatory steps and accordingly would be a faster and lower cost option.

3.7.8 Abbreviations

ABE: acetone, butanol, ethanol

HEA: hexane extracted algae

ILEA: ionic liquid extracted algae

LEA: lipid extracted algae

H2P: hexane 2-propanol (2:1 w/w)

3.7.9 Acknowledgements

The authors are grateful to the Natural Sciences and Engineering Research Council of Canada, China Scholarship Council (CSC), the Canada Foundation for Innovation, BioFuelNet Canada and the Alexander von Humboldt Foundation for financial support.

Chapter 4 – Summary and Conclusions

4.1 Summary

It was the objective of this thesis to develop a biorefinery process for the production of biodiesel and other biofuel products using oleaginous yeast or microalgae. First, a method for the quantification of lipids was significantly improved in order to study the heterotrophic cultivation of microalgae. Following the optimization of microalgae lipid production using glucose, a renewable source of glucose; derived from the pyrolysis of lignocellulosic biomass, was used for the sustainable cultivation of both microalgae and oleaginous yeasts. Once reproducible cultivation processes were established, a large screening study was conducted to identify room temperature ionic liquids capable of disrupting microalgae cell structure and aiding lipid extraction. Conditions for the wet extraction of biomass using ionic liquids was established. This was extended to allow the direct conversion of lipids to FAME by the addition of a homogenous base catalyst.

This thesis was composed of three specific objectives (i) cultivation and production of microalgae and oleaginous yeasts using renewable feedstocks, (ii) development of a water compatible lipid extraction method, and (iii) the recovery and use of residual solids for the production of value added products.

Significant contributions to the cultivation and production of microalgae and oleaginous yeast were made first through the improvement of the Nile Red assay. The modified method presented in this thesis improved the reproducibility of this high throughput assay and demonstrated that the most significant source of variance was the variable chlorophyll content of the cultures. This improved method will allow for more consistent reporting of microalgae lipid content throughout the microalgae biotechnology field. This assay was used to study the optimization of heterotrophic cultivation conditions for the microalgae *Chlorella vulgaris*. The design used required a small number of shaker flask cultivations (15 total) to study the effects of three factors on lipid production. The media optimization resulted in a 19.1-fold increase in lipid production. Cultivation in a stirred tank reactor identified aeration as a possible method of controlling

carbon flux between carbohydrate synthesis or lipid accumulation. Finally, microalgae were demonstrated along with oleaginous yeast to be capable of using pyrolysis derived glucose for a more sustainable approach to heterotrophic lipid production. Microalgae were found to be more sensitive to growth inhibition caused by the presence of inhibitory compounds produced in the pyrolysis process than *Rhodosporidium diobovatum*. However, increasing amounts of pyrolytic sugars were found to have a positive effect on increasing both biomass production and increased lipid production 2.8-fold compared to the control for *R. diobovatum* when using a complete media with 100% pyrolytic sugars. Increasing amounts of pyrolytic sugars had a similar effect on increasing lipid content of *C. vulgaris*, however, a concurrent decrease in biomass production meant the overall volumetric lipid production was unchanged. This was the first published report of both *C. vulgaris* and *R. diobovatum* on pyrolytic glucose and the only report studying the effects of increasing inhibitor concentrations on growth and lipid production.

The second objective of this work was to develop a water compatible process for lipid extraction from microalgae and oleaginous yeast. The initial significance of this work was the diverse nature of the ionic liquids tested for their ability to disrupt microalgae. Furthermore, this was the first study to use solely room temperature ionic liquids and non-imidazolium based ionic liquids. Furthermore, lipids were extracted from the algae biomass for only 15 minutes using hexane allowing the identification of ionic liquids which facilitated the extraction process while previous studies used extensive hexane extraction times probably greater than the time it would take hexane to extract lipids from biomass on its own. This work identified over 10 possible ionic liquids which would facilitate lipid extraction. One ionic liquid was chosen for further investigation and it was discovered that methanol was required for the extraction process to proceed in the presence of water. The final process required as little as 75 minutes to extract all available lipids at room temperature from biomass containing up to 82% water. As the resulting process was conducted with wet biomass at room temperature this represents a significant improvement over solvent extraction and drying practices currently used for lipid extraction from microalgae. Furthermore, the ionic liquid was reused four times without and decrease in efficiency and ionic liquid lose was minimal. This process was then further improved and extended to include lipid recovery from oleaginous yeasts

directly as FAME. Response surface methodology was used to optimize reaction conditions and study the interactions of the catalyst, methanol, and reaction temperature. All of the available lipids were converted to FAME within 2.5h directly from the whole dried biomass. However, when this work was extended to fresh yeast biomass, it was found that the optimized conditions resulted in poor yields. However, full conversion could be achieved in as little at 4h if the temperature was decreased and the catalyst loading was approximately doubled. Water contents of greater than 76% resulted in a decrease in the FAME yield, however at lower moisture contents the presence of the ionic liquid allowed the reaction to go to completion. Furthermore, the ionic liquid facilitated the recovery of over 40% of the homogenous catalyst depending on the reaction conditions. Catalyst consumption was greater when wet biomass was used. Overall, the catalyst loading ratio was lower than any previous reports of direct transesterification of oleaginous biomass. Furthermore, the reaction times were significantly shorter than any previous reports.

The final chapter built on the previous work by recovering residual solids from the developed ionic liquid extraction process and demonstrating their further application for the generation of additional value added biofuel products. This was contrasted with fermentation of the recovered solids using the traditional hexane extraction process. It was found that the composition of the recovered solids could be adjusted in the ionic liquid based process by the choice of anti-solvent used for precipitation. The use of ethanol resulted in the recovery of solids identical in composition to the hexane extracted solids. However, the use of water, resulted in increased purity of the carbohydrate fraction by the removal of proteins during the washing step due to the greater solubility of proteins in water. This control was possible as the ionic liquid extraction process results in the disruption of cellular structure, while hexane extraction leaves cell structure intact. The ionic liquid extracted solids were directly fermented from starch to acetone, butanol, and ethanol with the greatest conversion and highest productivity reported so far for algal based feedstocks. This work demonstrated the possible flexibility of microalgae biomass for the production of multiple biofuels products. This could be particularly significant for microalgae produced heterotrophically using waste waters which may not

reach high lipid content and whose growth conditions may favour carbohydrate production instead.

4.2 Scientific Contributions

Several significant contributions were made throughout this body of work to the scientific community.

Firstly, the Nile Red assay (*Section 3.2*) is the only high-throughput method for the characterization of lipids. The modifications developed herein represent a significant improvement in the reproducibility and accuracy of this method. Furthermore, the facile method of cell bleaching allows more accurate determination of optical cell densities even between cultures differing in chlorophyll content. If adopted by other phycologists, this would facilitate greater reproducibility of cultivation studies between research groups and aid in the advancement of the field.

Secondly, this work presents the greatest conversions of pyrolytic sugars to lipids (*Section 3.4*) reported thus far using oleaginous yeasts. Furthermore, this was the first report that the presence of increasing inhibitors amounts affects the lipid composition and consequently the future biodiesel properties. This work was also the first to investigate the positive effect of inhibitors on lipid accumulation in oleaginous yeasts and to investigate the combined effect of both nitrogen limitation and growth inhibitors on the cultivation of *R. diobovatum*. Finally, this work was the first to report growth of microalgae on pyrolytic sugars.

Thirdly, the work encompassing the development of ionic liquid based lipid extraction and transesterification processes (*Sections 3.5 and 3.6*) made several significant contributions. These include the largest screening study of ionic liquids for algal biomass processing to date. With the exception of a single ionic liquid, this was also the first investigation of non-imidazolium ionic liquids for this purpose. Additionally, the use of any ionic liquids for yeast biomass processing had not been reported prior to this work. Furthermore, this work was the first to investigate the effects of processes parameters on extraction and transesterification in a systematic way. Finally, this work was the first in depth investigation of the effects of water content on lipid extraction or transesterification when using fresh microalgae or oleaginous yeasts.

Finally, during the study of the recovery of residual solids for subsequent ABE fermentation (*Section 3.7*), this work was the first to report the effect of the anti-solvent choice on recovery of residual proteins and carbohydrates and compare them to traditional solvent extractions. Furthermore, the resulting ABE fermentation reported the highest conversion of glucose to biobutanol to date using algal substrates.

4.3 Conclusions

In conclusion, it was found in this work that the oleaginous yeast *R. diobovatum* is a more promising platform for heterotrophic lipid production than the microalgae *C. vulgaris*. They are a much more flexible species capable of utilizing a greater number of substrates and were much more resistant to inhibitors found in waste feedstocks.

The use of the ionic liquid $[C_2mim][EtSO_4]$ was found to allow for the direct extraction of lipids as biodiesel using wet biomass in a quick and simple process. It also facilitated the recovery of the homogenous catalyst in this process making it a promising novel downstream process for biodiesel production of microbial lipids. Furthermore, the ionic liquid based process allows for the simple recovery by precipitation of the lysed residual solids which can be used for the production of additional biofuels.

Overall, this work significantly contributed to our greater understanding of the cultivation of microalgae and oleaginous yeasts using renewable feedstocks, demonstrated the advantages of the low energy ionic liquid based biomass fractionation process developed, and demonstrated that multiple fuels products could be produced from a single feedstock in a biorefinery type scheme.

4.4 Future Work

The results presented in this thesis indicate that heterotrophic cultivation of microalgae from renewable feedstocks may be a viable pathway for glucose to lipid bioconversion. However, while microalgae were found to have the highest conversion of glucose to lipids in this study, their growth was also found to be highly sensitive to the inhibitory compounds found in pyrolysis derived sugars. One possible approach to lessening this sensitivity may be culture adaption which was not explored in this work.

Secondly, as glucose is not a readily consumed carbon source (low glucose consumption rate of < 5 $gL^{-1}d^{-1}$) for microalgae cultivation, this work could also be extended to study other alternative sources of organic carbon that are more readily consumed such as acetate or glycerol. In particular, pyrolysis conditions of lignocellulosic biomass can be adjusted to favour the generation of an acetate rich bio-oil rather than one rich in anhydrous sugars which might be more readily consumed by *C. vulgaris*. Additionally, glycerol is also a low cost waste feedstock produced in large quantities by the biodiesel industry. While it was shown in *Section 3.6* that waste glycerol could be used for oleaginous yeast cultivation, similarly, microalgae can also readily consume glycerol and it would be of interest to compare the performance of these two platforms directly.

The results of the ionic liquid based lipid extraction or transesterification process were demonstrated at the bench scale in this work. This was partly due to materials limitations, however, in order to true understand the techno-economic advantages of the developed process, the process would need to be scaled up. Therefore, future work on the developed process should include a larger scale process which studies the mass balance of the system in greater detail. However, the operation itself could also be improved as one particular disadvantages for the direct conversion process developed was the consumption of the catalyst. Furthermore, only three catalysts were explored for this process. Therefore, future work should include the assessment of additional catalysts for the wet transesterification process, particularly heterogeneous catalysts. Finally, due to the diverse chemical nature of ionic liquids, the task-specific design of an ionic liquid based catalyst for this process should be explored as it could reduce the number and amounts of materials needed for this process.

Finally, during the development of the ionic liquid based process it became obvious that ionic liquids could also be used to recover other types of high value products from the oleaginous yeast and microalgae, particularly the carotenoids. In this work, carotenoids appeared to be readily extracted by the alcohol ionic liquid mixture from the disrupted biomass. Upon the removal of the alcohol via evaporation, the carotenoids separated into a distinct phase from the ionic liquids and could be recovered in this manner. Furthermore, carotenoids present in the ionic liquid/alcohol mixtures were found to be much less sensitive to photobleaching than alcohol mixtures alone as they could be

kept in full light for several months and remained highly pigmented while alcohol extractions degraded within a day. While these phenomena were noted, they were not explored in enough detail to be presented in this work. However, recovery of carotenoids could be of advantage in order to increase the number of bioproducts recovered in this simple low energy process.

References

Abedini Najafabadi H, Vossoughi M, Pazuki G (2015) The role of co-solvents in improving the direct transesterification of wet microalgal biomass under supercritical condition. Bioresour Technol 193:90–96.

Abo-Shady AM, Mohamed YA, Lasheen T (1993) Chemical composition of the cell wall in some green algae species. Biol Plant 35:629–632.

Adamová G, Gardas RL, Nieuwenhuyzen M, Puga A V., Rebelo LPN, Robertson AJ, Seddon KR (2012) Alkyltributylphosphonium chloride ionic liquids: synthesis, physicochemical properties and crystal structure. Dalt Trans 41:8316–8332.

Adamová G, Gardas RL, Rebelo LPN, Robertson AJ, Seddon KR (2011) Alkyltrioctylphosphonium chloride ionic liquids: synthesis and physicochemical properties. Dalt Trans 40:12750–12764.

Ageitos JM, Vallejo JA, Veiga-Crespo P, Villa TG (2011) Oily yeasts as oleaginous cell factories. Appl Microbiol Biotechnol 90:1219–1227.

Aguirre AM, Bassi A (2014) Investigation of high pressure steaming (HPS) as a thermal treatment for lipid extraction from Chlorella vulgaris. Bioresour Technol 164:136–142.

Almeida JR, Modig T, Petersson A, Hähn-Hägerdal B, Lidén G, Gorwa-Grauslund MF (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates bySaccharomyces cerevisiae. J Chem Technol Biotechnol 82:340–349.

Alonzo F, Mayzaud P (1999) Spectrofluorometric quantification of neutral and polar lipids in zooplankton using Nile red. Mar Chem 67:289–301.

Amarasekara AS, Owereh OS (2010) Synthesis of a sulfonic acid functionalized acidic ionic liquid modified silica catalyst and applications in the hydrolysis of cellulose. Catal Commun 11:1072–1075.

Amaro HM, Guedes AC, Malcata FX (2011) Advances and perspectives in using microalgae to produce biodiesel. Appl Energy 88:3402–3410.

Ankit Rohatgi (2015) WebPlotDigitizer.

Arora R, Manisseri C, Li C, Ong MD, Scheller HV, Vogel K, Simmons B a., Singh S (2010) Monitoring and analyzing process streams towards understanding ionic liquid pretreatment of switchgrass (Panicum virgatum L.). Bioenergy Res 3:134–145.

Atabani AE, Silitonga AS, Badruddin IA, Mahlia TMI, Masjuki HH, Mekhilef S (2012) A comprehensive review on biodiesel as an alternative energy resource and its characteristics. Renew Sustain Energy Rev 16:2070–2093.

Atadashi IM, Aroua MK, Abdul Aziz AR, Sulaiman NMN (2012) The effects of water on biodiesel production and refining technologies: A review. Renew Sustain Energy Rev 16:3456–3470.

Azma M, Mohamed MS, Mohamad R, Rahim RA, Ariff AB (2011) Improvement of medium composition for heterotrophic cultivation of green microalgae, Tetraselmis

suecica, using response surface methodology. Biochem Eng J 53:187–195.

Balat M (2011) Potential alternatives to edible oils for biodiesel production – A review of current work. Energy Convers Manag 52:1479–1492.

Banerjee N, Bhatnagar R, Viswanathan L (1981) Inhibition of Glycolysis by Furfural in Saccharomyces cerevisiae. 226–228.

Banković-Ilić IB, Stamenković OS, Veljković VB (2012) Biodiesel production from non-edible plant oils. Renew Sustain Energy Rev 16:3621–3647.

Baranyi J, Roberts TA (1994) A dynamic approach to predicting bacterial growth in food. Int J Food Microbiol 23:277–294.

Beopoulos A, Nicaud J-M, Gaillardin C (2011) An overview of lipid metabolism in yeasts and its impact on biotechnological processes. Appl Microbiol Biotechnol 90:1193–1206.

Bertozzini E, Galluzzi L, Penna A, Magnani M (2011) Application of the standard addition method for the absolute quantification of neutral lipids in microalgae using Nile red. J Microbiol Methods 87:17–23.

Binder JB, Raines RT (2010) Fermentable sugars by chemical hydrolysis of biomass. 2010:1–6.

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917.

Bommareddy RR, Sabra W, Maheshwari G, Zeng A-P (2015) Metabolic network analysis and experimental study of lipid production in Rhodosporidium toruloides grown on single and mixed substrates. Microb Cell Fact 14:1–13.

Boyd AR, Champagne P, Mcginn PJ, Macdougall KM, Melanson JE, Jessop PG (2012) Bioresource Technology Switchable hydrophilicity solvents for lipid extraction from microalgae for biofuel production. Bioresour Technol 118:628–632.

Brandt A, Gräsvik J, Hallett JP, Welton T (2013) Deconstruction of lignocellulosic biomass with ionic liquids. Green Chem 15:550–583.

Brennan L, Owende P (2010) Biofuels from microalgae — A review of technologies for production, processing , and extractions of biofuels and co-products. Renew Sustain Energy Rev 14:557–577.

Bricaud A (2004) Natural variability of phytoplanktonic absorption in oceanic waters: Influence of the size structure of algal populations. J Geophys Res 109:C11010.

Canola Council of Canada (2016) Steps in oil and meal processing. http://www.canolacouncil.org/oil-and-meal/what-is-canola/how-canola-is-processed/steps-in-oil-and-meal-processing/.

Chen CL, Huang CC, Ho KC, Hsiao PX, Wu MS, Chang JS (2015a) Biodiesel production from wet microalgae feedstock using sequential wet extraction/transesterification and direct transesterification processes. Bioresour Technol 194:179–186.

Chen C-Y, Yeh K-L, Aisyah R, Lee D-J, Chang J-S (2011a) Cultivation, photobioreactor

design and harvesting of microalgae for biodiesel production: a critical review. Bioresour Technol 102:71–81.

Chen F, Johns MR (1991) Effect of C/N ratio and aeration on the fatty acid composition of heterotrophic Chlorella sorokiniana. J Appl Phycol 3:203–209.

Chen L, Sharifzadeh M, Mac Dowell N, Welton T, Shah N, Hallett JP (2014) Inexpensive ionic liquids: [HSO4]−-based solvent production at bulk scale. Green Chem 16:3098.

Chen M, Tang H, Ma H, Holland TC, Ng KYS, Salley SO (2011b) Effect of nutrients on growth and lipid accumulation in the green algae Dunaliella tertiolecta. Bioresour Technol 102:1649–55.

Chen W, Sommerfeld M, Hu Q (2011c) Microwave-assisted nile red method for in vivo quantification of neutral lipids in microalgae. Bioresour Technol 102:135–41.

Chen W, Zhang C, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. J Microbiol Methods 77:41–7.

Chen X, Hu L, Xing R, Liu S, Yu H, Qin Y, Li K, Li R, Li P (2015b) Ionic liquid-assisted subcritical water promotes the extraction of lipids from wet microalgae Scenedesmus sp. Eur J Lipid Sci Technol 117:1192–1198.

Cheng CH, Du TB, Pi HC, Jang SM, Lin YH, Lee HT (2011) Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO 2. Bioresour Technol 102:10151–10153.

Cheng H, Whang L, Chan K, Chung M, Wu S, Liu C-P, Tien S-Y, Chen S-Y, Chang J-S, Lee W-J (2015) Biological butanol production from microalgae-based biodiesel residues by Clostridium acetobutylicum. Bioresour Technol 184:379–385.

Chisti Y (2007) Biodiesel from microalgae. Biotechnol Adv 25:294–306.

Chisti Y, Moo-Young M (1986) Disruption of microbial cells for intracellular products. Enzyme Microb Technol 8:194–204.

Chiu S-Y, Kao C-Y, Tsai M-T, Ong S-C, Chen C-H, Lin C-S (2009) Lipid accumulation and CO2 utilization of Nannochloropsis oculata in response to CO2 aeration. Bioresour Technol 100:833–8.

Choi SA, Jung JY, Kim K, Kwon JH, Lee JS, Kim SW, Park JY, Yang JW (2014a) Effects of molten-salt/ionic-liquid mixture on extraction of docosahexaenoic acid (DHA)-rich lipids from Aurantiochytrium sp. KRS101. Bioprocess Biosyst Eng 37:2199–2204.

Choi S-A, Lee J-S, Oh Y-K, Jeong M-J, Kim SW, Park J-Y (2014b) Lipid extraction from Chlorella vulgaris by molten-salt/ionic-liquid mixtures. Algal Res 3:44–48.

Choi S-A, Oh Y-K, Jeong M-J, Kim SW, Lee J-S, Park J-Y (2014c) Effects of ionic liquid mixtures on lipid extraction from Chlorella vulgaris. Renew Energy 65:169–174.

Choix FJ, De-Bashan LE, Bashan Y (2012) Enhanced accumulation of starch and total

carbohydrates in alginate-immobilized Chlorella spp. induced by Azospirillum brasilense: I. Autotrophic conditions. Enzyme Microb Technol 51:294–299.

Cooksey KE, Guckert JB, Williams SA, Callis PR (1987) Fluorometric determination of the neutral lipid content of microalgae using Nile Red. J Microbiol Methods 6:333–345.

Couto RM, Simoes PC, Reis A, Da Silva TL, Martins VH, Sanchez-Vicente Y (2010) Supercritical fluid extraction of lipids from the heterotrophic microalga Crypthecodinium cohnii. Eng Life Sci 10:158–164.

da Silva AS, Lee S-H, Endo T, P.S. Bon E (2011) Major improvement in the rate and yield of enzymatic saccharification of sugarcane bagasse via pretreatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim] [Ac]). Bioresour Technol 102:10505–10509.

Daroch M, Geng S, Wang G (2013) Recent advances in liquid biofuel production from algal feedstocks. Appl Energy 102:1371–1381.

De la Hoz Siegler H, Ayidzoe W, Ben-Zvi a., Burrell RE, McCaffrey WC (2012) Improving the reliability of fluorescence-based neutral lipid content measurements in microalgal cultures. Algal Res 1:176–184.

De S, Dutta S, Saha B (2012) One-pot conversions of lignocellulosic and algal biomass into liquid fuels. ChemSusChem 5:1826–1833.

Demirbas A (2008) Biodiesel: A realistic fuel alternative for diesel engines. Springer Verlag, London

Demirbas A (2007) Biodiesel: A Realistic Fuel Alternative for Diesel Engines. Springer Science & Business Media

Desai RK, Streefland M, Wijffels RH, Eppink MHM (2015) Novel astaxanthin extraction from Haematococcus pluvialis using cell permeabilising ionic liquids. Green Chem 1261–1267.

Ding S-Y, Liu Y-S, Zeng Y, Himmel ME, Baker JO, Bayer E a (2012a) How does plant cell wall nanoscale architecture correlate with enzymatic digestibility? Science 338:1055–60.

Ding ZD, Chi Z, Gu WX, Gu SM, Liu JH, Wang HJ (2012b) Theoretical and experimental investigation on dissolution and regeneration of cellulose in ionic liquid. Carbohydr Polym 89:7–16.

Doan TTY, Sivaloganathan B, Obbard JP (2011) Screening of marine microalgae for biodiesel feedstock. Biomass and Bioenergy 35:2534–2544.

Dominguez de Maria P (2014) Recent trends in (ligno)cellulose dissolution using neoteric solvents: Switchable, distillable and bio-based ionic liquids. J Chem Technol Biotechnol 89:11–18.

Dürre P (2007) Biobutanol: an attractive biofuel. Biotechnol J 2:1525–34.

Ellis JT, Hengge NN, Sims RC, Miller CD (2012) Acetone, butanol, and ethanol production from wastewater algae. Bioresour Technol 111:491–495.

Engel P, Mladenov R, Wulfhorst H, Jäger G, Spiess AC (2010) Point by point analysis: how ionic liquid affects the enzymatic hydrolysis of native and modified cellulose. Green Chem 12:1959–1966.

Erickson B, Nelson JE, Winters P (2012) Perspective on opportunities in industrial biotechnology in renewable chemicals. Biotechnol Jou 7:176–185.

Ezeji T, Qureshi N, Blaschek H (2007) Butanol production from agricultural residues: impact of degradation products on Clostridium beijerinckii growth and butanol fermentation. Biotechnol Bioeng 97:1460–1469.

Fan G, Liao C, Fang T, Wang M, Song G (2013) Hydrolysis of cellulose catalyzed by sulfonated poly(styrene-co- divinylbenzene) in the ionic liquid 1-n-butyl-3-methylimidazolium bromide. Fuel Process Technol 116:142–148.

Feng D, Li L, Yang F, Tan W, Zhao G, Zou H, Xian M, Zhang Y (2011) Separation of ionic liquid [Mmim][DMP] and glucose from enzymatic hydrolysis mixture of cellulose using alumina column chromatography. Appl Microbiol Biotechnol 91:399–405.

Ferguson JL, Holbrey JD, Ng S, Plechkova N V., Seddon KR, Tomaszowska A a., Wassell DF (2011) A greener, halide-free approach to ionic liquid synthesis. Pure Appl Chem 84:723–744.

Folch J, Lees M, Stanley GHS (1956) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509.

Foley PM, Beach ES, Zimmerman JB (2011) Algae as a source of renewable chemicals: opportunities and challenges. Green Chem 13:1399.

Fu D, Mazza G, Tamaki Y (2010) Lignin Extraction from Straw by Ionic Liquids and Enzymatic Hydrolysis of the Cellulosic Residues. J Agric Food Chem 58:2915–2922.

Fujita K, Kobayashi D, Nakamura N, Ohno H (2013) Direct dissolution of wet and saliferous marine microalgae by polar ionic liquids without heating. Enzyme Microb Technol 52:199–202.

Fujita K, MacFarlane DR, Forsyth M (2005) Protein solubilising and stabilising ionic liquids. Chem Commun 70:4804–4806.

Gao K, Boiano S, Marzocchella A, Rehmann L (2014) Cellulosic Butanol Production from Alkali-Pretreated Switchgrass (Panicumvirgatum) and Phragmites (Phragmites australis). Bioresour Technol 174:176–181.

Gao K, Orr V, Rehmann L (2016) Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction. Bioresour Technol 206:77–85.

Gao K, Rehmann L (2014) ABE fermentation from enzymatic hydrolysate of NaOH-pretreated corncobs. Biomass and Bioenergy 66:110–115.

Garcia H, Ferreira R, Petkovic M, Ferguson JL, Leitão MC, Gunaratne HQN, Seddon KR, Rebelo LPN, Silva Pereira C (2010) Dissolution of cork biopolymers in biocompatible ionic liquids. Green Chem 12:367.

Garcia-perez M, Wang S, Shen J, Rhodes M, Lee WJ (2008) Effects of Temperature on the Formation of Lignin-Derived Oligomers during the Fast Pyrolysis of Mallee Woody Biomass. 2022–2032.

Garcia-Perez M, Wang XS, Shen J, Rhodes MJ, Tian F, Lee W-J, Wu H, Li C-Z (2008) Fast Pyrolysis of Oil Mallee Woody Biomass: Effect of Temperature on the Yield and Quality of Pyrolysis Products. Ind Eng Chem Res 47:1846–1854.

Gong J, You F (2015) Value-Added Chemicals from Microalgae: Greener, More Economical, or Both?

Greaves TL, Drummond CJ (2008) Protic ionic liquids: Properties and applications. Chem Rev 108:206–237.

Greenspan P, Fowler SD (1985) Spectrofluorometric studies of the lipid probe, nile red. J Lipid Res 26:781–9.

Griffiths MJ, Garcin C, van Hille RP, Harrison STL (2011) Interference by pigment in the estimation of microalgal biomass concentration by optical density. J Microbiol Methods 85:119–23.

Griffiths MJ, Van Hille RP, Harrison STL (2010) Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 45:1053–1060.

Grima EM, González MJI, Giménez AG (2013) Solvent extraction for microalgae lipids. In: Borowitzka MA, Moheimani NR (eds) Algae for Biofuels and Energy. Springer Netherlands, Dordrecht, pp 187–205

Grimi N, Dubois a., Marchal L, Jubeau S, Lebovka NI, Vorobiev E (2014) Selective extraction from microalgae Nannochloropsis sp. using different methods of cell disruption. Bioresour Technol 153:254–259.

Guckert JB, Cooksey KE (1990) Triglyceride accumulation and fatty acid profile changes in Chlorella (Chlorophyta) during high pH-induced cell cycle inhibition. J Phycol 26:72–79.

Guillard RRL (2005) Purification methods for microalgae. In: Andersen RA (ed) Algal Culturing Techniques. Elsevier Academic Press, pp 117–132

Guo H, Lian Y, Yan L, Qi X, Smith RL (2013) Cellulose-derived superparamagnetic carbonaceous solid acid catalyst for cellulose hydrolysis in an ionic liquid or aqueous reaction system. Green Chem 15:2167–2174.

Guo H, Qi X, Li L, Smith RL (2012) Hydrolysis of cellulose over functionalized glucose-derived carbon catalyst in ionic liquid. Bioresour Technol 116:355–359.

Haas MJ, McAloon AJ, Yee WC, Foglia TA (2006) A process model to estimate biodiesel production costs. Bioresour Technol 97:671–678.

Halim R, Danquah MK, Webley PA (2012) Extraction of oil from microalgae for biodiesel production : A review. Biotechnol Adv 30:709–732.

Haque MA, Bangrak P (2012) Factors Affecting the Biomass and Lipid Production from Chlorella sp . TISTR 8990 under Mixotrophic Culture. 9:347–359.

Hara, Radin (1978) Lipid extraction of tissues with a low-toxicity solvent. Anal Biochem 90:420–426.

Heinze T, Schwikal K, Barthel S (2005) Ionic liquids as reaction medium in cellulose functionalization. Macromol Biosci 5:520–525.

Held P (BioTek), Kerri R (BioTek) (2011) Determination of Algal Cell Lipids Using Nile Red. 1–5.

Heredia-Arroyo T, Wei W, Ruan R, Hu B (2011) Mixotrophic cultivation of Chlorella vulgaris and its potential application for the oil accumulation from non-sugar materials. Biomass and Bioenergy 35:2245–2253.

Hidalgo P, Toro C, Ciudad G, Navia R (2013) Advances in direct transesterification of microalgal biomass for biodiesel production. Rev Environ Sci Biotechnol 12:179–199.

Ho SH, Huang SW, Chen CY, Hasunuma T, Kondo A, Chang JS (2013) Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. Bioresour Technol 135:191–198.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 54:621–39.

Huang C, Chen X, Xiong L, Chen X, Ma L, Chen Y (2013) Single cell oil production from low-cost substrates: the possibility and potential of its industrialization. Biotechnol Adv 31:129–39.

Huang G-H, Chen G, Chen F (2009) Rapid screening method for lipid production in alga based on Nile red fluorescence. Biomass and Bioenergy 33:1386–1392.

Hussain a, Trudell P, Repta a J (1970) Quantitative spectrophotometric methods for determination of sodium hypochlorite in aqueous solutions. J Pharm Sci 59:1168–70.

Hutner SH, Provasoli L, Schatz A, Haskins C. (1950) Some Approaches to the Study of the Role of Metals in the Metabolism of Microorganisms. Proc Am Philos Soc 94:152–170.

Illman AM, Scragg AH, Shales SW (2000) Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme Microb Technol 27:631–635.

Iverson SJ, Lang SL, Cooper MH (2001) Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 36:1283–1287.

Jernigan A, May M, Potts T, Rodgers B, Hestekin J, May PI, McLaughlin J, Beitle RR, Hestekina C (2009) Effects of Drying and Storage on Year-Round Production of Butanol and Biodiesel from Algal Carbohydrates and Lipids using Algae from Water Remediation. Environ Prog 28:404–409.

John RP, Anisha GS, Nampoothiri KM, Pandey A (2011) Micro and macroalgal biomass : A renewable source for bioethanol. Bioresour Technol 102:186–193.

Jones DT, Woods DR (1986) Acetone-butanol fermentation revisited. Microbiol Rev 50:484–524.

Keris-Sen UD, Sen U, Soydemir G, Gurol MD (2014) An investigation of ultrasound effect on microalgal cell integrity and lipid extraction efficiency. Bioresour Technol 152:407–413.

Kim SJ, Dwiatmoko AA, Choi JW, Suh YW, Suh DJ, Oh M (2010) Cellulose pretreatment with 1-n-butyl-3-methylimidazolium chloride for solid acid-catalyzed hydrolysis. Bioresour Technol 101:8273–8279.

Kim Y, Choi Y, Park J, Lee S, Yang Y, Joo H, Park T, Hwan Y, Hyun S (2012) Ionic liquid-mediated extraction of lipids from algal biomass. Bioresour Technol 109:312–315.

Kim Y-H, Park S, Kim MH, Choi Y-K, Yang Y-H, Kim HJ, Kim H, Kim H-S, Song K-G, Lee SH (2013) Ultrasound-assisted extraction of lipids from Chlorella vulgaris using [Bmim][MeSO4]. Biomass and Bioenergy 56:99–103.

Kircher M (2015) Sustainability of biofuels and renewable chemicals production from biomass. Curr Opin Chem Biol 29:26–31.

Knothe G (2005a) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 86:1059–1070.

Knothe G (2005b) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. Fuel Process Technol 86:1059–1070.

Kumar RR, Rao PH, Arumugam M (2015) Lipid Extraction Methods from Microalgae: A Comprehensive Review. Front Energy Res 2:1–9.

Lakaniemi A-M, Tuovinen OH, Puhakka JA (2013) Anaerobic conversion of microalgal biomass to sustainable energy carriers – A review. Bioresour Technol 135:222–231.

Lateef H, Grimes S, Kewcharoenwong P, Feinberg B (2009) Separation and recovery of cellulose and lignin using ionic liquids: A process for recovery from paper-based waste. J Chem Technol Biotechnol 84:1818–1827.

Laurens LML (2013) Summative Mass Analysis of Algal Biomass – Integration of Analytical Procedures Laboratory Analytical Procedure (LAP). Golden, CO

Laurens LML, Cooksey KE, Sears J, Edwards M, Lundquist T, Behnke C, Schultz N, Howell S, Clapper G, Gardner R, Slaby E, Olivares J, McCormick R, Cattolico RA (2015) Industrial Algae Measurements.

Lee J, Yoo C, Jun S, Ahn C, Oh H (2010) Comparison of several methods for effective lipid extraction from microalgae. Bioresour Technol 101:S75–S77.

Lee SH, Doherty T V., Linhardt RJ, Dordick JS (2009) Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis. Biotechnol Bioeng 102:1368–1376.

Lee SJ, Yoon B, Oh H (1998) Rapid method for the determination of lipid from the green alga Botryococcus braunii. 12:553–556.

Lepage G, Roy CC (1984) Improved recovery of fatty acid through direct

transesterification without prior extraction or purification. J Lipid Res 25:1391–1396.

Li C, Cheng G, Balan V, Kent MS, Ong M, Chundawat SPS, Sousa L daCosta, Melnichenko YB, Dale BE, Simmons B a., Singh S (2011a) Influence of physico-chemical changes on enzymatic digestibility of ionic liquid and AFEX pretreated corn stover. Bioresour Technol 102:6928–6936.

Li C, Knierim B, Manisseri C, Arora R, Scheller H V., Auer M, Vogel KP, Simmons BA, Singh S (2010a) Comparison of dilute acid and ionic liquid pretreatment of switchgrass: Biomass recalcitrance, delignification and enzymatic saccharification. Bioresour Technol 101:4900–4906.

Li C, Zhao ZK (2007) Efficient acid-catalyzed hydrolysis of cellulose in ionic liquid. Adv Synth Catal 349:1847–1850.

Li Q, Du W, Liu D (2008a) Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol 80:749–756.

Li Q, Jiang X, He Y, Li L, Xian M, Yang J (2010b) Evaluation of the biocompatibile ionic liquid 1-methyl-3-methylimidazolium dimethylphosphite pretreatment of corn cob for improved saccharification. Appl Microbiol Biotechnol 87:117–126.

Li Y, Han D, Sommerfeld M, Hu Q (2011b) Photosynthetic carbon partitioning and lipid production in the oleaginous microalga Pseudochlorococcum sp. (Chlorophyceae) under nitrogen-limited conditions. Bioresour Technol 102:123–9.

Li Y, Horsman M, Wang B, Wu N, Lan CQ (2008b) Effects of nitrogen sources on cell growth and lipid accumulation of green alga Neochloris oleoabundans. Appl Microbiol Biotechnol 81:629–36.

Lian J, Chen S, Zhou S, Wang Z, O'Fallon J, Li C-Z, Garcia-Perez M (2010a) Separation, hydrolysis and fermentation of pyrolytic sugars to produce ethanol and lipids. Bioresour Technol 101:9688–99.

Lian J, Chen S, Zhou S, Wang Z, O'Fallon J, Li C-Z, Garcia-Perez M (2010b) Separation, hydrolysis and fermentation of pyrolytic sugars to produce ethanol and lipids. Bioresour Technol 101:9688–99.

Lian J, Garcia-Perez M, Chen S (2013) Fermentation of levoglucosan with oleaginous yeasts for lipid production. Bioresour Technol 133:183–9.

Liang Y (2013) Producing liquid transportation fuels from heterotrophic microalgae. Appl Energy 104:860–868.

Liang Y, Sarkany N, Cui Y (2009) Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol Lett 31:1043–1049.

Liang Y, Zhao X, Chi Z, Rover M, Johnston P, Brown R, Jarboe L, Wen Z (2013) Utilization of acetic acid-rich pyrolytic bio-oil by microalga Chlamydomonas reinhardtii: Reducing bio-oil toxicity and enhancing algal toxicity tolerance. Bioresour Technol 133:500–506.

Liu B, Benning C (2013) Lipid metabolism in microalgae distinguishes itself. Curr Opin Biotechnol 24:300–9.

Liu B, Zhao Z (Kent) (2007) Biodiesel production by direct methanolysis of oleaginous microbial biomass. J Chem Technol Biotechnol 82:775–780.

Liu Q-P, Hou X-D, Li N, Zong M-H (2012) Ionic liquids from renewable biomaterials: synthesis, characterization and application in the pretreatment of biomass. Green Chem 14:304.

Lotero E, Liu Y, Lopez DE, Suwannakarn K, Bruce D a., Goodwin JG (2005) Synthesis of biodiesel via acid catalysis. Ind Eng Chem Res 44:5353–5363.

Lowrey J, Brooks MS, McGinn PJ (2015) Heterotrophic and mixotrophic cultivation of microalgae for biodiesel production in agricultural wastewaters and associated challenges???a critical review. J Appl Phycol 27:1485–1498.

Luque L, Orr VCA, Chen S, Westerhof R, Oudenhoven S, Rossum G van, Kersten S, Berruti F, Rehmann. L (2016) Lipid accumulation from pinewood pyrolysates by Rhodosporidium diobovatum and Chlorella vulgaris for biodiesel production. Bioresour Technol 214:660–669.

Luque L, Westerhof R, Van Rossum G, Oudenhoven S, Kersten S, Berruti F, Rehmann L (2014) Pyrolysis based bio-refinery for the production of bioethanol from demineralized ligno-cellulosic biomass. Bioresour Technol 161:20–8.

Makareviciene V, Skorupskaite V, Andruleviciute V (2013) Biodiesel fuel from microalgae-promising alternative fuel for the future: a review. Rev Environ Sci Bio/Technology 12:119–130.

Malihan LB, Nisola GM, Mittal N, Seo JG, Chung WJ (2014) Blended ionic liquid systems for macroalgae pretreatment. Renew Energy 66:596–604.

Mansfield SD, Mooney C, Saddler JN (1999) Substrate and enzyme characteristics that limit cellulose hydrolysis. Biotechnol Prog 15:804–816.

Medina AR, Grima EM (1998) Downstream processing of algal polyunsaturated fatty acids. Biotechnol Adv 16:517–580.

Meher L, Vidyasagar D, Naik S (2006) Technical aspects of biodiesel production by transesterification—a review. Renew Sustain Energy Rev 10:248–268.

Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M (2009) Biodiesel production from oleaginous microorganisms. Renew Energy 34:1–5.

Milledge JJ (2011) Commercial application of microalgae other than as biofuels: A brief review. Rev Environ Sci Biotechnol 10:31–41.

Milledge JJ, Heaven S (2012) A review of the harvesting of micro-algae for biofuel production. Rev Environ Sci Biotechnol 12:165–178.

Moazami N, Ranjbar R, Ashori A, Tangestani M, Nejad AS (2011) Biomass and lipid productivities of marine microalgae isolated from the Persian Gulf and the Qeshm Island. Biomass and Bioenergy 35:1935–1939.

Molina Grima E, Belarbi EH, Acién Fernández FG, Robles Medina a., Chisti Y (2003)

Recovery of microalgal biomass and metabolites: Process options and economics. Biotechnol Adv 20:491–515.

Montero MF, Aristizábal M, García Reina G (2010) Isolation of high-lipid content strains of the marine microalga Tetraselmis suecica for biodiesel production by flow cytometry and single-cell sorting. J Appl Phycol 23:1053–1057.

Mosier N, Wyman C, Dale B, Elander R, Lee YY, Holtzapple M, Ladisch M (2005) Features of promising technologies for pretreatment of lignocellulosic biomass. Bioresour Technol 96:673–86.

Munch G, Sestric R, Sparling R, Levin DB, Cicek N (2015) Lipid production in the under-characterized oleaginous yeasts, Rhodosporidium babjevae and Rhodosporidium diobovatum, from biodiesel-derived waste glycerol. Bioresour Technol 185:49–55.

Mutanda T, Ramesh D, Karthikeyan S, Kumari S, Anandraj a, Bux F (2011) Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. Bioresour Technol 102:57–70.

Myers J a, Curtis BS, Curtis WR (2013) Improving accuracy of cell and chromophore concentration measurements using optical density. BMC Biophys 6:4.

Myers J, Graham J-R (1958) On the mass culture of algae. II. Yield as a function of cell concentration under continuous sunlight irradiance. Plant Physiol 345–352.

Nan Y, Liu J, Lin R, Tavlarides LL (2015) The Journal of Supercritical Fluids Production of biodiesel from microalgae oil (Chlorella protothecoides) by non-catalytic transesterification in supercritical methanol and ethanol : Process optimization. J Supercrit Fluids 97:174–182.

Nascimento IA, Marques SSI, Cabanelas ITD, Pereira SA, Druzian JI, de Souza CO, Vich DV, de Carvalho GC, Nascimento MA (2013) Screening Microalgae Strains for Biodiesel Production: Lipid Productivity and Estimation of Fuel Quality Based on Fatty Acids Profiles as Selective Criteria. BioEnergy Res 6:1–13.

Nguyen TAD, Kim KR, Nguyen MT, Kim MS, Kim D, Sim SJ (2010) Enhancement of fermentative hydrogen production from green algal biomass of Thermotoga neapolitana by various pretreatment methods. Int J Hydrogen Energy 35:13035–13040.

Nigam PS, Singh A (2014) Production of Oils and Fatty Acids. In: Batt CA, Tortorello M-L (eds) Encyclopedia of Food Microbiology, 2nd edn. Elservier, pp 792–803

Olkiewicz M, Caporgno MP, Font J, Legrand J, Lepine O, Plechkova N V, Pruvost J, Seddon KR, Bengoa C (2015) A novel extraction process for lipids from microalgæ for biodiesel production, using a phosphonium ionic liquid. Green Chem 2813–2824.

OriginOil (2010) Algae Harvesting , Dewatering and Extraction. In: World biofuels Markets.

Orr V, Plechkova N V, Seddon KR, Rehmann L (2016) Disruption and Wet Extraction of the Microalgae Chlorella vulgaris Using Room-temperature Ionic Liquids. ACS

Sustain Chem Eng 4:591–600.

Orr V, Rehmann L (2015) Improvement of the Nile Red fluorescence assay for determination of total lipid content in microalgae independent of chlorophyll content. J Appl Phycol 27:2181–2189.

Oudenhoven SRG, Westerhof RJM, Aldenkamp N, Brilman DWF, Kersten SR a (2013) Demineralization of wood using wood-derived acid: Towards a selective pyrolysis process for fuel and chemicals production. J Anal Appl Pyrolysis 103:112–118.

Pan J, Muppaneni T, Sun Y, Reddy HK, Fu J, Lu X, Deng S (2016) Microwave-assisted extraction of lipids from microalgae using an ionic liquid solvent [BMIM][HSO4]. Fuel 178:49–55.

Papanikolaou S, Aggelis G (2011) Lipids of oleaginous yeasts. Part II: Technology and potential applications. Eur J Lipid Sci Technol 113:1052–1073.

Paquet V, Croux C, Goma G, Soucaille P (1991) Purification and characterization of the extracellular alpha-amylase from Clostridium acetobutylicum ATCC 824. Appl Environ Microbiol 57:212–218.

Park JY, Park MS, Lee YC, Yang JW (2015) Advances in direct transesterification of algal oils from wet biomass. Bioresour Technol 184:267–275.

Patil PD, Gude VG, Mannarswamy A, Cooke P, Nirmalakhandan N, Lammers P, Deng S (2012) Comparison of direct transesterification of algal biomass under supercritical methanol and microwave irradiation conditions. Fuel 97:822–831.

Patil PD, Gude VG, Mannarswamy A, Deng S, Cooke P, Munson-McGee S, Rhodes I, Lammers P, Nirmalakhandan N (2011) Optimization of direct conversion of wet algae to biodiesel under supercritical methanol conditions. Bioresour Technol 102:118–122.

Paudel A, Jessop MJ, Stubbins SH, Champagne P, Jessop PG (2015) Bioresource Technology Extraction of lipids from microalgae using CO 2 -expanded methanol and liquid CO 2. Bioresour Technol 184:286–290.

Petkovic M, Ferguson JL, Gunaratne HQN, Ferreira R, Leitão MC, Seddon KR, Rebelo LPN, Pereira CS (2010) Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability. Green Chem 12:643.

Petkovic M, Seddon KR, Rebelo LPN, Silva Pereira C (2011) Ionic liquids: a pathway to environmental acceptability. Chem Soc Rev 40:1383–403.

Pinkert A, Marsh KN, Pang S, Staiger MP (2009) Ionic Liquids and Their Interaction with Cellulose. Chem Rev 109:6712–6728.

Plechkova N V, Seddon KR (2008) Applications of ionic liquids in the chemical industry.

Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochem Biophys Acta 975:384–394.

Posten C, Schaub G (2009) Microalgae and terrestrial biomass as source for fuels-A

process view. J Biotechnol 142:64–69.

Potts T, Du J, Paul M, May P, Beitle R, Hestekin J (2012) The Production of Butanol from Jamaica Bay Macro Algae. AIChE J 31:29–36.

Pu Y, Jiang N, Ragauskas AJ (2007) Ionic Liquid as a Green Solvent for Lignin. J Wood Chem Technol 27:23–33.

Ramos MJ, Fernández CM, Casas A, Rodríguez L, Pérez A (2009) Influence of fatty acid composition of raw materials on biodiesel properties. Bioresour Technol 100:261–8.

Ratledge C (2004) Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie 86:807–15.

Reddy HK, Muppaneni T, Patil PD, Ponnusamy S, Cooke P, Schaub T, Deng S (2014) Direct conversion of wet algae to crude biodiesel under supercritical ethanol conditions. Fuel 115:720–726.

Regestein L, Doerr EW, Staaden A, Rehmann L (2015) Impact of butyric acid on butanol formation by Clostridium pasteurianum. Bioresour Technol 196:153–159.

Remsing RC, Swatloski RP, Rogers RD, Moyna G (2006) Mechanism of cellulose dissolution in the ionic liquid 1-n-butyl-3-methylimidazolium chloride: a 13C and 35/37Cl NMR relaxation study on model systems. Chem Commun 1271–1273.

Ren H-Y, Liu B-F, Ma C, Zhao L, Ren N-Q (2013) A new lipid-rich microalga Scenedesmus sp. strain R-16 isolated using Nile red staining: effects of carbon and nitrogen sources and initial pH on the biomass and lipid production. Biotechnol Biofuels 6:143.

Rinaldi R, Meine N, vom Stein J, Palkovits R, Schüth F (2010) Which Controls the Depolymerization of Cellulose in Ionic Liquids: The Solid Acid Catalyst or Cellulose? ChemSusChem 3:266–276.

Rinaldi R, Palkovits R, Schüth F (2008) Depolymerization of cellulose using solid catalysts in ionic liquids. Angew Chemie - Int Ed 47:8047–8050.

Roleda MY, Slocombe SP, Leakey RJG, Day JG, Bell EM, Stanley MS (2013) Effects of temperature and nutrient regimes on biomass and lipid production by six oleaginous microalgae in batch culture employing a two-phase cultivation strategy. Bioresour Technol 129:439–49.

Rosenbaum M, Schröder U, Scholz F (2005) Utilizing the green alga Chlamydomonas reinhardtii for microbial electricity generation: a living solar cell. Appl Microbiol Biotechnol 68:753–6.

Safi C, Charton M, Pignolet O, Silvestre F, Vaca-Garcia C, Pontalier P-Y (2013) Influence of microalgae cell wall characteristics on protein extractability and determination of nitrogen-to-protein conversion factors. J Appl Phycol 25:523–529.

Saha BC, Iten LB, Cotta MA, Wu YV (2005) Dilute acid pretreatment, enzymatic saccharification and fermentation of wheat straw to ethanol. Process Biochem 40:3693–3700.

Samayam IP, Schall CA (2010) Saccharification of ionic liquid pretreated biomass with

commercial enzyme mixtures. Bioresour Technol 101:3561–3566.

Samorì C, Torri C, Samorì G, Fabbri D, Galletti P, Guerrini F, Pistocchi R, Tagliavini E (2010) Extraction of hydrocarbons from microalga Botryococcus braunii with switchable solvents. Bioresour Technol 101:3274–3279.

Samori C, Vet R, Pezzolesi L, Brilman DWF, Galletti P, Tagliavini E (2013) Effective lipid extraction from algae cultures using switchable solvents. Green Chem 15:353–356.

Seddon KR (1997) Review Ionic Liquids for Clean Technolog y *. 50:1–6.

Seoud O a El, Koschella A, Fidale LC, Dorn S, Heinze T (2007) Application of ionic liquids in carbohydrate chemistry: A window of opportunities. Biomacromolecules 8:2630–2647.

Sescousse R, Gavillon R, Budtova T (2011) Aerocellulose from cellulose-ionic liquid solutions: Preparation, properties and comparison with cellulose-NaOH and cellulose-NMMO routes. Carbohydr Polym 83:1766–1774.

Shill K, Padmanabhan S, Xin Q, Prausnitz JM, Clark DS, Blanch HW (2011) Ionic liquid pretreatment of cellulosic biomass: Enzymatic hydrolysis and ionic liquid recycle. Biotechnol Bioeng 108:511–520.

Singh J, Gu S (2010) Commercialization potential of microalgae for biofuels production. Renew Sustain Energy Rev 14:2596–2610.

Singh RN, Sharma S (2012) Development of suitable photobioreactor for algae production - A review. Renew Sustain Energy Rev 16:2347–2353.

Singh S, Simmons B a, Vogel KP (2009) Visualization of biomass solubilization and cellulose regeneration during ionic liquid pretreatment of switchgrass. Biotechnol Bioeng 104:68–75.

Sitepu I, Selby T, Lin T, Zhu S, Boundy-Mills K (2014a) Carbon source utilization and inhibitor tolerance of 45 oleaginous yeast species. J Ind Microbiol Biotechnol 41:1061–70.

Sitepu IR, Garay LA, Sestric R, Levin D, Block DE, German JB, Boundy-Mills KL (2014b) Oleaginous yeasts for biodiesel: current and future trends in biology and production. Biotechnol Adv 32:1336–60.

Sluiter A, Hames B, Ruiz RO, Scarlata C, Sluiter J, Templeton D, Energy D of (2004) Determination of Structural Carbohydrates and Lignin in Biomass. Golden, CO

Sluiter A, Sluiter J (2008) Determination of Starch in Solid Biomass Samples by HPLC Laboratory Analytical Procedure (LAP). Golden, CO

Spolaore P, Joannis-cassan C, Duran E, Isambert A, Génie L De, Paris EC (2006) Commercial Applications of Microalgae. J Biosci Bioeng 101:87–96.

Stoeglehner G, Narodoslawsky M (2009) How sustainable are biofuels? Answers and further questions arising from an ecological footprint perspective. Bioresour Technol 100:3825–3830.

Suh WI, Mishra SK, Kim TH, Farooq W, Moon M, Shrivastav A, Park MS, Yang JW

(2015) Direct transesterification of wet microalgal biomass for preparation of biodiesel. Algal Res 12:405–411.

Swatloski RP, Spear SK, Holbrey JD, Rogers RD (2002) Dissolution of Cellose with Ionic Liquids. J Am Chem Soc 124:4974–4975.

Tadesse H, Luque R (2011) Advances on biomass pretreatment using ionic liquids: An overview. Energy Environ Sci 4:3913.

Taher H, Al-Zuhair S, Al-Marzouqi AH, Haik Y, Farid M (2014) Effective extraction of microalgae lipids from wet biomass for biodiesel production. Biomass and Bioenergy 66:159–167.

Teixeira RE (2012a) Cutting-edge research for a greener sustainable future Green Chemistry Energy-efficient extraction of fuel and chemical feedstocks from algae †. 14:419–427.

Teixeira RE (2012b) Energy-efficient extraction of fuel and chemical feedstocks from algae. Green Chem 14:419–427.

Thang VH, Kanda K, Kobayashi G (2010) Production of Acetone-Butanol-Ethanol (ABE) in direct fermentation of cassava by Clostridium saccharoperbutylacetonicum N1-4. Appl Biochem Biotechnol 161:157–170.

Thliveros P, U??kun Kiran E, Webb C (2014) Microbial biodiesel production by direct methanolysis of oleaginous biomass. Bioresour Technol 157:181–187.

Trivedi TJ, Kumar A (2014) Efficient Extraction of Agarose from Red Algae Using Ionic Liquids. Green Sustain Chem 4:190–201.

Tsoutsos T, Bethanis D (2011) Optimization of the dilute acid hydrolyzator for cellulose-to-bioethanol saccharification. Energies 4:1601–1623.

Uju, Shoda Y, Nakamoto A, Goto M, Tokuhara W, Noritake Y, Katahira S, Ishida N, Nakashima K, Ogino C, Kamiya N (2012) Short time ionic liquids pretreatment on lignocellulosic biomass to enhance enzymatic saccharification. Bioresour Technol 103:446–452.

van der Wal H, Sperber BLHMHM, Houweling-Tan B, Bakker RRCC, Brandenburg W, López-Contreras AM (2013) Production of acetone, butanol, and ethanol from biomass of the green seaweed Ulva lactuca. Bioresour Technol 128:431–437.

Van Gerpen J (2005) Biodiesel processing and production. Fuel Process Technol 86:1097–1107.

van Wychen S, Laurens LML (2013) Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by in situ Transesterification Laboratory Analytical Procedure (LAP). Golden, CO

Vitz J, Erdmenger T, Haensch C, Schubert US (2009) Extended dissolution studies of cellulose in imidazolium based ionic liquids. Green Chem 11:417.

Wada M, Ike M, Tokuyasu K (2010) Enzymatic hydrolysis of cellulose I is greatly accelerated via its conversion to the cellulose II hydrate form. Polym Degrad Stab 95:543–548.

Wagner M, Hilgers C (2008) Scale-Up of Ionic Liquids. In: Ionic Liquids in Synthesis. Wiley VCH, pp 36–37

Wahidin S, Idris A, Shaleh SRM (2016) Ionic liquid as a promising biobased green solvent in combination with microwave irradiation for direct biodiesel production. Bioresour Technol 206:150–154.

Wang H, Gurau G, Rogers RD (2012) Ionic liquid processing of cellulose. Chem Soc Rev 41:1519.

Wang M, Yuan W, Jiang X, Jing Y, Wang Z (2014) Disruption of microalgal cells using high-frequency focused ultrasound. Bioresour Technol 153:315–321.

Wang P, Tao BY (1998) Soy Fatty Acid Oxidation with Sodium Hypochlorite Monitored by Nuclear Magnetic Resonance Spectroscopy. J Am Oil Chem Soc 75:9–14.

Wang S, Zhang Y, Dong H, Mao S, Zhu Y, Wang R, Luan G, Li Y (2011) Formic acid triggers the “acid crash” of acetone-butanol-ethanol fermentation by Clostridium acetobutylicum. Appl Environ Microbiol 77:1674–1680.

Wang Y, Guo W, Cheng C-L, Ho S-H, Chang J-S, Ren N (2016) Enhancing bio-butanol production from biomass of Chlorella vulgaris JSC-6 with sequential alkali pretreatment and acid hydrolysis. Bioresour Technol 200:557–564.

Wang ZT, Ullrich N, Joo S, Waffenschmidt S, Goodenough U (2009) Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless Chlamydomonas reinhardtii. Eukaryot Cell 8:1856–68.

Wood JA, Orr VCA, Luque L, Nagendra V, Berruti F, Rehmann L (2014) High-Throughput Screening of Inhibitory Compounds on Growth and Ethanol Production of Saccharomyces cerevisiae.

Xiao G, Gao L (2011) First Generation Biodiesel. In: Bernardes MADS (ed) Biofuel Production - Recent Development and Prospects. InTech, pp 45–64

Xiong W, Gao C, Yan D, Wu C, Wu Q (2010) Double CO(2) fixation in photosynthesis-fermentation model enhances algal lipid synthesis for biodiesel production. Bioresour Technol 101:2287–93.

Xiong Y, Zhang Z, Wang X, Liu B, Lin J (2014) Hydrolysis of cellulose in ionic liquids catalyzed by a magnetically-recoverable solid acid catalyst. Chem Eng J 235:349–355.

Xu A, Wang J, Wang H (2010) Effects of anionic structure and lithium salts addition on the dissolution of cellulose in 1-butyl-3-methylimidazolium-based ionic liquid solvent systems. Green Chem 12:268.

Yang Z (2009) Hofmeister effects: an explanation for the impact of ionic liquids on biocatalysis. J Biotechnol 144:12–22.

Yen H-W, Hu I-C, Chen C-Y, Ho S-H, Lee D-J, Chang J-S (2013) Microalgae-based biorefinery – From biofuels to natural products. Bioresour Technol 135:166–174.

Young G, Nippgen F, Titterbrandt S, Cooney MJ (2010) Lipid extraction from biomass using co-solvent mixtures of ionic liquids and polar covalent molecules. Sep Purif

Technol 72:118–121.

Yu X, Yang J, Lu H, Tu ST, Yan J (2014) Energy-efficient extraction of fuel from Chlorella vulgaris by ionic liquid combined with CO2 capture. Appl Energy 160:648–655.

Yu X, Zheng Y, Dorgan KM, Chen S (2011) Oil production by oleaginous yeasts using the hydrolysate from pretreatment of wheat straw with dilute sulfuric acid. Bioresour Technol 102:6134–40.

Yu Z, Chen X, Xia S (2016) The mechanism of lipids extraction from wet microalgae Scenedesmus sp. by ionic liquid assisted subcritical water. J Ocean Univ China 15:549–552.

Yuvraj, Vidyarthi AS, Singh J (2016) Enhancement of Chlorella vulgaris cell density: Shake flask and bench-top photobioreactor studies to identify and control limiting factors. Korean J Chem Eng 32:1–10.

Zakrzewska ME, Bogel-Łukasik E, Bogel-Łukasik R (2010) Solubility of Carbohydrates in Ionic Liquids. Energy & Fuels 24:737–745.

Zhang H, Wu J, Zhang J, He J (2005) 1-allyl-3-methylimidazolium chloride room temperature ionic liquid: A new and powerful nonderivatizing solvent for cellulose. Macromolecules 38:8272–8277.

Zhang Z, Zhao ZK (2009) Solid acid and microwave-assisted hydrolysis of cellulose in ionic liquid. Carbohydr Res 344:2069–2072.

Zhao H, Baker G a, Cowins J V (2009a) Fast enzymatic saccharification of switchgrass after pretreatment with ionic liquids. Biotechnol Prog 26:127–33.

Zhao H, Holladay JE, Brown H, Zhang ZC (2007) Metal Chlorides in Ionic Liquid Solvents Convert Sugars to 5-Hydroxymethylfurfural. Science (80-) 316:1597–1600.

Zhao H, Jones CIL, Baker GA, Xia S, Olubajo O, Person VN (2009b) Regenerating cellulose from ionic liquids for an accelerated enzymatic hydrolysis. J Biotechnol 139:47–54.

Zhao X, Davis K, Brown R, Jarboe L, Wen Z (2015) Alkaline treatment for detoxification of acetic acid-rich pyrolytic bio-oil for microalgae fermentation: Effects of alkaline species and the detoxification mechanisms. Biomass and Bioenergy 80:203–212.

Zhao X, Peng F, Du W, Liu C, Liu D (2012) Effects of some inhibitors on the growth and lipid accumulation of oleaginous yeast Rhodosporidium toruloides and preparation of biodiesel by enzymatic transesterification of the lipid. Bioprocess Biosyst Eng 35:993–1004.

Zheng H, Yin J, Gao Z, Huang H, Ji X, Dou C (2011) Disruption of Chlorella vulgaris cells for the release of biodiesel-producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl Biochem Biotechnol 164:1215–24.

Curriculum Vitae

Name:	**Valerie Orr**
Post-secondary Education and Degrees:	University of Waterloo, Waterloo, Ontario, Canada 2004-2009 B.Sc 2010-2012 M.ASc The University of Western Ontario, London, Ontario, Canada 2012-2016 Ph.D.

Honours and Awards:

[1] Natural Sciences and Engineering Research Council (NSERC) Post Graduate Fellowship (PDF) 2016-2018
[2] NSERC Alexander Graham Bell Canadian Graduate Scholarship 2014-2016
[3] EDJ Murray Biochemical Engineering Scholarship 2015
[4] Ontario Graduate Scholarship (OGS) 2013-2014
[5] HQP Exchange Award, BiofuelNet Canada, 2014

Related Work Experience:

- *Instructor - Computational Methods for Engineering*
  University of Western Ontario, London, On
- *Teaching Assistant*
  University of Western Ontario, London, On
- *MITACS Internship*
  University of Western Ontario, London, On
  Imperial Oil, Sarnia, On
- *Research Associate*
  University of Waterloo, Waterloo, On
- *Laboratory Technician*
  Merck Frosst Canada, Kirkland, Qc
- *Laboratory Technician*
  National Microbiology Laboratory, Winnipeg, Mb

Publications:

** Equal first author contribution*

[1] Luque*; L; **Orr, V***; Chen, S; Westerhof, R; Oudenhoven, S; van Rossum, G; Kersten, S; Berruti, F; Rehmann, L (2016) Lipid accumulation from pinewood pyrolysates by *Rhodosporidium diobavotum* and *Chlorella vulgaris* for biodiesel production. Bioresource Technol:214, 660-669. Impact factor 4.494

[2] Gao, K*; **Orr, V*;** Rehmann, L (2016) Butanol fermentation from microalgae derived carbohydrates after ionic liquid extraction. Bioresource Technol:206, 77-85. Impact factor 4.494

[3] **Orr, V**; Plechkova, N.V; Seddon, K.R; Rehmann, L (2016) Disruption and wet extraction of the microalgae *Chlorella vulgaris* using room-temperature ionic liquids. ACS Sustainable Chem Eng: 4(2) 591-600. Impact factor 4.642

[4] **Orr, V**, Rehmann, L (2016) Improvement of the Nile Red fluorescence assay for determination of total lipid content in microalgae independent of chlorophyll content. J Appl Phycol:28, 2181-2189. Impact Factor: 2.559

[5] Procentese, A., Johnson, E., **Orr, V**., Campanile, A.G., Wood, J.A., Marzocchella, A., Rehmann, L. (2015) Deep eutectic solvent pretreatment and subsequent saccharification of corncob. Bioresource Technol:192, 31-36. Impact Factor: 4.494

[6] Wood, J.A., **Orr, V.**, Luque, L., Nagendra, V., Berruti, F., Rehmann, L. (2014) High-throughput screening of inhibitory compounds on growth and ethanol production of *Saccharomyces cerevisiae*. BioEnergy Res: 8; 423-430. Impact Factor: 3.541

[7] Srirangan, K. *; **Orr, V. *;** Akawi, L.; Westbrook, A.; Moo-Young, M.; Chou, C.P. (2013) Biochemical and genetic strategies to enhance production of penicillin G acylase. Biotechnol Adv: 31; 1319-1332. Impact Factor: 9.015

[8] **Orr, V.;** Zhong, L.; Moo-Young, M.; Chou, C.P. (2013) Recent advances in bioprocessing: Application of membrane chromatography. Biotechnology Advances: 31; 450-465. Impact Factor: 9.015

[9] **Orr, V.;** Scharer, J.; Honeyman, H.; Moo-Young, M.; Fenner, D.; Crossley, L.; Suen, S.; Chou, C.P. (2012) Simultaneous clarification of Escherichia coli culture and purification of extracellularly produced penicillin G acylase using tangential flow filtration and anion-exchange membrane chromatography. Chromatogr B: 900 (1); 71-78. Impact factor: 2.729

[10] **Orr, V.;** Scharer, J.; Honeyman, H.; Moo-Young, M.; Fenner, D.; Crossley, L.; Suen, S.; Chou, C.P. (2012) Integrated Development of an Effective Bioprocess for Extracellular Production of Penicillin G Acylase in *Escherichia coli* and its Subsequent One-Step Purification. J Biotechnol: 161 (1); 19-26. Impact factor: 3.49

[11] Sukhija, K.; Pyne, M.; Ali, S.; **Orr, V**.; Abedi, D.; Moo-Young, M.; Chou, C.P. (2012) Developing an extended genomic engineering approach based on recombineering to knock-in heterologous genes to *Escherichia coli* genome. Mol Biotechnol: 51 (2); 109-118. Impact factor: 1.876